ROLE OF CELL-CELL ADHESION IN PROFILIN-1-DEPENDENT MODULATION OF BREAST CANCER CELL PROLIFERATION

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Hallmarks of progression of epithelial-derived tumors include downregulation of cell-cell adhesion, dysregulated cell proliferation, increased resistance to apoptosis and acquisition of motile and invasive phenotype. Profilin-1 (Pfn1) is a ubiquitously expressed actin-binding protein which is required for proliferation and migration of most normal cells, yet its expression is significantly downregulated in various types of adenocarcinoma including those originating in breast, pancreas and liver. Tumor-suppressive action of Pfn1 on breast cancer cells has also been previously documented in the literature. The present study shows that loss of Pfn1 expression in normal human mammary epithelial cells leads to junctional delocalization of E-cadherin with a concomitant reduction in cell-cell adhesion, reduced cell-matrix adhesion, increased cell proliferation and a hypermotile phenotype. These findings may provide a possible insight on why Pfn1 expression is downregulated in breast cancer cells. Using MDA-MB-231 as a model system for mesenchymal breast cancer cell type, we further show that overexpression of Pfn1 can restore adherence junctions and phenotypic reversion to an epithelioid-type through junctional stabilization of an endogenously expressed cadherin molecule. Pfn1 overexpression in MDA-MB-231 cells causes cell-cycle arrest at G1, inhibition in proliferation in vitro and tumor growth in vivo. Pfn1-induced growth inhibition of MDA-MB-231 cells is partly mediated by upregulation of p27kip1 (a CDK inhibitor) in cell-cell adhesion-dependent manner. We finally show that Pfn1 overexpression also sensitizes MDA-MB-231 cells to apoptosis suggesting the

survival of breast cancer cells can also be modulated Pfn1. Taken together, these findings highlight for the first time mechanistic insights underlying some of the tumor-suppressive properties of Pfn1.

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Abbreviation

ADP (adenosine diphosphate)

ADF (actin-depolymerization factor)

APC (adenomatous polyposis coli)

ATP (adenosine triphosphate)

BAD (Bcl-xL/Bcl-2-Associated Death Promoter)

cDNA (complementary DNA)

Cdk (Cyclin-dependent kinase)

CHX (cycloheximide)

DAPI (4',6-Diamidino-2-Phenylindole)

DNA (Deoxyribonucleic acid)

ECM (extracellular matrix)

EGFR (epithelial growth factor receptor)

EGTA (ethylenglycol bis (fl-aminoethyl) ether-N,N'-tetra- acetic acid)

FACS (fluorescence-activated cell sorter)

GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)

GFP (green fluorescence protein)

GSK (Glycogen synthase kinase)

IGF (insulin growth facto r)

IL (interleukin)

PARP (poly(ADP-ribose) polymerase)

PCR (polymerase chain reaction)

PI3-K (phosphoinositide 3-kinase)

PIP2 (Phosphatidylinositol 3,4-bisphosphate)

PIP3 (Phosphatidylinositol 3,4,5-trisphosphate)

pRb (retinoblastoma protein)

siRNA (small interfering RNA)

PTEN (Phosphatase and tensin homologue deleted on chromosome 10)

TNF (tumor necrosis factor)

UV (ultraviolet)

1.0 INTRODUCTION

1.1 INTRODUCTION OF CANCER

1.1.1 Characteristics of cancer

Cancer is one of the most fatal diseases in human. The unremitting clonal expansion of cancer cells kills by invading, subverting and eroding normal tissue. So far, there are more than 250 distinctive types of malignant tumors, and even more than thousands of subtypes within specific tissue or organ (Rojo 2007). Despite the diverse types or subtypes of cancer, all the phenotypes of the cancer cells share several essential acquired capabilities in common: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan 2000). These hallmark features of cancer cells are acquired through mutation of genes that govern and regulate the diverse aspects of cellular phenotypes. The most prominent evidence is the p53 tumor suppressor gene, which losses its function in most human cancer. Normally, p53 elicits either cell cycle arrest to give way to DNA repair or apoptosis due to the excessive damage to DNA when occurs in normal cells (Castro 2008), therefore, protein aberration may occur due to the damaged DNA when p53 losses its function to stop the cell proliferation. It has also been found that many other genes involved in DNA damage repair or

correct chromosomal segregation during mitosis are lost in cancer cells (Perez de Castro 2007, Yin 2007), which leads to genome instability and variability in tumor progression.

In the following section, we will introduce some hallmarks of the cancer cells.

1).Self-Sufficiency in Growth Signals: Usually, mitogenic growth signals are unexceptionally needed to stimulate the progression of cell cycle from a quiescent phase into an active proliferative phase. The mitogenic signals, including diffusible growth factors and extracellular matrix components, bind to specific transmembrane receptors to activate the cell cycling. This is how normal cells are stimulated to proliferate; however, it is not applicable to cancer cells. Many of the oncogenes in cancer cells can mimic those growth signals in one way or another, or even generate their own growth signals, to trigger the cell proliferation without the stimulation of those exogenous mitogenic signals. This reduced dependence on mitogenic growth factor disrupts the critically important homeostatic mechanism to maintain proper behavior of normal cells within a tissue.

2). Insensitivity to growth-inhibitory (antigrowth) signals: Since there are various growth signals, usually there are multiple anti-proliferative signals in normal cells to balance the cell growth. The anti-proliferative signals include soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix or on the membrane of adjacent cells. These anti-proliferative signals are received by transmembrane receptors which are coupled to the intracellular signaling circuits. During the transit of cell cycle through G1 phase, cells monitor their external environment to decide whether to move forward to G1 phase or to stay quiescent, or even to enter into a postmitotic state, which is based on the signals they receive. At the molecular level, almost all anti-proliferative signals take effect through the retinoblastoma protein (pRb). When phosphorylated, pRb can sequester and alter the function of E2F

transcription factors that control the expression of genes essential for cell cycle progression from G1 into S phase, therefore blocking the cell proliferation (Weinberg 1995). Any alteration in this signal transduction to pRb may lead to unregulated progression of the cell cycle, which is very common in cancer cells. One important anti-proliferative signal in normal cells is mediated by cell-cell adhesion which induces contact inhibition in most normal cells: the propagating cells cease to proliferate when they build up their cell-cell contact with their neighbor cells. Such contact mediated inhibition of cell growth is very important to prevent uncontrolled cell proliferation and maintain normal tissue size and function, but it is usually lost in tumor cells. Any alteration of those proteins that mediate cell-cell adhesion may lead to loss of contact inhibition of those growing cells. The details will be discussed in the following chapter.

3). Evasion of programmed cell death (apoptosis): Programmed cell death, or apoptosis, is an important machinery involved in tissue homeostasis. This is a highly regulated process by which organism eliminates damaged cells with no inflammatory response in vivo. However, cancer cells have the ability to escape from apoptosis so that they may expand their population in the involved tissue. Therefore, the acquired resistance to apoptosis is a hallmark of nearly all types of cancer cell. Usually, the apoptotic program is present in latent state in all cell types; once triggered by external signals, such as growth factor withdrawal, UV light exposure, or chemical treatment, this program is activated to transmit the apoptotic signal through a cascade reaction in a precisely choreographed series of steps to the nucleus of a cell, where DNA damage irreversibly occurs. In mammalian cells, there are two major signaling pathways through which the apoptosis signals can be transmitted: the intrinsic pathway which is initiated in mitochondria and the extrinsic pathway which is initiated through death receptor-mediated pathway (Ashkenazi 2002). The proteins involved in the apoptotic signaling transduction can also be

broadly categorized into two groups: sensor proteins and effector proteins. The sensor proteins are responsible to monitor the extracellular and intracellular signals to decide whether a cell is healthy or sick. The effector proteins are responsible for execution on receiving signals from the sensor proteins. The apoptosis effectors are an array of intracellular proteases, termed caspases, which act in the execution phase of the cascade events leading to cell death. Activation of these caspases is a central mechanism of apoptosis (Vaux 1999). Caspases are a family of cysteine proteases. They are synthesized as pro-enzymes and activated by internal cleavage. Two "gatekeeper" caspases, caspase-8 and -9, are activated by death receptors on the cell membrane, such as FAS, or by release of cytochrome C from mitochondria, respectively. After activation, the caspases cleave their specific substrate proteins; then, the cleaved substrate proteins, called "downstream" caspases, cleave numerous targets whose intactness are essential for cell survival. For example, caspase 3, one of the major downstream caspases (Nicholson 1995, Kumar 1997, Zakeri 2008), may cleave PARP. Since intact PARP is responsible for DNA repair in normal condition (Tewari 1995), the cleaved PARP losses the ability to repair damaged DNA as the result of caspase-3 activation, and therefore leads to cell death finally. The cascade events occurring in the cytoplasm are strictly regulated by many molecules within a cell. One set of the important modulators of the apoptotic response is the Bcl-2 family members, which have either pro-apoptotic (Bax, Bak, Bid, Bim) or anti-apoptotic (Bcl-2, Bcl-xL, Bcl-W) function. Here, it is worthy to mention a key member of this Bc1-2 family, the Bcl-xL/Bcl-2-Associated Death Promoter (BAD) molecule. It counters the anti-apoptotic effect of Bcl-xL, and is therefore a pro-apoptosis molecule. However, its pro-apoptotic effect is inactivated when BAD is phosphorylated by Akt pathway. This BAD inactivation by Akt pathway will be discussed in the next section. When the apoptotic signals is transduced into the nuclei and irreversible damage to

DNA occurs, some typical morphological changes can be observed in the apoptotic cells, such as loss of substrate attachment and round-up, blebbing of cellular membrane, disintegration of cytoplasmic and nuclear skeletons, degradation of chromosomes and fragmented nucleus. And finally, the apoptotic cells form multiple apoptotic bodies which are engulfed by nearby cells and disappear with no inflammatory irritation since cellular contents are not released. Actually, the possibility that evasion from apoptosis serves as a barrier to kill cancer cells was brought up in 1972 (Kerr 1972), and has now been widely accepted as one mechanism by which cancer cells escape from chemotherapy in the most of clinical cases. The ability of cancer cells to resist apoptosis may be achieved through some strategies, and the most common case is loss of a proapoptotic regulator gene, followed by the functional inactivation of its protein product (Harris 1996). As an important pathway to transmit anti-apoptotic signals, PI3K/AKT pathway is quite often involved in apoptosis resistance in a substantial fraction of human cancer cases (Kim 2007, Jin 2008). This survival signaling pathway can be activated by extracellular molecules such as IGF-1/2 or IL-3 (Evan 1998, Frasca 2008, Ekert 2008), intracellular signaling from Ras (Liou 2000, Jung 2005), or by loss of PTEN tumor suppressor (Li 2006, Seol 2008, Peacock 2009).

4). Tissue invasion and metastasis. Colonization of tumor cells in distant tissues is the most dangerous attribute of cancer, and this is the major cause of cancer death in most cases. The mechanism for the distant metastasis of cancer cells is complicated. Besides the phenotype changes mentioned above, these remarkable changes which enable cancer cells to invade and spread to the secondary site involve the interactions between the cells and their micro-environment, including the cell adhesion to the adjacent cells (which interaction is mediated by members of immunoglobulin and calcium-dependent cadherin families), and the cell adhesion to the immediate extracellular matrix (which is mediated by integrins). Such adhesion mediated

interactions convey the regulatory signals between the microenvironment and the cell. Integrin is the major protein that mediates cell-ECM interaction, through which the signals are transmitted via ‘outside-in’ and ‘inside-out’ mechanisms in various type of cells (reviewed by Giancotti 1999). As for the cell-cell adhesion, the most intensively investigated molecule is E-cadherin, a transmembrane protein ubiquitously expressed in all epithelial cells and a key mediator of adherence cell-cell adhesion. Due to the multiple roles of cell-cell adhesion in cell sorting, cell proliferation, cell survival and cell motility, the cadherin mediated cell-cell adhesion will be introduced in next chapter to elucidate how such cell adhesion influences cell behavior in normal cells and in cancer cells. In addition to the loss of cell adhesion, cell invasion through extracellular matrix needs proteolytic degradation of the ECM and basal membranes, which is accomplished by matrix metalloproteinases (MMPs). And, the proteolytic effect of MMPs is countered by its inhibitors. Thus, either up-regulated protease gene or down-regulated protease inhibitor genes, or activated enzymes from the inactive zymogen forms may facilitate invasion of cancer cells into nearby stroma (Price 2002).

Further understanding the hallmarks of human cancers can help us learn how those cancer cells behavior and how they escapes and invade into the surrounding tissues, but different cancer types have their own specific features in addition to these hallmark phenotypes. As one of most the life-threatening cancers, breast cancer has been paid more and more attention in the past decades.

1.1.2 Breast cancer

Among those malignant tumors, breast cancer is one of the most life-threatening cancers in women. According to the American Cancer Society (Cancer statistics 2009), there are more than

192,000 new cases of invasive breast cancer among women in US, which is even more than lung cancer (103,000), thus breast cancer has become the leading health threat to women.

Breast cancers are usually derived from the luminal epithelial cells because the luminal epithelial cells are functionally active and thus become the target of carcinogens (Gudjonsson 2004). Such epithelial originality is confirmed clinically by detection of epithelial markers in breast cancers (Zotter 1985, Sommers 1992, Grünewald 2000). When the transformed cells remain within the basement membrane, they are classified as non-invasive. An invasive breast cancer refers to the one in which cancer cells invade through the basement membrane into the adjacent normal tissue (Sainsbury 2000).

In the past decades, a lot of attention has beep paid to breast cancer due to its fatality in women. Although the exact molecular mechanisms of the tumorigenesis of breast cancer are unknown, it is well accepted that breast cancer cells undergo several genetic alterations that affect the mechanisms of carcinogenic transformation, as in any other cancers. These genetic alterations involve the cell behaviors in multiple aspects, such as growth factor and cell signaling pathways, the cell cycle and apoptosis, and cellular invasiveness, and angiogenesis (Kinzler 1997, Levin 1997). At the protein level, it is found that breast cancer cells escape from the molecular control at least by: (1) down-regulation of membrane proteins important for the communication and adhesion between epithelial cells, e.g. E-cadherin (Berx 2001, Kowalski 2003, Haass 2005), or some key regulators of cell cycle, e.g. p27 (Blain 2002, 2003); (2) Upregulation of growth factor receptors in the cell membrane, such as EGFR (Verbeek 1998, Grupka 2004).

In order to detect breast cancer and make a prognosis during cancer therapy, it is important to find some crucial molecules that regulate or determine the progress of breast cancer.

And, even more importantly, those molecules could also serve as the target molecules in the chemotherapy of breast cancer. Therefore, a large amount of study has focused on identifying and evaluating reliable markers for breast cancer. So far, some marker proteins have been well documented in breast cancer research and clinical application, such as Ki67, estrogen receptor, epithelial growth factor receptors, PBK/Akt, Bcl-2, Fos-related antigen 1, E-cadherin (or loss of cell-cell adhesion), Breast Cancer Susceptibility Gene 1, Lactoferrin and some resistance proteins (Esteva 2004, Gianocotti 2006). Here, more emphasis will be given to PBK/Akt and cell-cell adhesion in breast cancers.

1.1.2.1 Aberrant AKT signaling pathway

Akt is an important serine/threonine kinase involved in various cellular behaviors, such as cell proliferation and survival, glucose metabolism and transcriptional regulation, when activated through PI3-K pathway (Sansal 2004, Yoeli-Lerner 2006). The three Akt isoforms (Akt1, Akt2 and Akt3) are ubiquitously expressed in all cell types and tissues, although Akt3 seems to have some limited expression patterns. When stimulated by growth factors or insulin, the activated PIK pathway recruits Akt to the plasma membrane by direct binding with Akt pleckstrin homology (PH) domain through PIP_2/PIP_3. Complete Akt activation requires phosphorylation of Akt at Thr308 and Ser473. Then the phosphorylated Akt re-localizes to subcellular sites where it phosphorylates its downstream proteins that are involved in cell proliferation and cell apoptosis, such as Forkhead transcription factors (FOXO), pro-caspase-9, GSK-3, BAD, and p27. Such activated Akt is not detected in normal breast tissue but commonly found in many types of cancer, including breast cancer (Altomare 2005, She 2008). Actually, the mechanism by which Akt functions to promote cell survival is through phosphorylation of pro-apoptotic molecules, such as BAD, pro-caspase-9, and Apatf-1. When phosphorylated at Ser^{112} and Ser^{136} by Akt, the

pro-apoptotic protein BAD is inactivated by dissociation from Bcl-x_L and then translocated to the cytosol where it binds to 14-3-3 proteins. Further studies have found that the expression of a constitutively active Akt inhibits Bax conformation change and translocation to mitochondria, thus preventing the release of cytochrome c from mitochondria (Yamaguchi 2001), blocking intrinsic signaling pathway of apoptosis. Additionally, Akt can also phosphorylate transcription factors forkhead, FOXO, at threonine 32, serine 253 and serine 315, which are originally in the nucleus. As a result, phosphorylated FOXO proteins are sequestered in the cytoplasm and cannot act as transcription factors in the nucleus (Terragni 2008, Shankar 2008). Such inhibition of FOXO suppresses the expression of a key ligand of death receptor Fas (Kops 2000, Barthelemy 2004). Therefore, activated Akt assures cell survival through these mechanisms at both pre-mitochondrial and post-mitochondria levels, and this is one major mechanism that confers resistance to chemotherapy in breast cancer cells. The role of Akt in cell proliferation is mediated through the phosphorylation of p27 (p27 is an important Cdk inhibitor in cell cycle and induces G1 arrest when bound to nuclear Cdks). Akt can phosphorylate p27 at multiple binding domains, such as Ser10, Thr157, and Thr198 (Vervoorts 2008). Such phosphorylation of p27 redistributes p27 to the cytoplasm where p27 is sequestered from its target Cdks in the nucleus, effectively allowing Cdks to remain active to promote cell cycle progression. Taken together, Akt can positively regulate cell survival and cell proliferation when it is phosphorylated. The phosphorylation of Akt is regulated by other proteins, such as PTEN. PTEN is a major tumor suppressor, and it can inhibit phosphorylation of Akt by dephosphorylation of PIP3 to which Akt needs to bind before activation. And the balance between PTEN and PI3K determines PIP3 levels at the plasma membrane, which in turn regulates Akt activity. The literature demonstrates

that the PTEN-inactivating mutation is an important mechanism to allow excessive activation of Akt in breast cancers (Blanco-Aparicio 2007, Stemke-Hale 2008).

1.1.2.2 Deregulated cell-cell adhesion

Loss of cell-cell adhesion is the prerequisite for tumor cell invasion and metastasis, which is another characteristic of breast cancer cells. One of the most common causes of such change in cell-cell adhesion is downregulation of E-cadherin. E-cadherin is a transmembrane glycoprotein that mediates cell-cell adhesion to maintain the normal structure and function of the epithelium. Normal mammary epithelial cells express abundant E-cadherin protein. Numerous studies in breast cancers have found various alterations of E-cadherin that lead to dysfunctional cell-cell adhesion in cancer cells; for example, mutations or deletions of the E-cadherin gene itself, transcriptional repression of the E-cadherin gene (hypermethylation or chromatin re-arrangements in E-cadherin promoter region), and alterations of some E-cadherin intracellular binding partners (reviewed by Hirohashi 1998, 2003). Such aberrant E-cadherin expression is tightly associated with high grade, estrogen receptor negative and metastatic breast carcinomas, and it is usually followed by an unfavorable prognosis. Therefore, the aberration of E-cadherin is an important contributor to the malignance of breast cancer. In vitro studies showed that expression of the E-cadherin gene in highly invasive cancer cells dramatically suppressed their invasiveness, and conversely, introduction of E-cadherin-specific antisense RNA rendered noninvasive epithelial cells invasive (Vleminckx 1991, Mundy 1997). In vivo studies reveal that high expression level of E-cadherin is found in less invasive breast cancer cells, but relatively low levels of E-cadherin were associated with highly invasive human breast cancer cells (Sommers 1991, Mbalaviele 1996, Jeschke 2007, Silva 2008).

1.2 CELL-CELL ADHESION AND CELL PROLIFERATION

Cell–cell adhesion is very critical in maintaining the morphology and normal function of epithelial cells. Usually, epithelial cells need to adhere tightly to their neighbor cells through adhesive structures to ensure the morphological integrity and tensile strength of epithelial tissue. As the regulator in morphogenesis, cell-cell adhesion is indispensable in membrane integration, cell polarization and cell migration (Gooding 2004). Reduced cell-cell adhesion allows cancer cells to dissociate from the cell population, lose their polarity, and infiltrate the stroma in a disseminative manner. As the result of such dissociation, another prominent feature, loss of contact inhibition, has been shown to correlate with the disordered signal transduction from cell-cell adhesion to cell proliferation.

1.2.1 Cadherin/catenins mediated cell-cell adhesion

Epithelial cell–cell adhesion is mediated by a variety of membrane proteins, including classical cadherins, claudins/occludin, nectin and desomosomal cadherins (Nelson 2005). Intracellularly, these adhesion structures are linked either to intermediate filaments (desomosomes) or to microfilaments (adherence junctions and tight junctions). Such association with the cytoskeletal networks is necessary to stabilize the cell–cell adhesion and to integrate the cell–cell contacts with the morphological changes of epithelial cells. Here, we will introduce more about the adherens junction, i.e. cadherin-mediated cell-cell adhesion. Usually, the process of cell adhesion can be arbitrarily divided to following steps: mechanically weak interactions between individual cadherins on the membranes of two opposing cells, contact stabilization of such cell adhesion by recruitment of intracellular binding proteins, and formation of adhesion complex with the

cytoplasmic domain of cadherins, contact maturation of the adhesion due to the binding and further accumulation of actin filaments to the adhesion complex in the adhesion area. Here we will focus on the first two steps, the interaction of cadherin molecules and their interaction with their intracellular binding partners.

The cadherin superfamily is a set of transmembrane proteins with three distinct domains: the divergent extracellular domains, a hydrophobic transmembrane domain and an intracellular cytoplasmic domain. Based on domain composition, genomic organization and overall structures, the superfamily can be further divided into six subgroups (Gooding 2004): 1) classical or type I cadherins, including E-, N-, P-, R-, H-, and EP-cadherins; 2) highly related classical type II cadherins, such as VE-, K-cadherin, cadherin-7~12; 3) desmosomal cadherins; 4) protocadherins; 5) Flamingo cadherins and 6) FAT-like cadherins. Besides, there are some atypical cadherins in the superfamily. Among these cadherins, the classical cadherins are the best characterized in terms of both mechanism and function. A classical cadherin is defined by the presence of a conserved intracellular domain in structure. The extracellular binding domains of cadherin are necessary for Ca^{2+} binding and cadherin mediated cell-cell adhesion. The Ca^{2+} binding sites serve to rigidify the cadherin oligomers which subsequently form intercellular complexes to generate cell-cell adhesion and then additional lateral contacts to zipper up the cadherins into clusters. In this manner, multiple weak affinity interactions sum up to produce a very tight intercellular binding. The C-terminal cytoplasmic binding domain of classical cadherins mediates the interaction between cadherins with a set of cytoplasmic proteins, called catenins, to link cadherins with actin filaments. Two catenin molecules are implicated in this link: α- and β-catenin. β-catenin binds to the C-terminus of the intracellular domain of cadherins and the N-terminus of α–catenin (Delva 2009). Alpha-catenin binds directly to actin filaments,

and also binds to a number of proteins involved in actin binding, bundling and polymerization. Such intact cadherins-catenins-actin complexes are very important in stable cell-cell adhesion. When α- or β-catenin are absent, cadherin-catenin complexes fail to associate with the actin cytoskeleton, consequently leading to defective cell adhesion. In addition to α- and β-catenin, a third catenin, p120 catenin, binds to the classical cadherin intracellular domain at a site distinct from β–catenin and stabilizes cadherins (review by Pettitt 2005). Therefore, classic cadherins together with the three catenins form a core functional unit, the cadherin-catenin complex, which is a major component of the cadherin mediated adherens junction. Of course, an intact cadherin-catenin complex alone is not enough to maintain a stable cell-cell adhesion. As mentioned before, link of the adhesion complex to actin filament is important to stabilize the cell-cell adhesion, and any disruption of the adhesion complex and its link to actin filament would lead to dissociation of cell-cell adhesion. Interestingly, some experiments have measured the effect of actin filaments on cell-cell adhesion. In the presence of cytochalasin D, an actin polymerizing agent, cells exhibited little or very weak cadherin-mediated cell-cell adhesion (Hirano 1987, Angres 1996, Ko 2001). Thus, the weak extracellular interactions between cadherin molecules are strengthened by the crosslink of cadherin-catenin complex with intact actin cytoskeleton. Because the formation of actin filaments is dynamic process of actin polymerization, it determines that cell-cell adhesion is also a dynamic process, especially during cell migration or cell division in normal tissue and cancers. Here, we will discuss the role of the cadherin-catenin binding complex in cell-cell adhesion and the signaling pathways in which they may be involved.

1.2.1.1 E-cadherin and its regulation in disturbed cell-cell adhesion

E-cadherin is one of the first expressed adhesion molecules in human. As indicated by its name, E-cadherin is most widely distributed in epithelia. The encoding gene for E-cadherin, CDH1, is located on chromosome 16q22.1, and CDH1 is a key regulator of morphogenesis (Day 1999). Like other cadherins, E-cadherin is comprised of a large extracellular domain with 113 amino acid residues, a single transmembrane segment and a cytoplasmic domain that binds to β- or γ-catenin, thereby providing a mechanism for interaction with other intracellular proteins (Wijnhoven 2000, Conacci-Sorrell 2002). E-cadherin mediates homophilic cell-cell adhesion (the extracellular binding domain of cadherin on the surface of one cell links specifically to the extracellular binding domain of the same type of cadherin on the surface of the opposing cell), and the 113 amino acid ECD residuals are essential for such selective homophilic binding of E-cadherin (Takeichi 1988, Gooding 2004). When E-cadherin is active, epithelial cells, including epithelia-derived cancer cells, remain intact within the mutual connection.

Suppression of E-cadherin activity may trigger the release of cancer cells from the primary cancer foci. In vitro studies have shown that E-cadherin can suppress tumor invasion, which strongly suggests that E-cadherin is a tumor suppressor (Vleminckx 1991, Hoteiya 1999, Jeanes 2008), loss of which is an indicator of poor prognosis in breast cancer (Siitonen 1996, Yoshida 2001) and prostate cancer (Umbas 1994). E-cadherin has been found to be down-regulated in a wide variety of tumors derived from epithelial cells (Heijink 2007, Mandal 2008, Hudson 2008). The most commonly recognized mechanisms for the inactivated E-cadherin mediated cell-cell adhesion in human cancers include genetic alteration, reduced expression level and tyrosine phosphorylation of β-catenin (Hirohashi 1998). Abnormalities of E-cadherin expression is suggested to be significantly correlated with a lower patient survival rate (Lazar

2008). Silencing or reducing E-cadherin expression has been associated with germ line mutations, single nucleotide polymorphisms, frame shift and slice site mutations, gene deletion (Hirohash 1998, Reinhold 2007), and epigenetic events such as histone deacetylation, chromatin condensation and promoter region methylation in epithelial tumors (Reinhold 2007). Such epigenetic modifications, including hypomethylation/hypermethylation of global or gene-specific promoter region, chromatin modification and loss of imprinting, play crucial roles in cancer initiation and development (Reihold 2007). Among the factors contributing to epigenetic modifications, it is noteworthy to mention two well studied Zinc-finger transcription factors, Snail and Slug. Slug expression is definitely linked to the loss of the E-cadherin in breast cancer (Schmidt 2005). Snail represses E-cadherin expression at the transcriptional level by recruitment of Sin3A/Histone Deacetylase (HDAC1)/HDAC2 complex (Peinado 2004) and its phosphorylation causes its nuclear accumulation which further potentiates E-cadherin repression (Ko 2007). Other than these alterations to E-cadherin, some other changes to the cadherin-catenin-actin complex may attenuate a cell-cell adhesion. These other alterations, such as tyrosine phosprylation of β-catenin, will be introduced in the following section.

1.2.1.2 Dual role of β-catenin in cell-cell adhesion and Wnt signaling pathway

Beta-catenin is an ubiquitously distributed protein with multiple functions. Originally, β-catenin was identified as a cytoplasmic component at cell-cell adherens junctions, where it interacts with the cytoplasmic binding domains of cadherin molecule and links them via α-catenin to the actin cytoskeleton (Geiger 1995, Conacci-Sorrell 2002, Delva 2009). In addition to the alteration of E-cadherin, aberrant tyrosine phosphorylation of β–catenin is another important mechanism to inactivate E-cadherin mediated cell adhesion system, because phosphorylated β–catenin does not bind to E-cadherin. Strong tyrosine phosphorylation of β–catenin has been

detected with loss of the association between β–catenin and E-cadherin at the junctional site in those loosely attached breast cancer cells (Hiscox 2006, Zhao 2009). All these data suggest that tyrosine phosphorylation of β–catenin affects the cadherin-catenin binding complex, leading to unstable cell-cell adhesions.

In addition to its role in cell-cell adhesion, β-catenin is involved in the Wnt-signaling pathways (Wodarz 1998, Akiyama 2000, Persad 2001, Thompson 2007). Upon stimulation by growth factors, the accumulated β-catenin in the cytoplasm may translocate into the nucleus in association with the T cell factor (TCF)/lymphoid enhancer factor (LEF) of DNA-binding transcription factor family and induce the transcription and expression of specific genes (Miller 1996, Riese 1997, Akiyama 2000, Apte 2006). Cyclin D1 is one of the key transcriptional targets of β-catenin/TCF through a TCF-4/LEF-1 binding site in the cyclin D1 promoter (Shtutman 1999, Lin 2000). Without growth factor and differentiation signals, cytoplasmic β-catenin is degraded by a proteosome system, which is under the control of APC, GSK-3, and adapter proteins axin and conductin (Roh 2004, Barker 2008) through targeted phosphorylation of highly conserved serine and threonine residues and ubiquitination of the N-terminal of β-catenin (van Noort 2002, Su 2008).

Because of the dual role of β-catenin in cell adhesion and Wnt signaling pathway, intercellular adhesion can interfere with the signaling role of β-catenin by sequestering it in the plasma membrane at cell-cell adhesion site, while Wnt signaling may promote assembly of intercellular adhesion (Hinck 1994, Schambony 2004). Therefore, cell-cell adhesion may change cell proliferation through the crosstalk between cell-cell adhesion and Wnt signaling pathway.

1.2.1.3 Other catenins

1) Alpha-catenin is another adhesion protein in the binding complex that links β–catenin to actin filaments. It has high homology with vinculin, another actin binding protein that links membrane proteins to actin filaments at sites of cell-ECM and cell-cell adhesion. Alpha-catenin is also required for stable cell-cell adhesion. Cell lines that lack α-catenin do not form cell cluster even though they posses all other proteins in the adhesion binding complex, and the dissociation due to lack of α-catenin can be reversed by transfection of α-catenin cDNA (Hirano 1992). Another direct evidence to show the important role of α-catenin in E-cadherin mediated cell adhesion is the resistance to Triton-X extraction: the association of α-catenin with β-catenin and E-cadherin on the membrane coincides with the binding complex becoming Triton-insoluble (Hinck 1994). Junctional E-cadherin is susceptible to non-ionic detergent in tumor cells without α-catenin, but it becomes more detergent resistant upon expression of α-catenin (Watabe 1994). All these data confirm the pivotal role of α-catenin, as a key linker protein, in the cadherin-catenins-actin quaternary binding complex of cell-cell adhesion.

2) p120-catenin is a unique molecule in stabilizing cadherins in the cadherin-catenins binding complex (Reynolds 2007). Without physically binding to p120-catenin with the cytoplasmic tail, most of cadherins (type I and type II) are internalized and often degraded, implying the master regulator role of p120-catenin in the stabilization of cadherins as a post-translational regulation of cadherins. As introduced before, the stability of α-catenin and β-catenin is directly dependent on physical interactions with E-cadherin in the binding complex; therefore, the amount of E-cadherin is very crucial to stabilize the binding complex. In addition to stabilizing the cell-cell adhesion binding complex, p120-catenin physically or functionally

involves in a wide variety of oncogene, tumor suppressor proteins (such as Src kinases), receptor tyrosine kinases and phosphatases (reviewed by Reynolds 2007).

1.2.2 Role of cell-cell adhesion in cell migration and proliferation

Intact cell-cell adhesion is required for integrity of epithelial-based tissues. Loss of cell-cell adhesion causes epithelial cell dissemination and even neoplastic transformation in tumor progression. The role of cell-cell adhesion in cell migration is well established, especially in epithelial cells, since such adhesion force between cells limits free movement of cells. In addition to affecting cell migration, cell-cell adhesion also play a role in inhibiting cell proliferation, a process known as contact inhibition which is considered to be a major regulatory mechanism in tissue growth and development (Nakatsuji 2001, Cheng 2006). Loss of contact inhibition is a hallmark of cancer cells. Usually, contact inhibition induces cell cycle arrest at G1 phase accompanied by an increased expression level of p27, a cyclin-dependent kinase inhibitor (Polyak 1994, Kato 1997, Levenberg 1999). In spite of various important insights into the possible underlying mechanisms of contact inhibition, the pathway of the anti-proliferative signal from the cell-cell contact into nucleus is still elusive. Since this is a cell-cell adhesion mediated inhibition, some speculate that ß-catenin is an important signal transmitter because of its dual role in the cell-cell adhesion and pro-proliferative Wnt signaling pathway. In addition to β–catenin/Wnt pathway, some other signaling pathways have been found involved in contact inhibition, such as, MAPK pathway and PI3K/Akt pathways. Even with intensive effort to reveal the mechanisms, contact inhibition is still an elusive phenomenon and the molecular mechanisms in its signal transduction have not been comprehensively understood so far.

1.2.3 Cell proliferation and its regulators

Cell proliferation is the result of unremitting progression of cell cycle which comprises a series of tightly regulated events that drive the replication of DNA and cell division (Caldon 2006). Basically, cell cycle can be divided into four phases: G1 phase for preparation of DNA synthesis, S phase for DNA synthesis, G2 phase for DNA repair if there is any error during DNA synthesis, M phase for mitosis. In addition to these four phases, cells may enter G0 phase which is temporary or permanent quiescence phase in the cell cycle and from which resting cells may re-enter the cell cycle and go on to DNA replication and mitosis, when stimulated by mitogens.

The transition between these different phases in cell cycle is strictly controlled by the activity of specific CDKs, which generally remain at constant levels throughout cell cycle although their binding partners (i.e. cyclins) and post-translational modifiers (kinases and phosphatases) undergo periodic fluctuations to regulate DNA synthesis and mitosis. The active CDK is composed of a catalytic subunit and the cyclin regulatory subunit, and the activation of CDK is the central event of cell cycle transitions. Such activation is exquisitely regulated at several levels. One level of cell cycle regulation is cyclin proteins synthesized at a particular stage of the cell cycle. Thus a given catalytic subunit in CDK is not active until an appropriate cyclin is synthesized. Upon synthesized, the cyclin can assemble with an appropriate catalytic subunit and promote cell cycle progression to specific phase. For example, G1/S transition is modulated by cycle D1-CDK4/6 complex because cyclin D is synthesized during G1. In addition to this positive regulation system, there is another level of regulation system comprising a diverse protein family known as cyclin-dependent kinase inhibitors (CKIs) that can inhibit activation of CDKs. Two distinct classes of CKIs have been identified. One is CIP/KIP family which includes p21CIP1, p27KIP1 and p57KIP2 and inhibits multiple CDKs, and the other one

is INK4 family which includes p16INK4, p15INK4B, p18INK4C, and p19INK4D and specifically inhibits cyclin D/CDK4 or 6. The synthesis, degradation, and activity of these CKIs are regulated by mitogenic and anti-mitogenic signals. For example, p27KIP1 mediated cell cycle arrest in G1 phase may be induced by cell-cell contact or the transforming growth factor-β (TGF-β). In this intricate regulatory system, there are some cross-talks among them. For example, one of the mechanisms to control cyclin E/CDK-2 level is through CDK inhibitors, p21 and p27, which can stabilize cyclin E (Hwang 2005), but the availability of these CDK inhibitors is through their sequestration by cyclin D1/CDK4/6 and also through their cytoplasmic relocation as well as their overall protein level (Caldon 2006).

Finally, these transmitted signals converge on hypo-phosphorylated pRb in the nucleus. Hypo-phosphorylated pRb binds to E2F transcription factor and such binding prevents E2F from activating its target genes; in contrast, hyper-phosphoryated pRb releases E2F to activate a myriad of genes that are required to advance cell cycle progression from G1 to S phase (Kasten 1998). The phosphorylation of pRB is mediated by cyclin/Cdks: first by cyclin D/Cdk4/6 complex then by cyclin E/Cdk2 complex in cell cycle. The binding of p27 to cyclin E prevents its binding to pRB, consequently pRb can not be further phosphorylated and the cell cycle is therefore inhibited in presence of p27 in the nucleus.

A lot of studies show the aberration of G1/S transition is very common in breast cancer (Nielsen 1999, Loden 2003). Since cyclin D1 and p27 are the key regulators in G1/S transition, they have been widely studied in breast cancer.

Cyclin D1 is one of the most commonly overexpressed genes in breast cancers, especially in ER-positive breast cancers, where there is a high correlation between cyclin D1 amplification and poor prognosis (Sutherland 2004, Arnold 2005). Literature showed that overexpression of

cyclin D1 promotes tumor cell growth and confers resistance apoptosis in a pancreatic tumor cell line (Biliran 2005). And furthermore, the finding that cyclin D1 overexpression can result in tumorigenesis in mouse mammary fat pad with long latency suggests that cyclin D1 cannot induce tumor alone (Suthererland 2004).

p27 was first identified as a cyclin E/CDK2 inhibitor in the TGF-β induced G1 arrest (Robson 1999, Depoortere 2000), and its activity is regulated by its protein concentration, distribution in different cyclin/CDK complexes and its subcellular localization. Although there are reports of transcriptional regulation of p27 (Medema 2000, Dijkers 2000), p27 protein level is largely regulated at the post-transcriptional level, through modulation of protein stability and degradation (Yang 2000, Taube 2006, Chu 2007). Usually, p27 expression is highest in the nucleus of quiescent cells. As cells exit quiescence and progress into S phase upon mitogenic stimulation, p27 is exported from nucleus to cytoplasm and p27 protein level declines due to decreased translation of p27 mRNA and increased degradation by targeted proteolysis (Sherr 1999). Proteolysis through the ubiquitin-mediated degradation at the proteasome is the predominant mechanism of p27 degradation when p27 is phosphorylated at Thr187 by cyclin E/CDK2. Besides the total protein concentration, such redistribution of p27 in nucleus and cytoplasm determines its inhibitory activity in cell cycle since its target molecule, Cdk2, is a nuclear protein. The cytoplasmic distribution of p27 is mediated by its phosphorylation at ser10. When p27 is phosphorylated at ser10 by hKIS or Akt, Ser10-p27 is exported to cytoplasm and p27-induced G1 arrest is dismissed (Shin 2002, Boehm 2002). Recently, it has been found that phosphorylation of p27 at Thr157 and Thr198 by Akt impairs import of p27 into nucleus (Liang 2003, Shin 2005). Therefore, through phosphorylation at Ser10, Thr157 and Thr198, p27 is sequestered in cytoplasm, thereby losing its inhibitory activity. Another important mechanism to

regulate p27 level is ubiquitin-mediated degradation in proteasome. Stimulation of quiescent cells induces the expression of Skp2, a ubiquitin ligase, in late G1 phase, leading to the ubuquitinylation and subsequent degradation of p27 in vivo and vitro (Carrano 1999).

Because of its role in cell proliferation, p27 is a potential tumor suppressor gene. An overwhelming number of studies have shown that reduced p27 expression in primary cancer tissue is associated with high tumor grade, poor response to chemotherapies or target treatments in various types of tumors, including breast cancer (Nickeleit 2007). Clinical data further revealed that in cancer cells with reduced expression level or even normal expression level of p27, cytoplasmic localization of p27 is much more correlated with poor survival (Blain 2002, Alkarain 2004, Guan 2007), indicating that both p27 proteolysis and mis-localization contribute to the tumor suppressor role of p27 in tumorigenesis. Not only in cell proliferation, p27 also plays a role in apoptosis of cancer cells, although the exact effect is controversial. Some studies have found that down-regulation of p27 induces apoptosis in lung cancer cells (Yokoi 2003), but some others reported that re-expression of p27 induces apoptosis of tumor cells from breast, lung, kidney, and brain (Craig 1997, Li 2000, Katner 2002), and p27 overexpression induces apoptosis of cancer cells through elevation of BAX expression (Fujieda 1999). Therefore, the exact role of p27 in apoptosis is still elusive in cancer treatment.

1.3 THE ACTIN FILAMENTS AND THEIR REGULATION BY PROFILIN

All major functions that are deregulated in cancer, including cell proliferation, cell-cell adhesion and migration, require involvement of actin cytoskeleton which is under dynamic control of many actin-binding proteins.

1.3.1 Actin polymerization and its role in cell adhesion

Basically, actin exists as monomer and polymer in cells. Monomeric actin can be polymerized and assembled into filamentous protein (F-actin) by head-to-tail mode; meanwhile, F-actin can be de-polymerized and disassembled to actin monomer as well. Such conversion is regulated by a variety of proteins and the activity of these regulatory proteins is precisely controlled by various cellular signaling pathways (Pollard 2000, Paavilainen 2004). In non-muscular cells, these regulatory proteins can be divided into four major groups: filament nucleator protein (formin and Arp2/3 complex), monomer-binding proteins (profilin and thymosin-β4), pointed end depolymerization proteins (ADF/cofilin), filament cross-linker proteins (such as α-actinin and fascin), filament capping proteins (such as Cap Z, and CapG), filament severing proteins (gelsolin), and filament end-tracking protein (formins, VASP, N-WASP).

Because they attach to the plasm membrane, actin filaments are involved in many cell behaviors, as a bridge for signal transduction between the exterior and the interior of cells through in cell-cell and cell-ECM adhesion (Yamada 1997). To be specitifical their role in cell-cell adhesion, actin filaments provide strong anchor network to stabilize the adherens junction. Experiments with exogenous G-actin incorporation showed that these labeled G-actin is accumulated in the new formed cell-cell adhesion of epithelial cells and is co-localized with E-cadherin within five minutes after transfer of the cells from low-calcium medium to high-calcium medium (Ivanov 2005).

As a summary, intact actin filaments are very important to in the signal communication of cells to the microenvironment through their link to cell-cell and cell-ECM adhesion, and the dynamic regulation of actin polymerization may affect such cell adhesion.

1.3.2 Profilin in actin polymerization

As one of the first characterized actin-binding proteins (Carlsson 1977), profilin has been intensively studies in the past decades. Profilin is a small molecule (~14kD) and there are four different types genetically (I~IV), among which type I profilin is the most ubiquitously expressed in eukaryotes (Witke 1998). Profilin has a higher affinity for ATP-coupled G-actin than for ADP-coupled G-actin, forming a large reservoir of ATP-G-actin in cytoplasm. More importantly, profilin catalyzes nucleotide exchange on G-actin (Pollard 2000), therefore promoting ADP-ATP exchange in G-actin. When bound to the actin monomer, profilin inhibits the spontaneous nucleation of actin filament, and acts as a sequestering protein in the absence of free barbed end of actin filaments. However, profilin can also deliver the ATP-coupled G-actin to the free barbed end and promote the assembly of actin filaments when the barbed end is open (Pollard 2000, Pantaloni 1993). After delivering ATP-coupled G-actin to the barbed end, profilin dissociates with F-actin because of relatively weak binding of profilin with F-actin (Schutt 1993), and the free profilin binds to free G-actin in cytoplasm and starts another cycle.

In addition to actin molecule, profilin can also bind to some other molecules, such as phophoinositides and proline-rich ligands, through which profilin is involved in various signaling pathways, such as Rho/GTPases and PIP3-kinase pathways (Witke 2004).

1.3.3 Physiological role of profilin in cell behaviors

As a key actin-binding protein, profilin is actively involved in actin cytoskeleton relevant cell behaviors, such as cell shape, cell adhesion, and cell motility of non-muscle cells. Janke reported that profilin overexpression leads to an increased spreading of breast cancers (Janke 2000), but

Roy showed that even moderate overexpression of profilin in breast cancer cells is strong enough to impair the cell spreading (Roy 2004). These seemly contradictory results suggest that the differential behaviors might be due to the different effect of profilin on actin assembly, depending on profilin concentration in the cells.

In vivo studies show that profilin is so important that its deficiency in mammalian cells usually leads to various dysfunctions of cells, including cell migration, cytokinesis, endocytosis and transcriptional regulation (reviewed by Witke 2004). Analysis of profilin null mutation in yeast showed that mutant cells display aberrant phenotypes, such as slower growth, larger size, multiple nucleus and non-detectable actin filaments (Mogdolen 1988, Haarer 1990). In Drosophilium, profilin depletion leads to a recessive embryonic lethal phenotype (Verheyen 1994, Haugwitz 1994), which implies a stringent requirement for profilin in multicellular eukaryotes. Animal experiments show that Pfn1-null mice arrest at early stages of development, which is caused by the cell division defect in the Pfn1-null mice (Witke 2001). Profilin is therefore generally regarded as a key player of cytokinesis (Wilkes 2003, Glotzer 2005, Bottcher 2009).

Some other studies have shown that profilin aberration is involved in tumorigenesis and tumor progression. In tumor cells, the cell adhesion is decreased and locomotory activity is increased, which involve the strictly regulated interplay of actin cytoskeleton with the transmembrane proteins of junctional complex. There are more and more evidences showing the correlation between those pathological changes and an abnormal profilin level. In 2000, Janke reported lower expression of profilin in several tumorigenic breast epithelial cell lines, compared to their non-tumorigenic counterparts, which suggested that a partial loss of profilin-1 expression correlates with the tumorigenic phenotype in mammalian tumor cells (Janke 2000). Similar

results are also reported in migratory astrocytic tumor cells (Belot 2002) and hepatocarcinoma cells (Wu 2006). However, it's not always the case that profilin is lower in cancer cells. It is found that the expression level of profilin in human gastric cancer is significantly higher than in normal gastric tissue (Tanaka 1992, Schluter 1997). Therefore, the role of profilin in tumorigenesis is still elusive. Further study shows that overexpression of profilin in breast cancer cells suppresses tumorigenicity of those tumorigenic cells (Janke 2000). The complementation of profilin expression in the tumor cells with wild-type and mutant profilins showed that the actin-binding properties of profilin is required to rescue the transformed cell phenotype (Wittenmayer 2004). Due to the complex role of profilin in actin cytoskeleton and signal pathways, it is very difficult to predict the mechanisms how profilin works in tumorigenesis. Although some cell behaviors, such as those mentioned above, have been investigated in those profilin down-regulated tumor cells, some other hallmark cell behaviors of tumor cells, like cell-cell adhesion, cell proliferation and cell survival, have not been reported so far although they are important to help understand the mechanism how profilin is involved in tumorigenesis and how tumor formation is suppressed by overexpressing profilin in those tumor cells.

In general term, profilin may be considered to be a central hub that controls the framework of actin cytoskeleton and molecular interactions, as well as regulates the activity of ligands directly. And the alteration of profilin expression in cancer cells may contribute to the tumorigenesis and cancer developments, although the exact underlying mechanism is far from completely understanding yet.

2.0 HYPOTHESIS AND SPECIFIC AIMS OF THIS STUDY

Given that Pfn1 is a key regulator of actin polymerization in cells, I hypothesized that Pfn1 plays a role in the regulation of cell-cell adhesion in mammary epithelial and breast cancer cells and Pfn1-dependent regulation of cell-cell adhesion may have implication in its growth inhibitory effect on breast cancer cells (HYPOTHESIS). To test this hypothesis, I proposed the following aims:

1). To evaluate the effects of perturbation of Pfn1 expression on cell-cell adhesion of normal human mammary epithelial and breast cancer cells (AIM 1), and

2). To determine whether cell-cell adhesion plays a role in Pfn1-dependent regulation of breast cancer cell growth (AIM 2).

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3.0 MTERIALS AND METHODS

3.1 CELL CULTURE AND TRANSFECTION

Human Mammary Epithelial Cells (HMECs, from Cambrex, Walkersville, MD) were cultured in complete culture medium recommended by manufacturer. Because of its finite lifespan, HMECs used for experiments in this study were limited to 3~4 passages in culture after received from the manufacturer (i.e. up to P_{10}). MDA-MB-231 breast cancer cells were cultured in EMEM media supplemented with 10% FBS, sodium pyruvate and antibiotics. The GFP and GFP-Pfn1 mutant cells were cultured in regular serum plus medium with 1mg/ml G418.

Plasmid transfection of cells was perfomed using lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Stable clones of MDA-MB-231 cells were selected and maintained using the regular growth media containing 1mg/ml G418.

In **gene silencing** experiments, cells were transfected with either a control siRNA or a custom-designed Pfn1-siRNA: the custom-designed siRNA (sense strand: 5'-AGA AGG UGU CCA CGG UGG UUU-3'; antisense-strand: 5'-ACC ACC GUG GAC ACC UUC UUU-3') was synthesized by Dharmacon (Lafayette, CO) and was transfected into the cells using a proprietary reagent according to the manufacturer's protocol. Smart-pool of non-targeting control was purchased from Dharmacon (Chicago, IL). All siRNA transfections were performed at a 100 nM working concentration (except p27 siRNA, using 25nM) using transfection reagent available

through Dharmacon according to the manufacturer's instruction (Chicago, IL). After 24 hours, the transfection medium was replaced, followed by regular culture for additional 3 days.

The sources of other siRNA reagents were: R-cadherin siRNA (Santa Cruz, CA), p27 siRNA from Dharmacon (Chicago, IL).

3.2 PROTEIN EXTRACTION AND IMMUNOBLOTTING/IMMUNOPRECIPITATION

All protein manipulations were performed on ice to minimize protein degradation. The cell layer was washed twice with ice-cold DPBS, then lyzied with either 1x sample buffer or with RIPA buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 2mM EDTA, 50mM NaF, 1mM sodium pervanadate and protease inhibitors). The cell lysate was collected with a clean cell scraper. For the protein extracted with 1x sample buffer, the lysate was boiled in 100˚C for 5 minutes before measuring the protein concentration. For the protein extracted with RIPA buffer, the lysate was incubated in ice for 20 minutes with repeated vertex, then centrifuged at 13kg for 15 minutes at 4˚C to remove any insoluble debris, the protein in the supernatant was measured with DC protein assay (BioRad, CA), according to the recommened protocol.

Nuclear protein extraction: the monolayer cells were harvested and lyzed in buffer A first (10mM HEPES, pH7.9, 10mM KCl, 0.1mM EDTA, 0.4% NP-40, 1mM DTE with protease inhibitors) for 15 minutes, then centrifuge at 10krpm for 2 minutes. The cell pellet was washed twice with buffer A, then resupended in buffer B (20mM HEPES, pH7.9, 0.4M NaCl, 1mM EDTA, 10% glycerol and imM DTE with protease inhibitors). Violently vertex for two hours at

4˚C, then centrifuge at top speed for 2 minutes. The protein in supernatant was kept as nuclear protein for evaluation.

Actin-bound protein extraction: the monolayer cells were harvested and lyzed in Triton X-100 buffer (20mM Tris HCl, pH 7.5, 150mM NaCl, 0.5% Triton X-100, 1mM sodium orthovanadate, and protease inhibitors) for 10 minutes, and then spun down the cell pellet. The cell pellet was lyzed with 1x sample buffer, as the Triton X-100 insoluble fraction in the cells.

Immunoprecipitation (IP): the protein was extracted with modified RIPA buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 2mM EDTA, 50mM NaF, 1mM sodium pervanadate and protease inhibitors).1000 mg of lysate was first precleared with 30 ml of protein-G/ protein A-conjugated agarose beads. Precleared lysate was incubated with 3 mg of the indicated primary antibody overnight and then with 75 ml of the same beads for an additional two hours. Immunoprecipitated protein sample was washed three times with the IP lysis buffer, resuspended in 30 ml of 2x sample buffer and run on an SDS–PAGE.

Other antibodies: cyclin D1 (1:200) and p27 (1:500) from BD transduction lab (CA), GAPDH (1:1000) from Biogenesis (UK); R-cadherin (1:100), ser10-p27 (1:200) from Santa Cruz (CA).

3.3 INDIRECT IMMUNOFLUORESCENCE

All the cytoimmunochemical staining is done at room temperature. First, the treated cells were grown to subconfluence on 10ug/mL of collagen-coated coverslips, then fixed with 4% formaldehyde for 10 minutes and permeablized with 0.5% Triton-X 100 for 10 minutes. 1% BSA

in PBS was used to block non-specific binding, 60 minutes at room temperature. Different primary antibodies were used to detect the specific proteins in the fixed cells, 60 minutes at room temperature (E-Cadherin 1:100, pan-Cadherin 1:50, R-Cadherin 1:200, beta-Catenin 1:100, vinculin 1:100). After washing with 0.05% Tween-20 in PBS, the appropriate secondary antibody was added and incubated for 60 minutes at room temperature (TRITC conjugated goat anti-mouse 1:100, TRITC conjugated goat anti-rabbit 1:50). Cell nuclei were labeled by DAPI staining. All fluorescence images were acquired with a 60X objective using an Olympus IX-71 inverted microscope.

F-actin staining: When the cells were fixed with 4% formaldehyde for 10 min and permeabilized with 0.2% Triton-X 100 in PBS for 5 min, the cells were stained with rhodamine phalloidin (Molecular Probes, OR) for 30 min at room temperature. The stained cells were washed three times in PBS before mounting on slides.

3.4 FUNCTIONAL EVALUATION OF CELL-CELL ADHESION AND CELL-ECM ADHESION

This was accomplished by modified protocol of cell adhesion force by centrifugal assay (CAFCA, McClay 2001). In total, 20 000 cells were non-enzymatically dissociated, stained with 5 mM calcein AM (Invitrogen, Carlsbad, CA, USA) and then plated in triplicate on a dense monolayer of unlabelled cells in a 96-well plate (for cell-cell adhesion assay) or on collagen-precoated 96-well plate (for cell-ECM adhesion assay). After allowing cell attachment (30 min for cell-cell adhesion, 30 min and 60 min for cell-ECM adhesion), the culture plate was reversed and centrifuged at 200 g force for 2 min and then washed gently with PBS twice to remove the

unattached cells. Cell adhesion was scored by recording the fluorescence intensity of labeled cells that remained attached in the well using a plate fluorometer. Absolute fluorescence readings were background subtracted, averaged for the replicate set of samples from a total of four independent experiments to show significant difference statistically.

3.5 IN VITRO CELL MIGRATION ASSAY

Two commonly used assays were adopted for the in vitro evaluation of cell motility:

Wound healing assay: First, the bottom surface in each well of 24-well plate were pre-coated with 10ug/mL of type I collagen from rat tails, then washed with PBS twice to remove the non-binding collagen in each wells. Then, the treated cells were trypsinzed and seeded into each well of the 24-well plates and cultured in regular culture medium until they were confluent in the well plates. After washing off the floating cells in each well, an in vitro wound was introduced in the middle of the cell monolayer with a 2uL pipette tip, followed by extensive wash with DPBS twice. Cells were then incubated in regular culture medium for up to 6 hours. The wound closure in the cell monolayer was visualized under inverted microscope (Olympus), recorded and analyzed with Metamorph software. The wound closure was quantitatively evaluated by the change of area between edges of the migrating cells on two sides of the wound. At least 3 independent experiments were repeated for statistical analysis.

Transwell cell migration assay: The membranes of a 24-well transwell (8 mm pore size; Corning; Acton, MA) were pre-coated on both sides 10ug/ml type I collagen for one hour at room temperature. The pre-treated cells were serum-starved overnight and then seeded in at least triplicates onto the upper chamber of the transwell in 0.15% BSA containing basal media. The

lower chamber of the transwells was filled with the same media containing 10ng/ml EGF and 0.15% BSA to establish a chemotactic gradient. After an incubation period of six hours, the transwells were removed from the plate and non-migrating cells from the top of the transwells were gently scraped off with cotton swabs. Cells that had migrated onto the underside of the membrane were fixed and stained with 0.5% crystal violet solution for 10 min. The transwells were then thoroughly washed with water. To estimate the number of migrating cells on the button surface, an absorbance reading of the removed dye, was taken at 595 nm using an automated plate reader.

3.6 CELL PROLIFERATION ASSAY AND FACS ANALYSIS OF CELL CYCLE

MDA-MB-231 GFP cells and GFP-Pfn1 were cultured in regular culture medium until they were 60% confluent in culture dish.

Cell proliferation evaluation: The cells were plated at $3x10^4$ cells/well in 12-well plate. The cells were incubated overnight in regular culture medium. Then, the cells were serum starved with basal medium for 24 hours followed by 10% serum stimulation for additional 24, 48 and 72 hours. After serum starvation, triplicate cultures were trypsinized and the cell number was counted with trypan blue exclusion assay, as the starting cell number and the readouts at other time points were the cell number of the proliferating cells after serum stimulation.

FACS analysis: To evaluate the cell cycle of these treated cells, the relative DNA content of individual cell was measured by the level of fluorescence from propidium iodide (PI) bound to the DNA, using the fluorescence-activated cell sorter (FACS). Briefly, the treated cells were trypsinized and washed with DPBS twice before fixing with ice-cold 70% ethanol at 4C

overnight. Then ethanol was removed by centrifuge and wash with PBS. The same amount of cells was re-suspended with PI solution (50ug/ml PI with 100ug/ml DNAse free RNAse A, 0.1% TX-100 and 0.1mM EDTA in PBS), then kept in darkness for at least 45 minutes before FACS analysis using FacsCanto with Diva software (version 5.0.2, from BD). At least 10,000 cells were analyzed, and the FACS data was analyzed with the program FlowJo 5.7.2 (Tree Star Inc. OR) from three independent experiments.

3.7 RT-PCR

Total RNA was extracted from GFP cells and GFP-Pfn1 cells in 100cm culture dish with an RNeasy mini kit (QIAGEN, CA), according to the recommended RNA extraction protocol. The primers selected are as follows: R-cadherin, 5'- GTTGGGGCAGATGGGACAGT -3' (forward) and 5'-ACGTTGATGGGCGGGATGAC-3' (reverse); p27/KIP1, 5-TGCAACCGACGATTCTT CTACTCAA-3 (forward) and 5-CAAGCAGTGATGTATCTGATA AACAAGGA-3 (reverse); GAPDH, 5-CGGAGTCAACGGATTTGGTCGTAT-3 (forward), 5-AGGCTTCTCCATGGTGG TGAAGAC-3 (reverse). R-cadherin was amplified in the following condition: 95°C for 15 min followed by 40 cycles of 94°C for 30 s and 60°C for 30 s, and 72°C for 30 s; p27 RNA was amplified by 95°C for 15 min followed by 45 cycles of 95°C for 15 s and 60°C for 15 s. For GAPDH RNA amplification, 95°C for 15 min followed by 40 cycles of 95°C for 60s, 55°C 60s and 72°C for 60 s. The PCR products were visualized by 2% agarose gel.

3.8 CELL APOPTOSIS

In order to evaluate the cell survival of GFP cells and GFP-pfn1 cells, these cells were exposed to TNFα to induce apoptosis. Briefly, the cells were pre-treated with cycloheximide (10uM) for 30 minutes followed by addition of TNFα (40ng/ml) to the medium with cycloheximide.

Nuclear fragmentation: To identify the apoptotic cells, the cell nuclei were observed by DAPI staining. The cells were seeded on collagen-coated cover slip before treatment. Then, they were exposed to CHX or CHX+ TNFα for no more than 8 hours before fixing with 100% methanol. The nuclei of the apoptotic cells were stained with 1ug/mL of DAPI for 30 minutes at room temperature and visualized under the Olympus fluorescence microscrope.

FACS analysis: the cells were treated with the apoptotic inducer drugs for 18 hours before harvested and fixed using the same protocol for cell proliferation.

Immunoblotting: after 18 hours, all the treated cells including the floating cells and the attached cells were harvested and the protein was extracted from the cells using 1x sample buffer, and PARP (Cell Signaling, MA) blot was performed to evaluate the cleaved PARP in the treated cells.

3.9 IN VIVO STUDY OF TUMOR FORMATION

Two million MDA-231 cells, suspended in 200 uL of sterile PBS, were orthotopically injected into the right inguinal mammary fat pad of a 4-week old nude female mouse. After killing the animals at either 5–6- or 8-week time points after the injection, tissues from the lung, liver and spleen were harvested for histological staining. These experiments were performed in

compliance with an approved protocol by the Institutional Animal Care Committee of the University of Pittsburgh.

3.10 STATISTICAL ANALYSIS

All data were collected as mean ± standard deviation. Student t-test or one-way analysis of variance (ANOVA) was used to compare the cell behaviors in control group and experimental group, as indicated in specific experiment. The difference was considered significant when p value was less than 0.05.

4.0 RESULTS AND DISCUSSION

4.1 AIM-1: TO EVALUATE THE EFFECTS OF PERTURBATION OF PFN1 EXPRESSION ON CELL-CELL ADHESION OF NORMAL HUMAN MAMMARY EPITHELIAL AND BREAST CANCER CELLS

4.1.1 Downregulation of Pfn1 leads to reduced F-actin content in HMECs

To downregulate Pfn1 expression in normal human mammary epithelial cells (HMECs), we transfected HMEC with either Pfn1-siRNA or a non-targeting control siRNA Ninety-six hours after transfection, Pfn1 expression level in HMECs was successfully down-regulated by Pfn1-siRNA transfection (Fig.1).

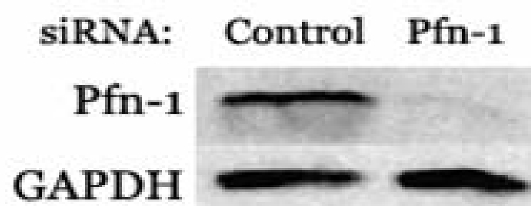


Figure 1 Pfn1 expression in HMECs is knocked down by siRNA transfected on 4th day.

We next evaluated whether silencing Pfn1 expression alters actin cytoskeleton of HMEC. Phalloidin staining showed silencing Pfn1 expression reduced F-actin in Pfn1 silenced cells (Fig 2A). Quantification of phalloidin intensity showed a significant 35% decrease of F-actin in Pfn1

depleted HMECs compared to that in the control cells (Fig 2B). However, the total expression level of actin was not affected by Pfn1 depletion (Fig 2C). These data suggest that Pfn1 acts as a promoter of actin polymerization in this case.

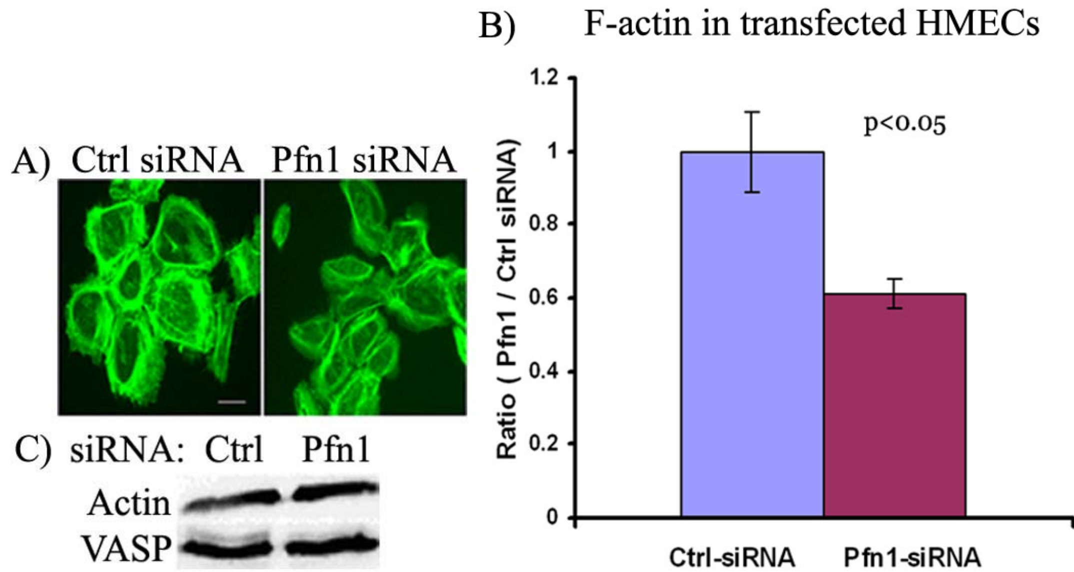


Figure 2 F-actin in HMECs is reduced by Pfn1-siRNA transfection. A) F-actin staining by phalloidin (60x); B) Quantification of phalloidin staining of the transfected HMECs; C) Total actin level in transfected HMECs.

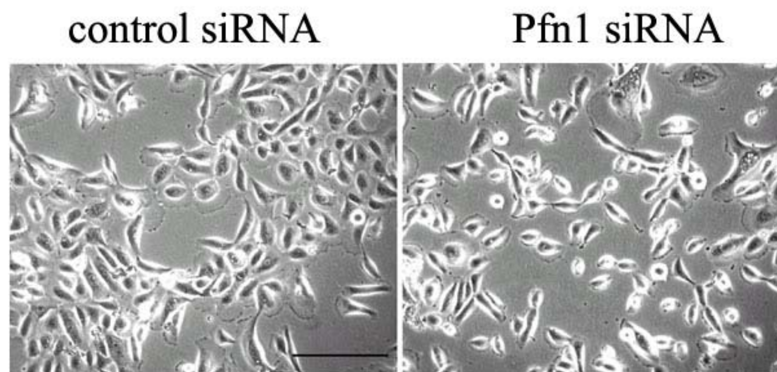


Figure 3 Pfn1 deficient HMECs are more disseminative (10x)

Morphological examination showed that Pfn1 deficient HMECs are more spindle shaped and disseminative from their neighboring cells, compared to the more typical clustered normal epithelial cells, under sub-confluent culture condition (Fig.3).

4.1.2 Pfn1 depletion leads to E-cadherin and β-catenin delocalization from AJ in HMECs

Usually, increased cell dissemination indicates unstable cell adhesion between cells. The morphologic observation of more disseminative Pfn1 deficient cells leaded us to the research question whether such disseminative phenomenon is due to altered cell-cell adhesion. Therefore, cell-cell adhesion of the transfected HMECs was evaluated qualitatively and quantitatively. Here, E-Cadherin and its intracellular binding partner, β-Catenin, were examined by immunostaining and immunoblotting (Fig. 4). The immunostaining data showed that Pfn1 silenced HMECs have much less E-cadherin and β-Catenin at the junctional area of the transfected epithelial cells, and also showed the overlapping of these Pfn1 depleted cells when they reached confluent (Fig. 4 A&C). However, immunoblotting showed comparable total protein levels of those adhesion proteins after silencing Pfn1 in HMECs (Fig. 4 B&D).

Because E-cadherin usually needs to bind to actin filaments in stable cell-cell adhesion of epithelial cells, it is necessary to verify whether such decreases of junctional E-cadherin and β-catenin are related to the decreased actin filament in Pfn1 deficient cells. Therefore, the fractionation of transfected HMECs was extracted by Triton-X-100. The cytoskeletal proteins were resistant to the Triton X-100 but lysed in 1x sample buffer. Consistent with immunostaining results, we found reduced E-cadherin and β-catenin in triton-insoluble fractions of cell lysates (the pool of cadherin/catenin complex attached to actin cytoskeleton) when Pfn1 expression was downregulated (Fig. 5).

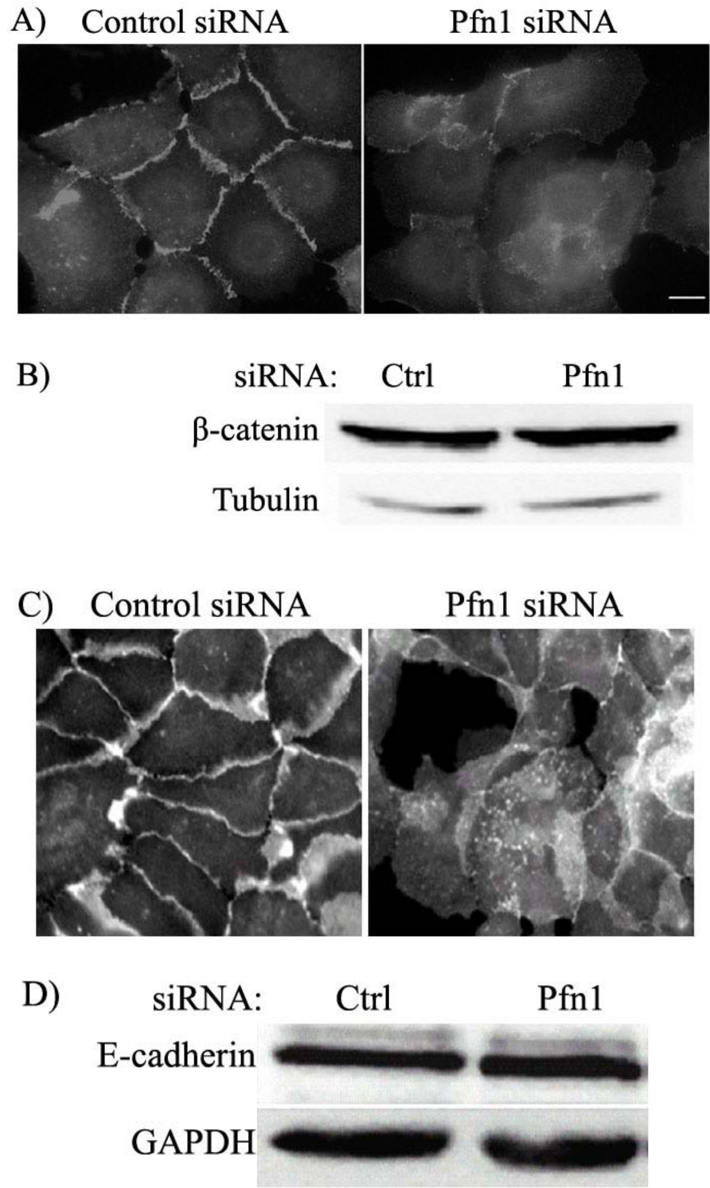


Figure 4 AJ mediator proteins are delocalized from cell-cell adhesion in Pfn1-siRNA transfected HMECs. β-catenin and E-cadherin staining of siRNA transfected HMECs (A & C, 60x); Protein level of total β-catenin and E-cadherin in siRNA transfected HMECs (B and D)

Taken together, the immunostaining and immunoblotting results suggested that reduction of junctional E-cadherin and β-Catenin in Pfn1 silenced HMECs is due to dissociation of these adherent proteins from actin filaments after siRNA transfection. Combined with the result

regarding F-actin in Pfn1 silenced HMECs, it is reasonable to postulate that actin filaments act as anchor substrates to recruit cadherin-catenin complex concentrated at the junctional area in cell-cell adhesion.

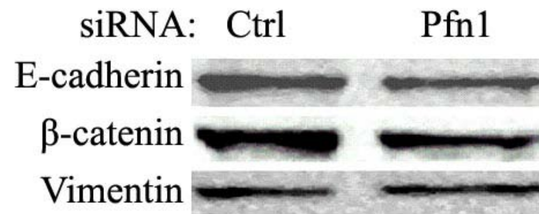


Figure 5 β-catenin and E-cadherin are dissociated from cytoskeleton of Pfn1 depleted HMECs (The blots show the protein level in triton-X 100 insoluble fraction of the cell lysate).

4.1.3 Cell-cell adhesion is significantly impaired in Pfn1 silenced HMECs

Morphologically, it was found that Pfn1 silenced HMEC lost junctional E-cadherin and β-catenin at cell-cell adhesion, so the following experiment was designed to test the functional cell-cell adhesion of HMECs when they lose E-cadherin at cell-cell adhesion. From the data readout of modified cell adhesion force by centrifugal assay, we could see the obvious difference of cell-cell adhesion. The results showed that even within the first 30 minutes of cell attachment phase, there was a significant reduction in the intercellular adhesion of HMEC (~46%) when Pfn1 expression was suppressed (Fig.6). Although centrifugation-based cell–cell adhesion assay adopted in this study is widely used, it suffers from a potential drawback that detachment of pre-coated cells from the underlying substratum, if present, can confound the results. However, there was no evidence of such disruption of the pre-coated monolayer formed by unlabelled cells when they were exposed to the same centrifugal force for either of the treatment groups. So, the experimental readouts truly represent cell–cell adhesion. Also, since this assay scores the number

of cells attached to a monolayer of cells as opposed to measuring the actual force, it is more reasonable to obtain the experimental readout shortly after cell-seeding rather than at longer time points where differences in the rate of cell proliferation between the two transfection conditions may perplex the results.

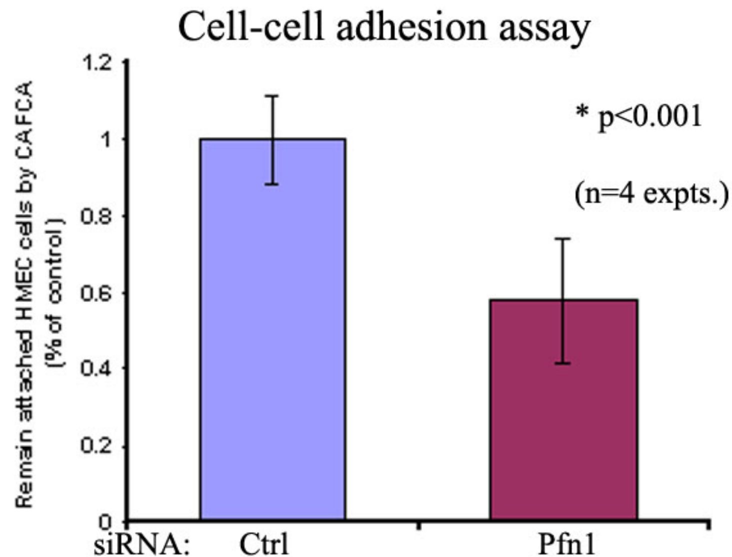


Figure 6 Cell-cell adhesion is impaired in Pfn1 deficient HMECs, evaluated by modified CAFCA.

4.1.4 Pfn1 depletion reduces cell-ECM adhesion in HMEC

As mentioned above, any changes in either cell-cell adhesion or cell-ECM adhesion may contribute to cellular disseminative phenotype. In order to assess cell–ECM adhesions of those disseminative cells, focal adhesion of HMECs on collagen-based substrate was examined. As the major protein in matured focal adhesion at cell-ECM interface, vinculin links F-actin to the plasma membrane and consequently links to the extracellular substrate through integrins. Here, the distribution and overall expression level of vinculin was examined in these siRNA transfected HMECs. In all of siRNA transfected HMECs, focal adhesion could be observed

under the fluorescent microscope after vinculin immunostaining with similar total protein expression level (Fig.7A&B). However, the size of vinculin, as an indicator of focal adhesion at the cell-ECM interface, is much smaller in Pfn1 depleted HMECs (Fig. 7C). The histogram

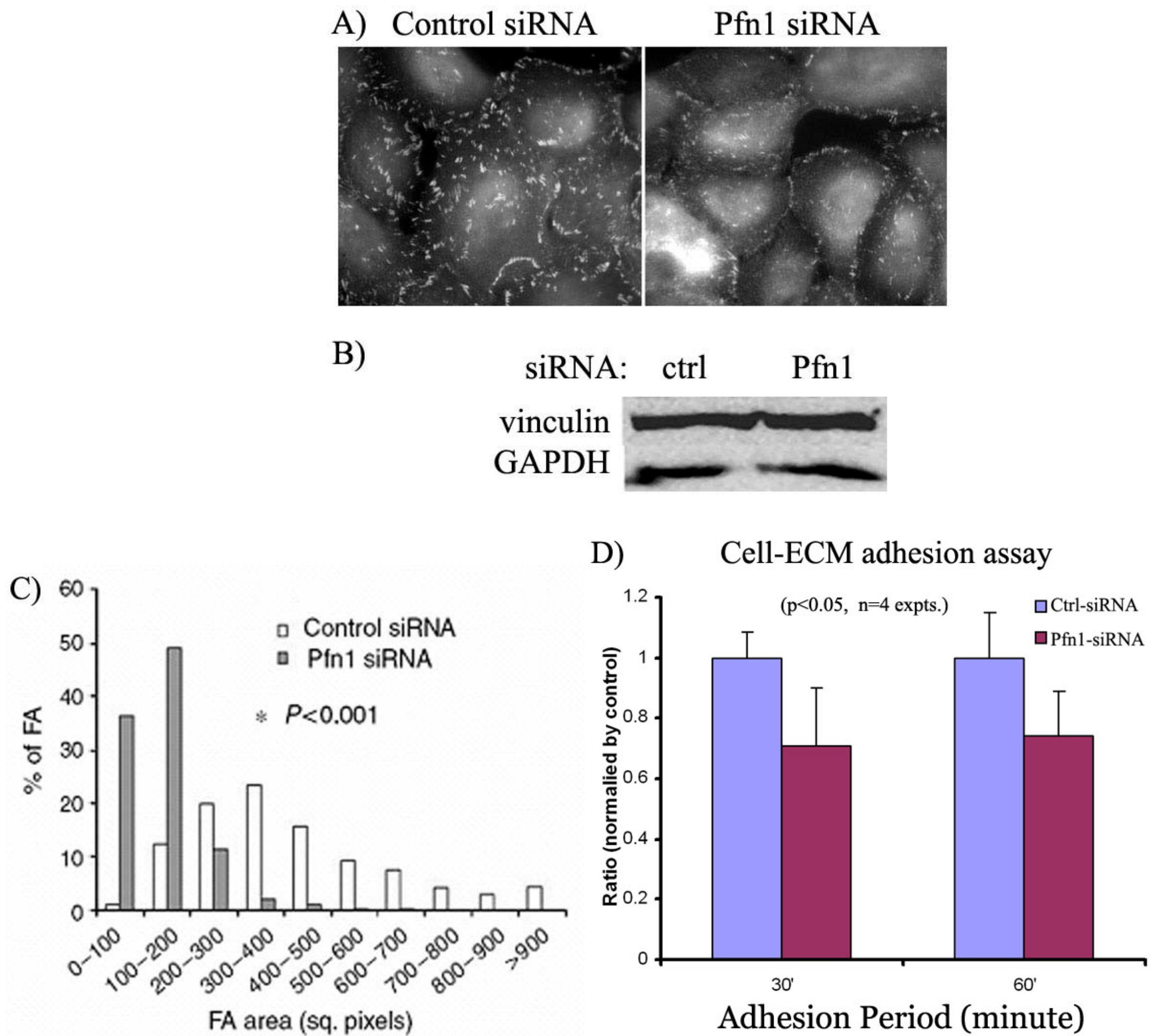


Figure 7 The Pfn1 down-regulation reduces cell-ECM adhesion of the transfected HMECs. A) The focal adhesion (vinculin staining) in the HMECs (60x); B) The protein level of total vinculin in HMECs**;** C) Quantification of focal adhesion area of the transfected HMECs; D) The cell-ECM adhesion by modified CAFCA.

illustrated the significant difference in the focal adhesion (FA) size between the control (average size: 425±237 square pixels; n=775 FAs from 48 cells) and Pfn1-siRNA treated cells (average size: 137±69 square pixels; n=788 FAs from 51 cells). And, the CAFCA result of cell-ECM adhesion confirmed weaker cell-ECM adhesion in Pfn1 silencing HMECs, compared to the control cells (Fig. 7D).

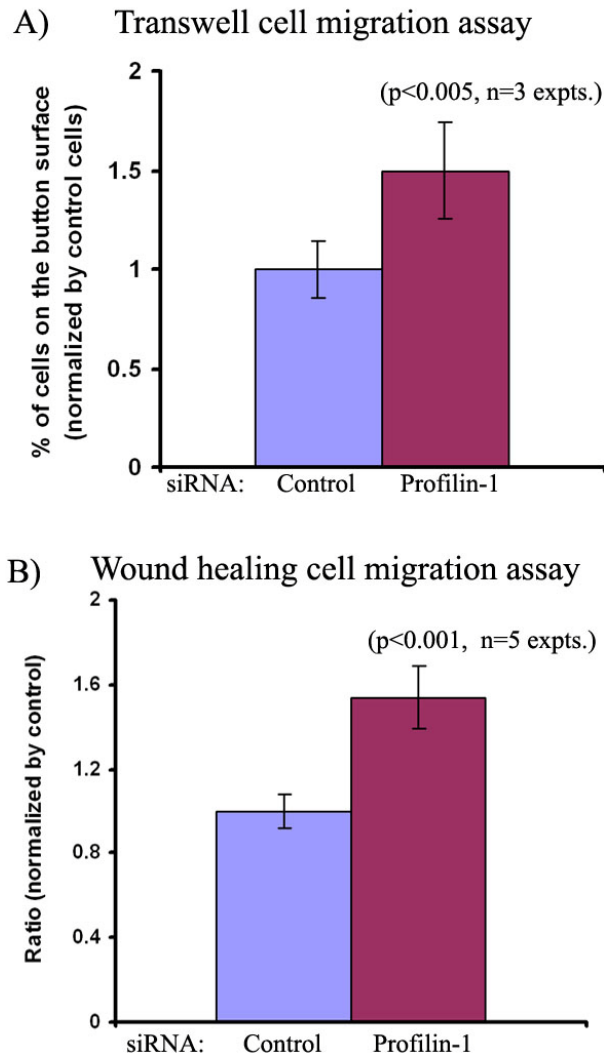


Figure 8 Pfn1 depletion enhances cell migration of HMECs, evaluated by transwell migration assay (A) and wound healing assay (B).

4.1.5 Pfn1 depletion increases HMEC motility

Based on all of the results mentioned so far, it is speculated that Pfn1 depletion endows enhanced cell motility to HMECs, as suggested by more disseminative cells in culture. The next step was to test whether cell motility is enhanced by silencing Pfn1 in HMECs. Two quantitative cell migration assays were adapted for this purpose, transwell migration assay and wound healing assay (Fig. 8). Both assays consistently indicated ~ 50% increase of cell migration after silencing Pfn1 expression level in HMECs.

4.1.6 Pfn-1 expression is successfully up-regulated in MDA-MB-231 cells by plasmid transfection

In all previous studies on HMECs, we found Pfn-1 down-regulation reduced cell-cell adhesion of normal epithelial cells. Therefore, we next asked whether increasing Pfn1 expression in mesenchymal breast cancer cells can enhance cell-cell adhesion.

Here, MDA-MB-231 cells were chosen in our study, because 1). MDA-MB-231 cells are well-characterized mesenchymal breast cancer cells, which lack E-cadherin expression (Fig 9A) and do not form cell-cell adhesion; 2). Pfn-1 level is significantly lower in MDA-MB-231 cells, compared to their normal counterpart, HMECs (Fig 9B); 3) our in vivo showed that over-expressing GFP-Pfn-1 suppresses tumorigenic ability of MDA-MB-231 in nude mice while control GFP expressers form robust tumor as expected (Fig 10).

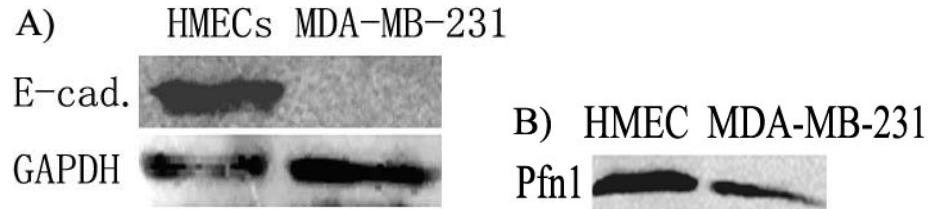
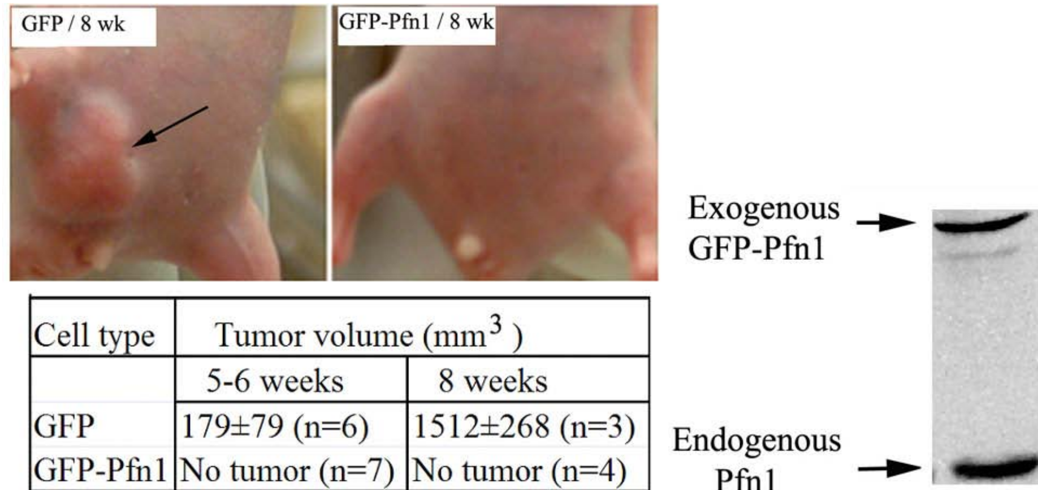


Figure 9 MDA-231 cells are null of E-cadherin expression, with lower Pfn-1 expression level.



Cell type	Tumor volume (mm^3)	
	5-6 weeks	8 weeks
GFP	179±79 (n=6)	1512±268 (n=3)
GFP-Pfn1	No tumor (n=7)	No tumor (n=4)

Figure 10 Pfn-1 over-expression inhibits in vivo tumor formation of MDA-231 cells (the western blot shows Pfn-1 expression level in GFP-Pfn1 cells).

4.1.7 Up-regulation of Pfn1 leads to cell cluster of cultured MDA-MB-231 cells

Morphological observation of GFP and GFP-Pfn1 cells at sub-confluent condition showed that GFP cells remains as single and isolated cells, as the parental MDA-MB-231 cells, but GFP-Pfn1 cells exhibit an epithelioid morphology, as indicated by the propensity to form discrete clusters in culture, and are also much more spread-out compared to GFP cells (Fig. 11A, the left two figures). Following this result, it was asked whether clustering of GFP-Pfn1 cells could be reversed by downregulating Pfn1 expression. To address this question, GFP-Pfn1 cells were treated with either non-targeting control- or Pfn1-siRNA. After transfection, the immunoblot

confirmed that Pfn1-siRNA treatment strongly suppressed the expression of both endogenous and exogenous Pfn1 in GFP-Pfn1 cells (Fig 11B). Morphological examination revealed that control siRNA transfected GFP-Pfn1 cells showed clustering phenotype similar to untransfected cells as expected and this clustering effect was completely eliminated when Pfn1 expression level was down-regulated in GFP-Pfn1 cells (Fig 11A, the right two figures). This observation demonstrated that clustering of GFP-Pfn1 expressers is specifically due to an increase in the overall Pfn1 content.

Although clustered cell population is a typical morphology in normal epithelial cells, it can not be concluded that Pfn1 overexpression reverses the phenotype of MDA-231 cells to normal epithelial cells. Usually, MDA-MB-231 cells, which are null of most major cadherin proteins (E- P-, and N-cadherin), do not form functional cell-cell adhesion and exhibit traits of

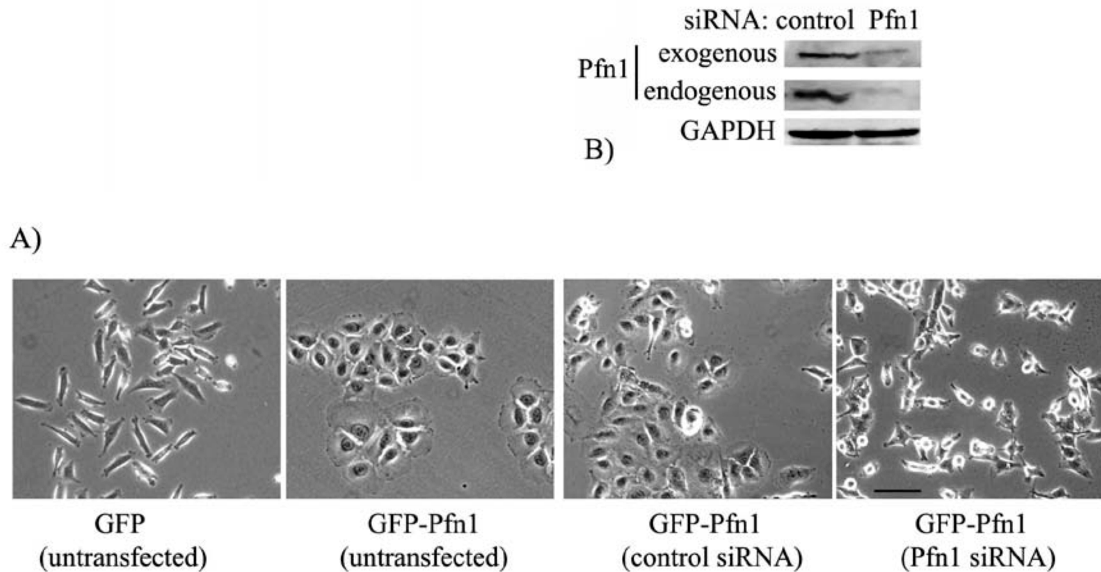


Figure 11 Pfn1-induced clustering of MDA-231 cells is reversible. A) Phase-contrast micrographs of GFP-Pfn1 expressers 72 hours after treatment with either control or Pfn1-siRNA (morphology of untransfected GFP and GFP-Pfn1 expressers are shown side-by-side for comparison). B) Pfn1 immunoblot showing strong suppression of endogenous and exogenous Pfn1 in GFP-Pfn1 expressers 72 hours after Pfn1-siRNA treatment.

post-epithelial-to-mesenchymal transition (post-EMT), such as robust vimentin and loss of cytokeratin expression. The lack of E-cadherin expression and comparable expression of vimentin (a mesenchymal marker) in GFP and GFP-Pfn1 cells were validated by immunoblotting analyses (Fig.12). Therefore, Pfn1 overexpression in our experiments introduces epithelial-like morphological traits but does not cause a true mesenchymal-to-epithelial transition (MET) of MDA-MB-231 breast cancer cell line.

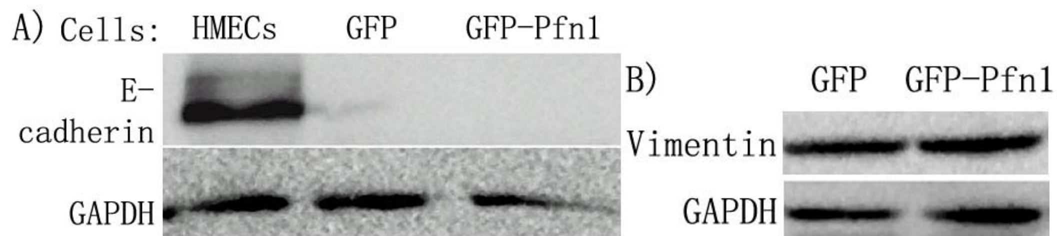


Figure 12 GFP and GFP-Pfn1 cells do not re-express E-cadherin protein (A) but express vimentin (B).

4.1.8 Clustering of GFP-Pfn1 cells is due to cadherin-medicated AJ

Even though this is not a true MET phenomenon, there is a clear morphological distinction between GFP cells and GFP-Pfn1 cells. Specifically, GFP-Pfn1 cells show propensity to form clusters in culture, while control GFP cells do not. Since clustered morphology indicates stronger cell-cell adhesion, it is necessary to identify the molecular mediator responsible for the clustered phenotype of Pfn1 overexpressing MDA-MB-231 cells, given that E-cadherin can not be involved in this cell adhesion.

Initially, it needs to be clarified whether clustered phenotype of GFP-Pfn1 cells is through restoration of AJ complex. Basically, AJ consists of two basic types: the nectin-afadin complex (in calcium-independent adhesion) and the classical cadherin-catenin complex (in

calcium dependent adhesion). Immunostaining of junctional molecules showed concentrated β−catenin (a marker for AJ) localization at cell-cell junctional area in GFP-Pfn1 cells but not in control GFP-expressing cells (Fig.13A), which implies strong cell-cell adhesion among those clustered GFP-Pfn1 cells. However, such cell clusters of GFP-Pfn1 cells were readily dissociated, accompanied with de-localization of β−catenin (Fig.13B), upon brief treatment of EGTA (a calcium chelator). Such response demonstrates that cell-cell adhesion in GFP-Pfn1 cells is Ca^{2+} sensitive adhesion. Therefore, the cell-cell adhesion in GFP-Pfn1 cells most likely involves classical cadherin-dependent AJ assembly.

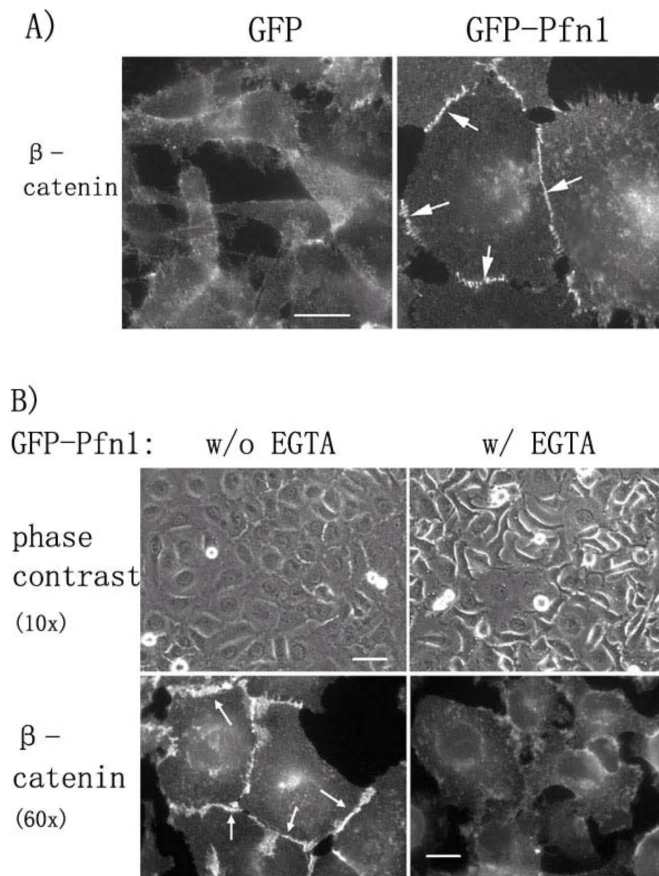


Figure 13 Pfn1overexpression restores AJ in MDA-231 cells. A) β-catenin staining of GFP and GFP-Pfn1 cells (60x, arrows shows the junctional localization); B) The morphology (the upper panel) and β-catenin staining of GFP-Pfn1 cells (the lower panel) 20 min after treatment of 2mM EGTA.

Indeed, immunostaining of pan-cadherin confirmed that cell-cell junctions of GFP-Pfn1 cells are positive for pan-cadherin immunoreactivity (note that the pan-cadherin antibody used here could recognize all members of cadherin family of proteins) (Fig. 14), which further suggests the cadherin-mediated AJ assembly between GFP-Pfn1 cells.

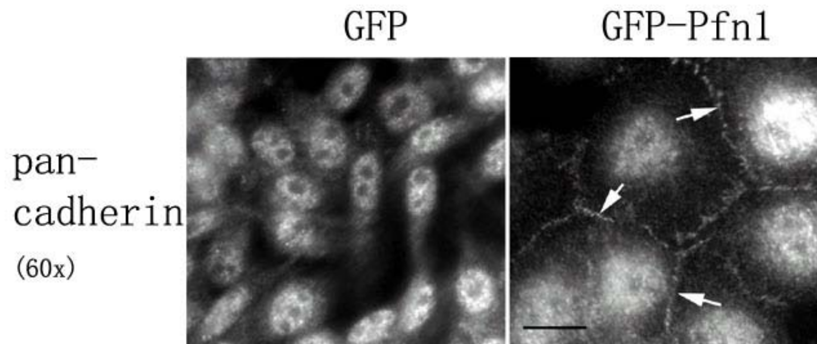


Figure 14 Pan-cadherin immunostaining of GFP and GFP-Pfn1 cells (arrows show junctional localization).

4.1.9 Intact actin filaments are critical in the restoration of cell-cell adhesion of Pfn1 overexpressing cells

It is known that stable AJ in epithelial cells is closely associated with circumferential belt of F-actin underneath the plasma membrane. Given that Pfn1 is a key regulator of actin polymerization in cells, it was speculated that Pfn1 overexpression may alter actin cytoskeletal structures in MDA-231 cells. However, due to its complex effect in actin polymerization, diverse cellular effects of over-expressed or microinjected Pfn1 have been reported in various cells, where an increase of overall F-actin content was noticed (Finkel et al, 1994) or de-polymerization of existing actin filaments occurred in other’s study (as could be expected from its G-actin-sequestering property –Balasubramanian 1994). In our studies, we have evaluated actin filaments in GFP and GFP-Pfn1 cells and found that over-expressing Pfn1 increases F-

actin, compared to GFP cells (Zou 2007), and one of the distinguishing features of GFP-Pfn1 cells is the formation of long actin cables that are organized as a peripheral F-actin belt as well as in a stress-fiber-like fashion (Fig. 15A, the left two figures). This fact suggests that a moderate level of Pfn1 overexpression can cause a marked change in the cytoskeletal organization of MDA-MB-231 cells, and such change is most likely via direct modulation of actin polymerization and/or bundling of actin filaments. Consistent with these overexpression data in MDA-MB-231 cells, it was found that silencing Pfn1 expression causes a marked reduction in circumferential F-actin cables in HMECs (Fig 2 A&C - note that the previous experiments showed that HMEC express Pfn1 at a much higher level compared to MDA-231 cells and exhibit junctional delocalization of E-cadherin upon Pfn1 depletion (Zou 2007)). Therefore, it is suggested that Pfn1 is a promoter of actin polymerization in the case of our study.

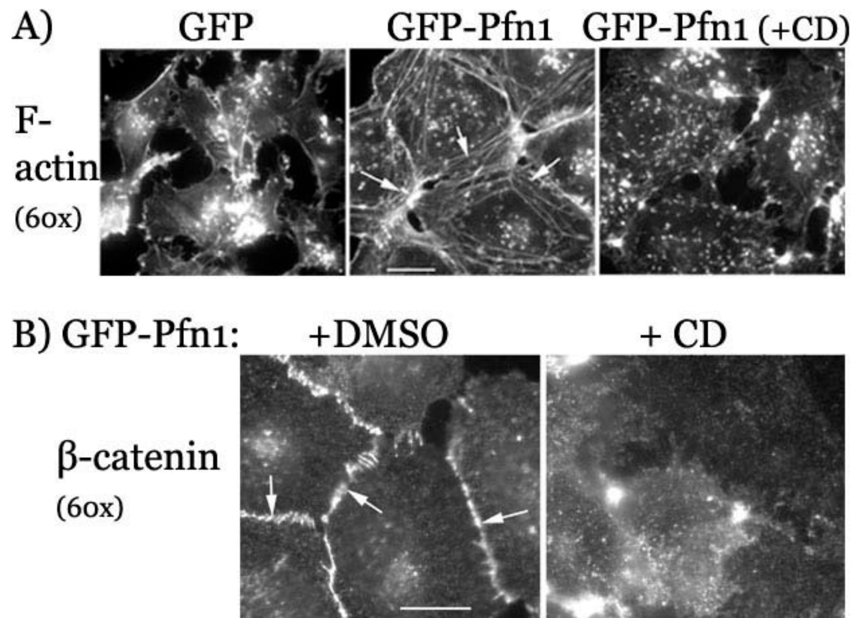


Figure 15 Perturbing Pfn1 expression alters actin cytoskeletal structures in MDA-231. A) Phalloidin staining shows circumferential F-actin cables in GFP-Pfn1 cells (middle – see arrows), but not in GFP cells (left). 500nM CD treatment deletes F-actin in GFP-Pfn1 cells (right). B) β-catenin immunostaining of GFP-Pfn1 cells 20 min after either DMSO (vehicle control) or 500 nM CD treatment (arrows show junctional localization).

Further study showed that these actin structures are critical for AJ formation since brief treatment of GFP-Pfn1 cells with cytochalasin-D (CD), which completely depolymerizes those F-actin cables as shown in Fig 15A (the right panel), also disrupts AJ in GFP-Pfn1 cells as judged by loss of junctional localization of β-catenin (Fig 15B – diluent control DMSO treatment preserves β-catenin localization at the cell-cell junctions as expected). Thus, all these data revealed the key role of cortical actin filaments in restoring AJ in GFP-Pfn1 cells.

The direct evidence to support that up-regulation of Pfn1 restore AJ in MDA-MB-231 cells was to evaluate the cell-cell adhesion when silencing Pfn1 expression in GFP-Pfn1 cells.

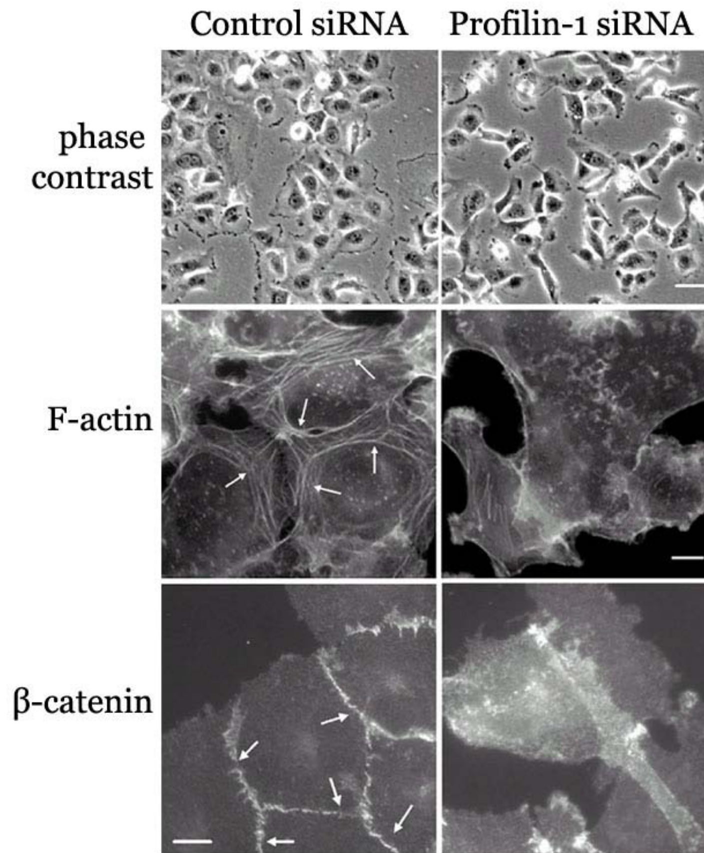


Figure 16 Silencing Pfn1 dissociates cell-cell adhesion of GFP-Pfn1 cells: the morphology (10x, the upper panel), the F-actin staining (60x, middle panel, see arrows) and β-catening staining (60x, lower panel, see arrows).

Following the morphological changes (Fig.16, upper panel), the parallel studies showed that cortical actin filaments were dramatically reduced and β-catenin was significantly delocalized from cell-cell junction when silencing Pfn1 in GFP-Pfn1 cells (Fig. 16, middle and lower panels). These data strongly suggest that it is the up-regulated Pfn1 that leads to the AJ formation between the GFP-Pfn1 cells through promoting actin polymerization in cells.

In order to further test it is the actin polymerizing action of Pfn1 that contributes to generation of circumferential F-actin cables and AJ formation in our case, we examined F-actin and β-catenin distribution in stable transfectants of MDA-231 cells overexpressing GFP-Pfn1-H119E, a mutant form of Pfn1 that is deficient in actin-binding (loss of actin-binding function of the mutant and generation of this mutant cell line have been previously described (Fig. 17, Zou 2007)). We found that GFP-Pfn1-H119E cells do not form cell cluster in culture (Fig 18A). These cells do not have circumferential belt of F-actin and they fail to form AJ as displayed by β-catenin immunostaining (Fig 18B). These data from the mutant cells demonstrated actin-binding is critical for Pfn1-induced epithelioid conversion of MDA-231 cells.

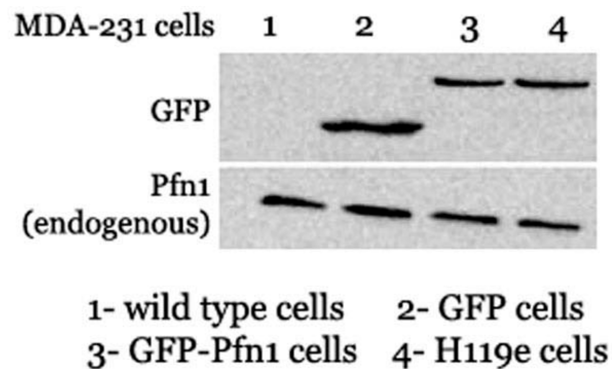


Figure 17 GFP-Pfn1 blot of different mutant MDA-231 cells.

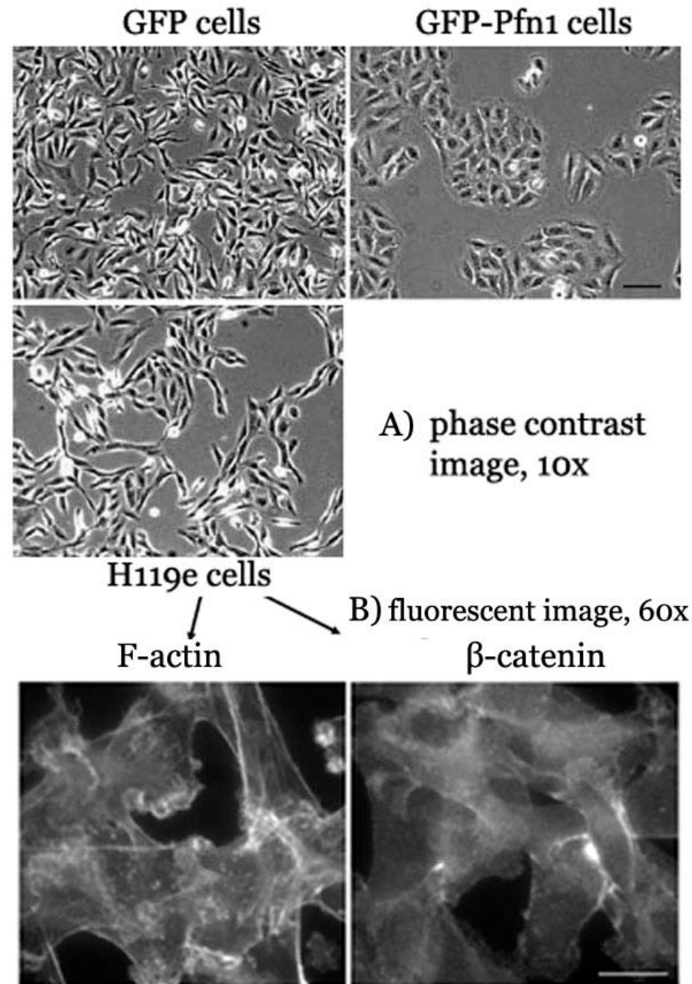


Figure 18 H119e cells in culture and the cell-cell adhesion between H119e cells. A) The morphology of cultured H119e cells, compared with GFP and GFP-Pfn1 cells; B) Phalloidin (left panel) and β-catenin (right panel) staining of GFP-Pfn1-H119E expressers of MDA-231 cells.

4.1.10 R-cadherin is the major mediator protein of cell-cell adhesion in GFP-Pfn1 cells

Since it is suggested that the cell cluster of GFP-Pfn1 cells is cadherin-mediated AJ, the next step of our experiments was to find out which cadherin is responsible for AJ formation in GFP-Pfn1 MDA-231 cells. Based on the fact that MDA-MB-231 cells are E-cadherin null breast cancer cells, other classical cadherins were screened by DNA microarray analysis, which revealed

MDA-231 cells express R (retinal)-cadherin, cadherin-11, cadherin 18 and cadherin 19 (data not shown here). Since R-cadherin is one of the classical cadherin family members mediating homophilic cell-cell adhesions, it becomes the focus of the following studies to learn whether R-cadherin is responsible for the cadherin-mediated AJ formation by up-regulating Pfn1 in the disseminative tumorigenic epithelial cancer cells.

First of all, immunostaining data showed junctional localization of R-cadherin in GFP-Pfn1 cells, but not in control GFP expressing cells (Fig. 19A). Subsequent co-immunoprecipitation experiments revealed increased β-catenin-R-cadherin complex formation in GFP-Pfn1 expressing cells compared to GFP expressers (Fig. 19B), which is consistent with the previous result in Fig. 14B), thus suggesting incorporation of R-cadherin in functional AJ complex. Interestingly, the total protein level of R-cadherin in GFP-Pfn1 cells was found significantly higher (~3-fold) than GFP cells (Fig. 19C, the ~3-fold number is based on relative densitometric analyses of R-cadherin and GAPDH bands averaged from 4 independent experiments). It is known that the membrane pool of E-cadherin is subjected to endosomal trafficking and degradation when cell-cell adhesions are disrupted (Reynolds 2007). In our case, the excess pool (i.e equivalent to the protein differential) of R-cadherin in GFP-Pfn1 cells appears to be membrane localized since the protein level R-cadherin in Pfn1 overexpressing cells is dramatically reduced and becomes nearly comparable to that in GFP expressers within a short time-frame (3 hours) after EGTA treatment (Fig 19D). Binding to p120-catenin protects cadherins from endocytosis and subsequent degradation thereby determining the cadherin level in cells. Although co-immunoprecipitation experiments revealed an overall increase in R-cadherin/p120-catenin complex in GFP-Pfn1 cells (Fig 19E), the total protein level of p120 was comparable between GFP and GFP-Pfn1 cells (Fig. 19F). Given a 3-fold higher total protein

content of R-cadherin in GFP-Pfn1 cells, the intrinsic binding capability between R-cadherin and p120-catenin does not appear to be affected by Pfn1 upregulation. These findings ruled out any

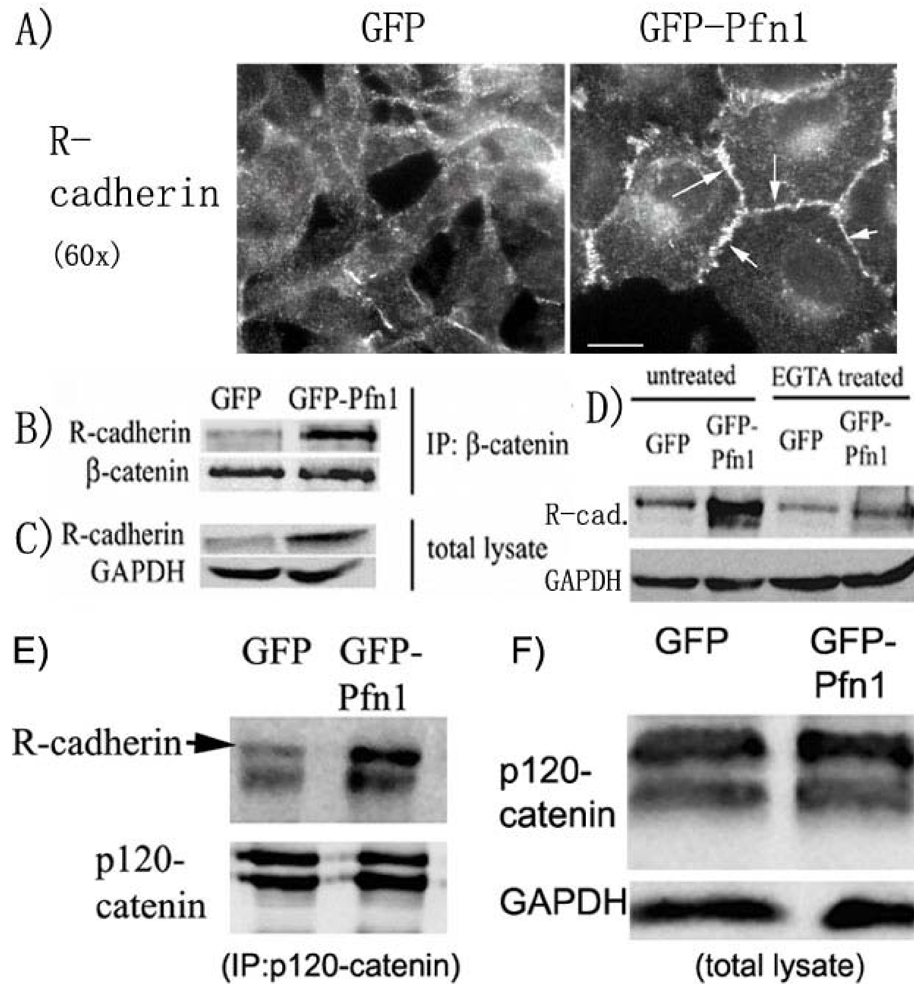


Figure 19 Pfn1 overexpression causes junctional accumulation of R-cadherin in MDA-231 cells. A) R-cadherin immunostaining of GFP and GFP-Pfn1 cells (arrows show junctional localization; scale bar – 20 μm); B) Co-immunoprecipitation analyses of R-cadherin/β-catenin complex formation in GFP and GFP-Pfn1 cells; C) R-cadherin level in total cell lysates; D) Relative R-cadherin levels between GFP and GFP-Pfn1 cells either untreated or following 3 hours of 2 mM EGTA treatment (GAPDH blot serves as the loading control); E) R-cadherin level in p120 IP lysate of GFP and GFP-Pfn1 cells; F) p120 in total lysate of GFP and GFP-Pfn1 cells.

specific effect of Pfn1 on p120-catenin-mediated R-cadherin stabilization mechanism and prompted us to examine whether increased R-cadherin protein in GFP-Pfn1 cells is due to any changes in the mRNA level.

Interestingly, both regular RT-PCR and real-time qRT-PCR experiments showed a similar ~3-fold higher transcript level of R-cadherin in GFP-Pfn1 expressing cells (Fig. 20) thus demonstrating that Pfn1 overexpression increases R-cadherin expression of MDA-231 cells at the mRNA level.

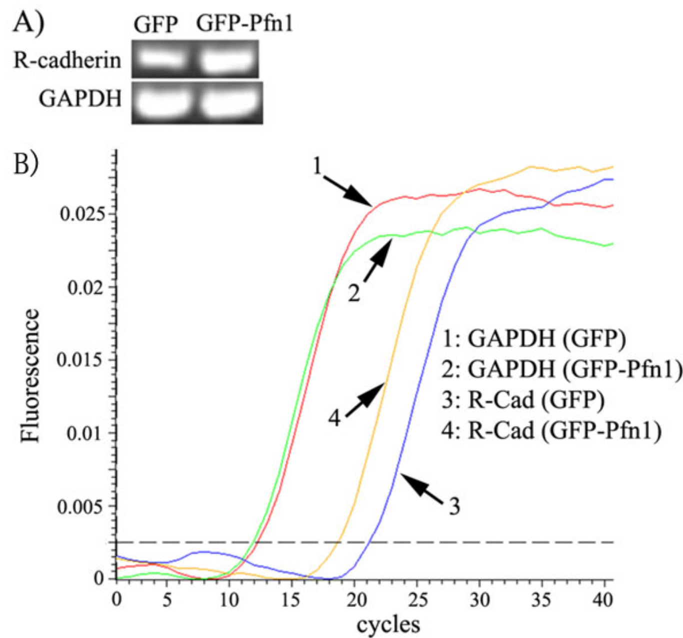


Figure 20 Pfn1 overexpression upregulates R-cadherin expression in MDA-231 cells at the mRNA level. A) RT-PCR data showing relative levels of R-cadherin and GAPDH mRNAs in GFP and GFP-Pfn1 cells. B) SYBR-green fluorescence tracings of GAPDH and R-cadherin RT-PCR products in real-time qRT-PCR experiments.

Finally, to more directly assess the role of R-cadherin in AJ formation in GFP-Pfn1 cells, GFP-Pfn1 cells were transfected with either R-cadherin-specific or control siRNA. 3 days after transfection, the protein level of R-cadherin was dramatically downregulated by R-cadherin siRNA treatment. Phase-contrast micrographs of transfected cells showed that R-cadherin

depleted cells failed to form clusters and presented as isolated cells in a sub-confluent culture (Fig 21A upper panel). This was further supported by immunostaining data which revealed that junctional localization of β-catenin in GFP-Pfn1 cells was dramatically reduced when R-cadherin expression was silenced (Fig 21A lower panel). Taken together, these data clearly demonstrate that Pfn1 overexpression restores AJ in the disseminative MDA-231 cells in R-cadherin dependent manner.

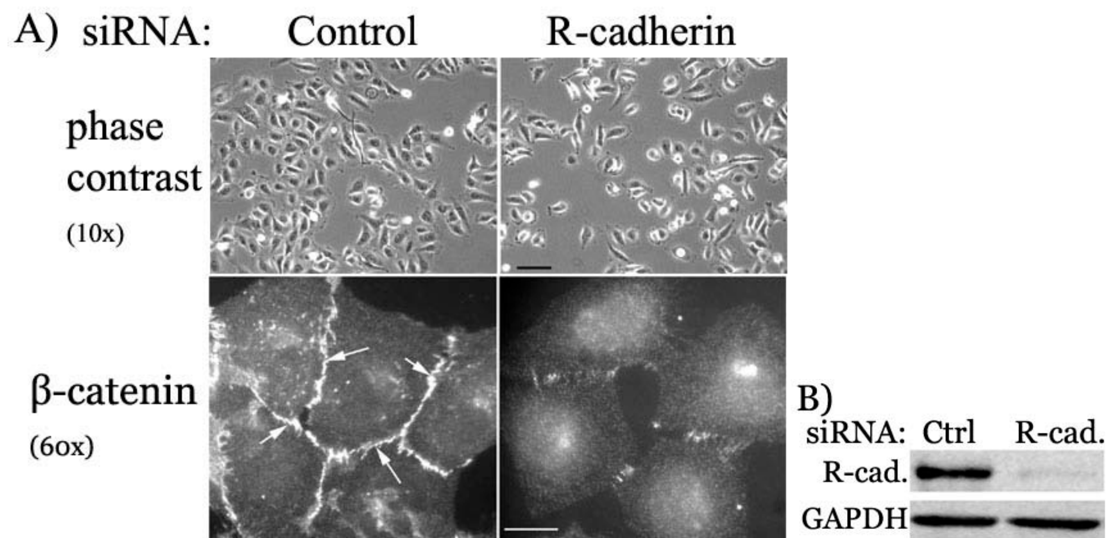


Figure 21 Pfn1 overexpression restores AJ in MDA-231 cells in R-cadherin-dependent manner. A) Phase contrast image (upper panel) and β-catenin immunostaining (lower panel, see arrows) of GFP-Pfn1 cells 3 days after R-cadherin siRNA transfection. B) R-cadherin level in GFP-Pfn1 cells 72 hours after either control or R-cadherin siRNA transfection.

4.1.11 Discussion

In this study, we evaluated the role of Pfn1, a tumor suppressor in breast cancer, in cell-cell adhesion of normal mammary epithelial cells and breast cancer cells. As we know, cell-cell adhesion is involved in cell shape, cell motility and intercellular signal communications.

Adherens Junction (AJ), one of the major cell-cell adhesions between epithelial cells, consists of different proteins forming extracellular adhesion contacts between cells and intracellular links to the actin cytoskeleton. Tight and intact links with cytoskeleton within cells are mandatory for stable cell-cell contacts in normal epithelial tissue. Loss of cell-cell adhesion is one of the hallmark features of transformed cells during Epithelial-Mesenchymal Transition (EMT), and also a prominent feature of breast cancer cells. In this study, we found depletion of Pfn1 in HMECs reduces cell-cell adhesion of normal epithelial cells, leading to enhanced cell migration and cell dissemination, acting together with impaired cell-ECM adhesion; and upregulation of Pfn1 in MDA-MB-231 cells restores the Ca^{2+}-dependent AJ between these aggressive mesenchymal breast cancer cells, and such restored AJ is mediated by R-cadherin, a type of classical cadherin that is endogenously expressed in the parental MDA-MB-231 cell line. Even though, such restoring AJ formation in GFP-Pfn1 cells does not indicate up-regulation of Pfn1 reverses the mesenchymal breast cancer cells to epithelial cells, since such morphological transformation did not occur through re-expression of E-cadherin in cells and the Pfn1 overexpressing cells remained robust expression of mesenchymal molecular marker protein, thus suggesting that it is not a classical MET-like process. And, Pfn1 depletion of HMECs is not a classical EMT phenomenon either though they demonstrate some aggressive phenotypes, since the Pfn1 depleted HMECs remain expressing epithelial protein marker as well.

4.1.11.1 Pfn1 depletion leads to the aggressive phenotypic cell behaviors of normal mammary epithelial cells

In epithelial tissue, E-cadherin is one of the major mediator molecules in cell-cell adhesion between epithelial cells. This study found that silencing Pfn1 expression in HMEC results in delocalization of E-cadherin from adherens junction, followed by impaired cell–cell

adhesion; combined with decreased cell–matrix adhesions, the attenuated cell adhesion leads to enhanced cell motility and prominent cell scattering, which are precursor to tumor cells dissemination in vivo. These findings in HMECs for the first time provide a possible insight into the role of depleted Pfn1 expression in breast cancer cells: downregulated Pfn1 expression level may contribute, at least in part, to tumorigenic transformation of normal mammary epithelial cells to aggressive breast cancer cells.

Classical cadherins in AJ are the major transmembrane molecules and initiate intercellular adhesion through homophillic binding between cadherins of opposing cells (Gumbiner 2005). They also bind directly or indirectly to cytoplasmic molecules, particularly catenin family members, which establish a link between actin cytoskeleton and regulate both cadherin stability and intracellular signaling pathways that control gene transcription (Perez-Moreno 2006). Specifically in HMECs, E-cadherin links with β–catenin or γ–catenin via its intracellular binding domain, then to actin cytoskeleton through the binding between β–catenin-α–catenin complex and actin filaments. Strong and stable cell-cell adhesion requires the linkage of E-cadherin to actin cytoskeleton through catenins (Adams 1998). However, stable cell-cell adhesion does not mean it is static. Previous studies have shown that the cell-cell adhesion is remodeled in real time although it is perturbed transiently in fast migrating MDCK cells (Pollack 1998, Le 1999). Therefore, cell-cell adhesion is a dynamic rearrangement of adhesion molecules, especially in early embryonic development, wound healing, and cancer cell dissemination (Adams 1998). One mechanism of the dynamic rearrangement of cell-cell adhesion is to continuously endocytose and recycle the cadherin-catenin complexes (Le 1999, 2002; Akhtar 2001, Palacios 2001, Xiao 2003). In this study, it is found that E-cadherin and its intracellular binding partner β–catenin are dislodged from cell-cell adhesion site in Pfn1-depleted HMECs,

reflected by immunostaining; the fractionation of cell lysate revealed that these two adhesion molecules are disassociated with actin cytoskeleton. Such dislodgement and dissociation vitiate the cell-cell adhesion, as demonstrated by centrifugal assay. Loss of cell-cell adhesion is also supported by the evidence of overlapping growth when these Pfn1 depleted cells reach confluence, which suggests that they lose cell-cell contact inhibition in culture. Actually, monolayer growth due to contact inhibition is a characteristic feature of normal epithelial cells in culture, and such overlapping cell growth is quite opposite to the typical cell growth of normal epithelial cells and indicates the aggressive phenotypes of these transformed epithelial cells.

4.1.11.2 The cell-cell adhesion was restored between GFP-Pnf1 cells, and such adhesion was mediated by R-cadherin molecule

Complementary to the findings in HMECs, the data present in this study revealed a novel finding that overexpression of Pfn1 is capable of restoring AJ complex in MDA-MB-231 cells, an E-cadherin-negative mesenchymal breast cancer cell line, which is not through re-expression of E-cadherin but in R-cadherin–dependent manner.

Originally, R-cadherin was identified in the retina of chicken (Inuzuka 1991), but it has been found in various tissues (Rosenberg 1997, Christofori 2003, Charrasse 2004, Johnson 2004). Structurally, R-cadherin is similar to E- and N-cadherin (Shan 2000). And, expression of R-cadherin in embryonic stem cells lacking E-cadherin can rescue striated muscle and epithelia, implicating the important role of R-cadherin in the formation of striated muscle and possibly also of epithelia (Rosenberg 1997). Here, our data demonstrate that R-cadherin can actually compensate for loss of E-cadherin function in forming AJ in breast cancer cells, and is consistent with a recent report by Hazan's group that also showed R-cadherin overexpression can induce AJ formation in MDA-231 cells (Agiostratidou 2009). Some literatures show that R-cadherin is

required for EMT in developing kidney and striated muscles (Rosenberg 1997, Goto 1998, Dahl 2002), and this is also consistent with high levels of R-cadherin expression in prostate and bladder carcinoma, rhabodomyosarcoma (Bussemakers 2000, Giroldi 2000, Kucharczak 2008). Meanwhile, it is noteworthy that in at least two variants of cancer (colorectal and gastric), R-cadherin expression is actually down-regulated (Miotto 2004). And, such down-regulation of R-cadherin in cancer samples may suggest its direct role in the pathogenesis of gastrointestinal neoplasms, possibly in consequence of reduced adhesiveness of epithelial cells that normally express R-cadherin. In this experiment, we found the disseminative MDA-MB-231 cells form cell cluster when over-expressing Pfn1 in those cells, and such epithelial-like cell-cell adhesion is mediated by R-cadherin with higher expression level and junctional localization in Pfn1 overexpressing cells, compared to in the control mesenchymal MDA-MB-231 cells. Taken together, it is suggested that R-cadherin may have context-dependent function.

Another interesting finding about R-cadherin in this study is that Pfn1 overexpression increases R-cadherin expression in MDA-231 cells at the mRNA level. However, it is not clear at this point whether mRNA upregulation occurs at the true transcriptional level or post-transcriptionally through enhanced mRNA stability. Either of these two scenarios is not unrealistic given that Pfn1 has been previously shown to affect gene transcription (Lederer et al. 2005) and a recent study has provided evidence of post-transcriptional upregulation of cadherin-11 by β-catenin and GSK3 β (glycogen synthase kinase 3β) in prostate and breast cancer cells (Farina et al. 2009). These issues will need to be addressed in future studies.

Our studies with HMECs and MDA-231 cells concomitantly indicate that cell-cell adhesion could be significantly altered by manipulating Pfn-1 expression, since down-regulation of Pfn1 in HMECs disrupted E-cadherin mediated cell-cell adhesion while up-regulation of Pfn1

in MDA-231 cells restored R-cadherin mediated cell-cell adhesion, thus strongly suggesting Pfn1 plays a key role in the cell-cell adhesion. Since we have noticed that actin filaments alter significantly following perturbed Pfn1 expression levels, specifically, less junctional actin filaments in Pfn1 depleted HMECs and more actin belt at the junctional area when upregulating Pfn1 in MDA-231 cells, therefore, we proposed that cell-cell adhesion of these mammary epithelial cells is manipulated by Pfn1 expression level through actin filament in these epithelial cells. In the study on MDA-231 cells, Pfn1 overexpression generates more cortical F-actin structure which is known to stabilize the cell adhesion by reinforcing cadherin-catenin complex, it is strongly suggested that restoration of AJ in MDA-MB-231 cells by Pfn1 overexpression is based on the role of Pfn1 in actin polymerization, which is verified in our studies. Specifically, we showed induction of circumferential F-actin bundles (characteristic features of epithelial cells with intact AJ) by Pfn1 overexpression in MDA-231 cells and conversely, a marked reduction of those F-actin structures in normal HMEC in response to Pfn1 downregulation. Our experiments with the actin-binding deficient mutant of Pfn1 (GFP-Pfn1-H119E) further demonstrated that actin-binding is critical for Pfn1 to facilitate formation of circumferential F-actin cables. Although it is reported that overexpression of R-cadherin led to restoring AJ formation (Agiostratidou 2009), obviously the endogenously expressed R-cadherin in the parent MDA-MB-231 cells is not efficient enough to establish the AJ between cells unless Pfn-1 expression level is up-regulated, as found in our study. Following the higher expression of Pfn1, more cortical actin filaments are induced to help anchor adhesion molecules between cells. The fact that cell-cell adhesion of GFP-Pfn1 cells was interrupted by brief application of actin polymerization inhibitor, cytochalasin-D (CD), suggested the pivot role of actin in AJ formation. As for the mechanism how Pfn1 overexpression stimulate formation of cortical F-actin belt in

MDA-MB-231 cells, it involves the actin polymerization at or in the proximity of AJ, initiated by the two major nucleation proteins, formin and Arp 2/3, both of which bind to Pfn1. These two actin-nucleation proteins associate with cadherin complex and are co-localized with cadherins in the new AJ (Kovacs 2002, Kobielak 2004, Verma 2004). Therefore, it is most likely that Pfn1 cooperates with either Arp2/3 and/or formin proteins to generate those cortical F-actin structures at AJ during actin polymerization, which need to be further evaluated. Furthermore, it will also be interesting to see whether enhanced interaction of Pfn1 with either of these proteins plays a role in AJ formation in Pfn1 overexpressing MDA-231 cells.

Based on the experiments, it is concluded that Pfn1 is at least one key molecule to restore the R-cadherin mediated AJ between the MDA-MB-231 cells. Such conclusion provides an exciting mechanism how Pfn1 acts as tumor suppressor, because loss of cell-cell is a prominent feature in breast cancer, and over-expression of Pfn1 can rescue the cell-cell adhesion between those invasive cancer cells.

4.2 AIM-2: TO DETERMINE WHETHER CELL-CELL ADHESION PLAYS A ROLE IN PFN1-DEPENDENT REGULATION OF BREAST CANCER CELL GROWTH

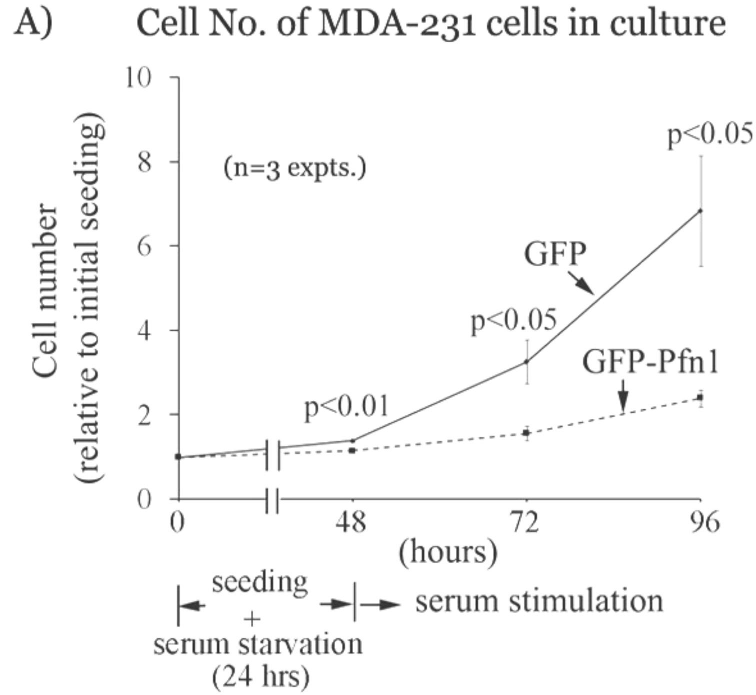
From the literature, we already know that Pfn1 is involved in multiple important cell behaviors, in addition to the regulation of actin polymerization. Actually, Pfn1 has been widely studied for its role in early stage embryonic lethality of Pfn1-/- mice, and it is suggested that Pfn1 plays a role in cell proliferation and/or survival (Witke 2001). Although in vivo study has found that overexpression of Pfn1 inhibits tumorigenicity of breast cancer cell lines (CAL-51) in xenograft

models (Janke 2000), the molecular mechanisms of how Pfn1 overexpression alters cell cycle progression and growth characteristics of breast cancer cells have not been elucidated yet.

In order to address these questions and explore the possible mechanism of Pfn1's suppressive role in breast cancer cells, the following study was designed to demonstrate how cell proliferation and cell survival are affected by Pfn1 perturbation in normal and tumorigenic mammary epithelial cells.

4.2.1 Overexpression of Pfn1 inhibits cell proliferation of MDA-MB-231 cells

Since it has been confirm that overexpression of Pfn1 suppresses breast cancer formation in vivo in our model, as shown in Fig 11, the next step is to explore some mechanisms for such in vivo effect. As we know, enhanced cell proliferation and cell survival are two hallmarks of cancer cells, so whether Pfn1 overexpression suppresses cell proliferation and cell survival of the MDA-231 cells came to our vision. First, cell proliferation was investigated in these Pfn1 overexpressing MDA-231 cells. After serum starved for 24 hours, GFP and GFP-Pfn1 cells were stimulated with 10% FBS for up to 3 days. Cell counting using trypan blue exclusion assay showed that GFP cells were about two-fold and three-fold higher than the readouts of GFP-Pfn1 cells at 24- and 48-hour after serum stimulation (Fig.22A). Complentary to this interesting finding, the Pfn1 down-regulated HMECs were also evaluated by cell counting after replating for up to 4 days, which showed that cell number of Pfn1 silencing HMECs is more than 80% greater than control-siRNA transfected cells on the 3rd day and more than two folds on the 4th day post-transfection (Fig.22B). Since trypan blue only filtrates into dead cell, trypan blue positive cells were excluded from cell counting. Therefore, trypan blue exclusion assay evaluates the proliferation curve of living cells after treatment.



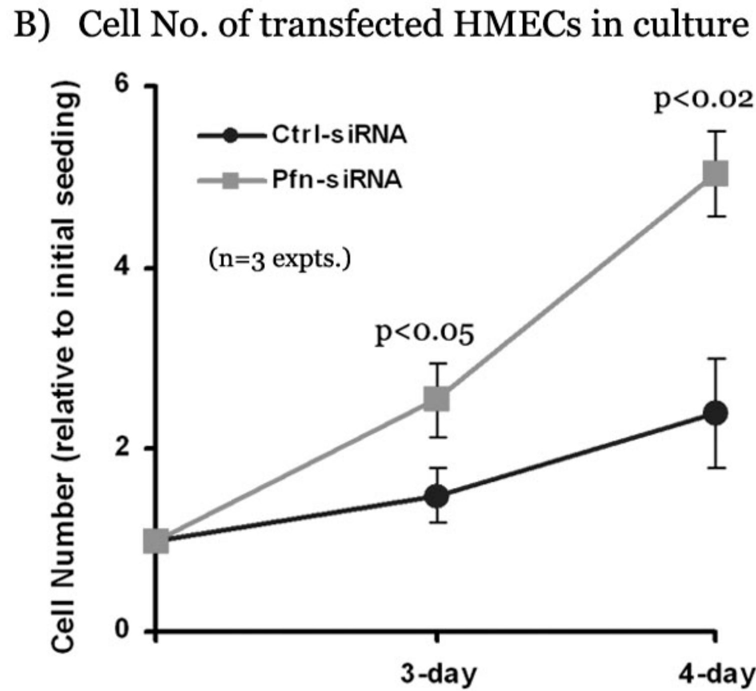
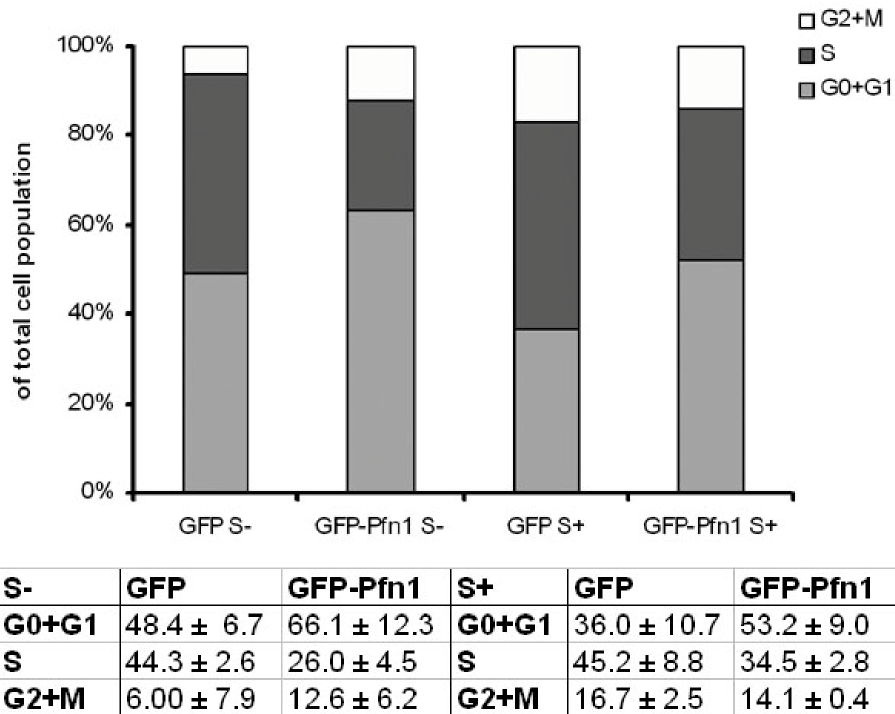


Figure 22 Relative growth kinetics of cell proliferation in response to serum stimulation. A) The cell number of MDA-231 cells in culture. B) The cell number of cultured HMECs after siRNA transfection.

Next, in order to determine the effect of Pfn1 overexpression on cell-cycle progression, the cell cycle profile of both GFP and GFP-Pfn1 cells in serum-starved vs. serum-stimulated condition was compared by FACS-based analysis of their DNA content (Fig.23). The FACS data showed the most pronounced differences between G0/G1 phase and S phase in the two cell lines: in serum starved condition, the percentages of GFP in G0/G1 phase and S phase are 48% and 44%, respectively; and the percentages of GFP-Pfn1 cells in G0/G1 phase and S phase are 66% and 26%, respectively; in serum stimulated condition, they are 36% and 45%, 53% and 34%, respectively. Although both cells displayed a general cell cycle progression in response to serum stimulation, there is a clear G0/G1 arrest in GFP-Pfn1 cells. Since there was no significant difference in G2/M phase between these two cell lines (based on the statistic analysis of FACS



S-	GFP	GFP-Pfn1	S+	GFP	GFP-Pfn1
G0+G1	48.4 ± 6.7	66.1 ± 12.3	G0+G1	36.0 ± 10.7	53.2 ± 9.0
S	44.3 ± 2.6	26.0 ± 4.5	S	45.2 ± 8.8	34.5 ± 2.8
G2+M	6.00 ± 7.9	12.6 ± 6.2	G2+M	16.7 ± 2.5	14.1 ± 0.4

Figure 23 Pfn1 overexpression causes G1 arrest of MDA-231 cells, indicated by the the FACS data from 3 independent experiments.

data, p values are 0.12 and 0.11 for serum starved and serum simulated condition, respectively), it suggests that Pfn1 over-expression does not lead to cytokinesis defect in MDA-MB-231 cells. These results from HMECs and MDA-MB-231 cells indicate that Pfn1 suppresses cell proliferation, which provides a plausible explanation why Pfn1 is suggested to be a tumor suppressor in breast cancer.

4.2.2 Total expression levels of cell cycle regulatory molecules are altered in GFP and GFP-Pfn1 cells, and p27 contributes to the cell cycle suppression of GFP-Pfn1 cells

Cell cycle progression of eukaryotic cells is tightly regulated by a series of protein complexes, categorized as cell cycle promoters and cell cycle inhibitors. One cell cycle promoter is cyclin D1, which is a positive regulator in G1-S transition, and another key cyclin-dependent kinase inhibitor is p27, which leads to G1 phase arrest in cell cycle. Studies in cultured cells have revealed that the down-regulation of p27 expression or up-regulation of cyclin D1 causes a shortened G1-phase and contributes to neoplastic transformation (Yamasaki 2004). In vivo studies demonstrate that mice over-expressing cyclin D1 in mammary epithelium develop mammary hyperplasia and mammary carcinomas (Wang 1994), while mice lacking cyclin D1 are resistant to breast tumors induced by the neu or Ras oncogenes (Yu 2001). As shown above, Pfn1 perturbation leads to the changes in cell cycle. In order to elucidate the mechanism of cell inhibition induced by Pfn1 upregulation in MDA-MB-231 cells, the regulators of G1-S transition were examined. Immunoblotting showed that the positive promoter molecule, cyclin D1, was significantly reduced in Pfn1 overexpressing MDA cells. In contrast, total protein expression levels of two inhibitors, p21 and p27, showed some differential results. p27 was dramatically increased in Pfn1 overexpressing cells, but p21 did not change much (Fig 24). Based on their

role in cell cycle, such changes are in accordance with the previous cell counting and flow cytometry evaluation of GFP and GFP-Pfn1 cells, which consistently indicates the G1/G0 phase arrest of GFP-Pfn1 cells.

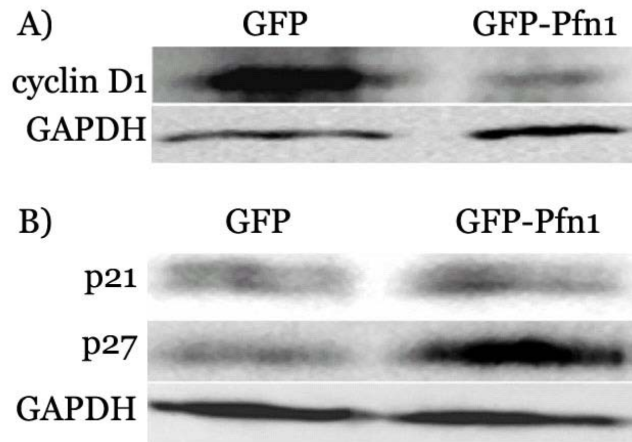


Figure 24 Pfn1 overexpression changes expression levels of cell-cycle markers in MDA-231 cells. Relative expression profile cyclin-D1 (A), p21 and p27 (B) between GFP and GFP-Pfn1 cells.

Although p27 expression level was higher in GFP-Pfn1 cells, there was no direct evidence to show whether elevated p27 level is responsible for the inhibited cell proliferation of GFP-Pfn1 cells. In order to dissect the role of p27, we silenced p27 expression by p27-siRNA, with non-target siRNA as control. Then the cell numbers of p27-siRNA transfected GFP and GFP-Pfn1 cells in culture was evaluated up to 48 hours after re-plating (corresponding to 72 hours after transfection). With silenced p27, GFP cells did not show dramatic change of cell proliferation as expected, since p27 level in GFP was very low even before p27-siRNA transfection. However, p27 silenced GFP-Pfn1 cells demonstrated substantial higher proliferation rate (1.3- and 1.7-folds increase at 24-hr and 48-hr after replating), compared to the control-siRNA transfected GFP-Pfn1 cells, although still lower than the transfected GFP cells

(Fig 25). Such changes in cell proliferation after knocking down p27 proved that p27 was at least partly responsible for the inhibited cell proliferation of Pfn1 overexpressing cells.

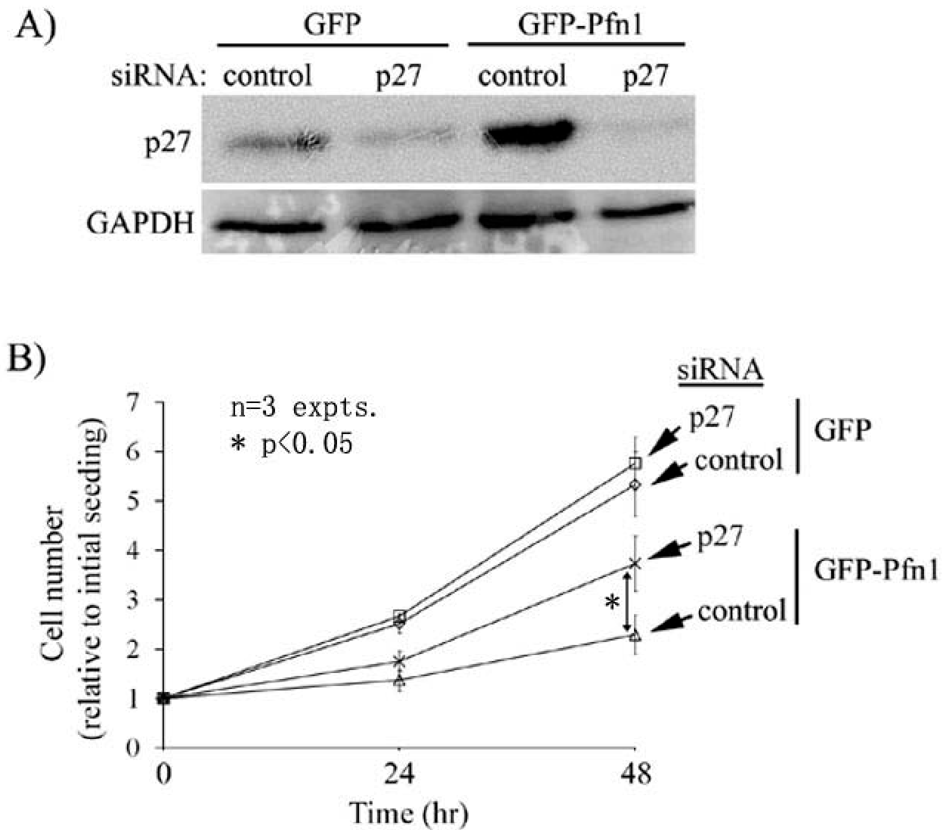


Figure 25 P27 upregulation is partly responsible for Pfn1-induced growth suppression of MDA-231 cells. A) P27 immunoblot demonstrating strong suppression of p27 expression 72 hours after 25 nM siRNA treatment. B) Effect of P27 silencing on the proliferation of GFP and GFP-Pfn1 expressers.

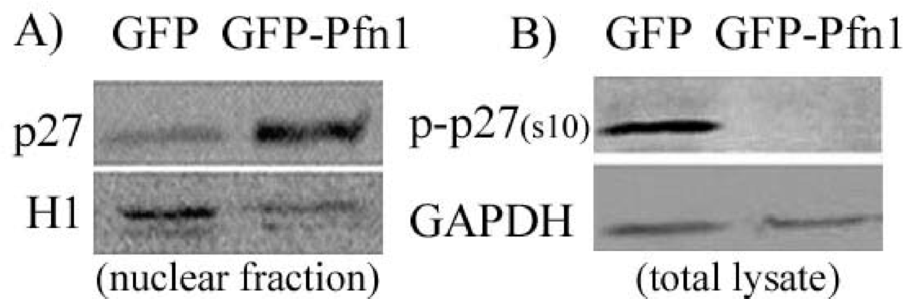


Figure 26 Pfn1 overexpression alters p27 localization in MDA-231 cells. A) protein level of p27 in nuclear fractionation of GFP and GFP-Pfn1 cell; B) phosphorylation of p27 at serine 10 in GFP and GFP-Pfn1 cells.

4.2.3 Pfn1 overexpression retains p27 in the nucleus of MDA-231 cells by inhibiting phosphorylation of p27 at serine10

Since p27 is a key inhibitor of Cdk2/cyclin E complex and it is at least partly responsible for G1 phase arrest of GFP-Pfn1 cells, the following experiments are focused on the role of p27 in the suppressed cell proliferation of GFP-Pfn1 cells.

First, we need to learn how p27 inhibits cell cycle progression of Pfn1 overexpressing cells. As it is known, the elevated total protein level of p27 contributes to its inhibitory activity by importing to the nucleus from the cytoplasm when activated because p27 needs to enter the nucleus and remain there to inhibit cell cycle progression. In this study, high p27 protein levels were detected in GFP-Pfn1 cells, but higher p27 expression does not necessarily indicate there are more nuclear p27 in the cell. To identify the sub-cellular localization of p27 protein in the Pfn1 overexpressing cells, fractionation of p27 was evaluated following total protein evaluation. Using standard high salt extraction protocol, nuclear protein was extracted. Immunoblotting results showed higher p27 expression in nucleus of Pfn1 overexpressing MDA cells compared to that in control cells (Fig 26A). Given that nuclear content of p27 is strongly correlated to its inhibitory effect on cell proliferation, the increased nuclear localization of p27 provides the molecular mechanism for G1 phase arrest of GFP-Pfn1 cells.

The nuclear localization of p27 from the cytoplasm is not only determined by the elevated total protein level, but it is also determined by its phosphorylation, especially at serine 10. Actually, serine 10 is known as the major phosphorylation site of p27 (accounts for an estimated 75% of total phosphate incorporation) (Kaldis 2007, Grimmler 2007). It is known that Ser10 phosphorylation promotes nuclear export of p27 to the cytoplasm. Such phosphorylation could be induced by activation of Akt pathway (Vervoorts 2006), which is also the mechanism

how Akt is involved in cell proliferation. Based on the parallel studies in our laboratory, which have shown that Pfn1 overexpression attenuates Akt phosphorylation (Das 2009), the Akt-induced phosphorylation of p27 may change correspondingly. In addition to exporting nuclear p27, phosphorylation of p27 at S10 residue can also indirectly regulate p27 level of proliferating cells through its proteolysis by kip1-ubiquitylation-promoting complex (KPC) in the cytoplasmic compartment. Not surprisingly, our immunoblotting showed much less phosphorylated Ser10 of p27 in GFP-Pfn1 cells, compared to its expression in the control GFP cells (Fig. 26B). This result implied that reduced Ser10 phosphorylation of p27 may lead to more nuclear retardation in GFP-Pfn1 cells, resulting in G1 phase arrest in cell cycle, which is consistent with our previous studies.

4.2.4 The increased p27 protein in GFP-Pfn1 cells is regulated at protein level instead of mRNA level

As all other proteins, p27 expression may be regulated at multiple levels, including transcriptional level or post-transcriptional level. Therefore, it is necessary to investigate how p27 is regulated in GFP-Pfn1 cells. First, the mRNA level of p27 in GFP and GFP-Pfn1 cells was evaluated by rt-PCR and real time rt-PCR. These results consistently showed similar mRNA level between GFP and GFP-Pfn1 cells (Fig. 27). Therefore, it is suggested that p27 is regulated at post-transcriptional level in stead of transcriptional level.

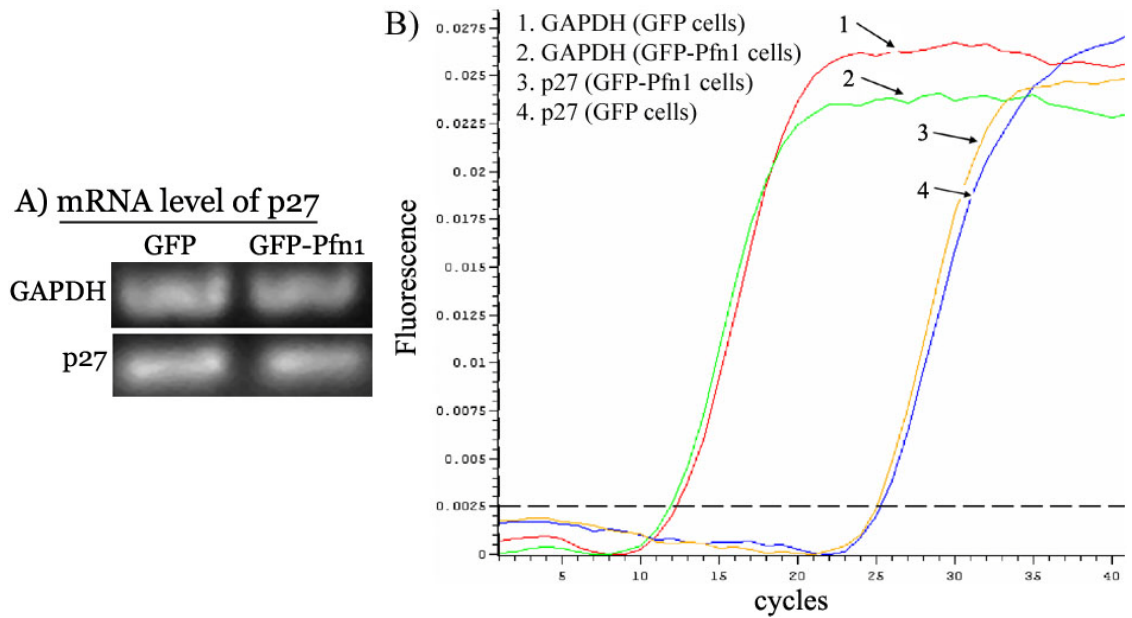


Figure 27 The transcriptional level of p27. A). Real time rt-PCR. RT-PCR data showing relative levels of p27 and GAPDH (loading control) mRNAs in GFP and GFP-Pfn1 expressers. B) SYBR-green fluorescence tracings of GAPDH and p27 RT-PCR products in real-time qRT-PCR experiments.

4.2.5 Cell-cell adhesion of Pfn1 overexpressing cells contribute, at least partially, to the higher expression level of p27 in MDA-231 cells (preliminary result)

It is known that higher expression of p27 may be induced by TGF-β stimulation and cell-cell contact. And, it is higher p27 expression that contributes to the cell-cell contact inhibition of cell proliferation in epithelial cells (Polyak 1994, Levenberg 1999). From our previous studies, we showed that Pfn1 overexpression restores R-cadherin mediated cell-cell adhesion of MDA-231 cells. If it is the cell-cell adhesion between GFP-Pfn1 cells that activates the inhibitory effect of p27, we need to test whether there is any change of p27 expression level when the cells are cultured at different density. We harvested GFP and GFP-Pfn1 cells at different confluences in culture dishes, and extracted total protein to evaluate the expression level of p27. Interestingly,

our preliminary results showed that p27 expression levels increase with higher cell density in culture (Fig.28), suggesting the regulatory role of cell-cell contact in p27 expression level.

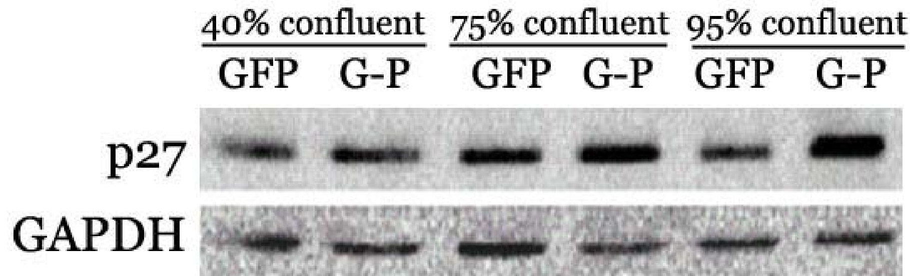


Figure 28 p27 expression level increases with higher confluence of GFP-Pfn1 cells in culture.

4.2.6 Pfn1 upregulation reduced cell survival of MDA-231 cells

In addition to increased cell proliferation, another prominent feature of cancer cells is their enhanced survival capability. Since Pfn1 is regarded as tumor suppressor of breast cancer (Janke 2000), and our parallel study found higher expression of PTEN (another tumor suppressor gene) in Pfn1 overexpressing cells (Das 2009), whether Pfn1 upregulation affects cell survival became our next goal. We treated GFP and GFP- Pfn1 cells with TNFα (tumor necrosis factor – a pro-apoptotic factor) and examined the relative apoptotic sensitivity of the two cell lines by nuclear staining, FACS based analyses of sub-G0/G1 population of cells and extent of PARP (poly-ADP ribose polymerase) cleavage (a caspase-3-mediated event and a hallmark of apoptosis). Nuclear staining demonstrated the more DNA condensation and fragmentation in Pfn1 overexpressing cells when exposed to TNFα. And, PI staining of apoptotic cells in subG0/G1 phase gives us a set of representative FACS profiles of DNA content of our two cell lines in response to TNF treatment. Quantitative analyses of FACS data showed a ~3 fold increase in sub-G0/G1

population of cells as a result of Pfn1 overexpression. PARP immunoblot also shows stronger TNF-induced cleaved PARP product in Pfn1 overexpressing cells (Fig. 29).

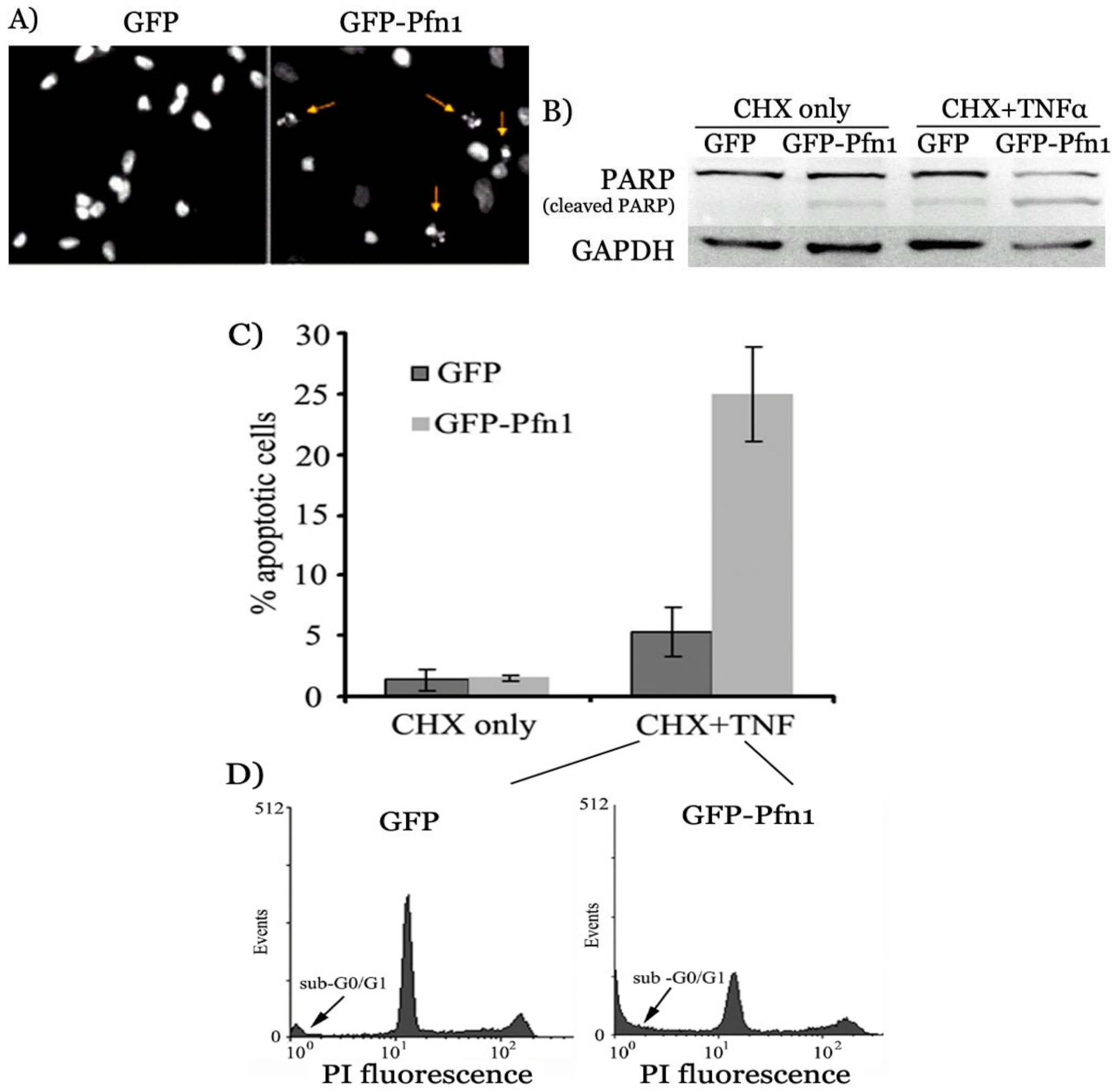


Figure 29 Pfn1 overexpression sensitizes MDA-231 cells to TNF-induced apoptosis. A) Nuclear staining by DAPI show DNA fragmentation in GFP-Pfn1 cells (20x, see arrows); B) PARP immunoblot shows stronger cleaved PARP band in GFP-Pfn1 cells; C) A bar graph comparing % apoptotic cells for the two cell lines following 18 hours of either co-treatment of TNF and CHX or CHX only; D). FACS profile of TNF-treated cells show a larger fraction of GFP-Pfn1 cells with sub G0/G1 nuclear content compared to GFP cells.

4.2.7 Discussion

Although tumor-suppressive action of Pfn1 on breast cancer cell lines has been previously reported (Janke 2000), the molecular mechanism by which Pfn1 upregulation suppresses growth of breast cancer cells were not elucidated in those studies. In this study we investigated for the first time how Pfn1 upregulation alters cell-cycle progression of MDA-231, a highly aggressive breast cancer cell line that previously showed evidence of tumor-suppression in response to Pfn1 overexpression. The two major findings which have been reported in this study are: a) Pfn1 overexpression causes G1 arrest and suppresses growth of MDA-231 cells partly via p27 upregulation, and 2) Pfn1 overexpression can also negatively influence survival of MDA-231 cells. These findings provide the possible mechanism underlying the tumor suppressive effect of Pfn-1 on breast cancer in vivo.

4.2.7.1 p27 is a major player in Pfn1 overexpression induced cell cycle arrest

Previous studies have implicated Pfn1's role in cell proliferation mostly in the context of cytokinesis. This is not surprising given that cytokinesis is an actin-driven process and Pfn1 is an important regulator of actin polymerization in cells. In our case, however, we failed to see any significant effect of Pfn1 overexpression on cytokinesis of MDA-231 cells. Instead, we established a critical involvement of p27 in the growth inhibitory pathway initiated by Pfn1 in breast cancer cells. This is a novel finding. Our experiments showed that transient silencing of p27 expression leads to a substantial yet partial rescue of Pfn1-induced growth inhibition of MDA-231 cells. Partial restoration of growth inhibition by silencing p27 expression is not surprising for at least two reasons. First, our experiments were performed in a transient transfection setting and only followed short-term, and therefore long-term effect of p27

suppression is not known. Second, it is possible that more than just p27 upregulation contributes to decreased mitogenesis of Pfn1 overexpressing cells (for example, we also observed a major decrease in cyclin-D1 level in MDA-231 cells as a result of Pfn1 overexpression).

We further demonstrated that Pfn1-induced p27 upregulation does not occur at the transcriptional level and showed further evidence of post-translational regulation by phosphorylation. Although the exact underlying mechanism is not completely clear, one possibility is that alteration in p27 phosphorylation may contribute to this phenomenon. We have evaluated the status of S10 phosphorylation (the major phosphorylation event) of p27 in this study and showed a dramatic inhibitory effect of Pfn1 overexpression on S10 phosphorylation in proliferating MDA-231 cells. Phosphorylation at S10 residue has a dual effect on p27 stability depending on the stages of cell cycle. S10 phosphorylation tends to stabilize p27 in cells arrested in G0 phase of cell cycle (Borriello 2006). However, when cells are exposed to mitogenic stimulation, S10 phosphorylation can indirectly lower p27 level by exporting it out of the nucleus and facilitating its degradation by KPC (Ishida 2001, Kamura 2004). Since in our case phosphorylation assessment was from extracts prepared from actively growing cultures in the presence of serum, reduced S10 phosphorylation is, at least, consistent with elevated level (both total and nuclear) of p27 in Pfn1 overexpressing cells. There are several kinases that are capable of phosphorylating p27 at S10 residue including AKT, KIS (kinase interacting stathmin) and MAPK (mitogen activated protein kinase). AKT can also phosphorylate other sites of p27 and there is strong experimental evidence of AKT's involvement in downregulating p27 through both transcriptional and post-translational regulation (Liang 2002, 2003). We have recently shown that Pfn1 overexpression upregulates PTEN (phosphatase and tensin homolog deleted on chromosome10) and suppresses AKT activation in two different breast cancer cell lines

including MDA-231 cells (Das 2009). Therefore, one possible pathway that Pfn1 overexpression upregulates p27 level in MDA-231 cells is through altering phospholipid signaling and suppressing AKT activation. Since cell-cell adhesion can significantly change p27 expression level (Motti 2005, Migita 2008) and we have recently shown that Pfn1 overexpression can restore adherence junctions in mesenchymal MDA-231 cells (Zou 2009), and our preliminary data from p27 expression at different seeding densities showed the increased p27 expression in higher seeding density of GFP-Pfn1 cells, implying the cell-cell contact dependent regulation of p27 protein level in Pfn1 overexpressing cells. Therefore, another likely possibility is increasing Pfn1 expression elevates p27 in MDA-231 cells secondary to changes in cell-cell adhesion. In fact, there is a crosstalk between cell-cell adhesion and AKT pathway, based on the finding that PTEN, the negative regulator of Akt activation, is involved in cell-cell adhesion mediated contact inhibition (Uegaki 2006). However, these two alternative possibilities will need to be explored in the future.

4.2.7.2 Pfn1 overexpressing cells are more susceptible to apoptosis when exposed to TNF-α

In addition to the cell proliferation, our apoptosis data revealed another important feature of GFP-Pfn1 cells – they are more susceptible to apoptosis when exposed to TNF-α, indicated by the nuclear fragmentation, more cells in subG0 phase, and the cleaved PARP in GFP-Pfn1 cells.

A seemly contradiction with this finding is that overexpressing Pfn1 restores cell-cell adhesion and usually cell-cell adhesion is thought to protect cells from apoptosis by promoting G1 phase arrest in cell cycle, and only those proliferating cells are more prone to apoptosis because DNA damage can hardly repair in the fasting growing cells, therefore a lower cell survival should be connected with higher proliferation. However, it is not always true. For example, activated Ras can promote cell proliferation and cell survival through the MAPK

signaling pathway (Lin 2002) and PI3-K pathway (Mannova 2005). It has also been reported that a spontaneous apoptosis was found in human HL-60 cells when they reached a high confluence in culture (Saeki 1997, Nishizawa 1998). Therefore, cell-cell contact can not always rescue the cells from apoptosis, and simultaneous regulation of cell proliferation and cell survival does not necessarily act synergistically in active cells. On the contrary, some literatures provide some clues for the possible explanation for our cell survival results – the elevated p27 level in Pfn1 overexpressing cells. Some studies have shown that re-expression or overexpression of p27 induce apoptosis of tumor cells including breast cancer cells, finally leading to reduced tumorigenicity in vivo (Craig 1997, Li 2000, Katner 2002, Zheng 2005). Further study found that such apoptosis induced by p27 overexpression is mediated through the elevated BAX molecule (Fujieda 1999). Therefore, elevated p27 level in our experiment may be a key answer for the impaired cell survival. Of course, further exploration is needed for direct evidence to show the role p27 expression in apoptosis.

Another most likely reason is PTEN/AKT pathway, which may be involved in the phenotypical changes of Pfn1 overexpressing cells. Particularly, PTEN status is critical determinant of apoptotic sensitivity of cancer cells to chemotherapeutic agents. Cancer cells with reduced PTEN expression and therefore having hyperactivated AKT signaling are resistant to chemotherapy and conversely, PTEN overexpression increases apoptotic susceptibility of cancer cells to chemotherapeutics (Yan 2006, Frattini 2007). Taken together with the data from a parallel study in our lab (Das 2009), it is highly speculated that suppressed AKT activation form the basis of reduced survival of Pfn1 overexpressing cells in the face of apoptotic insult. It will be interesting to determine if expression of constitutively active AKT rescues the survival defect of Pfn1 overexpressing MDA-231 cells.

5.0 SUMMARY AND FUTURE DIRECTION OF THE RESEARCH WORKS

5.1 SUMMARY OF THE RESULTS

1. Pfn1 depletion endows the aggressive phenotypes to normal epithelial cells, including loss of cell-cell adhesion and acquired cell motility and cell dissemination.
2. Up-regulation of Pfn1 restores cell-cell adhesion between MDA-MB-231 cells, which is mediated by R-cadherin.
3. Up-regulation of Pfn1 in MDA-MB-231 cells inhibits cell proliferation and cell survival. Increasing p27 protein in GFP-Pfn1 cells contributes to the inhibited cell proliferation. The dual role of Pfn1 in cell proliferation and cell survival lay down the potential mechanism for the tumor suppressive effect of Pfn1 in breast cancer.

5.2 FUTURE DIRECTION

1. To determine how Pfn1 upregulation alter p27 level in breast cancer cells:

i). To explore whether Akt pathway is involved in the Pfn1 overexpression modulated p27 upregulation.

ii). To Further test the role of cell-cell adhesion in p27 upregulation of Pfn1 overexpressing cells.

2. To test whether Pfn1 suppresses progression of pre-existing mammary tumor in vivo:

i). To test whether overexpression of Pfn1 in MDA-231 cells inhibits metastasis of these cells in vivo by tail vein injection.

ii). To test whether Pfn1 overexpression suppresses tumor progression of breast cancer formed by MDA-231 cells, in situ and metastasis.

BIBLIOGRAPHY

Adams, C. L. and W. J. Nelson (1998). "Cytomechanics of cadherin-mediated cell-cell adhesion." Curr Opin Cell Biol 10(5): 572-7.

Agiostratidou, G., M. Li, et al. (2009). "Loss of retinal cadherin facilitates mammary tumor progression and metastasis." Cancer Res 69(12): 5030-8.

Akiyama, T. (2000). "Wnt/beta-catenin signaling." Cytokine Growth Factor Rev 11(4): 273-82.

Alkarain, A., R. Jordan, et al. (2004). "p27 deregulation in breast cancer: prognostic significance and implications for therapy." J Mammary Gland Biol Neoplasia 9(1): 67-80.

Altomare, D. A. and J. R. Testa (2005). "Perturbations of the AKT signaling pathway in human cancer." Oncogene 24(50): 7455-64.

Angres, B., A. Barth, et al. (1996). "Mechanism for transition from initial to stable cell-cell adhesion: kinetic analysis of E-cadherin-mediated adhesion using a quantitative adhesion assay." J Cell Biol 134(2): 549-57.

Apte, U., G. Zeng, et al. (2006). "Activation of Wnt/beta-catenin pathway during hepatocyte growth factor-induced hepatomegaly in mice." Hepatology 44(4): 992-1002.

Arnold, A. and A. Papanikolaou (2005). "Cyclin D1 in breast cancer pathogenesis." J Clin Oncol 23(18): 4215-24.

Ashkenazi, A. (2002). "Targeting death and decoy receptors of the tumour-necrosis factor superfamily." Nat Rev Cancer 2(6): 420-30.

Balasubramanian, M. K., B. R. Hirani, et al. (1994). "The Schizosaccharomyces pombe cdc3+ gene encodes a profilin essential for cytokinesis." J Cell Biol 125(6): 1289-301.

Barker, N. (2008). "The canonical Wnt/beta-catenin signalling pathway." Methods Mol Biol 468: 5-15.

Barthelemy, C., C. E. Henderson, et al. (2004). "Foxo3a induces motoneuron death through the Fas pathway in cooperation with JNK." BMC Neurosci 5: 48.

Belot, N., R. Pochet, et al. (2002). "Extracellular S100A4 stimulates the migration rate of astrocytic tumor cells by modifying the organization of their actin cytoskeleton." Biochim Biophys Acta 1600(1-2): 74-83.

Berx, G. and F. Van Roy (2001). "The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression." Breast Cancer Res 3(5): 289-93.

Biliran, H., Jr., Y. Wang, et al. (2005). "Overexpression of cyclin D1 promotes tumor cell growth and confers resistance to cisplatin-mediated apoptosis in an elastase-myc transgene-expressing pancreatic tumor cell line." Clin Cancer Res 11(16): 6075-86.

Blain, S. W. and J. Massague (2002). "Breast cancer banishes p27 from nucleus." Nat Med 8(10): 1076-8.

Blain, S. W., H. I. Scher, et al. (2003). "p27 as a target for cancer therapeutics." Cancer Cell 3(2): 111-5.

Blanco-Aparicio, C., O. Renner, et al. (2007). "PTEN, more than the AKT pathway." Carcinogenesis 28(7): 1379-86.

Boehm, M., T. Yoshimoto, et al. (2002). "A growth factor-dependent nuclear kinase phosphorylates p27(Kip1) and regulates cell cycle progression." Embo J 21(13): 3390-401.

Borriello, A., V. Cucciolla, et al. (2006). "Retinoic acid induces p27Kip1 nuclear accumulation by modulating its phosphorylation." Cancer Res 66(8): 4240-8.

Bottcher, R. T., S. Wiesner, et al. (2009). "Profilin 1 is required for abscission during late cytokinesis of chondrocytes." Embo J.

Bussemakers, M. J., A. Van Bokhoven, et al. (2000). "Complex cadherin expression in human prostate cancer cells." Int J Cancer 85(3): 446-50.

Caldon, C. E., R. J. Daly, et al. (2006). "Cell cycle control in breast cancer cells." J Cell Biochem 97(2): 261-74.

Carlsson, L., L. E. Nystrom, et al. (1977). "Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells." J Mol Biol 115(3): 465-83.

Carrano, A. C., E. Eytan, et al. (1999). "SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27." Nat Cell Biol 1(4): 193-9.

Castro, M. A., R. J. Dalmolin, et al. (2008). "Evolutionary origins of human apoptosis and genome-stability gene networks." Nucleic Acids Res 36(19): 6269-83.

Charrasse, S., F. Comunale, et al. (2004). "Variation in cadherins and catenins expression is linked to both proliferation and transformation of Rhabdomyosarcoma." Oncogene 23(13): 2420-30.

Cheng, G., B. B. Youssef, et al. (2006). "Cell population dynamics modulate the rates of tissue growth processes." Biophys J 90(3): 713-24.

Chu, I., J. Sun, et al. (2007). "p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2." Cell 128(2): 281-94.

Conacci-Sorrell, M., J. Zhurinsky, et al. (2002). "The cadherin-catenin adhesion system in signaling and cancer." J Clin Invest 109(8): 987-91.

Conacci-Sorrell, M., J. Zhurinsky, et al. (2002). "The cadherin-catenin adhesion system in signaling and cancer." J Clin Invest 109(8): 987-91.

Craig, C., R. Wersto, et al. (1997). "A recombinant adenovirus expressing p27Kip1 induces cell cycle arrest and loss of cyclin-Cdk activity in human breast cancer cells." Oncogene 14(19): 2283-9.

Dahl, U., A. Sjodin, et al. (2002). "Genetic dissection of cadherin function during nephrogenesis." Mol Cell Biol 22(5): 1474-87.

Das, T., Y. H. Bae, et al. (2009). "Profilin-1 overexpression upregulates PTEN and suppresses AKT activation in breast cancer cells." J Cell Physiol 218(2): 436-43.

Day, M. L., X. Zhao, et al. (1999). "E-cadherin mediates aggregation-dependent survival of prostate and mammary epithelial cells through the retinoblastoma cell cycle control pathway." J Biol Chem 274(14): 9656-64.

Delva, E. and A. P. Kowalczyk (2009). "Regulation of cadherin trafficking." Traffic 10(3): 259-67.

Depoortere, F., I. Pirson, et al. (2000). "Transforming growth factor beta(1) selectively inhibits the cyclic AMP-dependent proliferation of primary thyroid epithelial cells by preventing the association of cyclin D3-cdk4 with nuclear p27(kip1)." Mol Biol Cell 11(3): 1061-76.

Dijkers, P. F., R. H. Medema, et al. (2000). "Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1)." Mol Cell Biol 20(24): 9138-48.

Ekert, P. G., A. M. Jabbour, et al. (2006). "Cell death provoked by loss of interleukin-3 signaling is independent of Bad, Bim, and PI3 kinase, but depends in part on Puma." Blood 108(5): 1461-8.

Esteva, F. J. and G. N. Hortobagyi (2004). "Prognostic molecular markers in early breast cancer." Breast Cancer Res 6(3): 109-18.

Evan, G. and T. Littlewood (1998). "A matter of life and cell death." Science 281(5381): 1317-22.

Farina, A. K., Y. S. Bong, et al. (2009). "Post-transcriptional regulation of cadherin-11 expression by GSK-3 and beta-catenin in prostate and breast cancer cells." PLoS One 4(3): e4797.

Finkel, T., J. A. Theriot, et al. (1994). "Dynamic actin structures stabilized by profilin." Proc Natl Acad Sci U S A 91(4): 1510-4.

Frasca, F., G. Pandini, et al. (2008). "The role of insulin receptors and IGF-I receptors in cancer and other diseases." Arch Physiol Biochem 114(1): 23-37.

Frattini, M., P. Saletti, et al. (2007). "PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients." Br J Cancer 97(8): 1139-45.

Fujieda, S., M. Inuzuka, et al. (1999). "Expression of p27 is associated with Bax expression and spontaneous apoptosis in oral and oropharyngeal carcinoma." Int J Cancer 84(3): 315-20.

Geiger, B., S. Yehuda-Levenberg, et al. (1995). "Molecular interactions in the submembrane plaque of cell-cell and cell-matrix adhesions." Acta Anat (Basel) 154(1): 46-62.

Giancotti, F. G. and E. Ruoslahti (1999). "Integrin signaling." Science 285(5430): 1028-32.

Giancotti, V. (2006). "Breast cancer markers." Cancer Lett 243(2): 145-59.

Giroldi, L. A., P. P. Bringuier, et al. (1999). "Changes in cadherin-catenin complexes in the progression of human bladder carcinoma." Int J Cancer 82(1): 70-6.

Glotzer, M. (2005). "The molecular requirements for cytokinesis." Science 307(5716): 1735-9.

Gooding, J. M., K. L. Yap, et al. (2004). "The cadherin-catenin complex as a focal point of cell adhesion and signalling: new insights from three-dimensional structures." Bioessays 26(5): 497-511.

Goto, S., E. Yaoita, et al. (1998). "Involvement of R-cadherin in the early stage of glomerulogenesis." J Am Soc Nephrol 9(7): 1234-41.

Grimmler, M., Y. Wang, et al. (2007). "Cdk-inhibitory activity and stability of p27Kip1 are directly regulated by oncogenic tyrosine kinases." Cell 128(2): 269-80.

Grunewald, K., M. Haun, et al. (2000). "Mammaglobin gene expression: a superior marker of breast cancer cells in peripheral blood in comparison to epidermal-growth-factor receptor and cytokeratin-19." Lab Invest 80(7): 1071-7.

Grupka, N. L., K. C. Lear-Kaul, et al. (2004). "Epidermal growth factor receptor status in breast cancer metastases to the central nervous system. Comparison with HER-2/neu status." Arch Pathol Lab Med 128(9): 974-9.

Guan, X., L. Chen, et al. (2007). "Protein profiling: a possible molecular mechanism to mislocalization and down-expression of p27(Kip1) in tumor cells." Med Hypotheses 69(3): 580-3.

Gudjonsson, T., R. Villadsen, et al. (2004). "Immortalization protocols used in cell culture models of human breast morphogenesis." Cell Mol Life Sci 61(19-20): 2523-34.

Gumbiner, B. M. (2005). "Regulation of cadherin-mediated adhesion in morphogenesis." Nat Rev Mol Cell Biol 6(8): 622-34.

Haarer, B. K., S. H. Lillie, et al. (1990). "Purification of profilin from Saccharomyces cerevisiae and analysis of profilin-deficient cells." J Cell Biol 110(1): 105-14.

Haass, N. K., K. S. Smalley, et al. (2005). "Adhesion, migration and communication in melanocytes and melanoma." Pigment Cell Res 18(3): 150-9.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell 100(1): 57-70.

Harris, C. C. (1996). "p53 tumor suppressor gene: from the basic research laboratory to the clinic--an abridged historical perspective." Carcinogenesis 17(6): 1187-98.

Haugwitz, M., A. A. Noegel, et al. (1994). "Dictyostelium amoebae that lack G-actin-sequestering profilins show defects in F-actin content, cytokinesis, and development." Cell 79(2): 303-14.

Heijink, I. H., P. M. Kies, et al. (2007). "Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity." J Immunol 178(12): 7678-85.

Hinck, L., W. J. Nelson, et al. (1994). "Wnt-1 modulates cell-cell adhesion in mammalian cells by stabilizing beta-catenin binding to the cell adhesion protein cadherin." J Cell Biol 124(5): 729-41.

Hirano, S., N. Kimoto, et al. (1992). "Identification of a neural alpha-catenin as a key regulator of cadherin function and multicellular organization." Cell 70(2): 293-301.

Hirano, S., A. Nose, et al. (1987). "Calcium-dependent cell-cell adhesion molecules (cadherins): subclass specificities and possible involvement of actin bundles." J Cell Biol 105(6 Pt 1): 2501-10.

Hirohashi, S. (1998). "Inactivation of the E-cadherin-mediated cell adhesion system in human cancers." Am J Pathol 153(2): 333-9.

Hirohashi, S. and Y. Kanai (2003). "Cell adhesion system and human cancer morphogenesis." Cancer Sci 94(7): 575-81.

Hiscox, S., W. G. Jiang, et al. (2006). "Tamoxifen resistance in MCF7 cells promotes EMT-like behaviour and involves modulation of beta-catenin phosphorylation." Int J Cancer 118(2): 290-301.

Hoteiya, T., E. Hayashi, et al. (1999). "Expression of E-cadherin in oral cancer cell lines and its relationship to invasiveness in SCID mice in vivo." J Oral Pathol Med 28(3): 107-11.

Hudson, L. G., R. Zeineldin, et al. (2008). "Phenotypic plasticity of neoplastic ovarian epithelium: unique cadherin profiles in tumor progression." Clin Exp Metastasis 25(6): 643-55.

Hwang, H. C. and B. E. Clurman (2005). "Cyclin E in normal and neoplastic cell cycles." Oncogene 24(17): 2776-86.

Inuzuka, H., S. Miyatani, et al. (1991). "R-cadherin: a novel Ca(2+)-dependent cell-cell adhesion molecule expressed in the retina." Neuron 7(1): 69-79.

Ivanov, A. I., D. Hunt, et al. (2005). "Differential roles for actin polymerization and a myosin II motor in assembly of the epithelial apical junctional complex." Mol Biol Cell 16(6): 2636-50.

Janke, J., K. Schluter, et al. (2000). "Suppression of tumorigenicity in breast cancer cells by the microfilament protein profilin 1." J Exp Med 191(10): 1675-86.

Jeanes, A., C. J. Gottardi, et al. (2008). "Cadherins and cancer: how does cadherin dysfunction promote tumor progression?" Oncogene 27(55): 6920-9.

Jeschke, U., I. Mylonas, et al. (2007). "Expression of E-cadherin in human ductal breast cancer carcinoma in situ, invasive carcinomas, their lymph node metastases, their distant metastases, carcinomas with recurrence and in recurrence." Anticancer Res 27(4A): 1969-74.

Jin, C. Y., G. Y. Kim, et al. (2008). "Induction of Apoptosis by Aqueous Extract of Cordyceps militaris Through Activation of Caspases and Inactivation of Akt in Human Breast Cancer MDA-MB-231 Cells." J Microbiol Biotechnol 18(12): 1997-2003.

Johnson, E., C. S. Theisen, et al. (2004). "R-cadherin influences cell motility via Rho family GTPases." J Biol Chem 279(30): 31041-9.

Jung, J. W., S. D. Cho, et al. (2005). "Ras/MAP kinase pathways are involved in Ras specific apoptosis induced by sodium butyrate." Cancer Lett 225(2): 199-206.

Kaldis, P. (2007). "Another piece of the p27Kip1 puzzle." Cell 128(2): 241-4.

Kamura, T., T. Hara, et al. (2004). "Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27(Kip1) at G1 phase." Nat Cell Biol 6(12): 1229-35.

Kasten, M. M. and A. Giordano (1998). "pRb and the cdks in apoptosis and the cell cycle." Cell Death Differ 5(2): 132-40.

Katner, A. L., P. Gootam, et al. (2002). "A recombinant adenovirus expressing p7(Kip1) induces cell cycle arrest and apoptosis in human 786-0 renal carcinoma cells." J Urol 168(2): 766-73.

Kato, A., H. Takahashi, et al. (1997). "Inactivation of the cyclin D-dependent kinase in the rat fibroblast cell line, 3Y1, induced by contact inhibition." J Biol Chem 272(12): 8065-70.

Kerr, J. F., A. H. Wyllie, et al. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer 26(4): 239-57.

Kim, N. H., K. Kim, et al. (2007). "PKB/Akt inhibits ceramide-induced apoptosis in neuroblastoma cells by blocking apoptosis-inducing factor (AIF) translocation." J Cell Biochem 102(5): 1160-70.

Kinzler, K. W. and B. Vogelstein (1997). "Cancer-susceptibility genes. Gatekeepers and caretakers." Nature 386(6627): 761, 763.

Ko, H., H. S. Kim, et al. (2007). "Nuclear localization signals of the E-cadherin transcriptional repressor Snail." Cells Tissues Organs 185(1-3): 66-72.

Ko, K. S., P. D. Arora, et al. (2001). "Cell-cell adhesion in human fibroblasts requires calcium signaling." J Cell Sci 114(Pt 6): 1155-67.

Kobielak, A. and E. Fuchs (2004). "Alpha-catenin: at the junction of intercellular adhesion and actin dynamics." Nat Rev Mol Cell Biol 5(8): 614-25.

Kops, G. J. and B. M. Burgering (2000). "Forkhead transcription factors are targets of signalling by the proto-oncogene PKB (C-AKT)." J Anat 197 Pt 4: 571-4.

Kovacs, E. M., M. Goodwin, et al. (2002). "Cadherin-directed actin assembly: E-cadherin physically associates with the Arp2/3 complex to direct actin assembly in nascent adhesive contacts." Curr Biol 12(5): 379-82.

Kucharczak, J., S. Charrasse, et al. (2008). "R-Cadherin expression inhibits myogenesis and induces myoblast transformation via Rac1 GTPase." Cancer Res 68(16): 6559-68.

Kumar, S. (1997). "The apoptotic cysteine protease CPP32." Int J Biochem Cell Biol 29(3): 393-6.

Lazar, D., S. Taban, et al. (2008). "The immunohistochemical expression of E-cadherin in gastric cancer; correlations with clinicopathological factors and patients' survival." Rom J Morphol Embryol 49(4): 459-67.

Le, T. L., S. R. Joseph, et al. (2002). "Protein kinase C regulates endocytosis and recycling of E-cadherin." Am J Physiol Cell Physiol 283(2): C489-99.

Le, T. L., A. S. Yap, et al. (1999). "Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics." J Cell Biol 146(1): 219-32.

Lederer, M., B. M. Jockusch, et al. (2005). "Profilin regulates the activity of p42POP, a novel Myb-related transcription factor." J Cell Sci 118(Pt 2): 331-41.

Levenberg, S., A. Yarden, et al. (1999). "p27 is involved in N-cadherin-mediated contact inhibition of cell growth and S-phase entry." Oncogene 18(4): 869-76.

Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell 88(3): 323-31.

Li, J., X. K. Yang, et al. (2000). "Overexpression of p27(KIP1) induced cell cycle arrest in G(1) phase and subsequent apoptosis in HCC-9204 cell line." World J Gastroenterol 6(4): 513-521.

Li, J. J., H. Y. Li, et al. (2006). "[Exogenous PTEN enhances apoptosis in pancreas cancer cell line ASPC-1 induced by hypoxia]." Zhonghua Zhong Liu Za Zhi 28(5): 345-8.

Liang, J. and J. M. Slingerland (2003). "Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression." Cell Cycle 2(4): 339-45.

Liang, J., J. Zubovitz, et al. (2002). "PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest." Nat Med 8(10): 1153-60.

Lin, C. C., M. H. Shyr, et al. (2002). "Thrombin-stimulated cell proliferation mediated through activation of Ras/Raf/MEK/MAPK pathway in canine cultured tracheal smooth muscle cells." Cell Signal 14(3): 265-75.

Liou, J. S., C. Y. Chen, et al. (2000). "Oncogenic ras mediates apoptosis in response to protein kinase C inhibition through the generation of reactive oxygen species." J Biol Chem 275(50): 39001-11.

Loden, M., F. Perris, et al. (2003). "C-erbB2, p27 and G1/S aberrations in human primary breast cancer." Anticancer Res 23(3A): 2053-61.

Magdolen, V., U. Oechsner, et al. (1988). "The intron-containing gene for yeast profilin (PFY) encodes a vital function." Mol Cell Biol 8(12): 5108-15.

Mandal, M., J. N. Myers, et al. (2008). "Epithelial to mesenchymal transition in head and neck squamous carcinoma: association of Src activation with E-cadherin down-regulation, vimentin expression, and aggressive tumor features." Cancer 112(9): 2088-100.

Mannova, P. and L. Beretta (2005). "Activation of the N-Ras-PI3K-Akt-mTOR pathway by hepatitis C virus: control of cell survival and viral replication." J Virol 79(14): 8742-9.

Mbalaviele, G., C. R. Dunstan, et al. (1996). "E-cadherin expression in human breast cancer cells suppresses the development of osteolytic bone metastases in an experimental metastasis model." Cancer Res 56(17): 4063-70.

Medema, R. H., G. J. Kops, et al. (2000). "AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1." Nature 404(6779): 782-7.

Migita, T., Y. Oda, et al. (2008). "Inverse relationship between E-cadherin and p27Kip1 expression in renal cell carcinoma." Int J Oncol 33(1): 41-7.

Miller, J. R. and R. T. Moon (1996). "Signal transduction through beta-catenin and specification of cell fate during embryogenesis." Genes Dev 10(20): 2527-39.

Miotto, E., S. Sabbioni, et al. (2004). "Frequent aberrant methylation of the CDH4 gene promoter in human colorectal and gastric cancer." Cancer Res 64(22): 8156-9.

Motti, M. L., D. Califano, et al. (2005). "Reduced E-cadherin expression contributes to the loss of p27kip1-mediated mechanism of contact inhibition in thyroid anaplastic carcinomas." Carcinogenesis 26(6): 1021-34.

Mundy, G. R. (1997). "Mechanisms of bone metastasis." Cancer 80(8 Suppl): 1546-56.

Nakatsuji, Y. and R. H. Miller (2001). "Density dependent modulation of cell cycle protein expression in astrocytes." J Neurosci Res 66(3): 487-96.

Nelson, W. J., F. Drees, et al. (2005). "Interaction of cadherin with the actin cytoskeleton." Novartis Found Symp 269: 159-68; discussion 168-77, 223-30.

Nicholson, D. W., A. Ali, et al. (1995). "Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis." Nature 376(6535): 37-43.

Nickeleit, I., S. Zender, et al. (2007). "p27kip1: a target for tumor therapies?" Cell Div 2: 13.

Nielsen, N. H., M. Loden, et al. (1999). "G1-S transition defects occur in most breast cancers and predict outcome." Breast Cancer Res Treat 56(2): 105-12.

Nishizawa, Y., K. Saeki, et al. (1998). "Potent inhibition of cell density-dependent apoptosis and enhancement of survival by dimethyl sulfoxide in human myeloblastic HL-60 cells." J Cell Physiol 174(1): 135-43.

Paavilainen, V. O., E. Bertling, et al. (2004). "Regulation of cytoskeletal dynamics by actin-monomer-binding proteins." Trends Cell Biol 14(7): 386-94.

Palacios, J., L. Catasus, et al. (2001). "Beta- and gamma-catenin expression in endometrial carcinoma. Relationship with clinicopathological features and microsatellite instability." Virchows Arch 438(5): 464-9.

Peacock, J. W., J. Palmer, et al. (2009). "PTEN loss promotes mitochondrially dependent type II Fas-induced apoptosis via PEA-15." Mol Cell Biol 29(5): 1222-34.

Peinado, H., E. Ballestar, et al. (2004). "Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex." Mol Cell Biol 24(1): 306-19.

Perez de Castro, I., G. de Carcer, et al. (2007). "A census of mitotic cancer genes: new insights into tumor cell biology and cancer therapy." Carcinogenesis 28(5): 899-912.

Perez-Moreno, M. and E. Fuchs (2006). "Catenins: keeping cells from getting their signals crossed." Dev Cell 11(5): 601-12.

Persad, S., A. A. Troussard, et al. (2001). "Tumor suppressor PTEN inhibits nuclear accumulation of beta-catenin and T cell/lymphoid enhancer factor 1-mediated transcriptional activation." J Cell Biol 153(6): 1161-74.

Pettitt, J. (2005). "The cadherin superfamily." WormBook: 1-9.

Pollack, A. L., R. B. Runyan, et al. (1998). "Morphogenetic mechanisms of epithelial tubulogenesis: MDCK cell polarity is transiently rearranged without loss of cell-cell contact during scatter factor/hepatocyte growth factor-induced tubulogenesis." Dev Biol 204(1): 64-79.

Pollard, T. D., L. Blanchoin, et al. (2000). "Molecular mechanisms controlling actin filament dynamics in nonmuscle cells." Annu Rev Biophys Biomol Struct 29: 545-76.

Polyak, K., J. Y. Kato, et al. (1994). "p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest." Genes Dev 8(1): 9-22.

Price, J. T. and E. W. Thompson (2002). "Mechanisms of tumour invasion and metastasis: emerging targets for therapy." Expert Opin Ther Targets 6(2): 217-33.

Reinhold, W. C., M. A. Reimers, et al. (2007). "Detailed DNA methylation profiles of the E-cadherin promoter in the NCI-60 cancer cells." Mol Cancer Ther 6(2): 391-403.

Reynolds, A. B. (2007). "p120-catenin: Past and present." Biochim Biophys Acta 1773(1): 2-7.

Riese, J., X. Yu, et al. (1997). "LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic." Cell 88(6): 777-87.

Robson, C. N., V. Gnanapragasam, et al. (1999). "Transforming growth factor-beta1 up-regulates p15, p21 and p27 and blocks cell cycling in G1 in human prostate epithelium." J Endocrinol 160(2): 257-66.

Roh, M. S., S. H. Hong, et al. (2004). "Gene expression profiling of breast cancers with emphasis of beta-catenin regulation." J Korean Med Sci 19(2): 275-82.

Rojo, F., L. Najera, et al. (2007). "4E-binding protein 1, a cell signaling hallmark in breast cancer that correlates with pathologic grade and prognosis." Clin Cancer Res 13(1): 81-9.

Rosenberg, P., F. Esni, et al. (1997). "A potential role of R-cadherin in striated muscle formation." Dev Biol 187(1): 55-70.

Roy, P. and K. Jacobson (2004). "Overexpression of profilin reduces the migration of invasive breast cancer cells." Cell Motil Cytoskeleton 57(2): 84-95.

Saeki, K., A. Yuo, et al. (1997). "Cell density-dependent apoptosis in HL-60 cells, which is mediated by an unknown soluble factor, is inhibited by transforming growth factor beta1 and overexpression of Bcl-2." J Biol Chem 272(32): 20003-10.

Sainsbury, J. R., T. J. Anderson, et al. (2000). "ABC of breast diseases: breast cancer." Bmj 321(7263): 745-50.

Sansal, I. and W. R. Sellers (2004). "The biology and clinical relevance of the PTEN tumor suppressor pathway." J Clin Oncol 22(14): 2954-63.

Schambony, A., M. Kunz, et al. (2004). "Cross-regulation of Wnt signaling and cell adhesion." Differentiation 72(7): 307-18.

Schmidt, C. R., Y. J. Gi, et al. (2005). "E-cadherin is regulated by the transcriptional repressor SLUG during Ras-mediated transformation of intestinal epithelial cells." Surgery 138(2): 306-12.

Schutt, C. E., J. C. Myslik, et al. (1993). "The structure of crystalline profilin-beta-actin." Nature 365(6449): 810-6.

Seol, D. W. (2008). "Up-regulation of IAPs by PI-3K: a cell survival signal-mediated anti-apoptotic mechanism." Biochem Biophys Res Commun 377(2): 508-11.

Shan, W. S., H. Tanaka, et al. (2000). "Functional cis-heterodimers of N- and R-cadherins." J Cell Biol 148(3): 579-90.

Shankar, S., Q. Chen, et al. (2008). "Inhibition of PI3K/AKT and MEK/ERK pathways act synergistically to enhance antiangiogenic effects of EGCG through activation of FOXO transcription factor." J Mol Signal 3: 7.

She, Q. B., S. Chandarlapaty, et al. (2008). "Breast tumor cells with PI3K mutation or HER2 amplification are selectively addicted to Akt signaling." PLoS ONE 3(8): e3065.

Sherr, C. J. and J. M. Roberts (1999). "CDK inhibitors: positive and negative regulators of G1-phase progression." Genes Dev 13(12): 1501-12.

Shin, I., J. Rotty, et al. (2005). "Phosphorylation of p27Kip1 at Thr-157 interferes with its association with importin alpha during G1 and prevents nuclear re-entry." J Biol Chem 280(7): 6055-63.

Shin, I., F. M. Yakes, et al. (2002). "PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization." Nat Med 8(10): 1145-52.

Shtutman, M., J. Zhurinsky, et al. (1999). "The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway." Proc Natl Acad Sci U S A 96(10): 5522-7.

Siitonen, S. M., J. T. Kononen, et al. (1996). "Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer." Am J Clin Pathol 105(4): 394-402.

Silva, E. M., M. D. Begnami, et al. (2008). "Cadherin-catenin adhesion system and mucin expression: a comparison between young and older patients with gastric carcinoma." Gastric Cancer 11(3): 149-59.

Sommers, C. L., S. E. Heckford, et al. (1992). "Loss of epithelial markers and acquisition of vimentin expression in adriamycin- and vinblastine-resistant human breast cancer cell lines." Cancer Res 52(19): 5190-7.

Sommers, C. L., E. W. Thompson, et al. (1991). "Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities." Cell Growth Differ 2(8): 365-72.

Stemke-Hale, K., A. M. Gonzalez-Angulo, et al. (2008). "An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer." Cancer Res 68(15): 6084-91.

Su, Y., C. Fu, et al. (2008). "APC is essential for targeting phosphorylated beta-catenin to the SCFbeta-TrCP ubiquitin ligase." Mol Cell 32(5): 652-61.

Sutherland, R. L. and E. A. Musgrove (2004). "Cyclins and breast cancer." J Mammary Gland Biol Neoplasia 9(1): 95-104.

Takeichi, M. (1988). "The cadherins: cell-cell adhesion molecules controlling animal morphogenesis." Development 102(4): 639-55.

Tanaka, M., H. Sasaki, et al. (1992). "Genes preferentially expressed in embryo stomach are predominantly expressed in gastric cancer." Cancer Res 52(12): 3372-7.

Taube, M. E., X. W. Liu, et al. (2006). "TIMP-1 regulation of cell cycle in human breast epithelial cells via stabilization of p27(KIP1) protein." Oncogene 25(21): 3041-8.

Terragni, J., J. R. Graham, et al. (2008). "Phosphatidylinositol 3-kinase signaling in proliferating cells maintains an anti-apoptotic transcriptional program mediated by inhibition of FOXO and non-canonical activation of NFkappaB transcription factors." BMC Cell Biol 9: 6.

Tewari, M., L. T. Quan, et al. (1995). "Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase." Cell 81(5): 801-9.

Thompson, M. D. and S. P. Monga (2007). "WNT/beta-catenin signaling in liver health and disease." Hepatology 45(5): 1298-305.

Uegaki, K., Y. Kanamori, et al. (2006). "PTEN is involved in the signal transduction pathway of contact inhibition in endometrial cells." Cell Tissue Res 323(3): 523-8.

Umbas, R., W. B. Isaacs, et al. (1994). "Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer." Cancer Res 54(14): 3929-33.

van Noort, M., M. van de Wetering, et al. (2002). "Identification of two novel regulated serines in the N terminus of beta-catenin." Exp Cell Res 276(2): 264-72.

Vaux, D. L. and S. J. Korsmeyer (1999). "Cell death in development." Cell 96(2): 245-54.

Verbeek, B. S., S. S. Adriaansen-Slot, et al. (1998). "Overexpression of EGFR and c-erbB2 causes enhanced cell migration in human breast cancer cells and NIH3T3 fibroblasts." FEBS Lett 425(1): 145-50.

Verheyen, E. M. and L. Cooley (1994). "Profilin mutations disrupt multiple actin-dependent processes during Drosophila development." Development 120(4): 717-28.

Verma, S., A. M. Shewan, et al. (2004). "Arp2/3 activity is necessary for efficient formation of E-cadherin adhesive contacts." J Biol Chem 279(32): 34062-70.

Vervoorts, J. and B. Luscher (2008). "Post-translational regulation of the tumor suppressor p27(KIP1)." Cell Mol Life Sci 65(20): 3255-64.

Vleminckx, K., L. Vakaet, Jr., et al. (1991). "Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role." Cell 66(1): 107-19.

Wang, T. C., R. D. Cardiff, et al. (1994). "Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice." Nature 369(6482): 669-71.

Watabe, M., A. Nagafuchi, et al. (1994). "Induction of polarized cell-cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a dispersed carcinoma line." J Cell Biol 127(1): 247-56.

Weinberg, R. A. (1995). "The retinoblastoma protein and cell cycle control." Cell 81(3): 323-30.

Wijnhoven, B. P., W. N. Dinjens, et al. (2000). "E-cadherin-catenin cell-cell adhesion complex and human cancer." Br J Surg 87(8): 992-1005.

Wilkes, D. E. and J. J. Otto (2003). "Profilin functions in cytokinesis, nuclear positioning, and stomatogenesis in Tetrahymena thermophila." J Eukaryot Microbiol 50(4): 252-62.

Witke, W. (2004). "The role of profilin complexes in cell motility and other cellular processes." Trends Cell Biol 14(8): 461-9.

Witke, W., A. V. Podtelejnikov, et al. (1998). "In mouse brain profilin I and profilin II associate with regulators of the endocytic pathway and actin assembly." Embo J 17(4): 967-76.

Witke, W., J. D. Sutherland, et al. (2001). "Profilin I is essential for cell survival and cell division in early mouse development." Proc Natl Acad Sci U S A 98(7): 3832-6.

Wittenmayer, N., B. Jandrig, et al. (2004). "Tumor suppressor activity of profilin requires a functional actin binding site." Mol Biol Cell 15(4): 1600-8.

Wodarz, A. and R. Nusse (1998). "Mechanisms of Wnt signaling in development." Annu Rev Cell Dev Biol 14: 59-88.

Wu, N., W. Zhang, et al. (2006). "Profilin 1 obtained by proteomic analysis in all-trans retinoic acid-treated hepatocarcinoma cell lines is involved in inhibition of cell proliferation and migration." Proteomics 6(22): 6095-106.

Yamaguchi, H. and H. G. Wang (2001). "The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change." Oncogene 20(53): 7779-86.

Yan, X., M. Fraser, et al. (2006). "Over-expression of PTEN sensitizes human ovarian cancer cells to cisplatin-induced apoptosis in a p53-dependent manner." Gynecol Oncol 102(2): 348-55.

Yang, H. Y., B. P. Zhou, et al. (2000). "Oncogenic signals of HER-2/neu in regulating the stability of the cyclin-dependent kinase inhibitor p27." J Biol Chem 275(32): 24735-9.

Yoeli-Lerner, M. and A. Toker (2006). "Akt/PKB signaling in cancer: a function in cell motility and invasion." Cell Cycle 5(6): 603-5.

Yokoi, S., K. Yasui, et al. (2003). "Down-regulation of SKP2 induces apoptosis in lung-cancer cells." Cancer Sci 94(4): 344-9.

Yoshida, R., N. Kimura, et al. (2001). "The loss of E-cadherin, alpha- and beta-catenin expression is associated with metastasis and poor prognosis in invasive breast cancer." Int J Oncol 18(3): 513-20.

Yu, Q., Y. Geng, et al. (2001). "Specific protection against breast cancers by cyclin D1 ablation." Nature 411(6841): 1017-21.

Zakeri, Z. and R. A. Lockshin (2008). "Cell death: history and future." Adv Exp Med Biol 615: 1-11.

Zhao, Y. and M. D. Planas-Silva (2009). "Mislocalization of cell-cell adhesion complexes in tamoxifen-resistant breast cancer cells with elevated c-Src tyrosine kinase activity." Cancer Lett 275(2): 204-12.

Zheng, J. Y., W. Z. Wang, et al. (2005). "Effect of p27(KIP1) on cell cycle and apoptosis in gastric cancer cells." World J Gastroenterol 11(45): 7072-7.

Zotter, S., A. Lossnitzer, et al. (1985). "Epithelial markers for paraffin-embedded human tissues. Immunohistochemistry with monoclonal antibodies against milk fat globule antigens." Virchows Arch A Pathol Anat Histopathol 406(2): 237-51.

Zou, L., M. Jaramillo, et al. (2007). "Profilin-1 is a negative regulator of mammary carcinoma aggressiveness." Br J Cancer 97(10): 1361-71.