PAR1 Action in Tumor Development and Metastasis

A dissertation submitted by

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ABSTRACT

Protease-activated receptor 1 (PAR1) is a G protein-coupled receptor that is not expressed in normal breast epithelia, but is up-regulated in invasive breast carcinomas. In the present study, we found that matrix metalloprotease-1 (MMP-1) activates the PAR1-Akt survival pathway in breast carcinoma cells. This process is blocked by a cell-penetrating lipopeptide 'pepducin', P1pal-7, a potent inhibitor of cell viability in breast carcinoma cells expressing PAR1. Both a MMP-1 inhibitor and P1pal-7 significantly promote apoptosis in breast tumor xenografts and inhibit metastasis to the lungs by up to 88%. Consistently, biochemical analysis of xenograft tumors treated with P1pal-7 or MMP-1 inhibitor demonstrated attenuated Akt activity. Dual therapy with P1pal-7 and taxotere inhibited the growth of MDA-MB-231 xenografts by 95%.

The correlation of PAR1 with invasive breast carcinoma and its ability to promote migration and invasion has been well documented. We have observed that ectopic expression of PAR1 in MCF-7 breast carcinoma cells induces a fibroblast-like morphological change and a corresponding enhancement of migration and invasion. Transcriptional profiling by genechip analysis revealed that PAR1 expression promotes the expression of mesenchymal proteins such as vimentin, laminin, and integrin, and down-regulates epithelial proteins such as E-cadherin, claudin, zona occludens and cytokeratin. PAR1 expression also induced a concerted shift in the expression of ErbB family of receptors and

ligands, promoting the constitutive activity of EGFR/HER2 signaling. Together, these observations suggest that PAR1 and EGFR cooperatively induce Epithelial-to-Mesenchymal Transition (EMT) in breast carcinoma cells. This proposed mechanism is supported by empirical evidence from the NCI-60 panel of breast carcinoma cells, which demonstrate 100% correlation of PAR1 expression with EGFR expression and EMT status.

We have demonstrated that PAR1 blockade induces apoptosis and chemosensization in the primary tumor, and inhibits metastasis to the lungs. Furthermore, we found that PAR1 induces EMT to promote breast cancer invasion and metastasis. Together, our results suggest that PAR1 is a critical target of breast cancer pathogenesis that can be exploited by novel inhibitors as exemplified by the pepducin technology.

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CHAPTER 1: Introduction

The primary goal of this dissertation is to discuss the molecular mechanisms underlying breast cancer pathogenesis and their applications for novel therapeutic regimens. Therefore, this first chapter will introduce the clinical and molecular aspects of cancer research with emphasis on breast cancer biology. Our group has identified Protease Activated Receptor-1 as a critical player in breast cancer development and metastasis. In the following two chapters, we will report our findings characterizing the role of PAR1 in breast tumor survival and metastasis, and induction of epithelial-to-mesenchymal transition (EMT). The final chapter will discuss the current challenges in the treatment of metastatic breast cancer and the potential utility of our discoveries.

Cancer Epidemiology

Cancer places a large burden on human life, causing significant morbidity and mortality. Over 1.4 million new cancer cases and approximately 0.5 million cancer deaths are projected for the US in 2009 (2). Cancer death rates are at a decline due to improved surveillance and treatment, however, a quarter of deaths in the US are still due to cancer, which grave statistics are only surpassed by those of cardiovascular diseases (2, 3).

Breast cancer is the most common malignancy in women (26% incidence in 2008) and is also a leading cause of cancer death (15%), second only to lung cancer (Figure 1.1) (1). Furthermore, breast cancer is the most prevalent cancer

Estimated New Cases*

Males			Females		
Prostate	186,320	25%	Breast	182,460	26%
Lung & bronchus	114,690	15%	Lung & bronchus	100,330	14%
Colon & rectum	77,250	10%	Colon & rectum	71,560	10%
Urinary bladder	51,230	7%	Uterine corpus	40,100	6%
Non-Hodgkin lymphoma	35,450	5%	Non-Hodgkin lymphoma	30,670	4%
Melanoma of the skin	34,950	5%	Thyroid	28,410	4%
Kidney & renal pelvis	33,130	4%	Melanoma of the skin	27,530	4%
Oral cavity & pharynx	25,310	3%	Ovary	21,650	3%
Leukemia	25,180	3%	Kidney & renal pelvis	21,260	3%
Pancreas	18,770	3%	Leukemia	19,090	3%
All Sites	**745,180**	**100%**	**All Sites**	**692,000**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	90,810	31%	Lung & bronchus	71,030	26%
Prostate	28,660	10%	Breast	40,480	15%
Colon & rectum	24,260	8%	Colon & rectum	25,700	9%
Pancreas	17,500	6%	Pancreas	16,790	6%
Liver & intrahepatic bile duct	12,570	4%	Ovary	15,520	6%
Leukemia	12,460	4%	Non-Hodgkin lymphoma	9,370	3%
Esophagus	11,250	4%	Leukemia	9,250	3%
Urinary bladder	9,950	3%	Uterine corpus	7,470	3%
Non-Hodgkin lymphoma	9,790	3%	Liver & intrahepatic bile duct	5,840	2%
Kidney & renal pelvis	8,100	3%	Brain & other nervous system	5,650	2%
All Sites	**294,120**	**100%**	**All Sites**	**271,530**	**100%**

Figure 1.1: Cancer incidence and death by site and sex. Adapted from Jemal, *et al*., 2008 (1).

in the world, with approximately 4 million women diagnosed with breast cancer in the past 5 years (3). Approximately 20 to 30% of women diagnosed with breast cancer eventually develop metastatic disease and these patients face extremely poor prognosis since current therapeutic regimens are unlikely to result in complete disease remission.

The Etiology of Cancer

"It begins to appear that almost everything one does to gain a livelihood or for pleasure is fattening, immoral, illegal, or, even worse, oncogenic"

- Robbins and Cotran, Pathologic Basis of Disease

At the root of carcinogenesis is a biological process common to all humans: cell division. Therefore, it would seem likely that everyone would be equally susceptible to various kinds of cancer. On the contrary, epidemiological studies reveal profound differences in cancer incidence and death rates around the world (3). Two major contributory factors to cancer, heredity and environment, explain these differences.

Environmental Factors

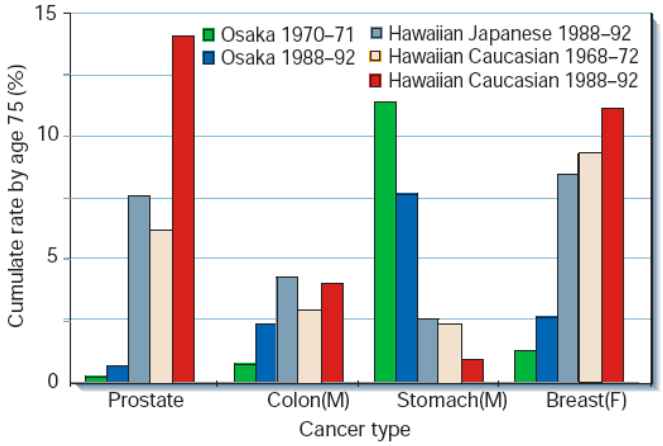


Figure 1.2: Cancer rates of Japanese immigrants to the US become similar to those in the local population. Adapted from Peto, J., 2001 (4).

The critical role of environmental factors in cancer is demonstrated very convincingly in a study comparing cancer incidence of immigrants and those of the local population (4). Public health records following cohorts of Japanese living in Japan (Osaka) and those who moved to the US (Hawaii) reveal that within a generation, cancer rates of immigrant Japanese converge with those of the endogenous population (Figure 1.2).

Furthermore, the carcinogenic effects of smoking cigarettes and its link to lung cancer (5) exemplifies the effect of lifestyle choice on cancer formation. In fact, recent studies indicate that obesity and lack of exercise (6), alcohol consumption (7), and sexual activity (8) all are closely associated with a variety of cancer types. The risk of breast cancer is also increased by environmental factors such as hormone replacement therapy (for the treatment of menopause symptoms), and reproductive patterns (low parity and first pregnancy at an older age) (4).

Pathogens are also critical mediators of carcinogenesis and account for approximately one-fifth of cancers worldwide (9). Hepatitis B and C are both carcinogenic and contribute to liver cancer formation (10). *Helicobacter pylori*, a bacteria that causes gastric ulcers, is a major factor for stomach cancer (11). Many forms of the human papillomaviruses (HPV) have also been shown to be a necessary factor in cervical cancer development (8).

Genetic Predisposition

Many common cancers are caused by exogenous insults to the human body, however, some cancers that run in families cannot be fully explained by environmental factors. Indeed, different human populations carry genetic abnormalities that predispose them to cancer at greatly different frequencies. Amongst inherited cancer syndromes, childhood retinoblastoma is the most conspicuous due the remarkably enhanced risk of cancer development. The inheritance of a mutation in the *RB* tumor suppressor gene accounts for approximately 40% of retinoblastomas and entails a 10,000-fold increased risk of cancer development (10, 12).

Women with a family history of breast cancer are at extremely high risk of developing breast cancer themselves, and may warrant prophylactic mastectomy or chemopreventive treatments. Familial breast cancer cases can be attributed to the highly penetrant genes *BRCA1* and *BRCA2,* which are inherited as autosomal dominant traits (13-15). The molecular mechanisms of such genetic predispositions to cancer are discussed later in this chapter.

Tumor Pathology

Transformation, Growth, Invasion and Metastasis of Tumors

A fertilized egg, through cycles of growth, division and differentiation, spawn all the cells in our body. Throughout our lives, many cells retain the ability to proliferate and participate in tissue regeneration in order to maintain the adult

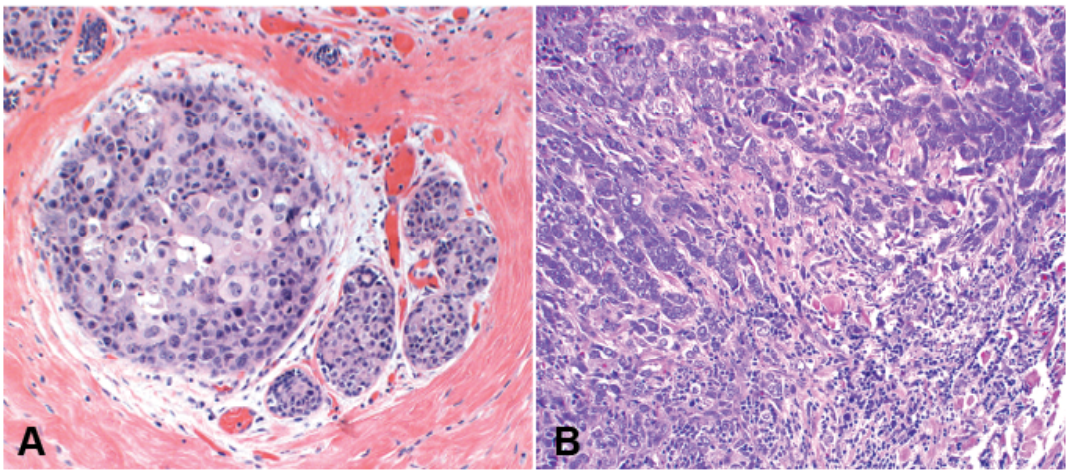


Figure 1.3: Benign vs. Malignant breast carcinoma. (A) Ductal carcinoma in situ (B) poorly differentiated invasive breast carcinoma. Adapted from Robbins and Cotran, Pathologic Basis of Disease, 2005 (10).

tissue. This retained autonomy, unfortunately, leaves room for mistakes that can result in uncontrolled cellular growth and dysfunction. Tumors, therefore, arise from normal cells within our own body that have gone through a malignant transformation. Epithelial cells give rise to carcinomas, which are the most common in human cancer cases and will be the focus of this dissertation. Other non-epithelial tumors include sarcomas of mesenchymal origin, leukemias and lymphomas of hematopoietic origin, and central and peripheral nervous system tumors (glioblastoma, schwannoma, etc.) of neuroectodermal origin (10).

Tumors can be largely divided into benign and malignant lesions. Benign tumors may display hyperplastic (excessive growth), metaplastic (displacement of one cell-type with another), or dysplastic (cytologically abnormal cells with some loss of differentiation) characteristics, but strictly stay within the basement membrane (Figure 1.3A) (10). Malignant tumors display the above abnormalities, but with a complete disregard for normal anatomic boundaries (Figure 1.3B). These tumor cells are able to invade through the basement membrane to infiltrate surrounding tissue and even form tumor implantations at distant sites.

Metastasis of tumor cells can occur by direct seeding of body cavities, or systemic dissemination through the circulatory system. Metastasis by direct seeding is exemplified by ovarian carcinomas spreading throughout the peritoneal cavity. Systemic dissemination can be distinguished by lymphatic spread and hematogenous spread. Although the division is not absolute, carcinomas tend to spread through the lymphatics, while sarcomas typically spread through arterial or venous vasculature. Anatomical proximity largely

determines routes of dissemination, and breast carcinomas frequently drain through the axillary lymph nodes, while lung carcinomas spread through tracheobronchial or mediastinal nodes (16). Lymph node involvement is a significant prognostic factor for carcinomas and is an important consideration in selection of therapeutic strategies.

Clinical Manifestations

The clinical manifestations of cancer vary widely depending on tumor type and location. Some of the most devastating effects of tumors present due to their impingements on adjacent organ structure and function. For example, breast tumors commonly metastasize to the lungs, liver and bone (17). Tumors in the lungs may present as shortness of breath and eventually lead to localized collapse and infection. The liver maintains metabolic homeostasis and its loss of function due to tumor burden have far-reaching consequences, including jaundice, edema, cognitive impairment, coagulopathy and failure of multiple organ systems (10). Bone metastasis can result in dysregulated calcium levels and increased susceptibility to fractures. In the case of bone marrow involvement, the compromised ability to make blood cells can lead to anemia, coagulopathy, and infection.

Cancer may also result in systemic complications owing to abnormal production of hormones and cytokines. For example, neoplasms of endocrine origin, such as β-cell adenoma of the pancreas can cause insulin overproduction

leading to fatal hypoglycemia (18). Cancer cachexia, characterized by profound loss of body mass, is commonly associated with elevated levels of inflammatory cytokines, such as TNF-α, IL-1, and IFN-γ (19). Idiopathic symptoms (hypercalcemia, myasthenia gravis, thrombophlebitis, etc.) not explained by the local/systemic effects of tumors are termed paraneoplastic syndromes and may also be debilitating to patients.

Molecular Principles of Neoplasia

Hallmarks of Cancer

Cancer is a disorder of cellular growth and function, and its ultimate etiology must be explained at the molecular level. A cellular model of carcinogenesis postulates that a normal cell must acquire the 6 hallmarks of cancer to become malignant (20):

1. Self-sufficiency in growth signals
2. Insensitivity to antigrowth signals
3. Evasion of apoptosis
4. Limitless replicative potential
5. Sustained angiogenesis
6. Tissue invasion and metastasis

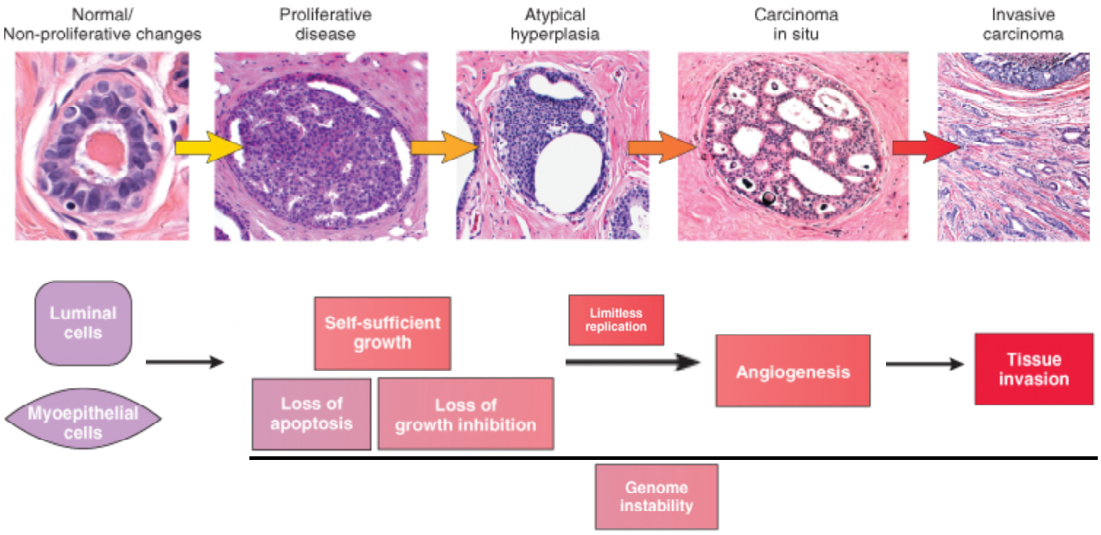


Figure 1.4: Schematic of breast cancer progression. Adapted from Pathologic Basis of Disease, Kumar, V., et al., 2005 (10).

The oncogenic characteristics listed above may be fully appreciated by following the discrete steps of tumor progression. The epithelium normally consists of tightly regulated cells that grow only as needed and stays within the boundaries set by the basement membrane. A tumor begins as a single cell that attains a survival advantage, which allows it to proliferate beyond the capacity of its neighbors. This advantage may be attained by a cell's ability to proliferate without growth signals (hallmark #1), or the ability to ignore antigrowth signals (hallmark #2) and evade apoptosis (hallmark #3). The clonal descendents of this pioneering cell may expand and manifest as premalignant lesions such as ductal hyperplasia of the breast.

The surrounding microenvironment can be hostile to the burgeoning tumor, and under selective pressure, the original population of mutant cells may undergo clonal expansion to attain other advantageous mutations. Subsequent accumulation of multiple mutations may alter cellular phenotype sufficiently to result in the atypical, de-differentiated morphological features observed in many carcinomas. The acquisition of mutations is not a trivial task considering the myriad of DNA surveillance and repair enzymes available. Therefore, as a permissive feature of oncogenesis, a cell must be genetically unstable. This would explain why p53, the master guardian of genomic integrity, is lost in majority of human cancers (21).

Normal cells have an internal clock keeping track of the number of times it has replicated. The telomeres at the ends of chromosomes shorten every time after a replication event due to the inability of the DNA polymerase to replicate

completely to the 3’ end of the chromosomal DNA (22). Once the telomere reaches a critical length, the cell enters replicative senescence or dies. Since tumor cells are replicating at significantly accelerated rates, they are bound to senesce or die-off unless telomere length is maintained. Indeed, majority of tumors gain telomerase activity (23) and acquire limitless replicative potential (hallmark #4).

Conservation of mass dictates that a tumor cell, regardless of its proliferative potential, cannot generate more cells without oxygenation and nutritional input. Therefore, once a tumor grows to a critical size (1-2 mm (24)), it must promote angiogenesis (hallmark #5) for the delivery of blood. Even after sustained angiogenesis, cells within a large tumor would favor the ability to invade and metastasize (hallmark #6) due to the inevitable spatial and nutritional constraints caused by its own growth. Figure 1.4 depicts a hypothetical case in which the 6 hallmarks of cancer may apply to breast cancer progression. The sequence of events does not imply the order in which the hallmarks were obtained, but the presumed necessity of each at the given stage of carcinogenesis.

The above hallmarks are pathological endpoints in which cancer cells reach after multiple rounds of evolution and clonal expansion. Prospective cancer cells have a vast arsenal of tumor suppressor genes and oncogenes to choose from to acquire the 6 hallmarks, leading to the overwhelming molecular heterogeneity of cancer.

Tumor Suppressor Genes

Tumor suppressor genes play a physiological role in regulating cell growth and therefore, their loss of function leads to tumor formation. Inactivating mutations in tumor suppressor genes and its associated predisposition to cancer are dominantly inherited and can be attributed to a large portion of hereditary cancers. *RB* was the first tumor suppressor gene discovered (25) and gave rise to Knudson's "two-hit" hypothesis (26, 27). In familial cases of retinoblastoma, a germline mutation in *RB* is inherited ("first hit"), and a second mutation occurs in a somatic cell ("second hit"), rendering the gene completely inactive in the newly oncogenic cell. In sporadic cases of retinoblastoma, both hits occur in a somatic (retinal) cell. Rb blocks cell-cyle progression by regulating the E2F transcription factor (28), and its inactivation endows the cell with ability to ignore growth inhibitory signals.

Tumor suppressor genes intimately tied to breast cancer formation are BRCA1, BRCA2, and p53 (13, 15, 29, 30). BRCA1 and BRCA2 repair damaged DNA by homologous recombination, although their exact molecular functions remain elusive (31, 32). The p53 protein is a very well characterized gatekeeper of genomic integrity. At cell cycle checkpoints, p53 scans for DNA damage and orchestrates repair responses by inducing cell cycle arrest. Otherwise, if the damage to the genome is too severe for proper repair, p53 prompts cellular apoptosis to prevent the propagation of an impaired genome (21) . Mutations in

all 3 gene are inherited in a dominant pattern and the “second hit” makes the affected cell susceptible to genomic corruption.

Cellular Oncogenes

Tightly regulated signals controlling cellular behavior are critical for the maintenance of tissue architecture and function. A prototypical signal transduction event begins with a growth factor binding to its cognate receptor at the cell membrane, triggering a cytoplasmic signaling network involving protein kinases and transcription factors. Activated components of the signaling circuitry are integrated and manifested as gene transcription, cellular growth, division, migration, and a variety of other biological events.

Many proteins involved in signal transduction have oncogenic potential and are considered proto-oncogenes. A gain-of-function mutation or simple over-expression can make these proteins independent of regulatory constraints, transforming them into cellular oncogenes. Here, oncogenic activation of the ErbB signaling pathway will be discussed in detail due to its particular relevance to breast cancer biology.

The ErbB receptors are a subclass of the receptor tyrosine kinase superfamily and consists of ErbB1/EGFR, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4 (Figure 1.5) (34). Their structural features include the ligand-binding ectodomain at the N-terminus, the hydrophobic transmembrane domain, and the cytoplasmic domain with tyrosine kinase activity at the C-terminus. Upon ligand

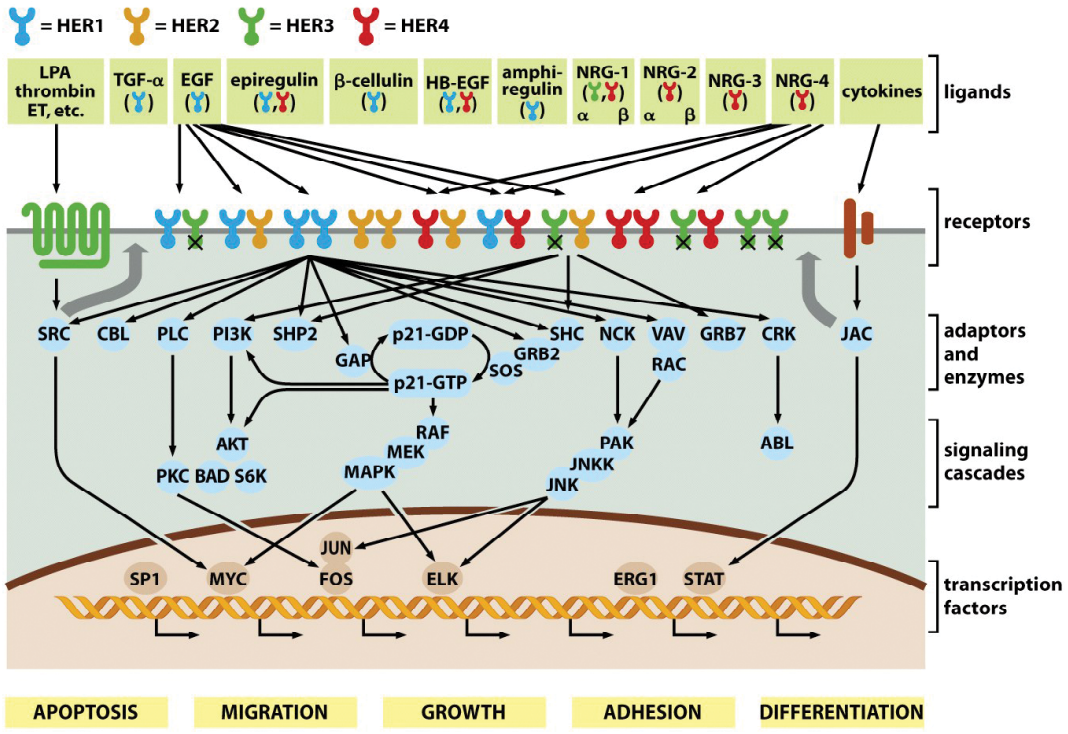


Figure 1.5: Schematic of the ErbB signaling network. Oncogenic activation of proteins at all steps of the signaling cascade have been implicated in human carcinogenesis. Adapted from The Biology of Cancer, Weingberg, R.A., 2007 (33).

binding, receptors homo- or hetero-dimerize and transphosphorylate each other at tyrosine residues. The phosphorylated receptors then act as docking sites for cytoplasmic proteins and initiate a complex network of signaling cascades. Exceptions to this general scheme are HER2 and HER3. HER2 has an extracellular domain not conducive to ligand binding and is considered an orphan receptor with innate propensity for heterodimerization. HER3 lacks tyrosine kinase activity in the cytoplasmic domain and can propagate ligand-mediated signaling only with the help of another ErbB family receptor (35).

The expression of EGFR, HER2, and HER3 are correlated with advanced stage breast cancer in patients and an aggressive cellular phenotype in tissue culture (35). In particular, immunostaining for HER2 in breast tumor specimen is now routine (36), for its prognostic value and possibility of treatment with an anti-HER2 monoclonal antibody, trastuzumab (37), or tyrosine kinase inhibitor (TKI), lapatinib (38). The role of HER4 in cancer remains elusive, although the current body of work suggests an anti-proliferative role, with likely involvements in breast tissue differentiation (39, 40).

Constitutive ErbB activity is possible as a direct consequence of over-expression and the increased probability of spontaneous receptor dimerization (41, 42). In fact, EGFR is found over-expressed in 30-48% of breast cancers, HER2 amplification is found in approximately 30% of invasive breast carcinomas, and HER3 is detected in up to 50% of reported cases (35). Activating mutations in EGFR have also been documented. The naturally occurring class III mutant of EGFR (EGFRvIII) lacks 267 amino acids in the ligand-binding domain and has

constitutive tyrosine kinase activity (43). EGFRvIII is found in patients with breast carcinoma (44), and has been shown to enhance motility in cell lines (43).

Under physiological conditions, receptor activity is determined by the spatial and temporal availability of their ligands. Ligands of ErbB receptors include (1) EGFR specific ligands (EGF, TGFa, amphiregulin), (2) EGFR/HER4 dual specificity ligands (HB-EGF, epiregulin, betacellulin), and (3) HER3/HER4 ligands (neuregulin 1-4) (Figure 5). Over-expression of growth factors by a cancer cell enables an autocrine signaling loop, rendering the receptor consitutively active. Indeed, an amphiregulin/EGFR autocrine loop has been implicated in the pathogenesis of inflammatory breast cancer (45, 46).

The PI3K-Akt cascade is one of several signaling pathways engaged by ErbB activation (Figure 5). Akt is a serine/threonine kinase and exerts its pleiotropic anti-apoptotic effects by inactivation of pro-apoptotic factors BAD, caspase-9, and forkhead transcription factors, and the activation of pro-survival genes through NF-κB. Akt also promotes mitotic progression by inactivation of GSK-3, p27, and p21, all negative regulators of the cell-cycle (47, 48). Akt defines a family of highly homologous isoforms Akt1, Akt2, and Akt3, all of which share a Pleckstrin homology (PH) domain, an alpha-helical linker, and a carboxy-terminal kinase domain (49). Artificial gain-of-function mutations in Akt, such as the membrane- targeting myristoylated Akt (50) and the phospho-mimetic Akt mutatant (Akt-T308D/S473D) (51) have been extensively used to study Akt function in cell culture and animal models. Naturally occurring mutations in Akt are relatively less common, however, an activating mutation in the PH domain

has been recently documented in breast, ovarian, and colon cancers (52). PTEN (phosphatase and tensin homolog at chromosome 10) is a tumor suppressor gene known to antagonize the PI3K/Akt cascade, and its loss-of-function is implicated in tumors of the breast, brain, lung, and skin (53). The PI3K/Akt pathway is not limited to ErbB activation, and can be downstream to a variety of growth factors, cytokines, and other cellular stimuli. In fact, there seems to be significant crosstalk between PAR1, ErbB, and the Akt pathway, and will be further discussed in chapters 2 and 3.

Taken together, we can begin to appreciate the pathological corruptibility of a signal transduction system necessary for physiological regulation of the cell. Because tumor cells can gain growth autonomy through a wide variety of novel molecular mechanisms, treatment of cancer is a particular challenge often plagued with therapeutic resistance.

Tumor-Stroma Interaction

Despite their intimidating capabilities, cancer cells without their stromal accomplice would have limited pathogenicity. For example, angiogenesis, a fundamental requirement for tumor growth, would not be possible without the recruitment of endothelial cells (Figure 6A). The ability of a tumor to recruit stromal cells to its microenvironment is well characterized by the desmoplastic reaction (formation of collagenous, scar-like stroma mediated by fibroblasts) commonly observed in breast carcinomas (Figure 1.6B) (55). In fact, a vast array

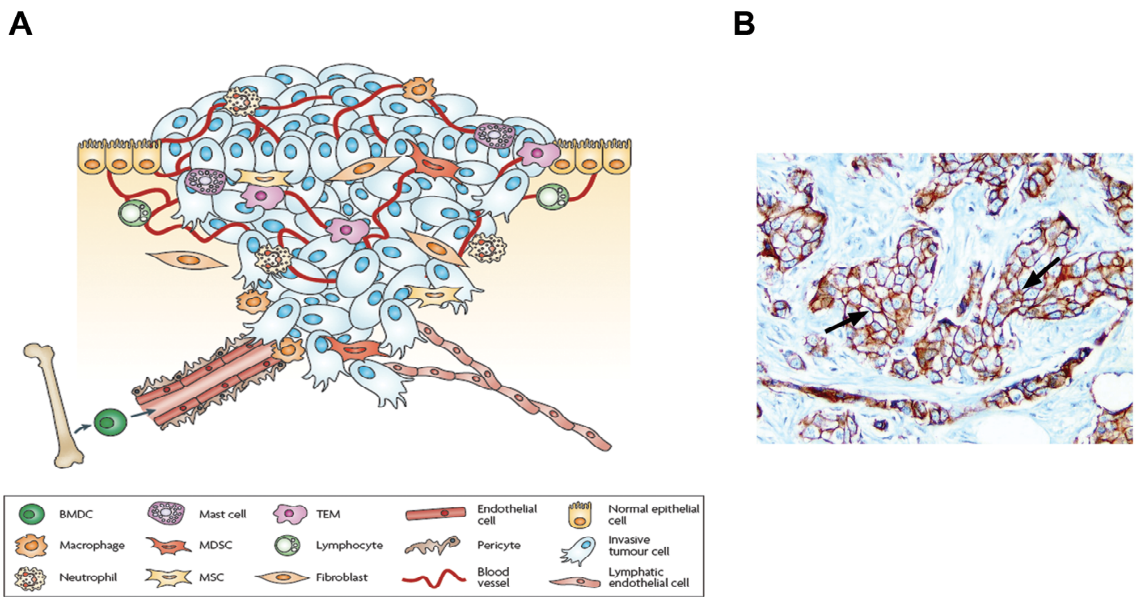


Figure 1.6: (A) Tumor microenvironment. BMDC (bone marrow derived cells), MDSC (myeloid-derived suppressor cells), TEM (Tie2-expressing monocytes), MSC (mesenchymal stem cells). Adapted from Joyce, J.A., *et al.* 2009 (54). (B) Desmoplasia observed in invasive breast carcinoma. Cancer cells are stained in brown and fibrous stroma is in blue. Adapted from The Biology of Cancer, Weingberg, R.A., 2007 (33).

of cells, including fibroblasts, endothelial cells, inflammatory cells, bone marrow derived cells (BMDC) and mesenchymal stem cells (MSC), are now recognized to be integral components of the tumor microenvironment (Figure 1.6A) (54).

Heterotypic signaling between tumor and stroma has been strongly implicated in invasion and metastasis. Of particular interest are the morphological changes induced in tumor cells in response to fibroblast-derived growth factors such as TGF-β and EGF. This phenomenon is known as epithelial-to-mesenchymal transition (EMT) and it endows the tumor cell with a fibroblast-like morphology, enhanced motility and resistance to apoptotic signals (56). EMT appears to play a significant role in PAR1-mediated breast tumor invasion and metastasis, and is further discussed in chapter 3.

Some cancer types rely heavily on stromal support even for basic tumorigenicity. In a recent report, mast cell recruitment to the tumor microenvironment was found to be necessary for neurofibroma formation. A truly remarkable ramification of this finding was that, a therapy specifically targeting the stromal mast cells (but not the tumor) was sufficient in reducing tumor burden in a patient (57). Stromal cells are genetically stable unlike their tumor counterparts, and are therefore unlikely to develop therapeutic resistance. Given the challenges of eradicating tumor cells, perhaps it is time to take a closer look at the less-potent stromal cells as novel therapeutic targets (52).

Cancer Therapy

Breast cancer is considered cured when the tumor is eradicated without threat of recurrence. Since cancer relapses are most common within the initial 5 years after treatment, 5-year survival is considered a cure for most practical purposes. Prognosis for metastatic breast cancer is particularly poor, with approximately 5-10% of patients surviving for 5 or more years (58).

Local Therapy

Surgical resection and radiation therapy are considered palliative treatments for metastatic breast disease. Mastectomy (surgical removal of the breast) and resection of metastatic lesions are considered when severe impingement of the local structure suggests an imminent catastrophe (bone fracture, spinal cord compression, severe pain, etc.). Under rare circumstances, tumor growth can be relatively indolent within multiple organs, in which case local therapy can have significant survival advantage and curative potential (59).

Systemic Therapy

Systemic therapy, which includes chemotherapy, endocrine therapy, and targeted therapy, is very effective in the adjuvant (systemic therapy after surgery as prophylaxis to recurrent disease) and neoadjuvent (reducing tumor volume to enhance the possibility of complete surgical resection) settings. Systemic therapy

is also the standard of care for patients with metastatic breast cancer, although chances for disease remission are extremely low (60).

Chemotherapy consists of general cytotoxic agents that target the most fundamental property of tumor cells: enhanced growth and proliferation. Drugs such as Anthracyclines (doxorubicin, daunorubicin), Antimetabolites (methotrexate, 5-fluorouracil), Alkylating agents (cyclophosphamide, chlorambucil), and Platinum compounds (carboplatin), all target DNA synthesis or promote DNA damage. Taxanes (docetaxel, paclitaxel) and Vinca Alkaloids (vincristine, vinblastine) target the dynamics of microtubule polymerization, effectively inhibiting proper mitosis (60). Insensitivity to DNA damage by loss of p53, and resistance to apoptotic signals by up-regulation of anti-apoptotic oncogenes such as Akt and Bcl-2 are common molecular mechanisms by which tumor cells gain resistance to chemotherapeutic drugs (61, 62). Due their intrinsic ability to attack fast-growing cells, these drugs also have significant toxicity against epithelial cells of the gastrointestinal tract and the hematopoietic cells in the bone-marrow. Such off-target toxicity causes unwanted side effects, such as nausea and susceptibility to infection.

Endocrine therapy is unique to hormone-sensitive carcinomas such as breast and prostate cancer. Estrogen and its nuclear receptors, ERα and β, have significant roles in breast cancer development. The possible utility of estrogen antagonism was first demonstrated when oopherectomy (surgical removal of ovaries) and the resulting ablation of estrogen caused tumor regression in patients (63). Selective estrogen receptor modulators (tamoxifen and roloxifene),

competitively inhibit the formation of the estrogen/ER complex, and prevent gene transcription events conducive to cancer progression (64). While tamoxifen is highly efficacious against breast carcinoma cells expressing ER, resistance quickly develops when tumor growth becomes independent of estrogen. Over-expression of HER2 and EGFR has also been implicated in endocrine therapy resistance (65).

Targeted therapy is a newer strategy in systemic therapy, in which an oncogene of functional significance is specifically targeted for cancer cell toxicitiy. HER2 antagonism with the blocking antibody Trastuzumab is the most prominent and successful example of targeted therapy for breast cancer treatment (66). The anti-apoptotic and growth-promoting potential of HER2 signaling are thought to be the underlying cause of many instances of chemotherapeutic resistance. In fact, the over-expression of HER2 in breast carcinoma cells induced resistance to taxanes, where as trastuzumab treatment sensitized HER2-expressing cells lines to taxane treatment (67, 68). From this perspective, targeted therapy becomes an invaluable method to induce chemosensitivity in cancer cells refractory to traditional chemotherapy. PAR1-mediated chemosensitization of breast carcinoma cells to taxotere (docetaxel) will be explored in the following chapter.

Protease Activated Receptor-1: a chemotherapeutic target in breast cancer

G-protein coupled Receptors (GPCRs)

GPCRs are structurally characterized by their seven transmembrane α –helical domains, which form 3 extracellular loops (e1-e3) and 3 intracellular loops (i1-i3). The extracellular N-terminus functions to bind ligands for receptor activation, while the intracellular C-terminus couples with heterotrimeric G-proteins (Gα, G$\beta\gamma$). The activated GPCR facilitates the exchange of GTP with GDP on the Gα subunit, resulting in the dissociation of the G$\beta\gamma$ subunit and subsequent signal transduction by both (69).

GPCRs form the largest and most functionally diverse family of signaling receptors, and act in response to a wide array of intercellular messengers and environmental cues (chemokines, neurotransmitters, peptide hormones, biogenic amines, bioactive lipids, light, temperature and pressure). Accordingly, GPCRs are intergral to many physiological processes, including the regulation of immune responses, fluid balance, vascular tone, cardiac contraction, pain, olfaction, vision, and taste (70). Many GPCRs also possess potent mitogenic potential and aberrant activity has been linked to malignant transformation (71). A leading example is the enhanced/ectopic signaling by protease-activated receptors (PARs), which can lead to cell proliferation, survival, angiogenesis, invasion, and metastasis in a variety of cancer types (72-74). Also to note is the Wnt-Frizzled receptor pathway and the induction of β-catenin mediated oncogenic gene transcription (75). Activating mutations involving many other GPCRs and G-proteins have been documented for carcinomas of the prostate, ovaries, lungs, and thyroid (33).

Protease-Activated Receptors (PARs)

PARs are a unique family of GPCRs that are irreversibly activated by the proteolytic cleavage and exposure of its cryptic, tethered ligand (76). PAR1, also known as the thrombin receptor, was the first protease-activated receptor to be discovered during the search for a mediator of thrombin activity on human platelets (77). Through its highly acidic hirudin-like sequence (78), PAR1 binds and positions thrombin, a serine protease, for efficient cleavage at the N-terminal peptide bond (R^{41}-S^{42}). The exposed tethered ligand with the amino acid sequence SFLLRN then swings over to engage the ligand-binding domain of the second extracellular loop (e2) and transduces signal through the plasma membrane by facilitating conformational changes in the transmembrane domains.

The persistence of thrombin signaling in the platelets of PAR1-knockout mice (79) prompted the discovery of PAR3 (80) and PAR4 (81), both also thrombin receptors but with varying signaling potential and kinetics. PAR3 is readily bound by thrombin through a hirudin-like sequence but does not signal to G-proteins, and appears to be a cofactor for PAR4 activation in mouse platelets (82). PAR4 is a low affinity thrombin receptor (no hirudin-like sequence), and promotes a subdued but prolonged thrombin signaling in human platelets (83). PAR2 is the only protease-activated receptor not cleaved by thrombin and is activated instead by trypsin, mast cell tryptase (84), and TF/FVIIa (85). PAR2,

however, may be under indirect thrombin control as it is transactivated by the PAR1 tethered ligand, SFLLRN (86).

The Role of PARs in Physiology and Pathophysiology

PARs are widely distributed throughout the organ system and can be found on platelets, immune cells, vascular endothelial cells, smooth muscle cells, fibroblasts, neuronal cells, and some epithelial cells (76). Therefore, in addition to the coagulation cascade, PARs function in the physiology and pathophysiology of the cardiovascular, nervous, gastrointestinal, respiratory, tegumentary, and immune systems.

Coagulation: PAR1 and PAR4 are expressed on human platelets (87, 88) and play a central role in platelet activation and the coagulation cascade. Thrombin cleaves and activates both PAR1 and PAR4 to promote platelet aggregation, degranulation, and shape changes. PAR1 is a “high-affinity” thrombin receptor, due to the hirudin-like sequence, with quick activation and shut-down in response to proteolytic cleavage. In contrast, PAR4 is a “low-affinity” receptor (lacking the hirudin-like sequence) and promotes a sustained response to thrombin cleavage (83). Antagonism of PAR1 or PAR4 may be a viable approach in treating inappropriate platelet aggregation and thrombus formation. Small molecule inhibitors of PAR1, such as RWJ-58259 (89) and SCH20581 (89), and PAR4 antagonists, such as the P4pal-i1 pepducin (90), have shown promising efficacy against thrombosis in animal models.

Cardiovascular System: Endothelial cells and vascular smooth muscle cells express PAR1, PAR2, and PAR4 (91, 92), and regulate vascular permeability and contractility. Activation of PAR1 promotes endothelial proliferation through direct intracellular signaling, through the release of vascular endothelial growth factor (VEGF), and through transactivation of PAR2 (76, 86, 93). The integral role of PAR1 in vascular biology is confirmed by the observation that $PAR1^{-/-}$ mice are 50% embryonically lethal due to bleeding at multiple sites (94), and furthermore, the embryos are rescued by conditional expression of PAR1 in endothelial cells (95). PAR1 also mediates vascular permeability and the protective and damaging roles of PAR1 during sepsis has recently been characterized by our group (96). PAR1 and PAR2 in vascular smooth muscle cells may be involved in vascular lesions (stenosis, ischemia reperfusion, atherosclerosis) and is actively pursued in our laboratory by Dr. George Koukos and Leila Sevigny.

Inflammation: PAR1 deletion and treatment with hirudin have both shown protective effects against glomerulonephritis in mice, indicating the involvement of thrombin, PAR1 and the coagulation cascade in inflammatory processes (97). PAR2 deletion in mice has also shown to decrease inflammation by reducing leukocyte rolling, adhesion, and invasion (97), and in particular, has shown significance in arthritis, allergic inflammation of the airway, and inflammatory responses in the GI (73, 76). Interestingly, both PAR1 and PAR2 also play anti-inflammatory roles as they can be protective during murine models of sepsis (96), and PAR2 activation can be protective against pulmonary neutrophilia,

gastric ulcers, and experimental colitis (76). Therefore, PAR antagonists and agonists as anti-inflammation therapy must be administered carefully under the appropriate disease context and timing.

Gastrointestinal, Respiratory, Tegumentary, and Nervous systems: PARs 1, 2, and 4 have been detected in the GI and are thought to regulate motility, secretion, and inflammatory processes (76, 98). In the respiratory system, PAR2 is of particular significance, and is expressed in the airway epithelia, endothelium, and smooth muscle cells. The current body of work suggests a role of PAR2 in bronchodilation, fluid secretion at bronchial surfaces, and airway remodeling in response to inflammation (76). In the tegumentary system, PAR1 and PAR2 are expressed in keratinocytes and regulate pigmentation and wound healing (99, 100). PAR activation in keratinocytes induces the expression of Cyr61, interleukin-6 (IL-6), interluekin-8 (IL-8), and GM-CSF, suggesting the role of PARs in inflammation and angiogenesis. PAR1 and PAR2 are also expressed in neuronal cells and all four PARs are expressed in glial cells (76, 101). PAR1 signaling regulates neuronal calcium levels, morphology, survival and proliferation. Interestingly, thrombin acts biphasically, with low levels of thrombin acting to protect neurons (102), while high concentrations are capable of inducing neuro-degeneration (103). PAR2 and PAR4 contribute to neural inflammation, and PAR2 specifically mediates hyperalgesia (increased sensitivity to pain) (104).

PAR1 and Cancer

PAR1 Expression: PAR1 has emerged as an oncogenic protein target due to its involvement in breast, endometrial, ovarian, colon, prostate, and skin malignancies (105-110) and its ability to induce malignant transformation in NIH-3T3 cells (111). The precise mechanism of PAR1 expression and its role in cancer progression is not completely understood. A study by Salah *et. al.* suggests that the elevated levels of PAR1 mRNA and protein in malignant epithelia are the result of increased transcription and not due to gene amplification events (112). The report further demonstrated that PAR1 expression can be induced by early growth response-1 (Egr-1), a transcription factor rapidly activated by growth factors, cytokines, and differentiation factors. Salah *et. al.* also demonstrated that p53 may act as a transcriptional repressor of PAR1. In this more recent study, PAR1 expression inversely correlated with wild-type p53 and directly correlated with mutant p53. Further, p53 was shown to directly bind to the *PAR1* promoter and its knockdown by siRNA resulted in PAR1 expression in the MCF-7 breast carcinoma cell line (113). The loss of p53, however, is a common feature in many carcinoma cells, while PAR1 over-expression is generally limited to advanced stage disease. This suggests that the p53 loss of function may be permissive for PAR1 over-expression, but not necessarily sufficient in many cancer types. The transcription factor, activator protein-2 (AP-2), has also been shown to be a transcriptional repressor of PAR1 (114) and may act in concert with p53 to modulate PAR1 expression.

Invasion and Metastasis: PAR1 is not expressed in normal breast epithelia or in benign hyperplastic lesions, but is over-expressed in invasive ductal carcinoma (109). Moreover, we have recently reported that over-expression of PAR1 in minimally invasive MCF-7 carcinoma cells is sufficient to promote invasion both *in vitro* and in nude mouse models (115). Of particular significance is our discovery that PAR1 is proteolytically cleaved and activated by fibroblast-derived MMP-1. MMP-1 expression is correlated with elevated metastatic capacity of breast cancer cells (116), and has been generally thought to promote metastasis by the breakdown of the restrictive extracellular matrix surrounding the tumor. Our findings demonstrate that MMP-1 not only “paves the road” for tumor invasion and metastasis, but it also has the ability to directly affect tumor cell behavior through PAR1.

After the invasive tumor cells intravasate and travel to distant sites, they must eventually attach to the vascular endothelium and extravasate to form metastatic colonies. Experimental models of pulmonary metastasis have demonstrated the significance of the thrombin/PAR1 cascade in tumor implantation at the lung. In a series of studies, Nierodzik *et. al.* have shown that pretreatment of tumor cells with thrombin enhances tumor attachment to platelets, von Willebrand Factor (vWF) and fibronectin (110), and promotes pulmonary metastasis through a PAR1 dependent mechanism (117).

Taken together, PAR1 expression and activation by context-dependent protease signals promote invasion and metastasis in animal models, which may indicate the pathological significance of PAR1 in the human disease.

Cell Survival and Proliferation: We have demonstrated that MCF-7 cells stably transfected with PAR1 become tumorigenic in nude mice without estrogen supplementation (115). Furthermore, PAR1 knockdown by short hairpin RNA (shRNA) has been shown to reduce melanoma tumor growth in mice models (118). PAR1 mediated survival has been attributed at least in part to the activation of a major survival factor Akt, and the down-regulation of the pro-apoptotic molecule, Bax. Thrombin mediated PAR1 activation also down-regulates Bim, another pro-apoptotic molecule, through MAPK and PI3K dependent pathways (119). The blockade of PAR1 survival pathways is not well characterized, and is further investigated in this dissertation.

Angiogenesis: The aggression of PAR1 positive cancer cells is augmented by their ability to secrete Cysteine-rich 61(Cyr61) to the surrounding stroma (120). Cyr61 is a member of the CCN (Cysteine-rich 61/Connective tissue growth factor/Nephroblastoma overexpressed) family of growth regulators and is implicated in tumorigenesis (121). In response to Cyr61 secretion, stromal fibroblasts express a variety of angiogenic factors, including MMP-1. Hence, in a self-sustaining paracrine fashion, PAR1 positive cancer cells stimulate the surrounding fibroblasts to produce a stromal environment conducive to growth and invasion. Activation of PAR1 also induces vascular endothelial growth factor (VEGF) expression, which promotes tumor angiogenesis (74). The ablation of tumor cell PAR1 in a tetracycline-inducible *in vivo* system lead to blood vessel regression (118), confirming the importance of PAR1-derived factors such as Cyr61 and VEGF in tumor angiogenesis. Furthermore, PAR1 is expressed on the

vasculature, and is possibly directly promoting angiogenesis through PAR1 mediated endothelial activation and proliferation (86, 122).

Sustained PAR1 signaling: In addition to aberrant expression and activation, prolonged PAR1 signaling due to defects in endocytosis seem to further enhance the pathological effects of PAR1 activation (123). Furthermore, PAR1 mediated transactivation of EGFR and HER2 has been shown to cause persistent MAPK activation and an increase in the invasive capacity of breast carcinoma cells (124).

Other PARs in Cancer: PAR2 has been found expressed in various tumor cells, including those of the breast (125, 126), prostate (108), and colon (127). The extrinsic pathway of coagulation seems to be of particular importance to PAR2 activation. Tissue factor (TF), the initiator of the extrinsic pathway is up-regulated the inflammatory states (128) and during tumor progression, and in fact, is over-expressed on the surface of several tumor cells (129). The TF-FactorVIIa complex in turn, is capable of activating PAR2. In addition, PAR2 may be also under thrombin regulation, as it can be transactivated by the cleaved tethered ligand of PAR1. PAR2 activity has been implicated in tumor cell proliferation, survival, migration, invasion, and the release of angiogenic factors (130). In a recent study involving polyoma middle T (PyMT) models of mouse mammary tumors, PAR2 was found to promote carcinoma development by enhancing tumor angiogenesis (126). The involvement of PAR3 and PAR4 in cancer remain elusive, although results from a recent study indicates that PAR4 may promote colon cancer cell proliferation through HER2 transactivation (131).

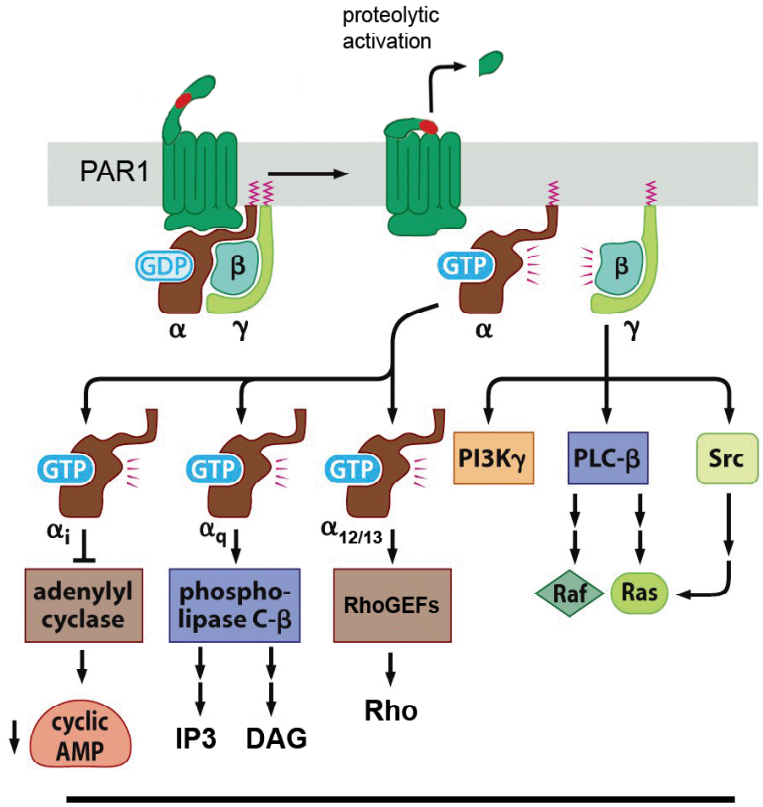


Figure 1.7: Schematic of PAR1 activation and signaling. Adapted from The Biology of Cancer, Weingberg, R.A., 2007 (33).

PAR1 Activation and Signal Transduction

Activation: PAR1 is activated by multiple proteases in addition to thrombin. Serine proteases intimately involved in coagulation and anti-coagulation, such as Factor Xa (FXa) (132), Activated Protein C (APC) (133), and plasmin (134) are known to target PAR1. While thrombin, FXa, and APC cleave and activate PAR1, plasmin can cleave PAR1 downstream to the tethered-ligand site, thereby desensitizing the receptor to any further proteolytic activation. Matrix Metalloprotease-1 (MMP1), a zinc-dependent endopeptidase capable of cleaving collagen (135) has also been shown to proteolytically activate PAR1 (115), but at a distinct site slightly upstream (D^{39}-P^{40}RSFLLRN) of the thrombin cleavage site (R^{41}-S^{42}FLLRN) (136). Synthetic peptides that mimic the tethered ligand can also activate PAR1 (SFLLRN, TFLLRN) (137) and are very useful tools to investigate PAR1 activity. The differences in signaling potential of Thrombin, MMP-1, and activating peptide are not well understood, however, some reports indicate that propensity for G-protein activation can shift depending on method of activation (138).

In addition to protease and peptide mediated activation, it is possible that PAR1 has a basal level of constitutive activity. GPCRs are not static structures, but rather, exist in a dynamic equilibrium between the active and relaxed states (139). The rotation of transmembrane domain III (TMIII) relative to TMVI indicates receptor activation and is conserved amongst many GPCRs (140, 141). The active state is stabilized with ligand binding, but short-term switches between

conformations can occur spontaneously without the presence of an agonist. This periodic activation and inactivation of GPCR results in an increased basal level of GDP/GTP exchange of G-proteins, leading to constitutive signaling just by the presence of GPCRs at the plasma membrane (69).

Signal Transduction: PARs interact with several subtypes of heterotrimeric G proteins, triggering a diverse set of downstream events leading to changes in cell shape, proliferation, migration, secretion, adhesion, and gene transcription (76). Specifically, PAR1 couples with 3 G-proteins ($G\alpha_q$, $G\alpha_{12/13}$, and $G\alpha_i$), giving it significant potential for pleiotropic signaling (Figure 1.7). The PAR1-$G\alpha_q$ pathway activates phospholipase C-β (PLCβ), which hydrolyzes phosphoinositides for the generation of secondary messengers inositol (1,4,5)-triphosphate (IP_3) and diacylglycerol (DAG) (142). IP_3 generation leads to mobilization of intracellular Ca^{2+}, while DAG activates Protein Kinase C (PKC) and gives PAR1 access to the mitogen activated protein kinase (MAPK) pathways and NFκB mediated gene transcription (143). $G\alpha_q$, therefore, mediates PAR1 control over cellular degranulation, proliferation and survival. PAR1 also signals through $G\alpha_{12/13}$, which activates the Rho pathway for cell shape change and motility (142). PAR1 mediated $G\alpha_i$ activation is not as well characterized, although current data suggests that, in addition to suppression of cAMP formation, $G\alpha_i$ might have a role in phosphoinositide-3 kinase (PI3K) activation through release of ADP (144). The resulting activation of Akt results in platelet integrin activation and aggregation (145). PAR1-mediated Akt activation has

been previously shown in melanoma cells, and has been implicated in tumor survival and progression (118).

The Gβγ subunit, upon dissociation from the Gα subunit, has independent signaling potential through the activation of protein tyrosine kinases, PI3K, and the MAPK cascade (146). Additionally, PAR1 is able to transactivate EGFR by the metalloprotease-dependent release of membrane bound ligands (HB-EGF) (147, 148) or through Src-dependent cytoplasmic pathways (149). Regardless of mechanism, access to EGFR signaling exponentially enhances the signaling capacity of PAR1 and further implicates its role in carcinogenesis.

Signal Termination and Endocytosis: Since PAR1 is irreversibly activated by proteolytic cleavage, its signal termination is accomplished by endocytic sorting and lysosomal degradation of the activated receptor (150). As a negative feedback mechanism, PAR1 activation results in G-protein receptor kinase-3 (GRK3) translocation to the plasma membrane, which then phosphorylates the cytoplasmic tail of PAR1 (151) and facilitates clathrin and dynamin dependent endocytosis (152). Defective endocytosis results in persistent PAR1 signaling, providing yet another mechanism of PAR1-mediated oncogenesis (123).

PAR1 antagonism and Pepducin Technology

Pepducins are cell-penetrating peptides that act as intracellular inhibitors or agonists of GPCRs (153-155). A generic pepducin consists of a lipid moiety attached to a peptide. Palmitate, a 16-carbon saturated fatty acid is most

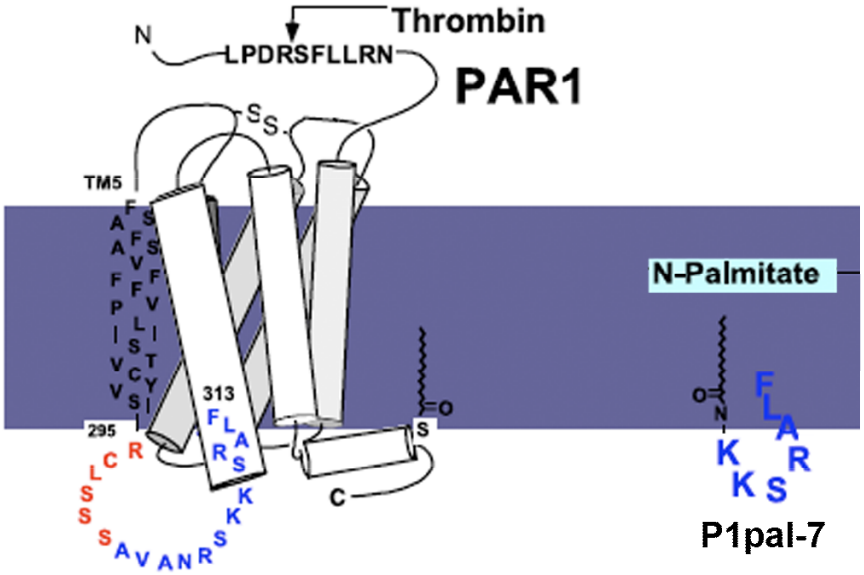


Figure 1.8: Pepducin Schematic. Adapted from Kuliopulos, A., *et al.,* 2003 (153)

commonly used for the generation of pepducins and confers cell-penetrating ability to the pepducin. The lipid moiety is also thought to anchor the pepducin to the inner leaflet of the plasma membrane, thereby increasing its effective concentration and protecting it from proteolytic degradation.

The peptide components are designed after the intracellular loops of GPCR and depending on the sequence, can act as an agonist or an antagonist. The third intracellular loop (i3 loop) of GPCRs have been identified as prominent players in GPCR/G-protein coupling (156), and therefore, peptide design has largely revolved around this particular domain of the GPCR. The peptide, upon intracellular delivery, is thought to compete with the GPCR for G-protein coupling.

The P1pal-7 pepducin (Figure 1.8) is a palmitoylated peptide with the amino acid sequence KKSRALF, based on the C-terminal portion of the PAR1 i3 loop, and is a full antagonist of PAR1 signaling (115, 157). Antagonist activity can be lost by mutation of amino acid residues, and P1pal-19EE is one such pepducin that is used as a negative control (157).

Pharmacokinetic and pharmacodynamic studies in mice indicate that pepducins have high biological activity due to prompt tissue penetration, slow elimination, and broad systemic distribution (158). The efficacy of P1pal-7 pepducins have been validated in a xenograft model in which PAR1-expressing breast carcinoma cells were implanted in the mammary fat pads of female nude mice. P1pal-7 was administered at 10 mg/kg every other day, which resulted in significant attenuation of tumor growth compared to vehicle (115).

Other PAR1 antagonists include RWJ-56110 and RWJ-58259 developed by Johnson & Johnson (159), and SCH-205831 and SCH-5303048 developed by Shering-Plough (160). All compounds act by competitively inhibiting the tethered-ligand binding site. RWJ compounds are no longer actively explored due to poor in vivo efficacy, but remain invaluable for their *in vitro* efficacy and specificity. SCH-5303048 is an orally available compound currently in phase-II clinical trials in the context of hemostasis and thrombosis (161), but its efficacy as a cancer therapeutic remains to be explored.

Experimental Approach and Specific Aims

We have identified PAR1 as a promising oncogenic target for breast cancer therapy and developed a PAR1 antagonizing pepducin on a candidate basis. PAR1 pleotropically signals through 3 G proteins and transactivates EGFR, giving it the potential to regulate a wide variety of cellular initiatives. Therefore, physiological manifestations of receptor antagonism must be further explored to validate PAR1 as an ideal drug target. We have employed 3 major strategies (162) to explore the role of PAR1 in breast cancer and the efficacy of receptor blockade.

1. Genetic approach: observe global transcriptional shifts as a result of PAR1 expression and PAR1 antagonism by genechip analysis.

2. Cell-based approach: observe functional significance of PAR1 by over-expression, knockdown, or pharmacological inhibition in breast carcinoma cell lines.
3. Animal Models: observe the *in vivo* significance and efficacy of PAR1 blockade by xenograft and experimental metastasis models in immuno-compromised mice.

Specific Aims

AIM 1. Investigate the role of PAR1 signaling in breast tumor survival and metastasis. The functional role of PAR1 on breast tumor survival and proliferation are not well-characterized. Here, we set out to investigate the MMP1/PAR1/Akt survival pathway and the apoptotic effects of pepducin-mediated receptor antagonism. *In vitro* and *in vivo* models were employed to explore the efficacy of PAR1 antagonists as chemosensitization agents to taxotere. Finally, the use of MMP1and PAR1 antagonists as potent inhibitors of metastasis was investigated in an animal model of experimental metastasis.

1.1 PAR1 over-expression enhances cancer cell survival through Akt signaling pathways.

1.2 Pepducin-mediated PAR1 blockade induces apoptosis and sensitizes breast carcinoma to taxotere administration.

1.3 Blockade of MMP-1/PAR1 cascade blocks breast tumor metastasis to the

lungs.

AIM 2. Investigate the role of PAR1 expression in epithelial mesenchymal transition (EMT). While the association of PAR1 with cancer invasion is well established, its precise molecular mechanism is still not completely understood. We have observed that ectopic expression of PAR1 induces a fibroblast-like morphological change in MCF-7 breast carcinoma cells. Here, we hypothesized that PAR1 expression induces EMT and employed a genechip approach to analyze global shifts in gene transcription events.

2.1 PAR1 expression induces epithelial mesenchymal transition (EMT).

2.2 PAR1 mediated changes in the ErbB receptor expression profile and its role in EMT induction.

CHAPTER 2: Investigate the role of PAR1 signaling in breast tumor survival and metastasis

The findings from this chapter have been accepted for publication in Cancer Research under the title: "Blockade of PAR1 signaling with cell-penetrating pepducins inhibits Akt-survival pathways in breast cancer cells and suppresses tumor survival and metastasis"

Introduction

Breast cancer is the most common malignancy in females in the United States and is a leading cause of cancer death second only to lung cancer (163). Metastatic disease has a particularly poor prognosis and current chemotherapeutic regimens are unlikely to result in complete remission (164, 165). Combining targeted inhibitors of oncogenic proteins with traditional cytotoxic agents has resulted in improved rates of patient response, however, given the heterogeneous nature of cancer and the high rate of reoccurrence (166, 167), there is still a need to identify novel oncogenic targets that can enhance chemotherapeutic vulnerability to resistant disease.

The PAR1 G protein-coupled receptor emerges as a promising oncogenic target because of its involvement in the invasive and metastatic processes of cancers of the breast, ovaries, lung, colon, prostate, and melanoma (105-110). Recent studies demonstrated that PAR1 promotes tumorigenicity, invasion and metastasis in breast and ovarian carcinoma xenograft models (115, 157) . PAR1 is activated by proteolytic cleavage and release of a tethered ligand by serine proteases such as thrombin, plasmin, factor Xa, and activated protein C (76, 134).

Recent studies identified MMP-1 as a novel protease agonist of tumor, platelet, and endothelial PAR1, however, the signaling components have not been characterized (115, 122, 136, 157). Overexpression of MMP-1 is associated with poor prognosis of breast cancer, colorectal and esophageal cancer

(168-170), and therefore, understanding the pathophysiologic role of MMP-1 in tumor progression is of great interest. Here we explore the significance of PAR1 and MMP-1 signaling and its blockade on downstream cell survival pathways in breast cancer cells and xenograft models.

To efficiently block PAR1 signaling, we developed a highly stable cell-penetrating pepducin, P1pal-7, that acts as an antagonist of PAR1-G-protein signaling (115, 154). In this study, we demonstrate the utility of P1pal-7 as an effective PAR1 antagonist in mouse models of breast cancer. P1pal-7 was cytotoxic only to breast carcinoma cells expressing PAR1 and blocked PAR1 mediated Akt signal. Dual therapy with P1pal-7 and taxotere inhibited the growth of MDA-MB-231 xenografts by up to 95% and induced apoptosis through an Akt dependent mechanism. Blockade of either MMP-1 or PAR1 significantly induced apoptosis in breast xenografts and also inhibited metastasis to the lung. These data implicate MMP1-PAR1-Akt axis as a promising new target for the treatment of breast cancer.

Results

P1pal-7 is Cytotoxic to Invasive Breast Cancer Cells Expressing PAR1

To investigate whether PAR 1 expression correlates with invasiveness of breast carcinoma cells, we conducted invasion assays using matrigel coated Boyden chambers. Three PAR1 expressing breast carcinoma cells, Bt549, MCF7-PAR1/N55 and MDA-MB-231, and two PAR1-null cells T47D and MCF-7 were tested for invasion through matrigel towards fibroblast conditioned medium and correlated with PAR1 cell surface expression (measured by flow cytometry). Total PAR1 protein levels were also confirmed by western blot (Supplemental Fig. 2.1A). There was a positive correlation ($R = 0.76$, $P < 0.05$) between PAR1 surface expression and cellular invasion through matrigel (Fig. 2.1A). The MCF7-PAR1/N55 is a clonal derivative of MCF-7 cells generated by the stable transfection of PAR1 (115, 120). A 20-fold increase in invasive capacity of N55 (compared to MCF-7) strongly supports the role of PAR1 in breast carcinoma cell invasion.

We also followed cell migration and proliferation by wound healing (scratch assay) of PAR1-expressing (N55, Bt549) and PAR1-null (MCF-7, T47D) cell lines. PAR1 expressing cell lines were able to close the wound within 72 hours, where as PAR1-null MCF-7 and T47D cells did not show any significant proliferation or migration into the wounded area (Supplemental Fig. 2.1B). Again, the difference in migration between the parental PAR1-null MCF-7 and PAR1-

expressing N55 (MCF7-PAR1) strongly supports the role of PAR-1 in cell movement and proliferation.

We then studied cellular proliferation to test for PAR1-mediated survival and proliferative advantages under nutrient-poor conditions. The high PAR1 expressing MDA-MB-231 cells proliferate 36-fold more quickly than the PAR1-null MCF-7 cells as compared over 7 days (Supplemental Fig. 2.1C). N55 (medium PAR1 surface expression) and N26 (low PAR1 surface expression) showed a 16-fold and 5-fold increase in proliferation, respectively, demonstrating a dose response in PAR1-mediated cell growth. We then treated two PAR1 expressing cell lines, MDA-MB-231 and N55, with PAR1 siRNA (115) that decreased cell viability by 75% and 40 %, respectively relative to the scrambled PAR1 control siRNA (Fig. 2.1B). We achieved almost complete inhibition of PAR1 surface expression with PAR1 siRNA as assessed by FACS analysis (Supplemental Fig. 1D).

Given that PAR1 siRNA decreased cell viability, we tested whether the PAR1 antagonist pepducin, P1pal-7, would confer cytotoxicity to breast carcinoma cells. A panel of breast cancer cells were treated with varying concentrations of P1pal-7 and cell viability was assessed using either MTT or trypan blue exclusion assays. PAR1 expressing cell lines (MDA-MB-231, BT549 and N55) were sensitive to P1pal-7, whereas both PAR1-null cell lines, MCF-7 and T47D, retained high cell viability (≥70%) for all P1pal-7 concentrations tested (Fig. 2.1C and Supplemental Fig. 2.2A-C). We observed a negative correlation ($R = 0.76$, $P < 0.05$; $R = 0.89$, $P < 0.016$) between cell viability and PAR1

expression in the presence of P1pal-7 with both MTT (Fig. 2.1D) and trypan blue exclusion assay (Supplemental Fig. 2.2B). Together, these results suggest that PAR1 promotes viability of breast carcinoma cells and renders the PAR1 expressing cells sensitive to the PAR1 pepducin, P1pal-7.

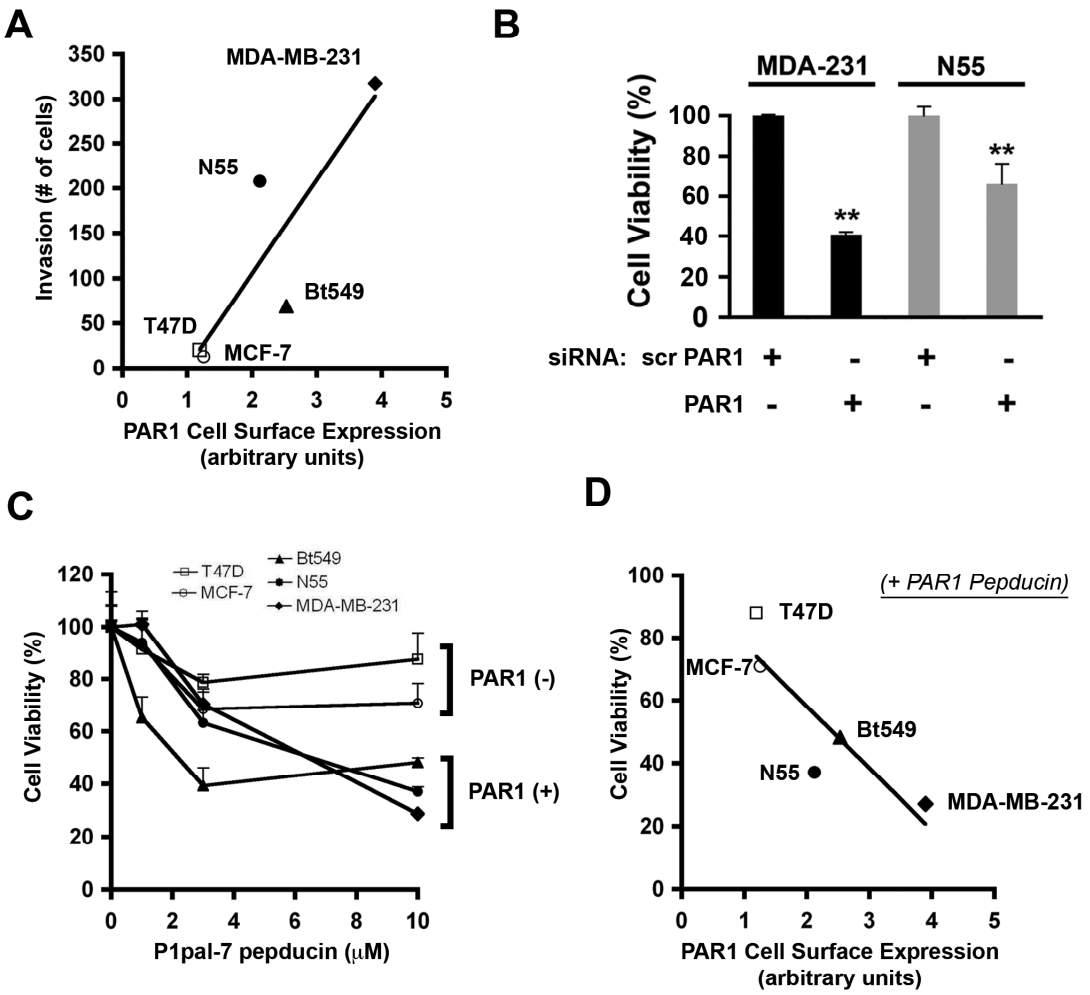


Figure 2.1. PAR1 expression enhances breast cancer cell invasion and survival and confers sensitivity to P1pal-7 pepducin. ***(A)*** MDA-MB-231, MCF7-PAR1/N55, MCF-7, T47D, BT549 breast cancer cell lines were evaluated for ability to invade through an 8 μm pore membrane coated with matrigel towards NIH-3T3 fibroblast conditioned medium ($R = 0.76$, $P < 0.05$). ***(B)*** MDA-MB-231 and MCF7-PAR1/N55 cells were transfected with siRNA against PAR1 and scrambled sequence PAR1 siRNA. After 72 h, cell viability was evaluated by the MTT assay. ***(C)*** Breast carcinoma cells were treated with P1pal-7 pepducin at

varying concentrations as indicated for 72 h and cell viability was evaluated by the MTT assay. ***(D)*** Cell viability at 10 μM P1pal-7 was correlated with relative PAR1 expression ($R = 0.76$, $P < 0.05$). PAR1 expression was analyzed by flow cytometry. Representative data (mean ± s.d.) from multiple experiments are shown. ** $p < 0.01$.

Synergistic Cytotoxicity of Pepducin-Taxotere Combination Therapy Activates Caspase-mediated Apoptosis

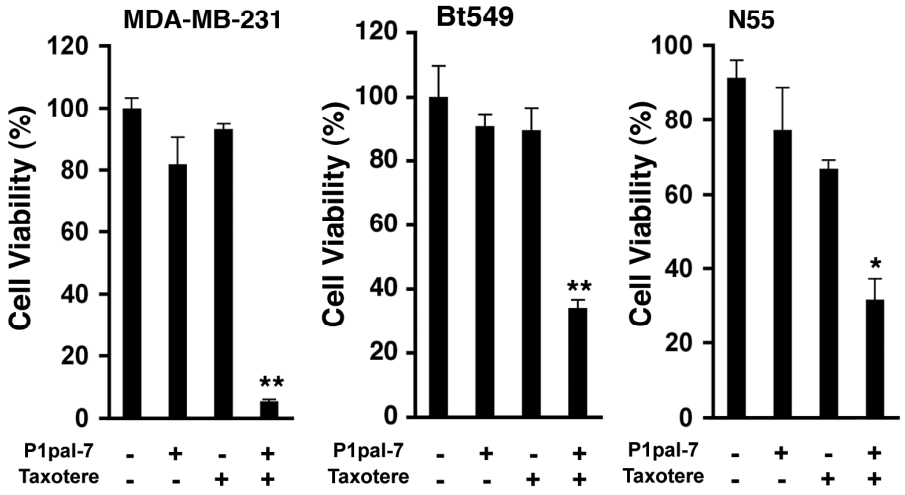
Docetaxel (taxotere) is considered as the standard-of-care chemotherapeutic agent for the treatment of metastatic breast cancer and other carcinomas. Therefore we tested whether addition of taxotere would provide synergistic effects with the PAR1 antagonist P1pal-7 on cell viability using sub-IC_{50} amounts of taxotere and P1pal-7. We have varied the concentration of P1pal-7 and found that IC_{50} for cell viability was 1.7 μM (Supplemental Fig. 2.3A) where as IC_{50} for taxotere was 1.1 nM (data not shown). Given together, P1pal-7 (1 mM) and taxotere (0.3 nM) decreased cell viability by 95%, 70%, and 70% in MDA-MB-231, Bt549, and N55 cells, respectively (Fig. 2.2A). Neither P1pal-7 nor taxotere alone significantly affected cell viability as evaluated by the MTT assay. The isobologram technique and the Chou and Talalay analysis (171) were employed to quantify the degree of synergy. At various concentrations of P1pal-7 and taxotere, the isobologram technique indicated strong synergism at a combination index (CI) of 0.17 (Supplemental Fig. 2.3B), which was further confirmed by the Chou and Talalay analysis (Supplemental Fig. 2.3C). This robust cytotoxic synergy between P1pal-7 and taxotere may suggest a promising therapeutic potential of combination therapy between PAR1 blockade and the standard-of-care therapy in breast cancer.

We then assessed the involvement of apoptotic pathways to better understand the molecular mechanism underlying the synergistic cytotoxicity

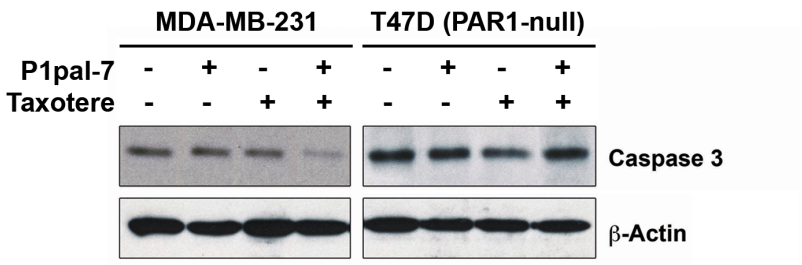
between P1pal-7 and taxotere. Elevated pan-caspase activity was detected in both MDA-MB-231 and N55 cells given combination treatment (Supplemental Fig. 2.4A, B). Specifically, caspase 3 cleavage and activation correlated closely with decrease in cell viability. 24 hours after treatment initiation, cell viability does not decrease and caspase 3 remains inactive (Supplemental Fig. 2.4C, D). However, after 72 hours of drug treatment, we observe near complete activation of caspase 3 (Fig. 2.2B) with a corresponding precipitous decrease in cell viability (Fig. 2.2A). Caspase 3 activation is not observed in T47D, a PAR1-null breast carcinoma cell line (Fig. 2.2B). Together, the above results suggest that the P1pal-7/Taxotere combination therapy causes synergistic cytotoxicity by induction of caspase 3-mediated apoptosis pathways in PAR1-expressing breast carcinoma cell lines.

Taxotere by itself confers cytotoxicity by interfering with the dynamics of microtubule assembly and thereby halting the cell cycle at the G2/M phase. We confirmed that when MDA-MB-231 cells were treated with taxotere, the G2/M peak increased significantly (65%) (Fig. 2.2C). However, P1pal-7 did not affect cell cycle distribution whether it was administered alone or in combination with taxotere. These results suggest that taxotere is conferring cytotoxicity to MDA-MB-231 through a cell-cycle arrest mechanism, whereas P1pal-7 is acting in a pathway independent of cell-cycle regulation.

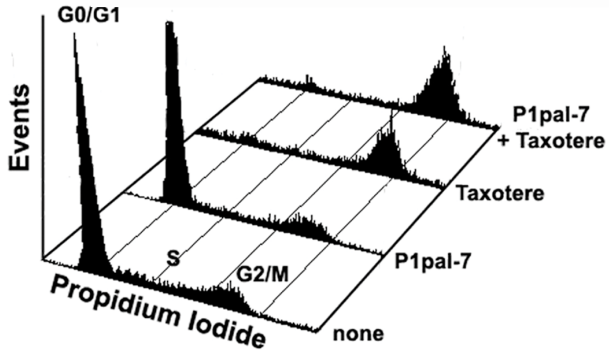
A



B



C



	G0/G1 (%)	S (%)	G2/M (%)
P1pal-7 + Taxotere	5.5	7.5	71.6
Taxotere	6.1	8.4	65
P1pal-7	65.1	10.8	19.7
vehicle	60.4	16.1	21

Figure 2.2. Dual treatment with P1pal-7 and taxotere synergistically inhibits cell viability and promotes apoptosis in PAR1-expressing breast carcinoma cells. ***(A)*** MDA-MB-231, Bt549 and MCF7-PAR1/N55 cells were treated with 1 μM P1pal-7, 0.3 nM taxotere, or both, incubated for 72 h and evaluated for cell viability by the MTT assay. ***(B)*** MDA-MB-231 and T47D (PAR1 null) cells were treated as indicated above. Lysates were immunoblotted with anti-Caspase 3. β-Actin was used as loading control. ***(C)*** MDA-MB-231 cells were treated with 5 μM P1pal-7, 100 nM taxotere or both and incubated overnight (18 h). Cells were then stained with propidium iodide and evaluated for cell cycle distribution by flow cytometry. Representative data (mean ± s.e.m.) from multiple experiments are shown. * $p < 0.05$, ** $p < 0.01$.

Activated form of Akt blocks P1pal-7 Apoptotic Effect in Breast Carcinoma Cells

Since synergistic inhibition of cell viability and enhanced apoptosis was dependent on PAR1, we examined the effects of PAR1 activation on Akt signaling in breast carcinoma cells. Akt, a serine/threonine kinase plays a prominent role in cellular growth, metabolism, proliferation, and survival (172), and is frequently hyperactive in many cancer types (173) including breast cancer (52, 174), and contributes to chemotherapy resistance (175). Akt has been established as a downstream component of the PAR1-G protein-PI3K axis in platelets (144, 176) and its phosphorylation in response to thrombin has been shown to occur in melanoma cells (118). Therefore, we hypothesized that P1pal-7 may regulate apoptosis by blocking the Akt survival pathway downstream of PAR1.

As predicted, treatment of MDA-MB-231 or N55 cells with thrombin caused a rapid and robust induction of Akt phosphorylation that peaked 5 min upon stimulation (Fig. 2.3A, Supplemental Fig. 2.5A). Consistent with proteolytic activation of PAR1, the exogenously added SFLLRN-activating peptide also induced Akt phosphorylation, but with slightly slower kinetics. PAR1-dependent Akt kinase activity was also demonstrated by the corresponding time-dependent phosphorylation of GSK3 (177, 178) by the SFLLRN agonist peptide (Fig. 2.3A). Thrombin mediated Akt phosphorylation is inhibited with P1pal-7, whereas P1pal-19EE, a negative control pepducin (154, 157), was without effect (Fig. 2.3B; Supplemental Fig. 2.5B). Likewise, a small molecule antagonist of PAR1,

RWJ-56110 (159) strongly inhibited Akt phosphorylation of the MDA-MB-231 cells (Fig. 2.3B). Inhibition of Akt phosphorylation by P1pal-7 or RWJ56110 resulted in corresponding decrease in Akt kinase activity as witnessed by the decrease in p-GSK3 (Supplemental Fig. 2.5C). P1pal-7 did not modulate insulin or EGF-induced Akt phosphorylation of MDA-MB-231 cells (data not shown). As anticipated, thrombin or SFLLRN were not able to induce Akt phosphorylation in the PAR1-null MCF-7 and T47D carcinoma cell lines (Supplemental Fig. 2.5D). PAR1 knockdown by siRNA caused the MDA-MB-231 cells to lose the ability to induce GSK3 activity in response to the PAR1 agonist (Supplemental Fig. 2.6B, 1D). Furthermore, gene silencing of Akt1, Akt2 or Akt3 in MDA-MB-231 cells identified Akt1 as the major isoform that signals to GSK3 downstream from PAR1 (Supplemental Fig. 2.6A, B).

Next, we explored the significance of Akt signaling in the context of P1pal-7/Taxotere cytotoxicity. Ectopic expression of the constitutively active, myristoylated Akt in MDA-MB-231 protected against P1pal-7 cytotoxicity and eliminated its synergistic interaction with taxotere (Fig. 2.3C). We then investigated the effects of Akt knockdown on apoptosis as measured by PARP cleavage. PARP is a nuclear protein and its cleavage by caspase 3 is a reliable readout for the occurrence of apoptotic event (179). We observe here that P1pal-7 and taxotere given together results in near complete cleavage of PARP (Fig. 2.3D). Akt knockdown by siRNA confers cytotoxicity as indicated by the appearance of cleaved PARP. Notably, the addition of P1pal-7 alone does not increase apoptosis, but the addition of taxotere resulted in near complete

cleavage of PARP. Furthermore, P1pal-7 and taxotere given together did not demonstrate significantly enhanced cytotoxity as observed previously. To summarize, the cytotoxic effects of Akt knockdown mimicked those of P1pal-7 and rendered further addition of P1pal-7 ineffective. These results strongly suggest that P1pal-7 confers cytotoxicity by blocking the PAR1-Akt survival pathway, and Akt blockade is a critical step for the synergistic interaction of P1pal-7 and taxotere.

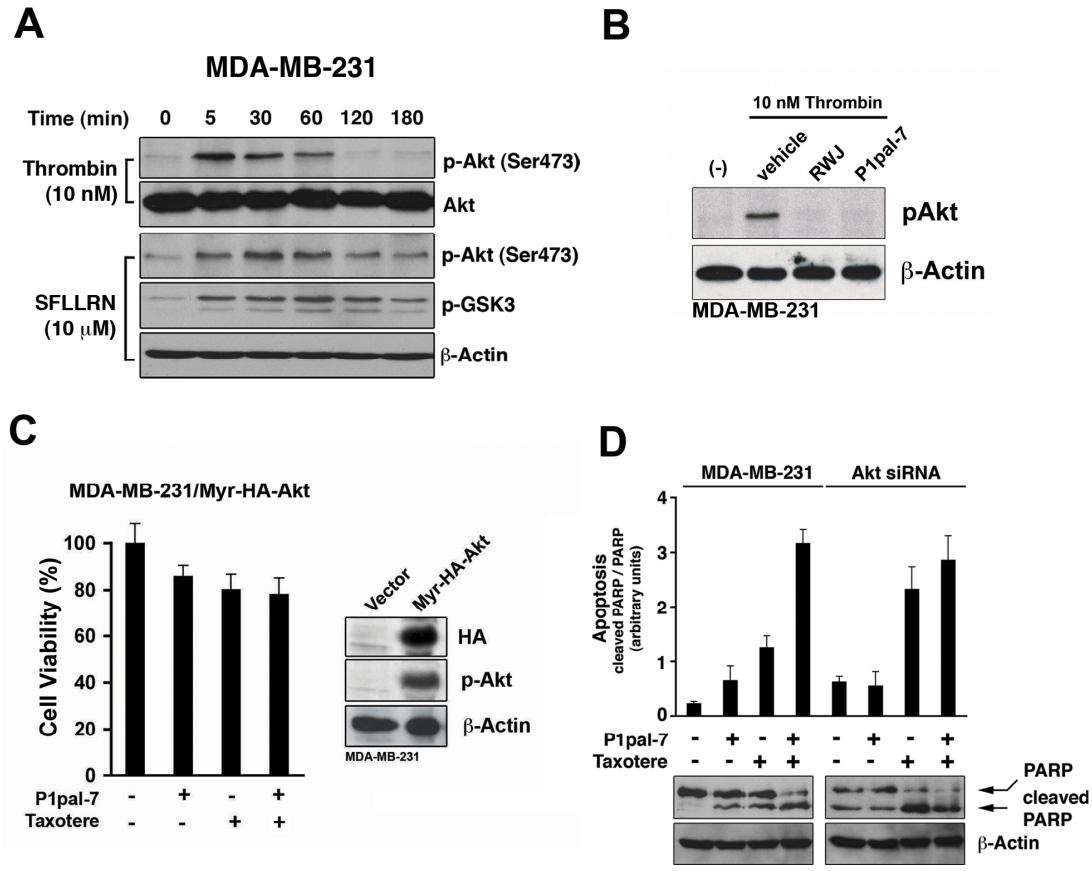


Figure 2.3. **PAR1-Akt signaling in breast carcinoma cells.** ***(A)*** MDA-MB-231 cells were starved overnight in serum free medium and stimulated with 10 nM Thrombin or 10 μM SFLLRN activating peptide over a period of 3 hours. Cell lysates were immunoblotted with anti-phospho-Akt (Ser473) or anti-phospho-GSK3 (Ser21/9). β-Actin and total Akt were used as loading controls. ***(B)*** MDA-MB-231 cells were pre-treated with 3 μM P1pal-7 and 5 μM RWJ5611 and subsequently stimulated with 10 nM Thrombin. Cell lysates were immunoblotted with anti-phospho-Akt (Ser473). β-Actin was used as loading control. ***(C)*** MDA-MB-231 cells were transiently transfected with Myr-HA-Akt or vector control.

Cells were treated with 1 μM P1pal-7, 0.3 nM taxotere, or both, incubated for 72 h and evaluated for cell viability by the MTT assay. Cell lysates were immunoblotted with anti-phospho-Akt (Ser473) and anti-HA tag to evaluate transfection efficiency. β-Actin was used as loading control. ***(D)*** MDA-MB-231 and MDA-MB-231 transfected with Akt siRNA were treated with 5 μM P1pal-7, 100 nM taxotere or both overnight (18 h). Cell lysates were immunoblotted with anti-PARP. β-Actin was used as a loading control. Bars represent densitometric measurements of PARP bands normalized to β-Actin. Representative data (mean ± s.d.) from multiple experiments are shown.

Dual Therapy Inhibits Growth and Amplifies Cell Death in Cancer Xenograft models

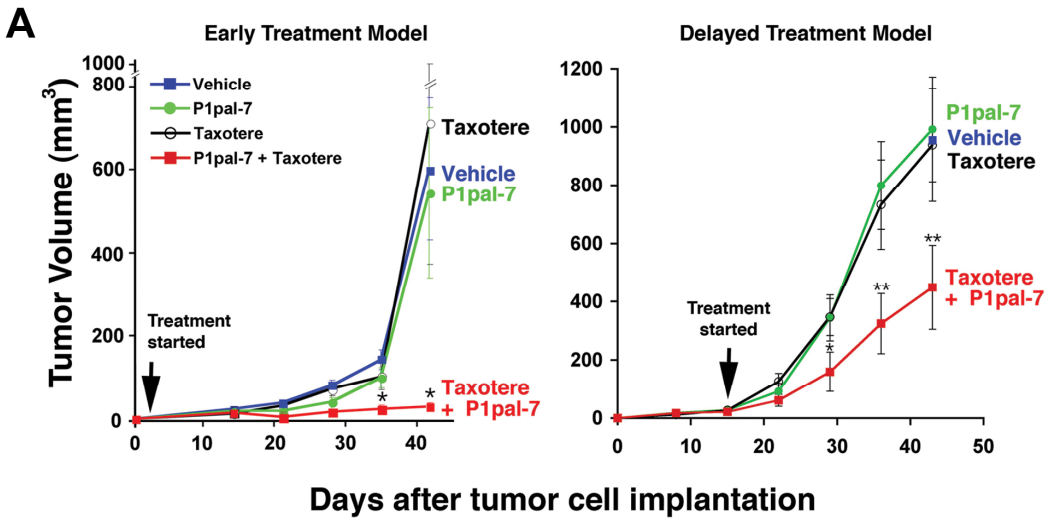
We tested whether the enhanced *in vitro* cytotoxicity of the P1pal-7-taxotere combination would be effective in estrogen-independent, aggressive breast cancer models in nude mice. MDA-MB-231 cells were inoculated isotopically into the mammary fat pads of female nude mice and treated with Vehicle (DMSO), P1pal-7, taxtore, or P1pal-7 + taxotere. As shown in Fig. 2.4A, P1pal-7 and taxotere monotherapy did not affect tumor growth relative to vehicle. However, dual administration of P1pal-7 and taxotere demonstrated striking synergistic inhibition of tumor growth. These results are consistent with our cell viability data.

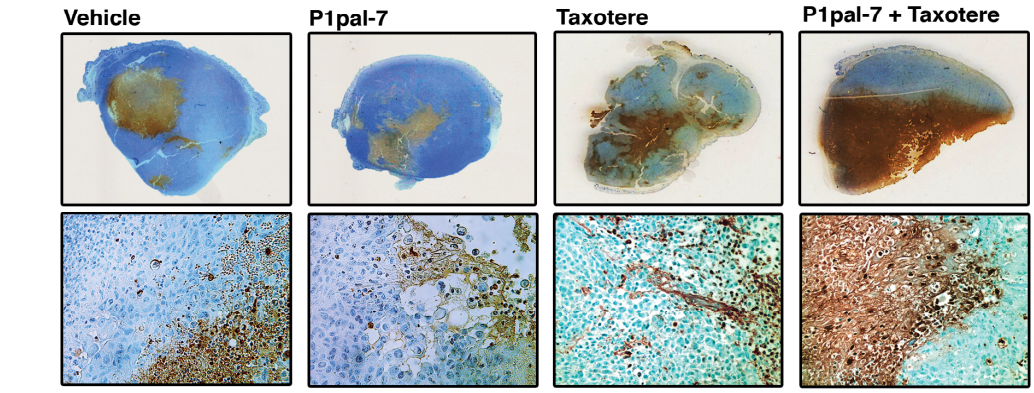
Next, we allowed the grafted breast carcinoma cells to form palpable tumors before initiating treatment (delayed treatment model) to test the efficacy of P1pal-7/taxotere combination therapy against established tumors. As in the early treatment model, tumor growth rates were similar in mice given delayed P1pal-7 or taxotere monotherapy as compared to vehicle (Fig. 2.4A). In contrast, delayed treatment with the combination of P1pal-7 and taxotere significantly attenuated growth rates. Visual inspection of the xenografts revealed a central area of tumor death in several of the mice treated with the combination therapy, whereas none of the mice that received mono-therapy or vehicle had necrotic lesions despite the considerably larger sizes of the tumors (Supplemental Fig. 2.7). This

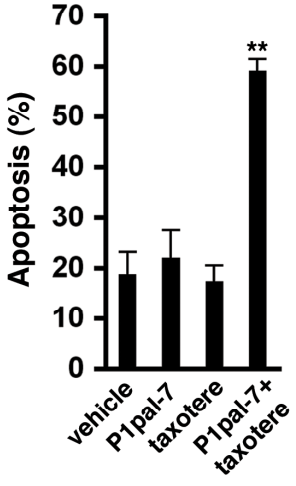
observation prompted an investigation of the apoptotic state and biochemical properties of the tumors.

The xenograft tumors were analyzed for apoptosis using TUNEL staining. The macroscopic and magnified views of the tumor sections (Fig. 2.4B) demonstrated a small central apoptotic core in the tumors of mice given either P1pal-7 or taxotere alone, or vehicle. In contrast, dual therapy resulted in massive segments of apoptosis extending well beyond the central region. The apoptotic areas were quantified and dual therapy yielded 60% apoptotic area on average whereas monotherapy or vehicle gave 20% apoptotic area (Fig. 2.4C).

In order to investigate the acute biochemical effects of PAR1 antagonists on tumor Akt activity, we allowed MDA-MB-231 tumors (8×10^6 cells orthotopically injected) to grow to 200 mm^3 before initiating a short-term 5 day treatment of P1pal-7 (10 mg/kg) or MMP-1 inhibitor FN439 (5 mg/kg) together with a single dose of taxotere (10 mg/kg). We found that the tumors of mice without PAR1 inhibition retained high levels of Akt phosphorylation, while addition of P1pal-7 or FN439 significantly attenuated Akt activity by 54% and 61%, respectively (Fig. 2.4D). Total Akt levels remain unchanged. This xenograft data suggests Akt as a pathophysiological effector molecule downstream to the MMP-1/PAR1 siganling cascade in tumors.


A
Early Treatment Model
Delayed Treatment Model
Tumor Volume (mm3)
Vehicle
P1pal-7
Taxotere
P1pal-7 + Taxotere
Treatment started
Taxotere
Vehicle
P1pal-7
Taxotere + P1pal-7
Days after tumor cell implantation


B
Vehicle
P1pal-7
Taxotere
P1pal-7 + Taxotere


C
Apoptosis (%)
vehicle
P1pal-7
taxotere
P1pal-7+ taxotere

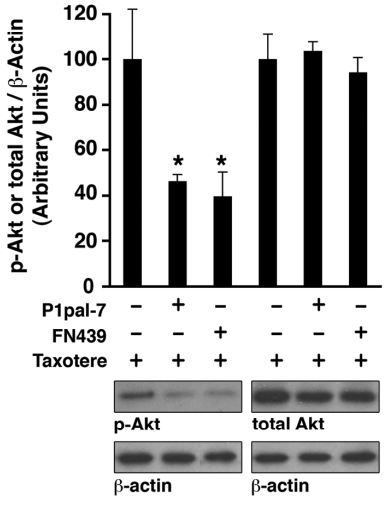

D
p-Akt or total Akt / β-Actin (Arbitrary Units)
P1pal-7
FN439
Taxotere
p-Akt
total Akt
β-actin
β-actin

Figure 2.4. Dual treatment with P1pal-7 pepducin and taxotere significantly attenuates growth of mice xenograft breast tumors by promoting apoptosis. ***(A)*** Early treatment model: MDA-MB-231 cells (4 x 10^6 cells) were injected into the mammary fat pads of female nude mice. 2 days after, injections with vehicle (10% DMSO), P1pal-7 (10 mg/kg), taxotere (10 mg/kg), or the combination were initiated (n = 5 mice per group). Delayed treatment model: MDA-MB-231/GFP cells (4 x 10^6 cells) were implanted. Treatment injections as above were initiated 15 days post implantation (n = 10-15 mice per group). Tumor volumes (mean ± s.e.m.) ***(B)*** Terminal deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) analysis of xenograft tumor sections. Top row: Macroscopic view of tumor sections with TUNEL. Bottom row: Representative fields of xenograft tumor sections (20X magnification). ***(C)*** Percentage of apoptotic area (mean ± s.e.m.) of tumor sections as quantified by the ImageJ software. % Apoptosis = (apoptotic area / total tumor section area). ***(D)*** Western blot analysis of MDA-MB-231 tumor homogenates (n = 5 mice per group) for Akt activity (p-Akt, Ser473; total Akt). β-actin was used as loading control. Bars represent densitometric measurements (by ImageJ) of phospho-Akt or total Akt bands normalized to total β-actin (mean ± s.e.m.). *$p<0.05$, **$p<0.01$.

P1pal-7 and MMP-1 inhibitor Accelerate Apoptosis of Breast Tumors

MMP-1 is an important mediator of cancer invasion and metastasis and has recently been identified as a novel PAR1 activating protease in cancer cells and platelets (115, 136). However, MMP-1/PAR1 signal transduction and its role in breast cancer cell survival remains unknown. Given that FN439 inhibited Akt phosphorylation in xenograft tumors (Fig. 2.4D), we predicted that the addition of exogenous MMP-1 to MDA-MB-231 cells will proteolytically activate PAR1 to mediate Akt phosphorylation. Indeed, we observed that 0.3 nM MMP-1 triggered Akt phosphorylation with a peak signal at 1 h that subsided by 3 h (Fig. 2.5A). This signal is blocked by P1pal-7 and FN439, suggesting that the Akt survival pathway is indeed engaged by the MMP-1/PAR1 cascade (Fig. 2.5B). We also observed that MMP1 derived from human fibroblast conditioned media is able to activate Akt in MDA-MB-231 cells (data not shown), implicating the role of tumor stroma in PAR1 mediated tumorigenesis, invasion, and metastasis.

We have previously studied the role of MMP-1 and PAR1 in tumor growth and showed that treatment of nude mice with P1pal-7 or FN439 inhibits growth of breast cancer xenografts (115). We also showed that MMP1 expression and collagenase activity were elevated in N55 tumors as compared to the control mammary pads. To determine whether MMP-1 and PAR1 contribute to cell survival during tumorigenesis, we tested the effect of PAR1 blockade (P1pal-7) and MMP-1 blockade (FN439) on tumor cell death using TUNEL, which detects DNA nicks formed during apoptosis. The brown color indicates positive TUNEL

staining and hence, apoptotic regions within the tumor (Fig. 2.5C). There was significant 2.1-fold and 3.4-fold increases in the number of cells undergoing apoptosis upon PAR1 or MMP-1 blockade (Fig. 2.5D), suggesting that the MMP-1/PAR1 cascade plays a role in protecting breast tumors from apoptotic insults.

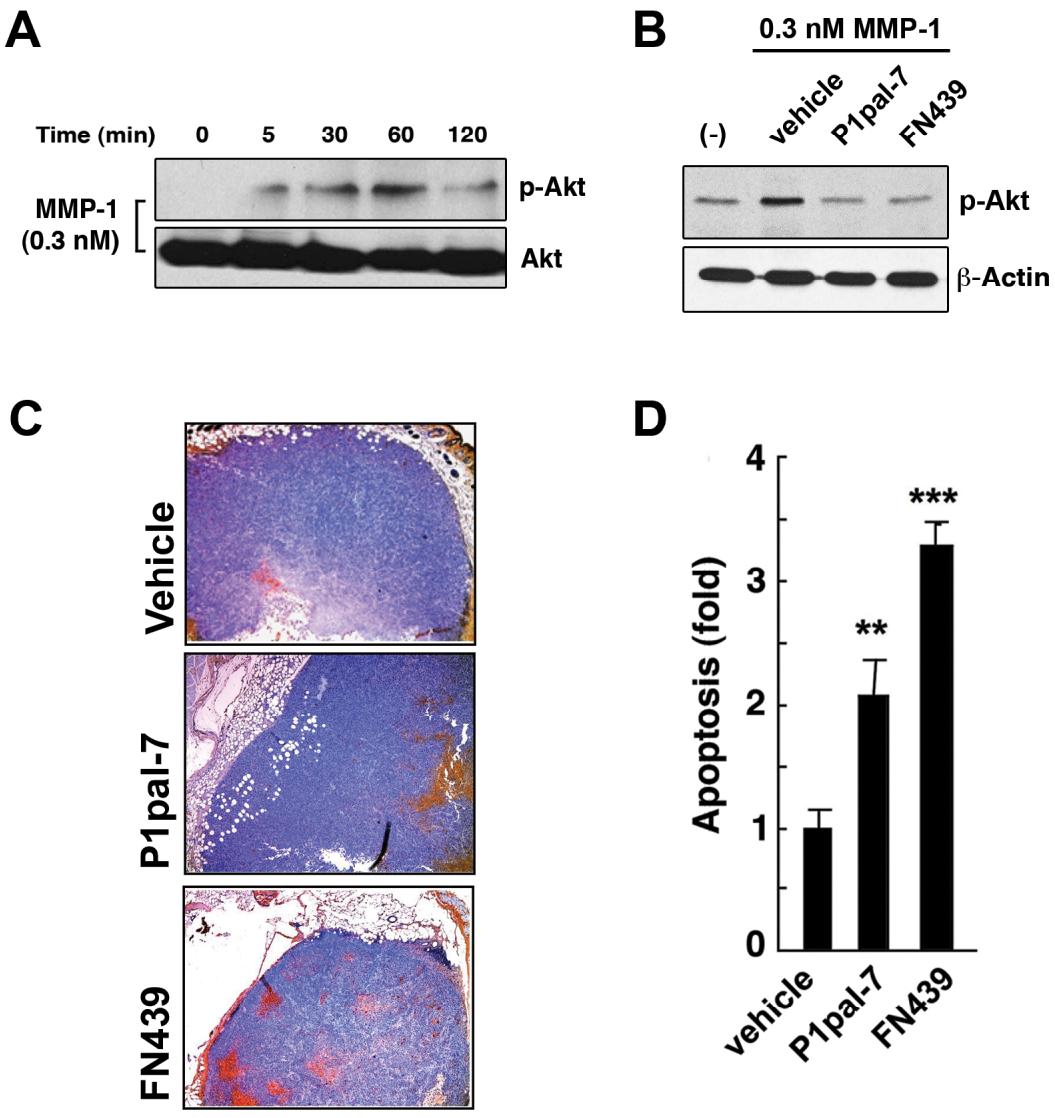


Figure 2.5. MMP1-PAR1-Akt signaling cascade promotes tumor survival in mice xenograft model. ***(A)*** MDA-MB-231 cells were starved overnight in serum free medium and stimulated with 0.3 nM MMP-1 over a period of 2 hours. Cell lysates were immunoblotted with anti-phospho-Akt (Ser473). Total Akt was used as loading controls. ***(B)*** MDA-MB-231 cells were pre-treated with 3 μM P1pal-7 and 3 μM FN439 and subsequently stimulated with 0.3 nM MMP-1. Cell lysates

were immunoblotted with anti-phospho-Akt (Ser473). β-Actin was used as loading control. ***(C)*** N55 cells (4 x 10^6 cells) were injected into the mammary fat pads of female nude mice. 2 days after, injections with vehicle (10% DMSO), P1pal-7 (10 mg/kg), or FN439 (5 mg/kg) were initiated (n = 10 mice per group). Tumors were explanted upon experiment termination and sectioned for TUNEL analysis. Representative fields (4X) are shown. ***(D)*** Tumor cells demonstrating apoptosis were counted (mean ± s.e.m.). **p < 0.01 ***p < 0.001.

MMP-1/PAR1 Blockade Inhibits Breast Tumor Metastasis to the Lung

The over-expression of both PAR1 and MMP-1 are strongly implicated in breast cancer invasion, metastasis and poor overall survival (109, 180). Here, we tested the efficacy of MMP-1 and PAR1 blockade in attenuating the metastatic propensity of breast carcinoma cells using an *in vivo* model of experimental metastasis. We introduced MDA-MB-231/GFP cells via the tail vein of female nude mice and treated them with vehicle (10% DMSO), P1pal-7 or FN439. After 6 weeks, mice were sacrificed and the lungs were extracted for analysis. The lungs of mice given vehicle treatment were profusely populated with macroscopic tumor nodules at the surface (Fig. 2.6A). In stark contrast, tumor nodules were significantly decreased or absent on the lung surfaces of mice treated with P1pal-7 or FN439. Histological analysis of lung sections also confirmed the efficacy of MMP-1 and PAR1 blockade against breast tumor metastasis. In order to ensure representative sampling of the lungs, 3 sections were made per lung at varying depths: the top 1/3, middle 1/3, and bottom 1/3 along the coronal plan of the lung. Counting the number of tumor nodules per lung section revealed a remarkable decrease in metastatic incidence in mice treated with P1pal-7 (75% decrease) or FN439 (88% decrease) (Fig. 2.6B). To our knowledge, this is the first report to demonstrate inhibition of metastasis by blockade of the MMP-1/PAR1 cascade.

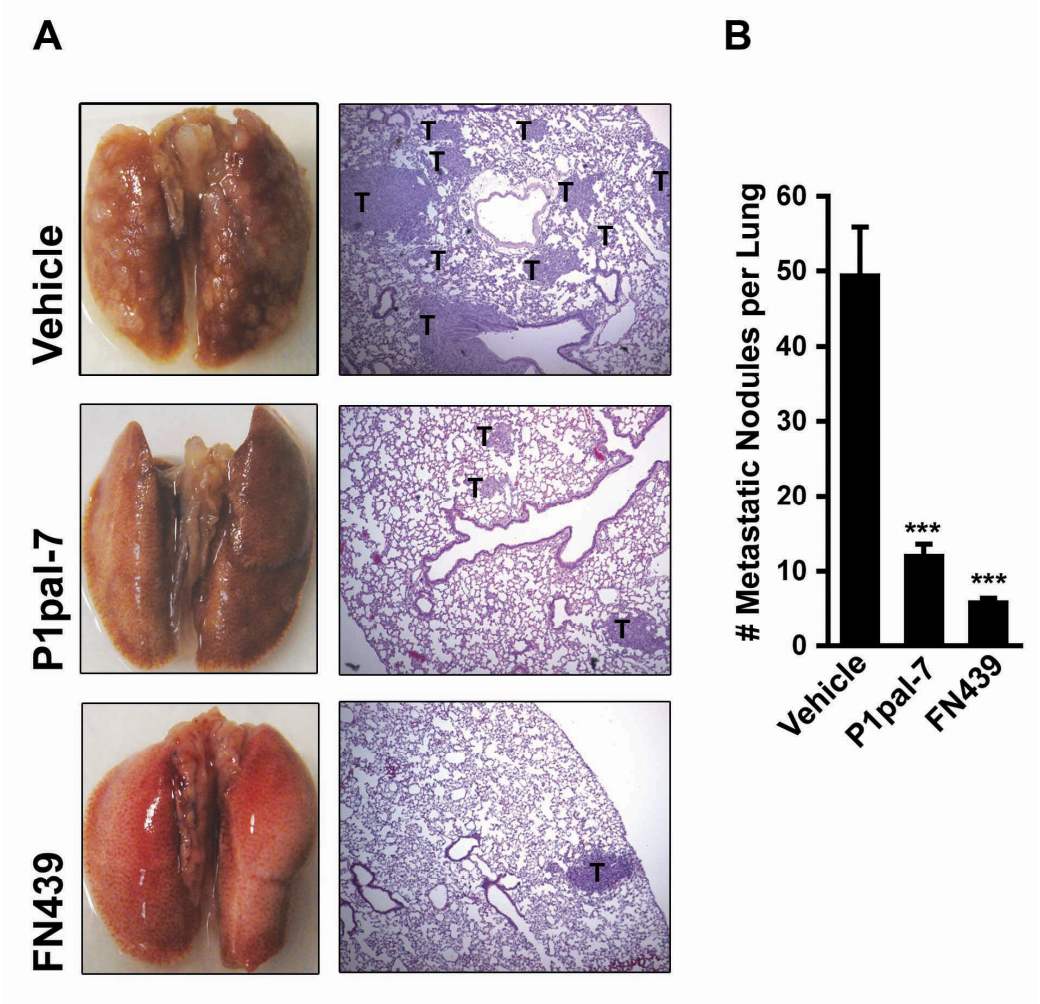


Figure 2.6. Effect of P1pal-7 pepducin on gene transcription in PAR1-expressing cells using microarray analysis. MMP-1/PAR1 Blockade Inhibits Breast Tumor Metastasis to the Lung ***(A)*** MDA-MB-231/GFP cells (2 x 10^6 cells) were introduced via the tail vein of female nude mice (n = 5-10 mice per group). Vehicle (10% DMSO), P1pal-7 (10 mg/kg), or FN439 (5 mg/kg) were administered 6 d/wk for 6 wks. Lungs were photographed (Left Column) and sectioned for H&E staining (Right Column; 4X magnification). T = Tumor Nodule. ***(B)*** Number of metastatic tumor nodules per lung as counted under a light microscope. ***p < 0.001.

Discussion

MMP-1 expression is a risk factor for overall survival of patients with invasive breast carcinoma (181). The source of MMP-1 could be stromal derived, or in some instances tumor derived (122, 182). Based on recent evidence, MMP-1 is a viable therapeutic target, however, inhibitors against MMPs have not been successful. For instance, marimastat (BB-2516), a broad spectrum MMP inhibitor, and trocade (Ro 32-3555), an MMP-1 selective inhibitor, have performed poorly in clinical trials largely due to toxicity or lack of efficacy (183). Accordingly, PAR1 may be good alternative target for the treatment of breast cancer. There is preliminary evidence from clinical trials investigating thrombosis that chronic blockade of PAR1 with a small molecule inhibitor (SCH205831 (160)) is safe. It remains to be determined whether SCH205831 can effectively block MMP-1/PAR1 mediated activation of breast cancer tumors. We show in this study, the efficacy of MMP-1/PAR1 blockade for the induction of tumor apoptosis and inhibition of metastasis to the lung.

In this report, we have examined the effects of PAR1 antagonism with a novel cell-penetrating lipopeptide, P1pal-7, on advanced stage breast cancer cells both *in vitro* and in animals. The data presented here suggest that PAR1 blockade by P1pal-7 may be a viable approach to impact PAR1-mediated survival pathways and may synergistically enhance cytotoxicity and apoptosis with anti-tumor agents, as exemplified by taxotere, in models of breast cancer.

PAR1 signals through Akt to confer its survival and anti-apoptotic effects. Akt is a very well established survival factor and exerts its pleiotropic anti-apoptotic effects by inactivation of pro-apoptotic factors BAD, caspase-9, and forkhead transcription factors, and the activation of pro-survival genes through NF-kB. (47, 48). Furthermore, Akt mediated inactivation of GSK3 leads to cell survival by preventing the degradation of the oncogenic protein BCL-3 (184). Hence, GSK3 phosphorylation was chosen as a representative readout of anti-apoptotic Akt activity. Akt defines a family of highly homologous isoforms Akt1, Akt2, and Akt3, all of which share a Pleckstrin homology (PH) domain, an alpha-helical linker, and a carboxy-terminal kinase domain (49). Functional redundancy and compensation exists amongst the three Akt isoforms, but emerging evidence also indicates significant functional differences. The distinct physiological functions of Akt1 and Akt2 have been studied in Akt isoform deficient mice, in which $Akt1^{-/-}$ mice demonstrated significant growth defects where as $Akt2^{-/-}$ mice exhibited defects in insulin-dependent glucose metabolism (185). In breast cancer cell lines, Akt family members have also been shown to have specific functions, in which tyrosine kinase receptor mediated Akt1 activity opposes cell migration and invasion, while Akt2 signaling favors hyperproliferation, invasion and migration (186-188). Akt3 seems to have a strong role in endocrine dysregulation, as demonstrated by its over-expression in $ER^{-/-}$ breast cancer cell lines (189) and its ability to induce estrogen resistance in MCF-7 cells (190). To further complicate matters, isoform functionality is not consistent across cell types, and the roles of Akt1 and Akt2 in migration are reversed in mouse

embryonic fibroblasts (191). Therefore, the characterization of isoform specific signalling is important when investigating and designing inhibitors against Akt or signalling mediators upstream of Akt. Here we show that under PAR1 regulation, Akt1 is the dominant isoform and the activity levels of Akt2 and Akt3 are more reserved. We further demonstrate that P1pal-7 mediated cytotoxicity is in part, a result of Akt1 blockage as evidenced by the loss of P1pal-7 sensitivity by Akt1 knockdown.

Combination treatment of breast tumors with P1pal-7 and taxotere significantly inhibited tumor growth and caused massive apoptosis. Our present study characterizes the involvement Akt in the context of PAR1 blockage and combination therapy. While investigating the role of PAR1 in growth and survival, we observed that breast cancer cells expressing PAR1 have increased proliferative potential, but are simultaneously vulnerable to PAR1 blockade. In fact, stable expression of PAR1 (MCF7-PAR1/N55) is sufficient in rendering P1pal-7 sensitivity to the MCF-7 cell line. PAR1 blockage also had cytotoxic effects against MDA-MB-231 and BT549, breast cancer cell lines naturally expressing high levels of PAR1, representing an advanced, endocrine therapy resistant form of breast cancer (192, 193). PAR1, hence, provides a novel mode of attack against advanced breast cancer models with aggressive phenotypes.

Materials and Methods

Reagents

N-palmitoylated peptides P1pal-7 and P1pal-19EE, and the PAR1 agonist peptide SFLLRN were synthesized as described previously (115, 154, 157). Taxotere (Docetaxel), MTT, propidium iodide and insulin were purchased from Sigma-Aldrich. RWJ-56110 was a generous gift from Johnson & Johnson. The plasmid pcDNA3-Myr-HA-Akt1 (plasmid #9008) was obtained from Addgene (194). Pro-MMP-1 and FN-439 were obtained from Calbiochem. Activation of pro-MMP-1 with APMA was performed as previously described (115, 157).

Cell Culture

MDA-MB-231, MCF-7, BT549, and T47D breast cancer cells were obtained from the National Cancer Institute. The MCF-7 cells stably expressing PAR1 (MCF7-PAR1/N55 and N26 clones) were generated in our lab as previously described (13). All cells were maintained in RPMI 1640 or DMEM supplemented with 10% FBS, 0.2% sodium bicarbonate, 1% penicillin and streptomycin in 5% CO_2 at 37 ^{o}C. Fibroblast conditioned medium was derived from WI-38 cells as described previously **(12)**. MMP-1 was measured using the proMMP-1 ELISA kit (R&D Systems) following manufacturer's protocols.

Western Blot Analysis

Cells were suspended in TNE lysis buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% NP-40), supplemented with 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and Halt Phophatase Inhibitor Cocktail (Pierce). Cells were lysed on ice for 30 minutes and protein content was measured by the Bradford assay. Lysates (40-50 μg) were resolved on a 12% SDS-PAGE and transferred to polyvinylidene difluoride or nitrocellulose membranes and blocked with 5% milk in TBST (0.1% Tween-20 in TBS). Membranes were incubated overnight at 4 ^{o}C with the appropriate primary antibodies: anti-total Akt, anti-Akt1, anti-Akt2, anti-phospho Akt (Ser473), anti-phospho GSK3 (Ser21/9), anti-Caspase-3, anti-PARP, anti-HA-tag, anti-β-Actin (Cell Signaling Technology) and anti-Akt3 (Upstate Cell Signaling Solutions), anti-PAR1 (Santa Cruz Biotechnology). Densitometry measurements for band quantification was performed using the ImagJ software.

Small Interfering RNA: Small interfering (si)-RNA against:

PAR1	(5'-GGCUACUAUGCCUACUACU-3') (115)
scrambled PAR1	(5'-GCUAAGUUGCACCUACCUAUA-3')
Akt1	(5'AAGGAGGGUUGGCUGCACAAA- 3')
Akt2	(5'-AACUUCUCCGUAGCAGAAUGC - 3')
Akt3	(5'-AACUGGAGGCCAAGAUACUUC - 3')
firefly luciferase	(5'-CGTACGCGGAATACTTCGA- 3')

were synthesized by Dharmacon (Lafayette, Colorado). MDA-MB-231 cells were transfected overnight with 0.2 μM siRNA using Oligofectamine (Invitrogen) in

serum free RPMI 1640 without antibiotics. Cells were recovered for 24 h in 10% FBS before used in assays.

Apoptosis Assays

Apoptosis was evaluated by caspase activity. Caspase-3 activativity was assessed by caspase-3 cleavage and Poly ADP-ribose polymerase (PARP) cleavage using western blot analysis. The CaspACE in situ marker (FITC-conjugated pan-caspase inhibitor) was purchased from Promega . Cells were labeled with 10 μM CaspACE for 30 minutes and analyzed by flow cytometry.

Cell Cycle Analysis

Cell cycle distribution was analyzed by DNA staining with propidium iodide . Cells were harvested and fixed in 100% ethanol overnight at 4^{o}C. Cells were then stained with propidium iodide (PI) solution (50 μg/ml PI, 10 mM Tris pH 7.5, 5 mM $MgCl_2$, 20 mg/mL RNase A) for 30 minutes at 37 ^{o}C. DNA content was quantified by flow cytometry.

Invasion and Wound Healing Assays

Invasion assays were conducted using Transwell chambers (Corning) with 8 μm pore membranes coated with Matrigel as described previously (12, 23). Wound healing assays were conducted by seeding cells on to glass slides. Confluent monolayers were wounded using a 200 μl pipette tip. The wounded areas were

examined under a light microscope. Fibroblast conditioned medium was derived from NIH-3T3 cells as described previously (15).

Cell Proliferation Assay

Cell proliferation was assessed by counting the viable cells under a light microscope with Trypan Blue staining.

MTT Assay

Cells in 96 well plates were subjected to various treatment conditions or vehicle (0.2% DMSO) for 72 h. MTT reagent was added at a concentration of 0.5 mg/mL and allowed to incubate at 37 ^{o}C for 5 h. The resulting formazan crystals were dissolved with 100% DMSO and absorbance measured on a SPECTRAmax 340 microplate reader (Molecular Devices).

PAR1 surface expression

Breast carcinoma cells were labeled with the PAR1 polyclonal SFLLR-Ab, and a FITC-goat anti-rabbit Ab (Zymed) and quantified by FACS as previously described (115, 120, 134, 195).

Human Breast Cancer Xenograft in Nude Mice

All experiments were conducted in full compliance with the Institutional Animal Care and Use Committee of Tufts Medical Center. Female NCR nu/nu mice (Taconic Farms) each received mammary fat pat injections (cells suspended in

100 mL serum free RPMI with 20 µg/ml Matrigel) or tail-vein injections (cells suspended in 200 mL PBS). Vehicle (10% DMSO), P1pal-7 and FN439 are administered by subcutaneous injections (100 mL) every other day, and Taxotere is administered by intraperitoneal injections (100 mL) once a week unless otherwise indicated.

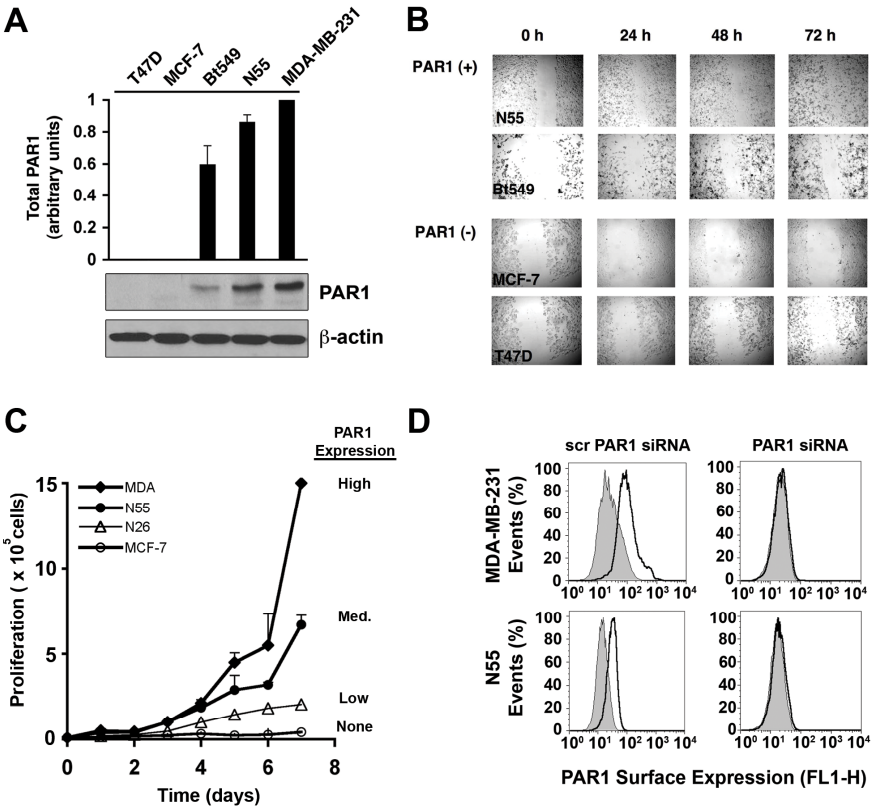
Tumor measurements: Tumor length (L) and width (W) were measured with a caliper and volume was calculated by the equation: $V = (L \times W^2)/2$. Images of xenograft tumors were taken using a Xenogen IVIS 200 Biophotonic Imager.

Histology: Formalin fixed tumors were sent to IDEXX Laboratories for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis. Formalin fixed lungs were paraffin embedded, sectioned at 3 representative depths along the coronal plane, and stained with hematoxylin and eosin at the Department of Pathology, Tufts Medical Center. Metastatic tumor nodules were counted throughout the entire lung section at all 3 depths under a light microscope. Microscopy images were captured with a light microscope and SPOT digital camera (Diagnostic Instruments).

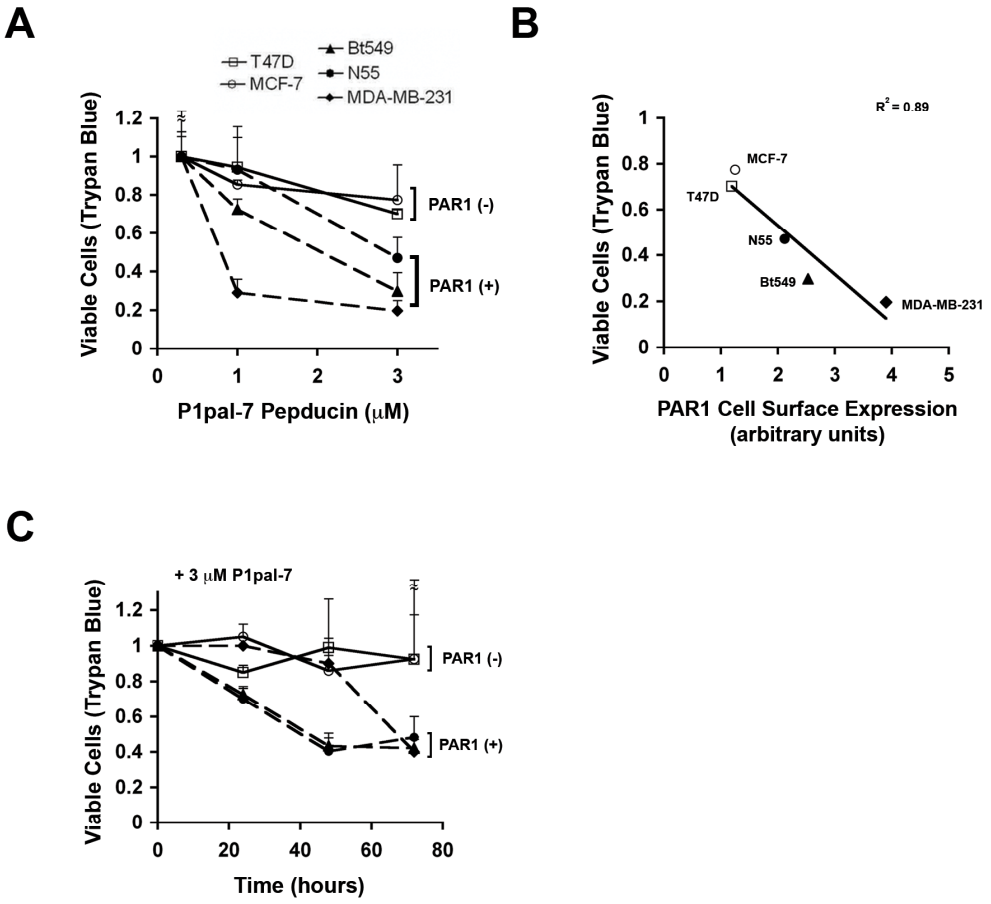
Statistical Analysis

All quantified xenograft and *in vitro* assay results are presented as mean ± s.d. or ± s.e.m. Comparisons were made with the Student's *t* test. Statistical significance was defined as * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

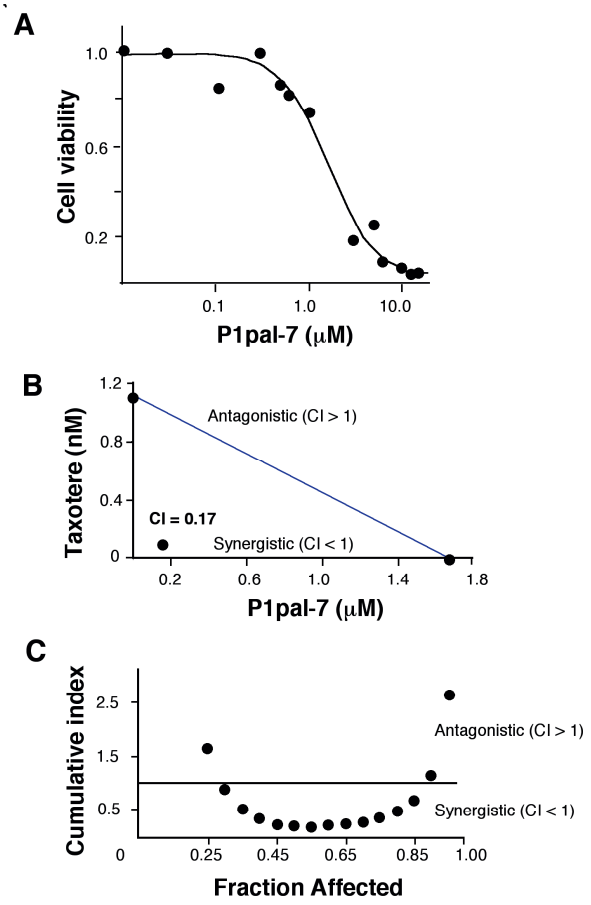
Supplemental Figures



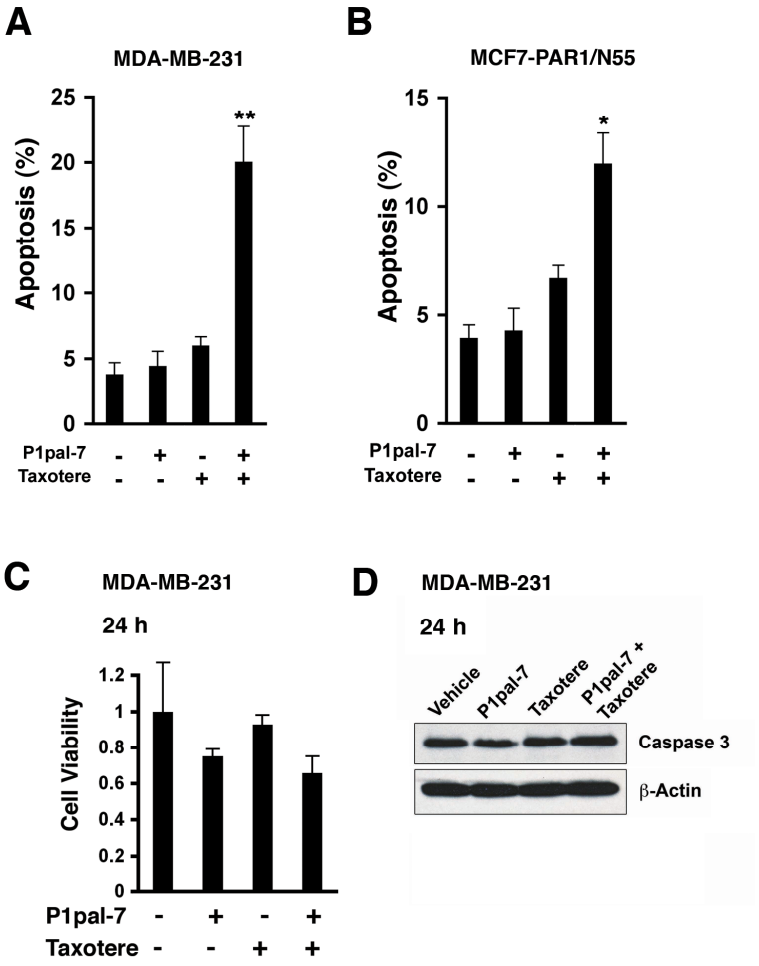
Supplemental Figure 2.1. ***(A)*** MDA-MB-231, MCF7-PAR1/N55, Bt549, MCF-7, and T47D cell lysates were analyzed with the western blot analysis. Bars represent densitometric measurements of PAR1 bands normalized to β-Actin (mean ± s.d.). ***(B)*** MCF7-PAR1/N55, Bt549, MCF-7, and T47D cells were grown to confluence and the cell monolayer was wounded with a pipette tip. Images of the wounded areas were collected at 0, 24, 48, and 72 h. ***(C)*** Proliferation of MDA MB-231, MCF7-PAR1/N55 and N26, and MCF-7 cells were counted under microscope for 7 days using a hemocytometer. ***(D)*** PAR1 expression was analyzed by flow cytometry in MDA-MB-231 and N55 cells after siRNA treatment. Representative data from multiple experiments are shown.



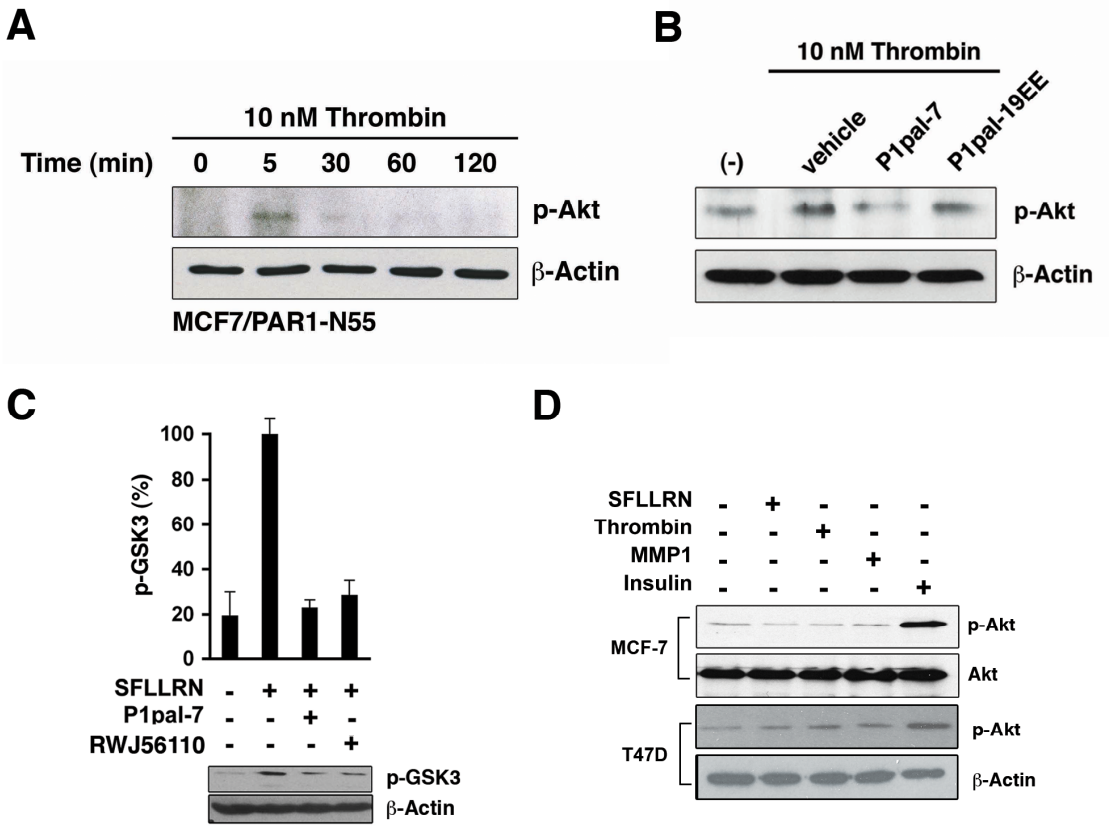
Supplemental Figure 2.2. ***(A)*** MDA-MB-231, N55, BT549, MCF-7 and T47D cells were treated with P1pal-7 pepducin at varying concentrations as indicated for 72 h and cell viability was evaluated by counting Trypan Blue stained cells with a hemocytometer. ***(B)*** Cell viability at 3 μM P1pal-7 was correlated with relative PAR1 expression (R = 0.89, P = 0.016). PAR1 expression was analyzed by flow cytometry. ***(C)*** MDA-MB-231, N55, BT549, MCF-7 and T47D cells were treated with P1pal-7 pepducin at 3 μM and cell viability was evaluated at 0, 24, 48, and 72 h by counting Trypan Blue stained cells with a hemocytometer. Representative data from multiple experiments are shown.



Supplemental Figure 2.3. ***(A)*** MDA-MB-231 cells treated with indicated concentrations of P1pal-7 were measured for cell viability using MTT assay. ***(B, C)*** Graphical representation of the CI analysis. $C_{P1pal\text{-}7,\ 50\%}$ and $C_{taxotere\ 50\%}$ are plotted with respect to $IC_{50,\ P1pal\text{-}7}$ and $IC_{50,\ taxotere}$. MDA-MB-231 cells were treated with P1pal-7 (0.03 - 15 μM) and Taxotere (0.01 - 300 nM) for 72 h and evaluated for cell viability by the MTT assay. $CI_{P1pal\text{-}7/taxotere}$ calculation. $C_{P1pal\text{-}7,\ 50\%}$ and $C_{taxotere\ 50\%}$ are the concentrations of P1pal-7 and taxotere used in combination to inhibit viability by 50% (IC_{50}). $IC_{50,\ P1pal\text{-}7}$ and $IC_{50,taxotere}$ are concentrations of drugs given individually to achieve 50% viability (IC_{50}). A CI value < 1 indicates synergy, = 1 indicates additive interaction and > 1 indicates antagonism.

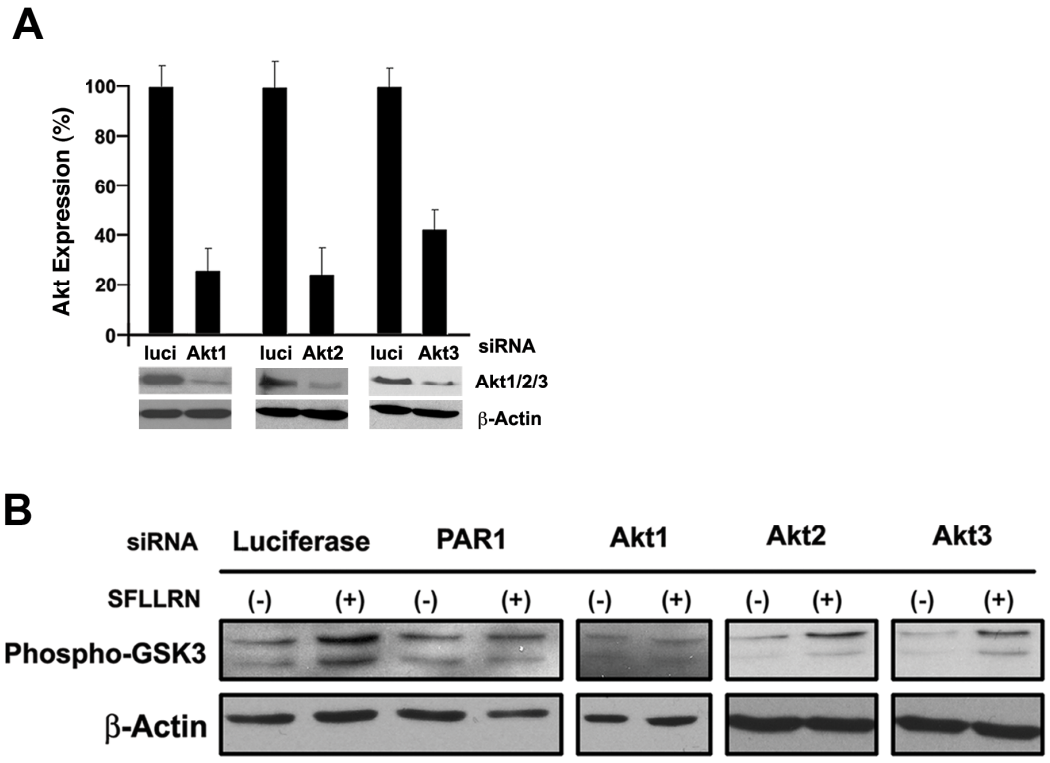


Supplemental Figure 2.4. ***(A)*** MDA-MB-231 and ***(B)*** MCF7-PAR1/N55 cells were treated with 5 μM P1pal-7, 100 nM taxotere or both and incubated overnight (18 h). Cells were labeled with FITC conjugated pan-caspase inhibitor and detected by flow cytometry. ***(C)*** MDA-MB-231 cells were treated with 1 μM P1pal-7, 0.3 nM taxotere, or both, incubated for 24 and evaluated for cell viability by the MTT assay. ***(D)*** MDA-MB-231 cells were treated as in (C). Lysates were immunoblotted with anti-Caspase 3. β-Actin was used as loading control. Representative data from multiple experiments are shown.

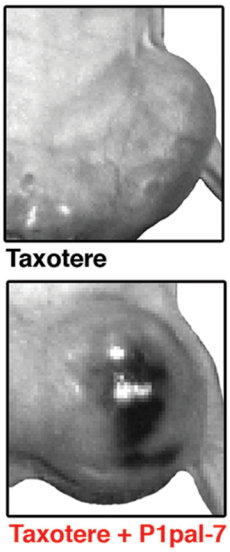


Supplemental Figure 2.5. ***(A)*** MCF7-PAR1/N55 cells were starved overnight in serum free medium and stimulated with 10 nM Thrombin over a period of 2 hours. Cell lysates were immunoblotted with anti-phospho-Akt (Ser473). β-Actin was used as loading control. ***(B)*** MDA-MB-231 cells were pre-treated with 3 μM P1pal-7 and 3 μM P1pal-19EE and subsequently stimulated with 10 nM thrombin. Cell lysates were immunoblotted with anti-phospho-Akt (Ser473). β-Actin was used as loading control. ***(C)*** MDA-MB-231 cells were pre-treated with 3 μM P1pal-7 and 5 μM RWJ5611 and subsequently stimulated with 10 μM SFLLRN. Cell lysates were analyzed with the western blot analysis. Bars represent densitometric measurements of phospho-GSK3 bands normalized to β-Actin (mean ± s.d.). ***(D)*** MCF-7 and T47D cells were starved overnight in

serum free medium and stimulated with 0.3 nM MMP-1, 10 nM Thrombin, 10 μM SFLLRN activating peptide or 100 nM insulin. Cell lysates were immunoblotted with anti-phospho-Akt (Ser473). Total Akt or β-Actin was used as a loading control. Representative data from multiple experiments are shown.



Supplemental Figure 2.6. ***(A)*** MDA-MB-231 cells were transfected with siRNA against Luciferase, Akt1, Akt2, and Akt3 and were immunoblotted with antibodies against Akt1, 2, and 3 for siRNA efficiency. β-Actin was used for loading control. ***(B)*** MDA-MB-231 cells were transfected with siRNA against Luciferase, PAR1, Akt1, Akt2, and Akt3 and were subsequently stimulated with 10 μM SFLLRN for 1 h. Cell lysates were immunoblotted with phospho-GSK3 to analyze Akt activity. β-Actin was used for loading control. Representative data from multiple experiments are shown.



Supplemental Figure 2.7. Ventral aspect of representative tumors immediately prior to experiment termination.

CHAPTER 3: Investigate the role of PAR1 expression in epithelial mesenchymal transition (EMT)

A manuscript with the title: “Protease-activated receptor-1 and epidermal growth factor receptor cooperatively facilitate epithelial to mesenchymal transition in human breast carcinoma cells” has been prepared from the findings in this chapter.

Introduction

Tumor invasion and metastasis is the greatest cause of mortality in breast cancer patients, and current therapeutic regimens are unlikely to result in complete remission (165). The invasion-metastasis cascade is a hallmark of cancer and results in the delivery of carcinoma cells to distant sites through a number of distinct cellular initiatives (20, 196).

Epithelial-Mesenchymal Transition (EMT) is a process critically involved in embryogenesis, wound-healing, and metastatic competence of tumor cells (197). Epithelial cells form polarized layer structures adjoined by specialized cellular junctions. In contrast, mesenchymal cells do not form organized structures and are highly motile. EMT, therefore, is a process in which epithelial cells take on a mesenchymal phenotype through a controlled set of transcriptional events and gain the ability to roam away from its tightly regulated niche. The seminal event in EMT is the loss of E-cadherin, a homotypic adherens junction molecule ubiquitously expressed in epithelial cells (20, 198). E-cadherin expression can be lost through a variety of genetic and epigenetic mechanisms, including the up-regulation of transcriptional repressors such as SNAI1, SNAI2/SLUG , ZEB1, and ZEB2/SIP1 (199-202). In addition to its functional role in cell junction formation, E-cadherin partitions b-catenin to the cytosol. Once E-cadherin expression is lost, b-catenin is free to translocate to the nucleus and mediate transcriptional events orchestrating the EMT program (203). Other transcription factors such as TWIST1, FOXC2, and NFkB are also implicated in the EMT program, and are

possibly linked to signaling pathways activated by FGF, PDGF, EGF, TGFb, BMP, and Wnt (56, 204, 205).

EMT is fully characterized by the loss of epithelial structural/adhesion proteins, such as cytokeratin, desmoplakin, zona occludens and claudin, replaced by their mesenchymal counterparts, N-cadherin, OB-cadherin, integrin, vimentin, fibronectin, laminin, and smooth muscle actin (198). After a complete epithelial-to-mesenchymal transition, cancer cells obtain a fibroblast-like phenotype, newly equipped with the ability to invade and resistance to apoptosis. Inhibition of EMT, is therefore, an attractive approach to cancer therapy that can prevent metastasis and reduce therapeutic resistance. The myriad of signaling pathways involved in EMT makes identifying an effective target a challenging task, and resolution of the critical molecular mechanisms underlying EMT is of great importance.

Protease Activated Receptor 1 (PAR1) is a pleiotropic G-protein coupled receptor over-expressed in invasive breast carcinoma but not in normal breast epithelium (109). We have recently demonstrated that forced over-expression of PAR1 enhances the invasive potential and tumorigenicity of a non-invasive, hormone-dependent breast carcinoma cell line (MCF-7) (115). The involvement of PAR1 in vasculogenesis during embryological development by regulating endothelial-mesenchymal transition has been recently been shown (206), but the role of PAR1 in cancer-related EMT has not yet been explored. In this report, we demonstrate the ability of PAR1 to induce EMT in MCF-7 cells. Interestingly, PAR1 expression was associated with a concerted shift in the ErbB receptor

expression profile, with significant up-regulation of EGFR and down-regulation of HER3 and HER4. We found that EGFR signaling is constitutively activated in MCF-7/PAR1 cells and is critically involved in mediating PAR1-induced EMT.

Results

PAR1 induces epithelial to mesenchymal transition in the MCF-7 breast carcinoma cell line. The MCF-7 breast carcinoma cell line represents an early-stage, hormone-sensitive (ER+) breast cancer model. We have previously demonstrated that MCF-7 cells stably expressing PAR1 (MCF-7/PAR1) display significant increases in migration, invasion, and tumorigenicity (115). Here, we correlate the invasive capacity of MCF-7/PAR1 cells with changes in cellular morphology. Consistent with the lack of motility, we observed that MCF-7 cells retain an epithelial morphology with clustered growth and significant cell-cell adhesion (Fig. 3.1*A*). In contrast, MCF-7/PAR1 cells obtained a spindle-shaped, fibroblast-like morphology with decreased cellular contacts. To further characterize the phenotypic differences, we labeled the cells for E-cadherin (epithelial adherens junction protein) and Vimentin (mesenchymal intermediate filament) and looked for changes associated with PAR1 expression (Fig. 3.1*B*). MCF-7 cells displayed a typical epithelial phenotype with E-cadherin localized at cell-cell junctions. We observed no expression of vimentin or PAR1. In association with PAR1 expression, we saw that that MCF7/PAR1 cells gained vimentin, which was polymerized into filamentous cytoskeletal structures. These cells further demonstrated a complete loss of E-cadherin expression both at the cell surface and in the cytoplasm.

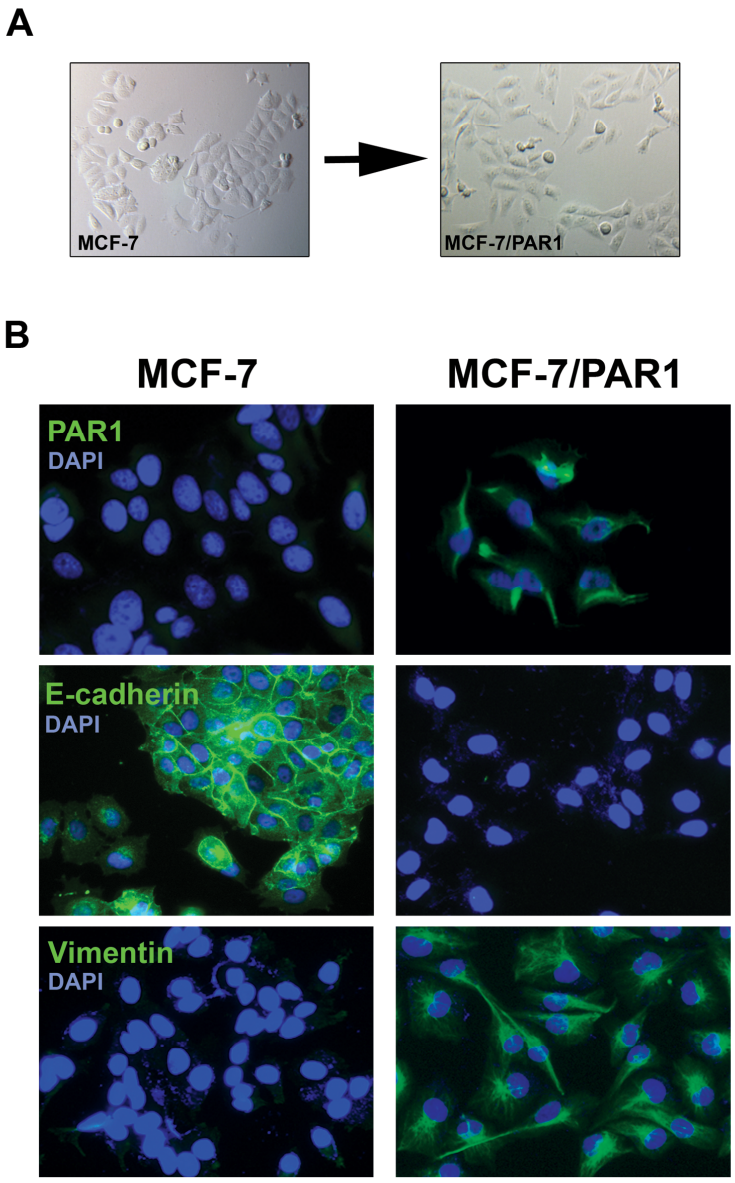


Figure 3.1. PAR1 over-expression in MCF-7 cells induces a mesenchymal phenotype. *(A)* Light microscopy images of MCF-7 cells and MCF-7/PAR1 cells (4X). *(B)* Immunofluorescence photomicrographs (40X) of MCF-7 and MCF-7/PAR1 cells stained for PAR1, E-cadherin, or Vimentin (green). Nuclear DNA was stained with DAPI (blue). Representative data from multiple experiments are shown.

Given the above changes in cellular phenotype and protein expression, we used the Human Genome U133A 2.0 Array (Affymetrix) containing an 18,400 probe set representing 14,500 human transcripts to investigate global changes in gene transcription in response to PAR1 expression (Fig. 3.2*A*). Hierarchical gene clustering by euclidean distance revealed that, of all transcripts analyzed, Vimentin experienced the greatest transcriptional induction (+3988-fold; $p < 1x10^{-3}$). Conversely, E-cadherin and Estrogen Receptor/Trefoil Factor demonstrated the greatest transcriptional reduction (-1383, -1681, and -3794-fold decrease, respectively; all $p < 1x10^{-3}$). Trefoil factor is a pro-angiogenic estrogen-responsive gene, and its down-regulation reemphasizes the loss of ER activity (207). The significant down-regulation of ER is in agreement with our recent finding that MCF-7/PAR1 cells are able to form tumors in the mammary fat pads of female nude mice without estrogen supplementation (115).

Overall, we observed here a remarkable transcriptional shift highly indicative of an epithelial-to-mesenchymal transition. In MCF-7/PAR1 cells, the loss of epithelial junctional proteins (E-cadherin [adherens junction], claudin 3/ zona occluden 3 [tight junction], desmoplakin [desmosome]) and epithelial intermediate filaments (cytokeratin 8/18/19) was accompanied by the gain of mesenchymal intermediate filament (vimentin), mesenchymal adhesion molecules (OB-cadherin, integrin a6), and stromal proteins (collagen, laminin, fibronectin) (Fig. 3.2*A*). Furthermore, transcriptional regulators of the EMT program, such as ZEB1 and SNAI2/SLUG are up-regulated, suggesting their functional relevance in PAR1-mediated EMT. The expression levels of other

prominent EMT regulators, such as ZEB2/SIP1, SNAI1, TWIST1, and NFkB, did not significantly change (data not shown; all microarray data discussed in this report will be deposited to NCBI gene expression and hybridization array data repository [GEO, www.ncbi.nlm.nih.gov/geo]).

Protein levels of representative EMT markers were also confirmed by western blot (Fig. 3.2*B*). Here, we analyzed the EMT status of 2 independent clones of MCF-7 cells stably expressing PAR1 (clone #2 was used for the genome array). The mesenchymal state of both clones indicates that the observed shift in transcriptional events is specific to PAR1 expression and not the artifact of a fortuitous gene-insertion event. In fact, we see that both clones display significant similarities with MDA-MB-231, a highly invasive breast cancer cell line expressing high levels of endogenous PAR1. Clones of MCF-7 cells stably transfected with inactive PAR1 mutatant (MCF-7/PAR1-310E) retain an epithelial phenotype, indicating that PAR1 activity is necessary for EMT induction (Supplemental Figure 3.1). Taken together, we have demonstrated here that the ectopic expression of PAR1 in MCF-7 cells induces a hormone-refractory, mesenchymal phenotype representative of advanced stage metastatic breast carcinoma.

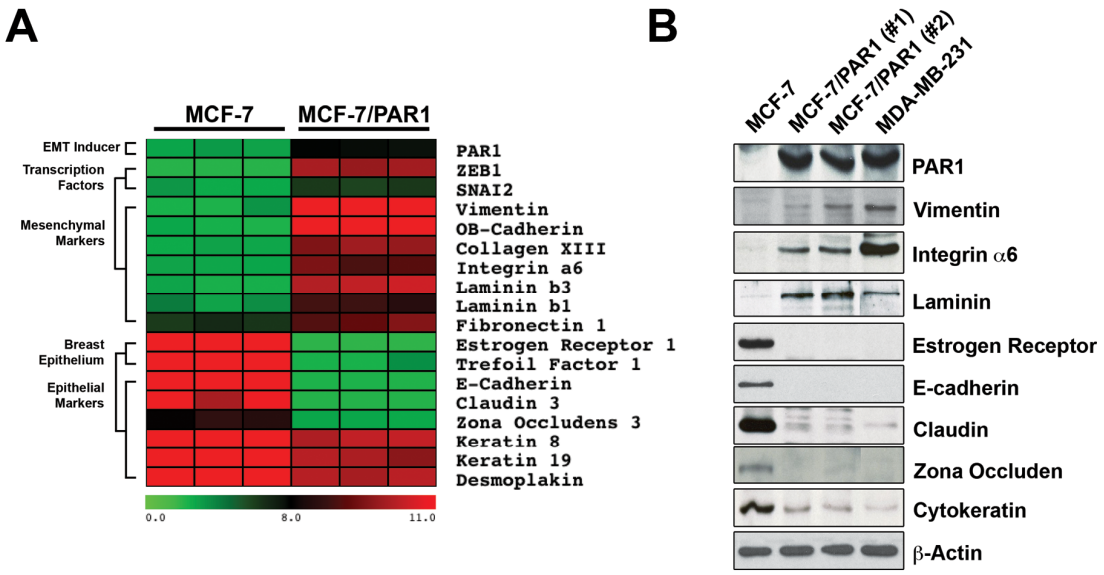


Figure 3.2. PAR1 induces epithelial to mesenchymal transition. *(A)* Gene expression profile of MCF-7 and MCF-7/PAR1 cells, maintained in phenol red free RPMI and 10% charcoal stripped FBS to prevent inadvertent ER stimulation. Cells were quiesced overnight in serum free media before RNA isolation. Purified total RNA was analyzed in triplicates using the Human Genome U133A 2.0 Array (Affymetrix) representing 14,500 human transcripts. Representative EMT-marker genes were selected after hierarchical clustering by euclidean distance. *(B)* MCF-7, MCF-7/PAR1 (Clones #1 and #2), and MDA-MB-231 cells were lysed and immunoblotted for PAR1, mesenchymal markers (vimentin, integrin α6, laminin) and epithelial markers (estrogen receptor α, E-cadherin, claudin 3, zona occludens-3 and cytokeratin 8/18). β-actin was used as loading control. Representative data from multiple experiments are shown.

PAR1 mediates a concerted shift in ErbB receptor and ligand expression. Advanced stage, hormone-refractory breast carcinoma are frequently associated with aberrant expression and activity of ErbB receptors (35). The ErbB receptors are a subclass of the receptor tyrosine kinase (RTK) superfamily and consists of ErbB1/EGFR, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4 which may homo- or heterodimerize upon ligand binding (34). Ligands of ErbB receptors include (1) EGFR specific ligands (EGF, TGFa, amphiregulin), (2) EGFR/HER4 dual specificity ligands (HB-EGF, epiregulin, betacellulin), and (3) HER3/HER4 ligands (neuregulin 1-4). HER2 has an extracellular domain not conducive to ligand binding and is considered an orphan receptor with innate propensity for heterodimerization (35). The ErbB signaling network is involved in the EMT program during cardiac valve formation (208).

We observed in MCF-7/PAR1 cells, a significant shift in the ErbB receptor/ligand expression profile (Fig. 3.3*A,B*). While MCF-7 cells expressed moderate levels of HER2, 3, and 4, MCF-7/PAR1 cells gained EGFR (+225-fold; $p < 1x10^{-4}$) and down-regulated HER3 (-384-fold; $p < 5x10^{-5}$) and HER4 (-88-fold; $p < 1x10^{-3}$). There was no significant change in HER2 (1-fold; $p = 0.5$) expression. Furthermore, up-regulation of EGFR/HER4 dual specificity ligands epiregulin (+184-fold; $p < 1x10^{-4}$) and HB-EGF (+111-fold; $p < 5x10^{-3}$) were observed. These results were confirmed at the protein level by western blot (Fig. 3.3*C*). Interestingly, we see here another striking similarity between MCF-7/PAR1 cells and the already mesenchymal MDA-MB-231 cells, possibly suggesting a PAR1-mediated induction of an ErbB signaling network with propensity for EMT. In fact,

amongst the NCI-60 panel of breast cancer cells, those expressing PAR1 (HS-578T, Bt-549, and MDA-MB-231) are phenotypically mesenchymal [vimentin (+), E-cadherin (-), fibroblast-like morphology] and also express EGFR. Conversely, PAR1-null cells (MCF-7 and T47D) are epithelial [vimentin (-), E-caherin (+), rounded morphology with extensive cell-cell contacts] and lack EGFR (Fig. 3.3*D*). Finally, P1pal-7 pepducin mediated blockade of PAR1 activity (115, 120, 157) reduces EGFR protein levels, implicating PAR1 in the regulation of EGFR expression (Fig. 3.3*E*).

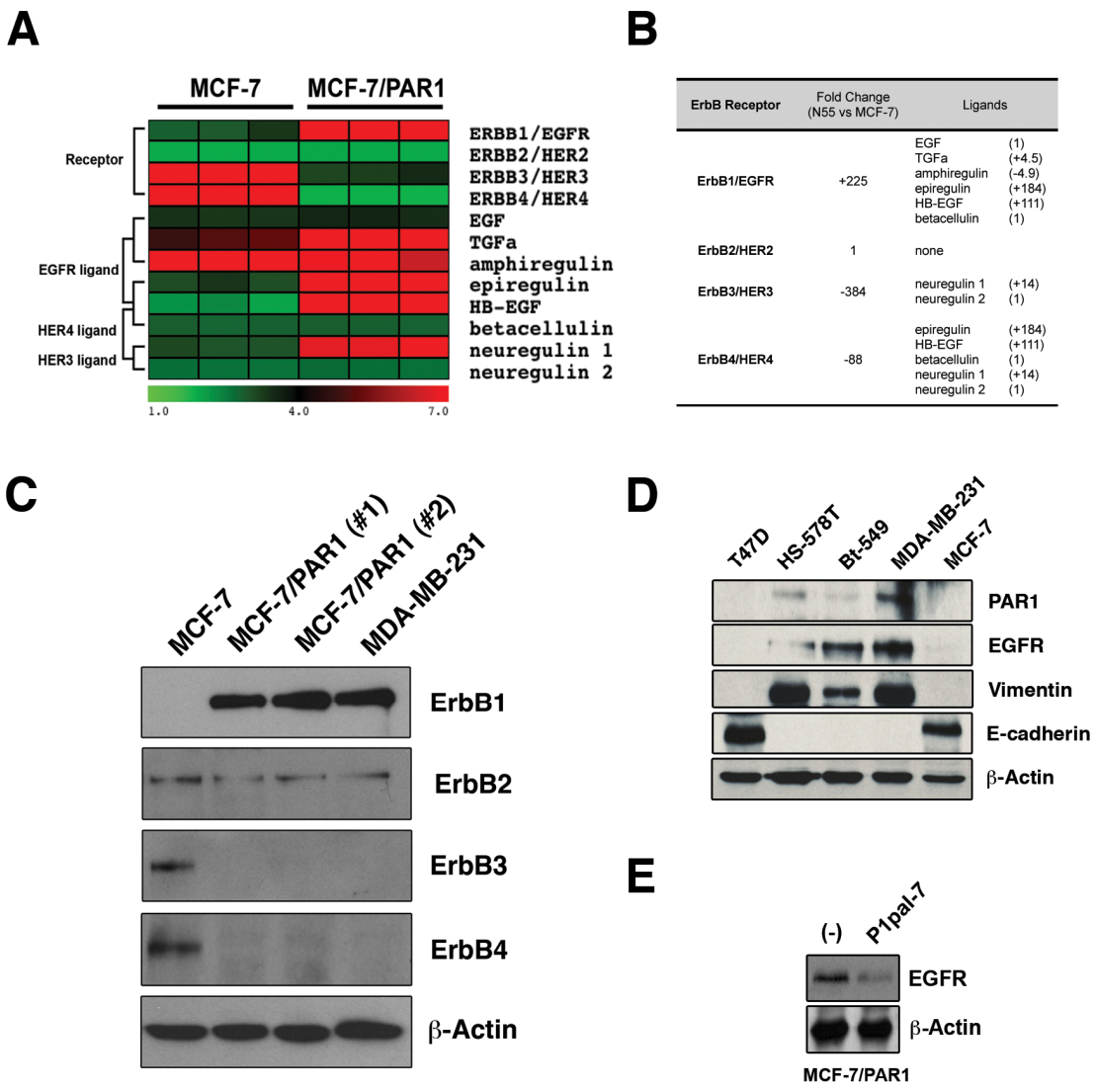


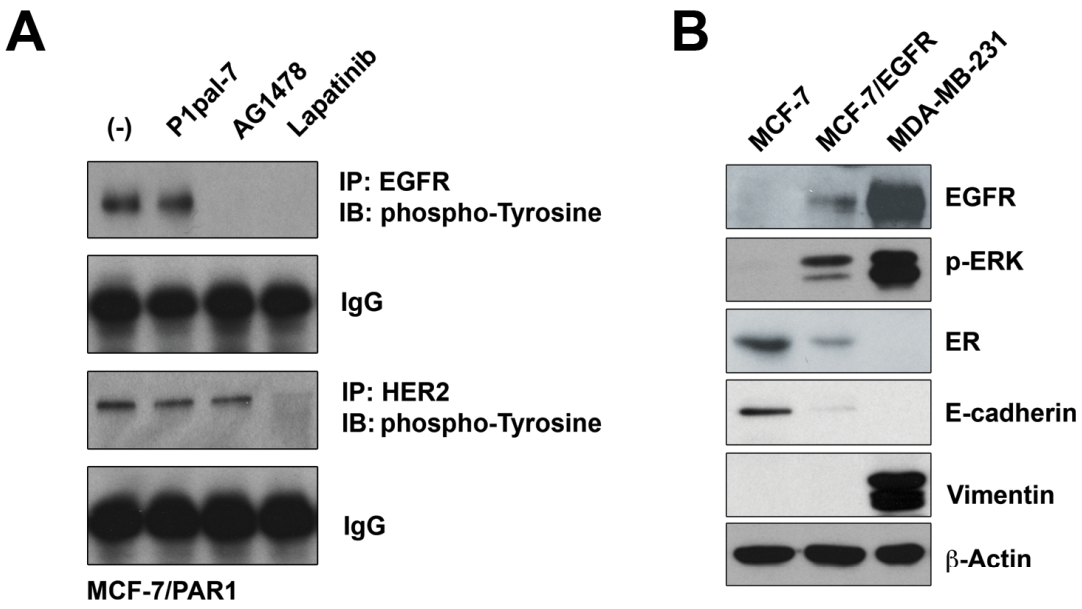
Figure 3.3. PAR1 mediates a concerted shift in ErbB receptor and ligand expression. *(A, B)* Expression profile of ErbB receptors and ligands in MCF-7 and MCF-7/PAR1 cells analyzed by the Human Genome U133A 2.0 Array (Affymetrix). *(C)* MCF-7, MCF-7/PAR1 (Clones #1 and #2), and MDA-MB-231 cells were lysed and immunoblotted for ErbB1/EGFR, ErbB2/HER2, ErbB3/ HER3, ErbB4/HER4. *(D)* NCI-60 panel of breast carcinoma cells were lysed and

immunoblotted for PAR1, EGFR, vimentin, and E-cadherin. *(E)* MCF-7/PAR1 was treated with P1pal-7 (3 μM) or vehicle (0.2% DMSO) for 5 days. Cells were lysed and immunoblotted for EGFR. β-actin was used as loading control for panels C-E. Representative data from multiple experiments are shown.

PAR1 and EGFR cooperatively promote EMT. The above results demonstrate the over-expression of EGFR and shedding of HER3/HER4 in MCF-7/PAR1 cells. Here, we tested if this shift in ErbB profile has optimized the cells for constitutive EGFR and HER2 signaling. Under serum-starved conditions, we see that both EGFR and HER2 are phosphorylated at tyrosine residues (Fig. 3.4*A*), indicating sustained receptor activity in the absence of exogenous stimulus. EGFR tyrosine phosphorylation is reversible by the tyrphostin AG1478 compound, a selective EGFR tyrosine kinase inhibitor (TKI) (209) and lapatinib, an EGFR/HER2 dual TKI (210), but is not sensitive to PAR1 antagonism by P1pal-7. HER2 tyrosine phosphorylation is reversible by lapatinib but not by AG1478, suggesting the independence of HER2 activity from EGFR tyrosine kinase activity.

Given the significant up-regulation and constitutive activity of EGFR in MCF-7/PAR1 cells, we then investigated the extent to which EGFR alone can promote EMT in MCF-7 cells. Over-expression of EGFR in MCF-7 cells (MCF-7/EGFR) resulted in the activation of the MAPK cascade, as observed by ERK1/2 phosphorylation and a concomitant decrease in ERa and E-cadherin (Fig. 3.4*B*). The ErbB/MAPK cascade has previously been shown to down-regulate ERa in hormone-sensitive breast carcinoma cells (211, 212). Furthermore, unliganded ERa has been established as a transcriptional activator of E-cadherin and its obstruction leads to E-cadherin promoter methylation and suppressed expression (213). Our result here integrates the findings above and demonstrates the EGFR/MAPK cascade as a potent mediator of a partial EMT-like response by modulation of unliganded ERa activity.

The reduction of E-cadherin/adherens junction in MCF-7/EGFR cells manifests by the scattered growth of cells at sub-confluence as compared to MCF-7 cells, which tend to grow in tight clusters (Fig. 3.4*C*). EGFR expression alone, however, is not sufficient in conferring neither fibroblast-like morphology (Fig. 3.4*C*) nor vimentin expression (Fig. 3.4*B*) as seen in MCF-7/PAR1 cells. In fact, vimentin, but not ERa or E-cadherin, appears to be under direct PAR1 regulation, since short-term PAR1 expression by transient transfection induced vimentin expression in MCF-7 cells (Fig. 3.4*D*). Conversely, P1pal-7 down-regulated vimentin in MCF-7/PAR1 cells, further suggesting the regulatory role of PAR1 on vimentin expression (Fig. 3.4*E*). As expected, EGFR obstruction by AG1478 does not alter vimentin expression. E-cadherin is not regained after AG1478 treatment, signifying the involvement of epigenetic modifications as a result of ERa down-regulation (213). Together, the above results indicate that PAR1 and EGFR cooperatively regulate the complete EMT program to induce a hormone-refractory, invasive phenotype in MCF-7 cells.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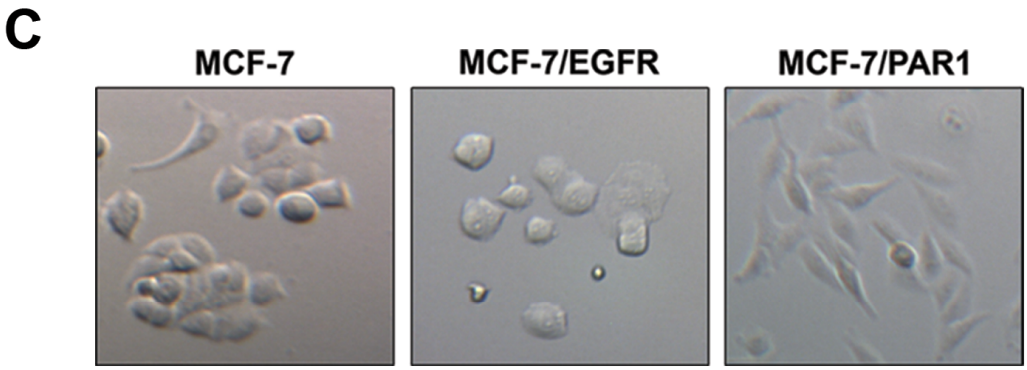
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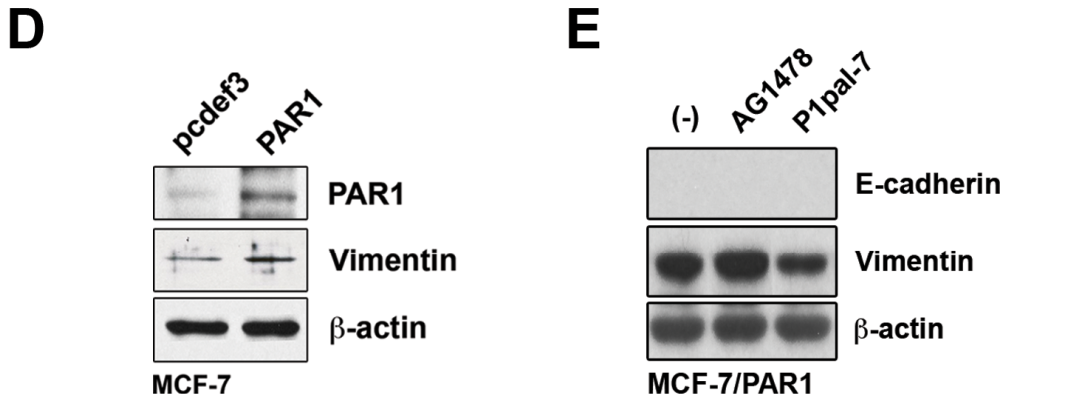
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β-actin
MCF-7/PAR1

Figure 3.4. Constitutively active EGFR is an integral mediator of PAR1-induced EMT. *(A)* MCF-7/PAR1 cells were treated overnight with vehicle (0.2% DMSO), P1pal-7 (3 μM), AG1478 (5 μM), or Lapatinib (5 μM). EGFR or HER2 was immunoprecipitated and probed for phospho-Tyrosine. IgG band was used as loading control. *(B)* Total lysates of MCF-7, MCF-7/EGFR, and MDA-MB-231 cells were immunoblotted for EGFR, phospho-ERK1/2, ERα, E-cadherin, and vimentin. β-actin was used as loading control. *(C)* Light microscopy images of MCF-7, MCF-7/EGFR, and MCF-7/PAR1 cells (4X). *(D)* MCF-7 cells were transiently transfected with vector (pCDEF3) or PAR1. 48 hours after transfection, cells were lysed and immunoblotted for PAR1, ER, E-cadherin or Vimentin. β-actin was used as loading control. *(E)* MCF-7/PAR1 cells were treated 3 days with vehicle (0.2% DMSO), AG1478 (5 μM), or P1pal-7 (3 μM). Cells were lysed and immunoblotted for E-cadherin and Vimentin. β-actin was used as loading control. Representative data from multiple experiments are shown.

Discussion

PAR1 is an oncogenic protein clinically associated with invasive breast carcinoma and characterized as a potent inducer of cancer cell migration, invasion, survival and metastasis (109, 115). In this study, we have demonstrated that PAR1 mediates its oncogenicity at least in part through the ability to engage the EMT program. PAR1 expression in MCF-7 induced a sustained EMT response, in which epithelial proteins were extensively down-regulated and mesenchymal proteins were acquired. PAR1 pleiotropically signals through 3 G proteins (G_a, G_q and $G_{12/13}$) and can engage multiple transcriptional pathways (76), although those precisely involved in EMT have yet to be explored. Transcriptional regulators of EMT, Zeb1 (202) and SNAI2 (200) were both up-regulated in MCF-7/PAR1 cells, indicating their functional relevance. The pathological manifestations of such transcriptional shifts were observed in our previous study in which MCF-7/PAR1 cells obtained hormone-independent *in vivo* tumor growth and ability to invade into surrounding tissue (115).

We have also demonstrated a PAR1 associated shift in ErbB receptor/ligand expression profile. The expression of EGFR, HER2, HER3 and their cognate ligands are generally correlated with advanced disease in patients and an aggressive cellular phenotype in tissue culture (35). The role of HER4 in cancer remains elusive, although the current body of work suggests an anti-proliferative role, with likely involvements in breast tissue differentiation (39, 40). The net outcome of ErbB signaling depends on the combination of receptors

expressed and ligands available, making the ErbB network highly versatile and vastly modifiable. In fact, past work has shown that a growth-inducing neuregulin signal can be transformed into a differentiative signal by HER2 down-regulation in breast carcinoma cells (214). With PAR1 expression, we observed an up-regulation of EGFR and a concomitant down-regulation of HER3 and HER4. In the context of dual-specificity ligand up-regulation (epiregulin and HB-EGF), we speculate that HER4 down-regulation was necessary for optimal EGFR signaling. Indeed, ectopic HER4 expression in MDA-MB-231 cells decreased phosphorylated ERK1/2 levels (Fig. S2), suggesting an antagonistic role of HER4 against the EGFR/MAPK cascade.

Allying to further amplify ErbB activity is the ability of GPCRs to transactivate RTKs. Specifically, PAR1 is able to transactivate EGFR and HER2 by a metalloprotease-dependent release of membrane bound HB-EGF (34, 70, 124, 148). The ErbB receptor/ligand expression profile and EGFR/HER2 constitutive activity suggest that the MCF-7/PAR1 cells may also be primed for GPCR/RTK cross-talk. Constitutive ErbB activity is also possible as a direct consequence of over-expression and the increased probability of spontaneous receptor dimerization (41, 42).

The presence of EGFR is often associated with an ER-negative phenotype in breast carcinoma cells. Acquisition of tamoxifen resistance in MCF-7 cells induces augmentation of the EGFR signaling pathway and EMT-like behavior (215). Conversely, hyper-activation of the EGFR/MAPK cascade has been repeatedly shown to down-regulate ERa expression (211, 216, 217).

Furthermore, the loss of unliganded-ERa activity has been shown to restrict E-cadherin transcription by epigenetic modifications (213). Together, our results and the results of others underscore the significance of an EGFR/ ERa mediated mechanism for the initiation of EMT.

In summary, we describe here, a novel approach taken by PAR1 to regulate EMT. The cooperative activity of PAR1 and EGFR to induce a fully mesenchymal phenotype suggests the utility of PAR1 and EGFR as prognostic markers for metastatic behavior and therapeutic resistance in breast cancer. Furthermore, our results provide mechanistic rationale for the use of a PAR1 inhibitor possibly in conjunction with EGFR antagonists for breast cancer therapy.

Materials and Methods

Reagents. N-palmitoylated peptide P1pal-7 (AA sequence: KKSRALF) was synthesized as described previously (153, 155, 157). AG1478 (Calbiochem), PD98059 (Cell Signaling), and Lapatinib (LC laboratories) were all dissolved in DMSO and stored at -20^{o}C.

Cell Culture. Breast carcinoma cell lines MCF-7, MDA-MB-231, T47D, Bt-549, HS-578T were all obtained from the National Cancer Institute. MCF-7 cells stably expressing PAR1 (MCF-7/PAR1) were generated in our laboratory as previously described (115, 120). Transient transfections were done using polyethylenimine 25kD linear (Polysciences). MCF-7/EGFR and MDA-MB-231/HER4 were generated by infecting with retroviruses (Phoenix retrovirus expression system, Orbigen, Inc.) and selected with 1-2 mg/mL puromycin as previously described (218). pBABE-EGFR was obtained from Addgene (Plasmid #11011 (219)) and pBABE-puro, and pBABE-HER4 were generous gifts from Dr. Nancy Hynes at Friedrich Miescher Institute. All cells were maintained in RPMI1640 or DMEM (supplements: 10% FBS, 1% penicillin/streptomycin) in 5% CO_2 at 37^{o}C.

Western Blot Analysis and Immunoprecipitation. Cells were washed in ice-cold PBS and lysed in T-PER (tissue protein extraction reagent) supplemented with Halt protease and phosphatase inhibitor cocktail (all from Pierce). Protein content was measured by Bradford Assay. 30-50 mg protein was resolved on

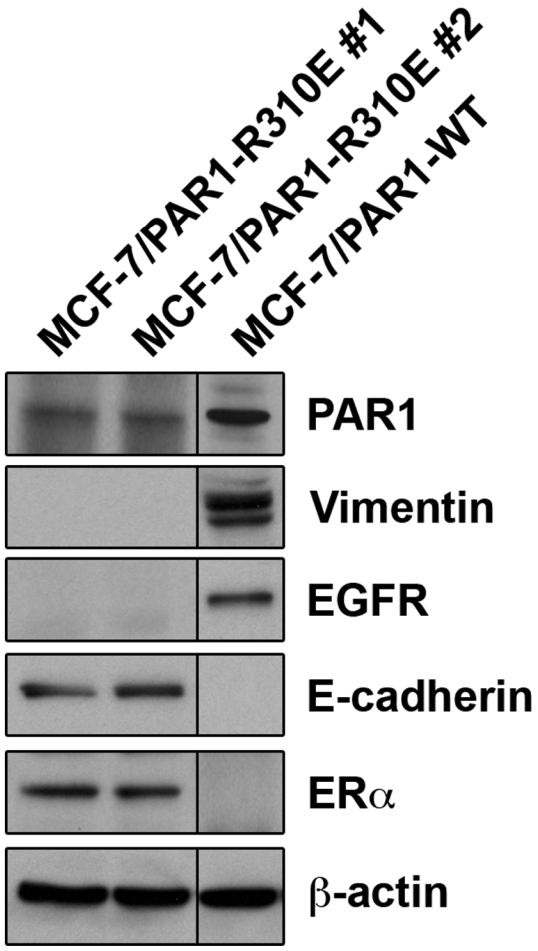
12% SDS-PAGE and transferred to nitrocellulose membranes. For immunoprecipitation, 2 mg of antibody (EGFR or HER2) was conjugated to Protein A sephrose beads (GE Healthcare) and incubated with cell lysate (300-500 mg protein) overnight at 4^{o}C. Beads were washed 3 times with lysis buffer and incubated at 95 ^{o}C for 5 minutes with sample buffer. After centrifugation, the supernatant was resolved on 12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in TBST (0.1% Tween-20 in TBS) and incubated overnight with the following antibodies: PAR1-ATAP2, EGFR, ER, Laminin (Santa Cruz), Vimentin V9, HER2 Ab-17 (Thermo Scientific), HER3, HER4, phospho-Tyrosine, E-cadherin, Integrin a6, Keratin 8/18, phospho-ERK1/2, total ERK1/2, b-actin (Cell Signaling), Claudin 3 (Abcam), Zona Occluden-3 (Chemicon).

Immunofluorescence Analysis. Cells were cultured on 8 chamber polystyrene vessel tissue culture treated glass slides (BD Falcon) and fixed with 4% formaldehyde, permeablized with 0.1% Triton X-100, and blocked with 1% BSA for 30 minutes. Cells were stained with PAR1-ATAP2, Vimentin, or E-cadherin and subsequently with Alexa Fluor 488 (Molecular Probes). Slides were washed and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) and visualized by fluorescence microscopy and image-captured by SPOT digital camera (Diagnostic Instruments).

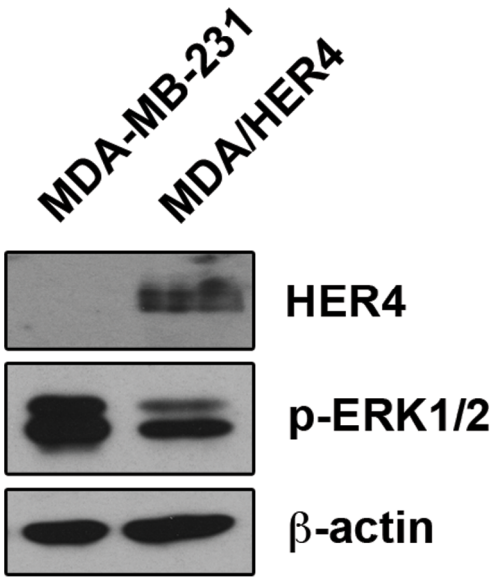
Affymetrix Microarray and Data Analysis. The microarray analysis was carried

out at the Genomics Core of the Tufts Center for Neuroscience Research, Tufts University School of Medicine. MCF-7 and MCF-7/PAR1 cells were quiesced overnight in serum-free media and RNA was isolated using the RNEasy kit (Qiagen). Target RNAs from each cell line (MCF-7, MCF-7/PAR1; n = 3) were fragmented and independently hybridized to Human Genome U133A 2.0 Array (Affymetryx). Genechips were then washed and stained with Streptavidin R-phycoerythrin (Molecular Probes) and following washes, were scanned using the GeneChip scanner. Data analysis was conducted using the Bioconductor suite of programs. The 3'/5' RNA degradation plot was used to confirm the quality of the RNA samples hybridized to microarrays. Background correction, normalization and summarization of the raw probe intensities were carried out using the GCRMA protocol with default options. LIMMA module and topTable function was used to generate the list of differentially expressed genes. TIGR Multiexperiment Viewer (MeV) was used for statistical analysis and heatmap production.

Supplemental Figures



Supplemental Figure 3.1. MCF-7/PAR1-R310E clones #1 and #2, and MCF-7/PAR1 cells were lysed and immunoblotted for PAR1, EGFR, vimentin, E-cadherin, and ERa. β-actin was used as loading control.



Supplemental Figure 3.2. MDA-MB-231/HER4 cells were lysed and immunoblotted for HER4 and phospho-ERK1/2. β-actin was used as loading control.

CHAPTER 4: Discussion

PAR1 signaling in breast tumor survival and metastasis

We have recently demonstrated that PAR1 expression promotes migration and invasion, and that the MMP1/PAR1 cascade promotes tumorigenesis in breast cancer models (115). In this study, we found that PAR1 expression enhances survival and proliferation of breast carcinoma cells, but concomitantly makes them susceptible to pepducin mediated PAR1 blockade. Further validation of PAR1 as a viable therapeutic target emerged as we discovered the ability of PAR1 pepducins to chemosensitize aggressive breast carcinoma cells to taxotere. The synergistic interaction of P1pal-7 and taxotere were observed in both *in vitro* assays and in a xenograft animal model. Finally, we demonstrated that the blockade of the MMP1/PAR1 pathway significantly attenuates breast cancer metastasis to the lung.

Combination Therapy

Combining chemotherapeutic drugs is a strategy applied in cancer therapy in order to achieve increased patient response and to overcome drug resistance. Furthermore, because of the synergistically enhanced therapeutic effects, doses of the individual drugs may be lowered to attenuate the morbidity caused by the side effects. Ideally, two drugs acting on distinct biochemical pathways are chosen to prevent antagonistic competition or overlap between the drug mechanisms. Recent clinical trials demonstrate that combining targeted inhibitors

of oncogenic proteins such as HER2 (trastuzumab) and Bcr-Abl (imatinib mesylate) with traditional cytotoxic agents such as taxanes or antimetabolites, are extremely effective with much higher rates of patient response (220, 221). The successes of these trials provide proof-of-principle that targeted combination therapy is a viable approach in battling malignancies. However, current combination therapies are not impenetrable to the heterogeneity and resilience of human cancer, and it still leaves a significant population of unresponsive patients needing alternate therapy. Given the heterogeneous nature of cancer, there is a concerted effort to identify novel oncogenic targets that can enhance chemotherapeutic vulnerability to resistant disease. We demonstrated in this study the potential of a novel combination therapy involving PAR1 blockade in the context of taxotere administration.

Effects on Angiogenesis

The efficacy of P1pal-7 and taxotere given together was clearly observed by significantly smaller tumors and later confirmed by biochemical studies indicating increased levels of apoptosis and decreased Akt activity. The cytotoxicity of P1pal-7 is ostensibly due to the blockade of the MMP1/PAR1/Akt survival cascade of the tumor, however, its effect on the tumor microenvironment has not been explored. PAR1 expression in prostate carcinoma cells has been shown to increase VEGF expression and promote tumor angiogenesis (74). Furthermore, both thrombin (86) and MMP-1 (122) has been shown to activate

endothelial cells through PAR1. Therefore, it seems that PAR1 induces tumor vascularization by direct stimulation of endothelial cells (MMP-1/Thrombin) and through an indirect paracrine mechanism (VEGF). Accordingly, by blocking PAR1 with P1pal-7, we may be attacking both tumor cells and endothelial cells recruited to the tumor stroma. This scenario is strongly supported by our recent observation that P1pal-7 administration has significant impact on angiogenesis and ascites formation in ovarian cancer xenograft models (157). The relative impact of targeting either cell types is unclear, and it may be interesting to compare the effects of specifically attacking endothelial cells with avastin (anti-VEGF monoclonal antibody) versus attacking both tumor and endothelial cells with P1pal-7. Alternately, syngeneic transplantation of EO 771 murine breast adenocarinoma cells (transformed with PAR1) (222), into $PAR1^{-/-}$ C57/BL6 mice (223) would model a system in which only tumor cells are susceptible to PAR1 antagonism. Endothelial cells, unlike tumor cells are genetically stable and are not prone to mutations. Therefore, if P1pal-7 is indeed attacking both tumor and endothelial cells, resistance to therapy may be slower to develop. In this study, we have demonstrated that PAR1 antagonism has cytotoxic effects on breast tumor cells, sensitizes tumors to taxotere chemotherapy, and prevents lung metastasis by monotherapy. Taken together with our speculations about the anti-angiogenic effects of P1pal-7, the discoveries made here strongly validate PAR1 as a desirable drug target for breast cancer therapy.

MMP-1 vs. Thrombin

We have demonstrated here that both MMP-1 and Thrombin engage the PAR1/Akt survival pathway, however the distinct roles of the two proteases remain unclear.

In MDA-MB-231 cells, we observed that thrombin quickly activates Akt and phosphorylation is rapidly reversed by 2 hours. In contrast, MMP-1 activation of Akt revealed slower kinetics, with Akt phosphorylation peaking at 1 hour. The differences here may be accounted by the lack of the hirudin-binding region on MMP-1. While thrombin binds PAR1 through the hirudin-like sequence and gets positioned optimally for tethered ligand cleavage, MMP-1 relies heavily on the fortuitous orientation of its catalytic domain and the PAR1 exodomain. Furthermore, the tethered ligand created by thrombin is SFLLRN whereas the one created by MMP-1 is PRSFLLRN (136). The precise differences in signal transmission through these distinct tethered ligands are not well characterized in cancer cells. A recent study by Balckburn *et al.* explored the differential effects of MMP-1 and thrombin on gene expression in endothelial cells and their results suggest that MMP-1 and thrombin induce overlapping, but distinct subsets of pro-angiogenic genes (182). The two proteases act through complementary mechanism to facilitate angiogenesis, and are not necessarily redundant by virtue of their common receptor, PAR1.

Interestingly, work by Hu *et al*. demonstrated that thrombin blockade with hirudin does not affect growth of established tumors, but is able to inhibit spontaneous metastasis (224). We have demonstrated that blockade of PAR1 or

MMP-1 significantly attenuates tumor growth (115) and pulmonary metastasis. This discrepancy may be attributed to the different roles of thrombin and MMP-1 in xenograft models. In the primary tumor environment, MMP-1 generated by the tumor stroma or the tumor itself may play a dominant role in activating PAR1 and hence, blocking MMP-1/PAR1 results in attenuated tumor growth. However, in the blood stream, thrombin may be of greater significance due to its enhanced availability. Many tumors cells, including MDA-MB-231 express tissue factor at their surface (129), and have the potential of activating the coagulation cascade once in the blood stream. The activated thrombin, then promotes PAR1 cleavage on the tumor cells, endothelial cells and platelets to enhance metastasis at distant sites. This is supported by the attenuation of experimental metastasis by hirudin treatments in mice models (110, 117, 224). Currently, a graduate student in our group (Caitlin Foley) is working to uncover these differences by creating carcinoma cell lines expressing PAR1 mutants that are cleaved specifically by thrombin (unresponsive to MMP-1) or by MMP-1 (unresponsive to thrombin).

PAR1 expression induces EMT

The correlation of PAR1 with invasive breast carcinoma and its ability to promote migration and invasion has been well documented (109, 115, 124). However, the precise molecular mechanism leading to cellular motility is not well characterized. We demonstrated here that the ectopic expression of PAR1 in MCF-7 breast carcinoma cells induces a morphological change indicative of

epithelial-to-mesenchymal transition (EMT). Transcriptional profiling by genechip analysis and confirmation of protein levels by western blot revealed that PAR1 expression promotes EMT in MCF-7 cells through an EGFR dependent mechanism. This observation is supported by empirical evidence from the NCI-60 panel of breast carcinoma cells, which demonstrate 100% correlation of PAR1 expression with EGFR expression and EMT status. Furthermore, in a recent publication, Hernandez et al. reported that 52% of patients with high-grade breast carcinoma co-expressed PAR1 and EGFR (225). They also reported that the expression of PAR1 is correlated significantly with increased metastasis and mortality. Together, our experimental observations and those from patient data suggest that the co-expression of PAR1 and EGFR may promote a significant selective advantage during cancer progression.

When over-expressed individually, neither PAR1 nor EGFR were able to promote complete EMT. Our results suggest that EGFR promotes the loss of epithelial properties (E-cadherin, Estrogen receptor), while PAR1 promotes the acquirement of mesenchymal properties (vimentin, fibroblast-like morphology). The induction of the EGFR/MAPK cascade has been associated with the loss of ER expression (211, 216, 217) and conversely, acquisition of tamoxifen resistance has been associated with EGFR signaling (215). The loss of ER, in turn, prevents E-cadherin expression and promotes EMT-like phenotypic changes (213). Therefore, our observations here with the MCF-7/EGFR cells recapitulate and integrate the findings of others. Whether or not PAR1 directly regulates EGFR and Vimentin transcription is unknown. While preliminary data

suggest that PAR1 blockade by P1pal-7 reduces EGFR and Vimentin levels, a more rigorous investigation of EGFR and Vimentin promoter activity in response to PAR1 regulated transcription factors is necessary. Of particular interest is PAR1 regulation of AP-1 transcription factor c-Jun (226), since the EGFR and Vimentin are both direct transcriptional targets of c-Jun (227-229).

The involvement of EMT mediating transcription factors in the PAR1/ EGFR mediated EMT needs further investigation. Genechip results demonstrate the up-regulation of 2 major transcriptional repressors of E-cadherin, Zeb1 (199) and SNAI2 (200). Both transcription factors are strongly implicated in EMT and are known to down-regulate a variety of structural proteins of the adherens junctions, tight junctions, and desmosomes. Thrombin activation of PAR1 has been shown to up-regulate TWIST (230), another master regulator of EMT (205), however, TWIST transcript levels did not change in MCF-7/PAR1 cells. NF-κB is another transcription factor profoundly involved in cancer progression (204), and is a down-stream mediator of PAR1 activity (143). Although NF-κB does not directly affect E-cadherin expression or EMT, its activity is associated with the up-regulation of EMT regulators such as SNAI2, Zeb1, and TWIST (204).

Constitutive Activity of PAR1

MCF-7/PAR1 cells were cultured in FBS-enriched media, but in the absence of PAR1 activating proteases. These growth conditions imply that the observed global shifts in gene transcriptional events are the result of constitutive

PAR1 activity. GPCRs exist in a dynamic equilbrium, switching between active and inactive states spontaneously. While agonist binding maximally stabilizes the active conformation, spontaneous periodic switching is sufficient in producing a basal level of G-protein activity (69, 139). Constitutive activity of PAR1 is implied from the general properties of GPCR, but has not been characterized specifically. PAR1 couples to $G\alpha_q$, $G\alpha_{12/13}$, and $G\alpha_i$, and intrinsic receptor activity can be measured by GDP/GTP exchange at the G proteins. The BODIPY FL-GTP consists of a fluorescent group attached to a GTP analog and the BODIPY fluorophore. Fluorescence is only observed after G-protein binding because under free conditions, BODIPY quenches GTP fluorescence (231). Such a compound can aid in following constitutive PAR1 activity and its response to pharmacological inhibition (P1pal-7) or genetic knockdown (siRNA, shRNA). Antagonists of PAR1, such as the RWJ and SCH compounds, target the ligand binding domain (e2) of PAR1 and can only inhibit ligand-mediated PAR1 activity. The unique properties of P1pal-7 allow it to block the receptor from the inside of the cell, making its inhibitory mechanism independent of ligand activation of PAR1. Therefore, pepducins may emerge as a highly efficacious, unique class of inhibitors that can inhibit PAR1 constitutive activity, making it highly desirable as a chemotherapeutic agent.

PAR1 mediated Gene Transcription

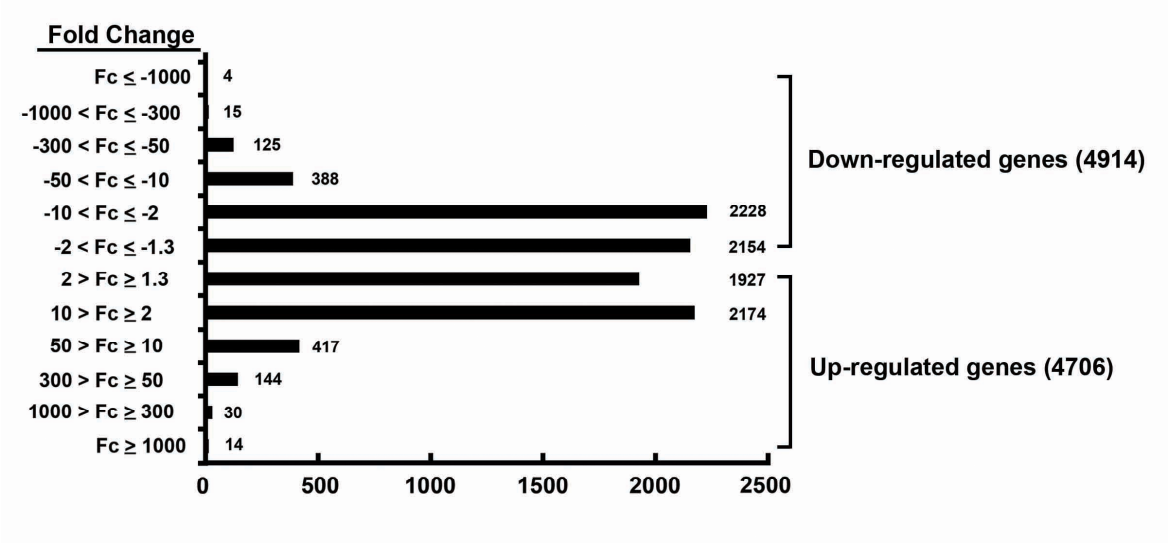


Figure 4.1. **Number of genes up/down-regulated by PAR1 expression shown as a distribution of fold changes.** Analysis of triplicate samples of MCF-7/PAR1 cells and MCF-7 cells were carried out. Mean changes in mRNA levels for individual genes were regarded significantly different if the *p* value was <0.05 and if the fold-change was greater or less than 1.3.

A microarray containing a total of 18,400 sets of probes representing 14,500 human transcripts was used to monitor changes in gene expression as a result of PAR1 expression. The salient discovery here was the major shift in gene transcriptional events indicating the occurrence of EMT. However, a total of 4914 genes were down-regulated and 4706 genes were up-regulated in MCF-7/PAR1-N55 cells when compared to the parental MCF-7 cell line (Figure 4.1). While the most significant shifts were observed in the genes involved in EMT, many other genes, ostensibly unrelated to EMT, were affected as well.

Lactate dehydrogenase (LDH), for example, was up-regulated by 3186-fold, which is one of the greatest upward shifts, second only to vimentin (3988-fold). LDH is an enzyme critical to glucose metabolism during glycolysis and catalyzes the final conversion of pyruvate to lactate during anaerobic respiration. In contrast, fructose 1,6-bisphosphatase (F1,6-BP), a metabolic enzyme critical for gluconeogenesis, was down-regulated by 609-fold in MCF-7/PAR1 cells. The opposing changes in these 2 enzymes suggest a shift in metabolic events, strongly favoring energy expenditure over energy storage. Since PAR1 expression confers enhanced proliferative potential, such a change in metabolic propensity may be favorable and even necessary for cellular propagation.

Another interesting transcriptional shift was observed with urokinase-type plasminogen activator (uPA) (+409-fold) and its receptor, uPAR (+114-fold). In MDA-MB-231 cells, constitutive ERK activity is a result of uPA vbinding to uPAR (232), and furthermore, uPA secretion is mediated by EGFR activation (233). We have observed that MCF-7/PAR1 cells also have constitutive ERK activity (Figure

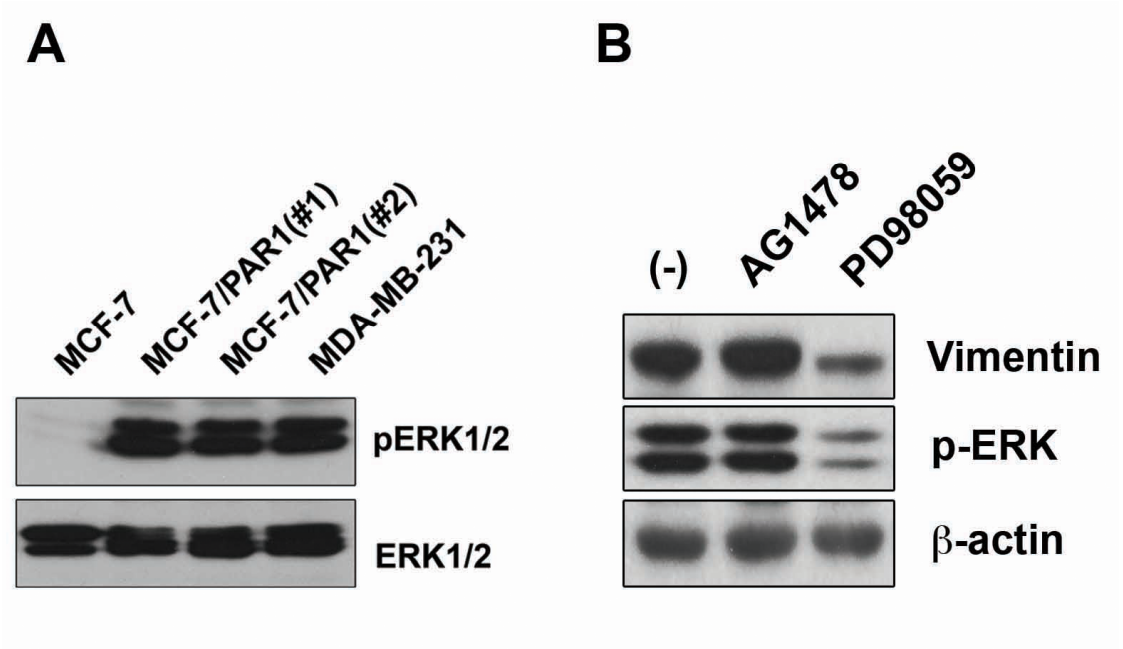


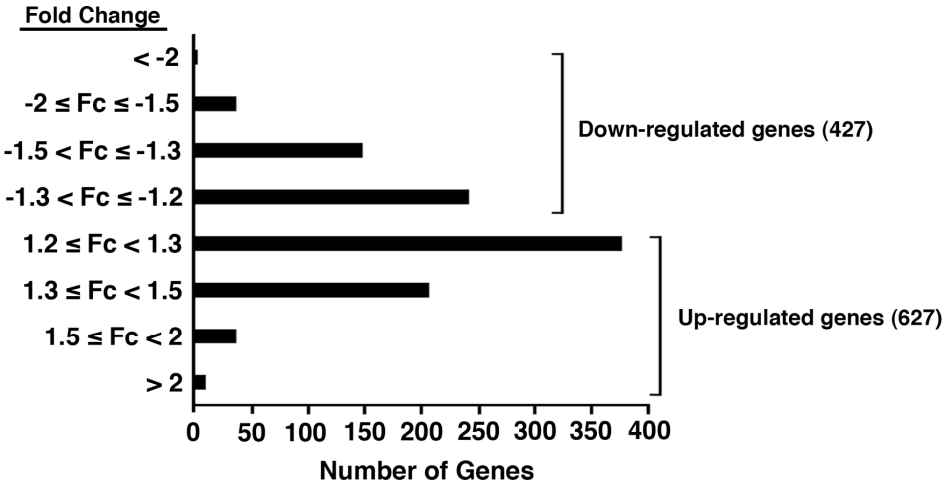
Figure 4.2. Constitutive ERK activity promotes vimentin expression in breast carcinoma cell lines. ***(A)*** MCF-7, MCF-7/PAR1 (clones #1 and #2), and MDA-MB-231 cell lysates were resolved and blotted for pERK1/2. Total ERK1/2 was using as loading control. ***(B)*** MCF-7/PAR1 (clone #2) was treated with AG1478 (5 μM) or PD98059 (50 μM) for 3 days. Cell lysates were resolved and blotted for Vimentin and phospho-ERK. β-actin was used as loading control. Representative data from multiple experiments are shown.

4.2A), possibly induced by the up-regulated uPA/uPAR signaling cascade. Significantly, antagonism of ERK activity by PD98059 reduced vimentin expression (Figure 4.2B), suggesting its involvement in EMT. We have observed that PAR1 expression alone is capable of inducing vimentin expression. However, the uPA/uPAR/ERK axis may also be a critical component in maintaining high levels of vimentin for sustained EMT. Whether the expression levels of uPA and uPAR are under direct PAR1 transcriptional control remains unknown. Exploring the relative significance of PAR1 and uPA mediated vimentin expression is of great interest when considering viable targets of EMT reversal.

Given that PAR1 activity results in the up- and down-regulation of a wide variety of genes, we also examined whether blockade of PAR1 with P1pal-7 would affect gene expression in breast carcinoma cells (FIgure 4.3A). PAR1-MCF7 (N55) breast carcinoma cells were treated with 3 μM P1pal-7 or vehicle for 18 h before RNA isolation. Distributional analysis of fold changes (Fc) in gene expression gave an approximate Gaussian distribution of 1054 genes in total, with 627 up-regulated genes and 427 down-regulated genes (Fig. 4.3A). There were 12 genes that varied by >2-fold and 84 genes varied by >1.5-fold (Fig. 4.3A-B). The fold-changes in mRNA levels were independently verified by quantitative PCR for several genes of interest (Fig. 4.3B).

The genes that were found to be down-regulated by P1pal-7 include those associated with cell survival, invasion, signaling and gene transcription. The mRNA levels of the three Akt isoforms were not affected by P1pal-7, indicating that PAR1 regulates Akt activity independently of gene transcription. Interestingly,

A



B

DOWN-REGULATED BY P1pal-7					
Category	**Gene**	**Symbol**	**Function in cancer**	**qPCR**	**Fold Change**
Cell Adhesion, Matrix and Tumor invasion	Four and a half LIM domains 1	FHL1	form focal adhesions; involvement in tumor invasion	-1.8	-2.5
	Kin of IRRE like (Drosophila)	KIRREL	actin filament polymerization and cell motility		-1.9
	Matrix metalloprotease 3	MMP3	cancer invasion and metastasis; angiogenesis	-1.7	-1.7
	Metalloprotease inhibitor 4	TIMP4	MMP inhibition; growth promoting effects	-1.5	-1.6
	Factor XIII	F13A1	polymorphism associated with oral cancer generation		-1.8
Survival&Apoptosis	Apolipoprotein C-1	APOC1	anti-apoptotic effects in pancreatic cancer cells		-1.6
	Retinoid X receptor, alpha	RXRA	nuclear receptor; involved beta-catenin degradation		-1.6
Cell Signaling and Transcription	Chloride intracellular channel 3	CLIC3	association with mitogen activated protein kinase family ERK7; regulation of cell growth	-1.5	-2.3
	SAM pointed domain containing ets transcription factor	SPDEF1	overexpression in breast tumors; independent risk factor for nodal involvement	-1.4	-1.9
	suppressor of variegation 3-9 homolog 1	SUV39H1	H3 lysine 9 methyltransferase; epigenetic changes involving chromatin remodeling and transcriptional regulation		+1.5

UP-REGULATED BY P1pal-7					
Category	**Gene**	**Symbol**	**Function in cancer**	**qPCR**	**Fold Change**
Stress Response	Metallothionein 1F	MT1F	induced by oxidative stress, heavy metal; scavenge free radicals, reactive oxygen species (ROS); role in zinc homeostasis suggests influence on activity of p53, caspase 3 and Ca/Mg-dependent endonuclease		+2.7
	Metallothionein 1X	MT1X		+4.0	+2.5
	Metallothionein 2A	MT2A		+4.9	+2.3
	Metallothionein 1A	MT1A			+2.2
	Metallothionein 1E	MT1E		+3.3	+2.2
Cell Signaling and Transcription	cAMP responsive element binding protein 5	CREB5	cross-talk of pro/anti-apoptotic pathways in leukemia		+1.8
	v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	transcription factor regulating osteogenesis; human oncogene c-fos		+1.6
	phosphonoformate immuno-associated protein 5	PFAAP5	interaction with HPV18 E6 oncogene		+1.6

Figure 4.3126126. **Effect of P1pal-7 pepducin on gene transcription in PAR1-expressing cells using microarray analysis.** Analysis of triplicate samples of P1pal-7 and vehicle treated N55 (MCF7-PAR1) cells were carried out. Mean changes in mRNA levels for individual genes were regarded significantly different if the *p* value was <0.05 and if the fold-change was greater or less than 1.2. ***(A)*** Number of genes up/down-regulated by P1pal-7 shown as a distribution of fold changes ($[N55\text{-}PAR1 + P1pal\text{-}7]_{mRNA}$ / $[N55\text{-}PAR1]_{mRNA}$) in gene expression. Messenger RNA isolated from N55-PAR1 breast carcinoma cells (± 3 μM P1pal-7) was analyzed by the Affymetrix Human Genome 133 array. ***(B)*** Functional role of cancer-related genes with significant changes in expression ($p < 0.05$) . Fold change as measured by the microarray analysis was validated by qPCR.

P1pal-7 downregulated the expression of the histone methyl-transferase Suv39H1 by 50%. Suv39H1 controls histone H3 lysine 9 methylation (234, 235) and chromatin activity. Six members of the metallothioneins (MT) family (1E, 1A, 2A, 1X, 1F and 1G) were significantly upregulated by P1pal-7 (Fig. 4.3B). MTs are a family of stress response proteins (236) and regulate cellular zinc levels. Expression of MT transcripts have also recently been described to be under the control of the zinc-finger protein, PLU-1/JARID1B in breast cancer cells (237). Therefore, an increase in MT expression might have an effect on zinc-dependent enzyme activities that may modulate a variety of cellular processes.

Interestingly, genes down-regulated by P1pal-7 do not significantly coincide with those up-regulated by PAR1 expression. This may be because PAR1 expression has induced the constitutive activity of multiple signaling pathways, including those involving EGFR and uPAR, thereby confounding the microarray results with gene transcriptional changes not directly regulated by PAR1. Here, we quantified the global effects of P1pal-7 on gene expression and found several unanticipated pathways under PAR1 control, possibly conferring lasting anti-tumor effects independently of its acute cytotoxicity through cell signaling regulation.

We have explored the effects of PAR1 expression and its blockade by P1pal-7 through genechip analysis. However, we have yet to investigate the effect of thrombin or MMP-1 mediated PAR1 activation on gene transcription. Albrektsen *et al.* have explored the effect of short-term PAR1 activation (1 h and 6 h) using the activating peptide TFLLRNPNDK (AP) on MDA-MB-231 cells

(238). Of the 26 genes up-regulated by AP stimulation, 10 genes coincided with those up-regulated in MCF-7/PAR1 cells: Cystein rich protein 61 (Cyr61), Connective tissue growth factor (CTGF), Long pentraxin (PTX3), Granulocyte-macrophage colony stimulating factor (GM-CSF), Urokinase-type plasminogen activator (uPA), Serpine 1, Cyclin-dependent kinase 5 regulatory subunit 1 (p35), Cyclin-dependent kinase inhibitor 1A (p21), Cyclin-dependent kinase inhibitor 1C (p57, Kip2), Unknown, chromosome 5 open reading frame 13. The up-regulation of Cyr61 confirms our recent report demonstrating the induction of Cyr61 upon expression and activation of PAR1 in MCF-7 cells (120). The induction of uPA by AP stimulation suggests that uPA is under direct PAR1 control, and may be a mediator of PAR1-induced vimentin expression. Interestingly, 5 genes did not show any changes, and 4 genes were down-reguated (7 genes were not available for comparison in the MCF-7/PAR1 microarray). These discrepancies may be explained by the differential signaling of proteolytically activated PAR1 and constitutively active PAR1, the duration of receptor activity, and the confounding over-expression of other signaling pathways in MCF-7/PAR1 cells.

The effects of thrombin signaling have also been explored in endothelial cells. Wu *et al.* have demonstrated that thrombin induces the expression of tissue factor (TF), PDGF-A, ICAM-1, u-PA, and Egr-1, all markers selected as indicators of endothelial activation (239). Egr-1 is a transcription factor that promotes PAR1 expression (112), and therefore, thrombin-mediated over-expression of Egr-1 may be a mechanism by which endothelial cells renew their own cellular surfaces with un-cleaved, protease-sensitive PAR1. The constitutive activity of PAR1 in

MCF-7/PAR1 does not involve proteolytic cleavage of PAR1, and therefore, may not result in Egr-1 induction as observed from our microarray results. Overall, in the MCF-7/PAR1 cells, TF, PDGF and uPA were up-regulated, but ICAM-1 levels were slightly decreased. The commonalities in TF, PDGF, and uPA regulation may indicate the conservation of PAR1 function across cell types, however, the discrepancies also suggest the flexibility of PAR1 function under varying cellular contexts.

Conclusion

In this report, we have explored the potential of PAR1 as a target for breast cancer therapy. PAR1 antagonism by the P1pal-7 pepducin has shown to promote tumor apoptosis and reduce metastasis to the lung. The efficacy of P1pal-7 has been demonstrated in the context of monotherapy and in combination therapy with Taxotere in xenograft mice models. We have also demonstrated that PAR1 and EGFR cooperatively induce EMT to promote an invasive, fibroblast-like phenotype of advanced breast carcinoma.

The current study closes with several unanswered questions. What are the effects of PAR1 antagonism on tumor angiogenesis? What are the distinct roles of MMP-1 and thrombin in cancer progression? How do PAR1 and EGFR interact to promote breast cancer progression? What are the differences between PAR1 constitutive activity and protease-activated PAR1? Much remains to be explored in this exciting field.

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