MOLECULAR MECHANISMS UNDERLYING PROFILIN-1'S REGULATION OF TUMORIGENIC POTENTIAL OF BREAST CANCER CELLS

by

Chang Jiang

B.E., Harbin Institute of Technology, 2006

M.S., Harbin Institute of Technology, 2008

M.S., Indiana University Purdue University Indianapolis, 2010

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This dissertation was presented

by

Chang Jiang

It was defended on

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and approved by

Lance A Davidson, Ph.D., Associate Professor, Department of Bioengineering

Sanjeev Govinddas Shroff, Ph.D., Professor, Department of Bioengineering

Shivendra Singh, Ph.D., Professor, Department of Pharmacology and Chemical Biology

Dissertation Director: Partha Roy, Ph.D., Associate Professor, Department of Bioengineering

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A hallmark of oncogenic transformation is disruption of the actin cytoskeleton, which is partly attributed to altered expression and/or activity of proteins that bind to and regulate the state of polymerization of actin in cells. Profilin-1 (Pfn1) is a key regulator of actin polymerization that is downregulated in human breast cancer. Previous studies have also shown Pfn1 has a tumor-suppressive effect on triple-negative breast cancer (TNBC) cells with mesenchymal features, and Pfn1-induced growth suppression of TNBC cells is partly mediated by upregulation of cell-cycle inhibitor $p27^{kip1}$ (p27). The aim of the first part of this dissertation was to elucidate the molecular mechanism of how Pfn1 elevation causes p27 accumulation in TNBC cells. It is demonstrated that Pfn1 overexpression leads to accumulation of p27 through promoting activation of AMP-activated kinase (AMPK) and AMPK-dependent phosphorylation of p27 on T198 residue (a post-translational modification that leads to increased protein stabilization of p27) secondary to a cadherin-mediated epithelial morphological reversion. These findings not only elucidate a potential mechanism of how Pfn1 may inhibit proliferation of mesenchymal TNBC cells, but also highlight a novel pathway of cadherin-mediated p27 induction and therefore cell-cycle control in cells. The aim of the second part of this dissertation was to investigate whether Pfn1 is a classic tumor-suppressive protein. Matrigel-based 3D-outgrowth assays that measure tumor-initiating capacity of breast cancer cells demonstrated that either depletion or elevation of Pfn1 expression impairs formation of filopodia-like structures (a feature that determines dormant vs

proliferative phenotype) and outgrowth of TNBC cells suggesting that a balanced Pfn1 expression is most conducive for tumor-initiating capacity of TNBC cells. Furthermore, perturbing Pfn1 expression affects expression of stem cell-associated genes thereby impacting the overall stem-like phenotype of TNBC cells. Finally, the outgrowth-deficient phenotype of TNBC upon Pfn1-depletion can be rescued by modulating ECM component which suggests that alteration in cell-matrix adhesion and downstream signaling underlies the growth-related phenotypic changes induced by loss of Pfn1. Based on these results, we conclude that Pfn1 expression level is an important determinant for stemness and outgrowth potential of TNBC cells.

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PREFACE

The current work cannot be completed without the support of a group of great people.

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ABBREVIATIONS AND ACRONYMS

2D: Two-dimensional

3D: Three-dimensional

ABPs: Actin-binding proteins

ADP: Adenosine-5’-diphosphate

AJ: Adherens junction

AKT: A serine-threonine kinase

ALDH1: Aldehyde dehydrogenase 1

AMP: Adenosine monophosphate

AMPK: AMP-activated kinase

Arp2/3: Actin-related protein 2/3 complex

ATP: Adenosine-5’-triphosphate

bFGF: Basic fibroblast growth factor

BME: Basement membrane extract

BSA: Bovine serum albumin

capZ: Capping protein from the Z-disc of muscle

Cdc42: Cell division control 42

CSC: Cancer stem cell

CTCs: Circulating tumor cells

Cre: Cre recombinase

ECM: Extracellular matrix

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EMT: Epithelia to mesenchymal transition

Ena: Enabled

ER: Estrogen receptor

FA: Focal adhesions

F-actin: Filamentous actin

FAK: Focal adhesion kinase

FBS: Fetal bovine serum

FLP: Filopodia-like protrusions

G-actin: Globular-Actin

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFP: Green fluorescent protein

HER2: Human epidermal growth factor receptor 2

HUVEC: Human vascular endothelial cells

JMY: Junction-mediating and –regulatory protein

KO: Knockout

KD: Knockdown

Lpd: Lamellipodin

MDA-231: MDA-MB-231

mDia: Mammalian Diaphanous-related formins

MET: Mesenchymal to epithelia transition

MMPs: Matrix metalloproteinases

NK cells: natural killer cells

NPF: Nucleation Promoting Factor

N-WASP: Neural Wiscott-Aldrich syndrome protein

p27: $P27^{kip1}$

Pfn: Profilin

Pfn1: Profilin-1

Pfn2: Profilin-2

PI3K: Phosphatidylinositol 3-kinase

PI3P: Phosphatidylinositol-3-phosphate

PI(3,4)P2: Phosphatidylinositol-3,4-bisphosphate

PI(4,5)P2: Phosphatidylinositol-4,5-bisphosphate

PIP3: Phosphatidylinositol-3,4,5-triphosphate

PLP: Poly-L-proline

PPI: Phosphoinositide

PR: progesterone receptor

PTEN: Phosphatase and tensin homolog deleted on chromosome 10

RTKs: Receptor tyrosine kinases

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA: Small interfering RNA

TNBC cells: Triple-negative breast cancer cells

TGF-β: Transforming growth factor-beta

VASP: Vasodilator-stimulated phosphoprotein

VEGF: Vascular endothelial growth factor

WASP: Wiskott-Aldrich syndrome protein

1.0 INTRODUCTION

1.1 BREAST CANCER AND CANCER METASTASIS

Breast cancer is the most commonly diagnosed non-skin cancer in women worldwide and it is the second life-threatening cancer after lung and bronchial cancer (Cancer Facts and Figures 2015, American Cancer Society). It has been estimated by The National Cancer Institute that there will be 231,840 new cases of breast cancer and an estimated 40,290 people will die of this disease in the US in 2015. Breast cancer is defined as the cancer that develops from the breast tissue. A normal breast contains breast tissue, fat tissue, lymph nodes, blood vessels, and connective tissue. The breast tissue is made up of lobes and ducts. Lobes are glands with the functions of generating and storage of milk, which consist of multiple smaller sections named lobules. Ducts refer to the tubes connecting the lobes, lobules and nipple, and they carry milk from the lobules to the nipple. Breast cancers are derived from either ductal or lobular epithelial cells of the breast. Ductal carcinoma, which initiates from ductal epithelial cells, is the most common type of breast cancer (Breast Cancer, National Cancer Institute, 2014).

According to the hormone receptor and human epidermal growth factor receptor 2 (HER2) status, breast cancer can be classified by the expression status of the three major receptors: estrogen receptor (ER), progesterone receptor (PR) and HER2. For instance, ER-positive (ER+) breast cancer cells have the receptor protein which can bind to hormone estrogen, and ER+

breast cancer cell growth and proliferation may largely depend on the availability of estrogen. PR-positive (PR+) breast cancer cells have the receptor protein that can bind to hormone progesterone and PR+ breast cancer cell growth and proliferation may largely depend on the availability of progesterone. HER2 positive (HER2+) describes breast cancer cell with overexpression of HER2 on the cell surface, which promotes cell growth. Breast cancer cells without these receptors are named basal-like or triple-negative breast cancer (TNBC) cells. As genomic study progresses, genome analysis has further distinguished breast cancer into four major molecular 'intrinsic' subtypes (Luminal A, Luminal B, Basal-like and HER2 type) and other three less common molecular subtypes (Normal breast-like, Claudin-low and Molecular apocrine). The general characteristics of theses subtypes are listed as following (1-6):

- Luminal A: ER+ and/or PR+, HER2-, low Ki67 (a cellular marker for proliferation), tends to have the best prognosis.
- Luminal B: ER+ and/or PR+, HER2+ or HER2- with high Ki67.
- HER2 type: ER-, PR-, high expression of HER2, tends to have poor prognosis.
- Basal-like: ER-, PR-, HER2-, tends to be aggressive and with poor prognosis compared with luminal A and luminal B subtypes, with high frequency of TP53 mutation.
- Normal breast-like: similar gene expression pattern as normal breast, may contain normal breast tissue.
- Claudin-low: ER-, PR-, HER2-, with properties of cancer stem cells, low expression of cell-cell junction proteins, tends to have poor prognosis.
- Molecular apocrine: ER-, androgen receptor positive, androgen responsive.

Breast cancer cells with hormone receptors on their surface can be treated with drugs to block the binding of hormone to the receptors, and this has led to better prognosis in luminal A subtype of breast cancer. Similarly, HER2 enriched subtype has been shown to be responsive to monoclonal antibody treatment, leading to an improved prognosis. By contrast, TNBC/basal-like subtype is associated with the worst prognosis, demonstrated by shorter survival time, high risk of recurrence, and early developed metastases (7).

Over 90% breast cancer caused death is due to metastasis (8), which is defined as a multi-step process for the malignant cells to depart from the original site, to spread through lymphatic system and/or blood stream, and to colonize in other organs of the body (9,10). Primary breast cancer cells have been reported to metastasize to various non-adjacent organs. The most common initial site of breast cancer metastasis is bone, and the most preferable sites of breast cancer metastasis are bone, lungs, liver and brain (11). Due to its high morbidity and fatality (12), it is crucial to elucidate the cellular and molecular mechanisms of breast cancer metastasis and to target molecular markers of metastasis, which pave the way for developing new therapeutic strategies to prevent and combat metastasis.

Breast cancer metastasis is multi-step process involving numerous signaling pathways and a series of interactions between breast cancer cells and the microenvironment (schematically illustrated in Figure 1). To begin with, breast cancer cells need to detach from primary tumor by becoming locally invasive and migratory. This step is governed by the epithelial-to mesenchymal transition (EMT). EMT is controlled by a series of factors in response to stimuli such as transforming growth factor-beta (TGF-β), Wnt and hypoxia, rendering breast cancer cells to lose epithelia markers, such as E-cadherin, to gain mesenchymal markers, such as vimentin and N-cadherin, and to promote the expression/activity of matrix metalloproteinase (MMPs). Loss of E-

cadherin destructs the cell-cell interaction among epithelial breast cancer cells at the site of origin, while gain of mesenchymal cadherin allows adhesion of cancer cells with stromal cells. Expression of N-cadherin is reported to lead to rearrangement of cytoskeleton and to promote lamellopodia (flat, broad sheet-like protrusions rich in branched actin filaments at the leading edge) and filopodia (thin actin-rich projections extending beyond the leading edge of plasma membrane to the external environment) formation, which ultimately increases the cancer cell motility (13,14). Enhanced activity of MMPs mediates the degradation of basement membrane and other extracellular matrix (ECM) of surrounding connective tissue. Collectively, cancer cells dissociate from primary tumor site, breach basement membrane, spread beyond the basement membrane, invade the surrounding stroma and reach the lymphatic routes or blood vessel [INVASION]. The disseminated cancer cells then cross the barrier of pericyte and endothelial cells of the microvessels, facilitated by the secreted growth factors, cytokines and MMPs. The tumor cell angiogenesis also promotes this process, since the tumor stimulated newly formed blood vessels usually tend to have relatively less pericyte coverage, loose endothelial structure and are under dynamic reconfiguration, rendering them prone to leakiness, and vulnerable for penetration [INTRAVASATION]. After entering into the circulation system, the circulating tumor cells (CTCs) must survive through resisting 1) shear stress from hemodynamic flow, 2) innate immune attack, and 3) anoikis due to lack of attachment (15,16) in the circulation system. This is achievable since, 1) CTCs form clusters with platelets, protecting them from shear stress in the blood flow and predation of natural killer (NK) cells; 2) transporting in circulation system is a fairly quick process, which may allow CTCs to escape from potential attrition [CIRCULATION]. Once traveling to and trapped at the microvessels of a distant organ, CTCs cross the pericyte and endothelial cell layers of the vessels, penetrate into the stromal

microenvironment, in a similar way as the intravasation process. However, in general, additional genes and extra factors are involved for CTCs to disrupt the microvessels and invade the distant organs, since the normal microvessels at the distant organs have more functional and tight structures compared with the leaky vasculature generated by angiogenesis at the primary site [EXTRAVASATION]. When lodged in the stroma of the distant organs, the extravasated breast cancer cells face a dramatically different microenvironment from the primary site, which majority of the cells find hard to adapt to. These differences may include growth factors and cytokines, ECM components, stroma cells, and tissue organizations. As a result, only a limited number of cells can survive by receiving pro-survival signals in the foreign microenvironment. These survival breast cancer cells largely stay quiescence due to incapability of engaging the integrin β1, focal adhesion kinase (FAK) and Src pathways in the ECM of the foreign microenvironment (17-19) [DORMANCY]. Once they overcome the incompatibility by successfully modifying and interacting with the foreign microenvironment, some of these survivors eventually extract the signaling to trigger their oncogenic proliferation pathways, leading to an outgrowth at the secondary site, and ultimately a macroscopic metastases is formed at the distant organ [COLONIZATION].

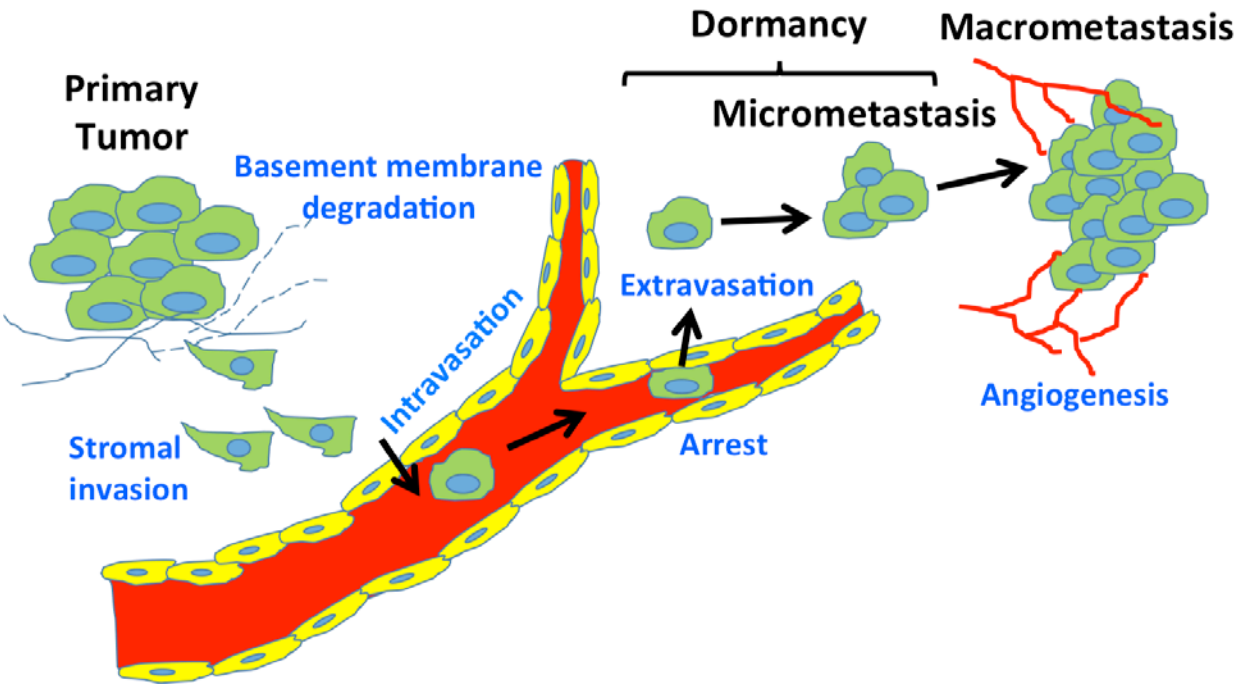


Figure 1. Schematic illustration of breast cancer metastatic cascade. Breast cancer carcinoma cells are depicted in green.

Among these steps, the early dissemination of tumor cells are fairly prevalent (10), the attrition during circulation is not significant (20,21), while it is the post-extravasations steps that are challenging and remarkably inefficient (15,22). In fact, it has been estimated that < 0.01% of cancer cells entering into the circulation system could finally lead to a lesion at the secondary site (10,23). Therefore the biggest hurdle for metastasis completion seems to occur during colonization process, which includes the outgrowth from a solitary cell, and the formation of macroscopic metastasis from micrometastasis. However, it remains unknown why massive disseminative cancer cell death occurs at the barrier of the secondary sites (24).

1.2 THE ORIGINS OF METASTATIC TUMORIGENESIS

Despite the fact that metastasis is an utterly inefficient process, the metastatic lesions eventually arise in distant organs in the body of cancer patients, which raises the question regarding the origins of the metastatic traits arise from the tumor populations. The tumor heterogeneity and regenerative capacity lead to the creation of two major prevailing models: clonal evolution model and cancer stem cell model.

1.2.1 Clonal evolution model

The clonal evolution model is a prevailing model to depict the tumor progression process, which has received widespread acceptance. Just like the concept of Darwinian evolution, animals undergo evolution by ecosystem selection; in the clonal evolution model, tumor cells are under the selection of tumor microenvironment. Normal cells with genetic and epigenetic alterations on tumor suppressor genes and oncogenes give rise to neoplasm growth. These mutant cells in the neoplasms as well as normal cells compete for local resources such as oxygen, growth factors, glucose, as well as space. Then a cell in the neoplasms obtains a certain kind of heritable mutation, rendering it advantageous in proliferation and/or survival over other cells in both the neoplasms and the normal cells. This cell is selected by the microenvironment to become a progenitor of a population of cells with this mutation, called a clone. This clone expands in the neoplasm due to a better fitness. Then new variants arise among the successors of this clone, and initiate the next round of selection. Such cycles of mutation and clonal selection result in tumor heterogeneity and foster the expansion of the fittest clone in tumor population.

The clonal evolution model well explains the chemo-resistance, cancer relapse, and the metastatic tissue tropism, etc. In addition, this model has been well exemplified by the gene analysis study of colorectal tumor progression (25,26), in which the mutations from benign tissue to adenoma, and from adenoma to carcinoma have been identified. However, the Darwinian view seems to be contradictory in explaining metastasis.

According to clonal evolution model, the tumor progression starts with the cells that are capable of forming tumor at the primary site. Then certain genetic alterations endow advantageous phenotypes to the descendant population of these cells, which may include the capabilities of acquiring mitogenic signaling, active migration, inducing angiogenesis and resisting apoptosis. Subsequently, a small fraction of cells in the large population obtain more genetic alterations, which enable them to metastasize and to outgrowth at the secondary site in a distant organ. If this holds true, since the primary sites and the metastatic sites have distinct selection pressure towards cancer cells, the primary tumor and metastases should have sufficient difference in the genetic alterations. However, both gene expression and genomic analysis have revealed strikingly similarity between primary tumor and the metastases. In addition, no specific metastasis driving mutations so far has been identified (27-31). Instead, the mutations associated with metastasis are often the ones within the primary tumor (32). The second inconsistency is that, the colonization step in metastasis is always rate limiting. The clonal evolution model cannot explain why the clonal selection at the final step of metastasis is so hard to achieve (33). Nevertheless, it has to be admitted that tumor progression is, in general, an evolutionary process (34). However, the clonal evolution theory probably needs amendment to eventually explain the origins of metastatic traits.

1.2.2 Cancer stem cell model

Another model to depict the tumor progression process is the cancer stem cell (CSC) model. The CSC model proposes that the growth and progression of many cancers are driven by a minority fraction of cancer cells (35), which can surmount all the obstacles during metastasis and successfully form tumors at secondary site in distant organs. This small fraction of cancer cells are contained within cancer cells with stem cell properties of self-renewal and differentiation, so called cancer stem cells (CSCs), or tumor-initiating cells (36). Unlike in the clonal evolution model, where all the undifferentiated cells in population have the potential to become tumorigenic; in CSC model, only CSCs have the capacity to lead to tumor formation.

The CSC is first precisely defined in a 2006 American Association for Cancer Research Workshop. CSC was defined as a cell within a tumor that possesses the capacity to self-renew and to give rise to the heterogeneous lineages of cancer cells that comprise the tumor (37). It was first discovered as a population of leukemia-initiating cells exhibiting stem cell-like properties in 1994 (38). In later 1990s, a subpopulation of leukemic cells expressing surface maker $CD34^{+}CD38^{-}$ were isolated, which were able to initiate tumor in mice upon xenotransplantation (39). Then in 2002, human cortical glial tumors were found to contain neural stem-like cells (40). The next year, breast CSCs were first isolated and characterized (41). From then on, more discoveries of CSCs from different organs were reported, including brain cancer (42), colon cancer (43), pancreatic cancer (44), prostate cancer (45), ovarian cancer (46) and thyroid cancer (47).

CSCs have three general properties. First, they share characteristics with stem cell or progenitor cells. CSCs have the capability of self-renewal to maintain the heterogeneous growth. CSCs also have the capability of differentiation to facilitate themselves adapting to foreign

tissues, for instance, EMT during intravasation process, mesenchymal to epithelia transition (MET) when forming micrometastases. Besides, CSCs have the homeostatic control ability to balance self-renewal and differentiation (36). Pathways involving the stem cell-like property in CSC include the Wnt signaling pathway, the JAK/STAT, the Hedgehog pathway, the Notch pathway, the bone morphogenetic proteins/transforming growth factor β (BMP/TGF-β) pathway and the HIPPO-YAP/TAZ pathway (48-50). Second, CSCs possess special surface markers, which distinguish them from normal cancer cell population. The well-established breast cancer stem cell markers include CD44, CD24, and aldehyde dehydrogenase 1 (ALDH1), CD133 and Breast Cancer Resistance Protein (BCRP, also called ABCG2) (51). The exact functions of these markers are unknown yet, although they have provided a way to characterize and isolate breast CSC population, making it possible for risk prediction of a patient caring malignant tumor. The third property of CSC is its ability to promote tumorigenicity. It has been reported by AI-Hajj et al in 2003 that as few as 100 cells isolated tumorigenic cells baring $CD44^{+}CD24^{-/low}$ and Lineage negative cell were able to form tumor in mice, indicating the high efficiency of breast CSCs in tumorigenesis (41). Many studies have indicated that CSC promotes metastasis, angiogenesis and lymphangiogenesis (41,52,53).

CSC model also explains the chemo-resistance and cancer relapse, since conventional chemotherapy mainly targets differentiated and differentiating tumor cells, and small subpopulations of CSCs may escape from it. In addition, compelling experimental evidence support the CSC model as reported in various human cancers as mentioned above. Nevertheless, the following two points have to be kept in mind: 1) the rare sub-population of CSCs have the potential to develop tumor does not mean they would develop one within patients. More clinical investigations are required to validate the role of CSCs in tumor propagation; 2) whether the

CSC model can be generalized to all cancers remains unknown. The current studies show that some cancers follow the CSC model, while others seem not (54). More examination is required in all cancers to determine the frequency and the key molecular markers for CSCs to develop cancer (35,55).

1.2.3 Clonal evolution model versus cancer stem cell model

The clonal evolution model and the CSC model are not mutually exclusive. The clonal evolution model focus primarily on the genetic alteration of cancer cells; while the CSC model provides a 'hierarchy' perspective to tumor propagation, which focus primarily on the differentiation status of cancer cells. Thereby, the tumor heterogeneity can be explained by both of the two models, just from different perspectives. Moreover, no matter which model is fit to depict tumor progression, all tumor starts with the cell that obtains that first inheritable oncogenic mutation. In other words, all cancers emerge as a result of clonal evolution. Furthermore, a tumor population may comprise several clones of CSCs, which undergo clonal evolution. The aggressive clone of CSCs may be selected and become the dominant one in the population (55).

The 'seed and soil' hypothesis proposed by the English surgeon Stephen Paget to describe metastasis process in 1889 has gained wide acceptance. It may reflect the relationship between the clonal evolutional model and the CSC model. To date, the 'seed' has been referred to as CSCs; the 'soil' has been referred to as a combination of the stroma, the microenvironment, the factors in the host place, and the niche (56). The metastatic traits may arise from the interplay between the intrinsic CSC property and the extrinsic selecting pressure. Therefore, a harmonious combination of the insights derived from both the clonal evolution model and the CSC concept

may provide a comprehensive perspective for us to eventually understand the origins of the metastatic traits.

1.3 ROLE OF ACTIN BINDING PROTEINS IN CANCER

Tumor metastasis is a multi-stage cascade, including detaching from primary tumor, invasion, intravasation, circulating in blood vasculature or lymphatic system, extravasation, and invading into distant secondary organ. One of the critical steps, which distinguishes malignant tumor and benign tumor, is the process of dissemination away from the primary site and invasion towards surrounding connective tissues, which requires active cell migration. Cell migration is triggered by a variety of extracellular stimuli, such as growth factors, gradients of chemokines and ECM components. In response to these stimuli, cell migration is initiated by forming membrane protrusions at the leading edge. There are mainly four types of protrusions cells adapt to migrate: lamellipodia (57), filopodia, invadopodia (F-actin based protrusions, which locally secrete protease for matrix degradation, crucial for invasion process) (58) and blebs (membrane bulge structures with actin cortex, which are formed due to high intracellular hydrostatic pressure) (59,60). These protrusions are underpinned by the dynamics of actin polymerization. Next, the protrusions are stabilized by development of cell and ECM contact points through transmembrane receptors linking components in ECM and intracellular actin cytoskeleton. A typical example of such transmembrane receptor is integrin, which is a heterodimeric protein, with both intracellular and extracellular domains. By connecting ECM and intracellular components, integrin not only serves as an anchor, a traction sites, but also a mediator for signal transduction from ECM into the cell, thereby leading to reorganization of cytoskeleton inside the

cell. While the leading edge of the cell is attached to ECM, the cell and substratum adhesion at the rear edge begins to disassemble. The force generated by myosin II with actin filaments network encourages the disassembly of the adhesion at the rear side, and drives the cell retraction towards the forward direction (61). Overall, each step in cell migration is critically involved with actin cytoskeleton remodeling, which is under dynamic controls of a large number of actin-binding proteins (ABPs). Oncogenic transformation causes alterations of the actin cytoskeleton through deregulating the ABPs.

Based on their functions in actin assembly, ABPs are classified into the following categories: 1) actin nucleation proteins, such as actin-related protein 2/3 (Arp2/3) complex, formins, as well as Junction-mediating and regulatory protein (JMY); 2) nucleation-promoting factors, such as neural Wiskott-Aldrich syndrome protein (N-WASP), the WASP family verprolin homology proteins (WAVE1-3), JMY, SPIN90 and cortactin; 3) actin filaments elongators, such as ENA (enabled)/VASP (vasodilator stimulated phosphoprotein) and formins; 4) capping proteins, such as gelsolin and capZ; 5) debranching and severing protein, such as cofilin, coronin, glidal maturation factor (GMF) and gelsolin; 6) actin-bundling proteins, such as fascin, α-actinin, filamin and cortactin; 7) G-actin sequestering protein, such as thymosin β4 and 8) profilin (Pfn), which can promote the exchange ADP for ATP on G-actin, making it competent to be added on the barbed end of F-actin (57,62,63); 10) motor proteins, such as myosin II.

The deregulation of ABPs mainly involved with 1) deregulation of the expression levels of ABPs; 2) deregulation of the activities of ABPs; and 3) genetic mutation of ABPs (64). For instance, the proteins of Arp2/3 complex play pivotal roles in actin organization. They bind to pre-formed actin filaments, nucleating branch networks, which is critical for lamellipodium and

filopodium formation and increased cell motility. The proteins in Arp2/3 complex are upregulated in breast cancer, colorectal cancer, pancreatic cancer, and head and neck squamous cell carcinoma (65-69). The WASP/WAVE family proteins are the key regulators of the activity of Arp2/3 complex (70). Consequently, they are critically required for cell migration and tumor invasion. N-WASP is required for formation of invadopodia in carcinoma cells (71), and overexpression of N-WASP has been noted in colorectal cancers and esophageal squamous cell carcinoma (72,73). Similarly, WAVE proteins are observed to be upregulated in several cancer tissues, such as breast cancer and prostate cancer (74-76). The Ena/VASP family proteins are mostly localized at the leading edge of the cells, such as tips of filopodia and focal adhesions (77). They are pivotal regulators of actin filament nucleation and assembly. Ena/VASP family proteins facilitate and enhance the elongation of actin filaments (57). Ena/VASP expression has been found to be upregulated in breast cancer and lung adenocarcinomas (78,79). Similar to Ena/VASP family proteins, formin family proteins also play key roles in actin filament nucleation and elongation. In particular, formins are responsible for generating long F-actin structures, such as filopodia. Members of this family, mammalian Diaphanous-related formin 1-3 (mDia1, mDia2 and mDia3) have been shown to be required for invadopodia formation and are critically involved in tumor invasion (80). Overexpression of mDia3 has been observed in leiomyosarcomas (81). One the contrary, the cofilin and gelsolin represent another family of ABPs. They promote F-actin depolymerization at the barbed-end. Cofilin binds and severs F-actin under the control of Serine 3 phosphorylation. It has been indicated that active cofilin severs F-actin, generating more new free barbed-ends, ultimately promotes lamellipodium or filopodium formation and cell migration (82-84). Significantly increased expression of cofilin has been reported in invasive mammary tumors and prostate cancer (84,85). Different from

cofilin, gelsolin not only severs F-actin, but also caps the barbed ends. Decreased expression of gelsolin has been found in most cancers (86,87). Filamin family proteins crosslink actin filaments into orthogonal branching, allowing actin to form three-dimensional (3D) networks. It has been reported that prostate cancer metastasis correlates with low nuclear and high cytoplasmic filamin A expression (88). Fascin is another F-actin cross-linking protein, which organizes F-actin into parallel bundles. Fascin is reported to be a significant independent prognostic indicator of poor outcome in cancers of the liver, ovary, lung, pancreas, colon, brain, as well as head and neck squamous cell carcinoma (89,90). Cortactin is a multi-functional actin cross-linking protein with the ability of activating and stabilizing Arp2/3-mediated actin nucleation, which plays critical roles in lamellipodium and invadopodia formation. Overexpression of cortactin has been reported in breast cancer, head and neck squamous cell carcinoma and colon cancer (91-93). Thymosins are a family of small ABPs, preferentially bind to ATP-G-actin, blocking actin polymerization. Thymosin β4 is the major actin sequester protein with the function of maintenance of the balance of G-actin and F-actin in most mammalian cells. Overexpression of thymosin β4, thymosin β10 and thymosin β15 have been reported in thyroid tumor, breast cancer and melanoma. In addition, thymosin β4 has been shown to promote metastasis and angiogenesis (94). Profilin (Pfn) is another important actin-monomer binding protein. As the ubiquitously expressed member in mammalian cells, Profilin-1 (Pfn1) facilitates the exchange of ADP to ATP on G-actin, and shuttles it to the barbed-end of growing F-actin. Downregulation of Pfn1 has been reported in several carcinomas including breast cancer (95-98). Taken together, oncogenic transformation induced ABP mediated actin cytoskeleton remodeling is often correlated with acquisition of a motile phenotype of cancer cells.

Besides, actin cytoskeleton remodeling also plays a vital role in governing tumor-initiating capacity of cancer cells. For example, metastatic outgrowth of breast cancer cells is critically influenced by signaling triggered by the extracellular matrix (ECM) component of the tumor microenvironment and this involves changes in actin cytoskeletal architecture. Specifically, ECM-induced activation of integrin receptors potentiates actin stress-fiber assembly through a FAK-Src-ERK signaling axis. Preventing actin stress-fiber assembly or blocking integrin signaling dramatically inhibits FAK-Src-ERK signaling and subsequent metastatic outgrowth of disseminated breast cancer cells (17-19,99,100). Adhesion-mediated triggering of proliferation switch in disseminated breast cancer cells is enabled by the formation of F-actin-rich filopodia-like protrusions (FLPs) as schematically illustrated in Figure 2. Disseminated cancer cells that fail to initiate FLPs become incompetent in metastatic outgrowth and therefore remain dormant. FLPs are also abundant in a sub-population of breast cancer cells that exhibit stem-like characteristics and have high tumor-initiating potential, further suggesting that FLP abundance could be a general deterministic feature for tumorigenic ability of breast cancer cells (101,102).

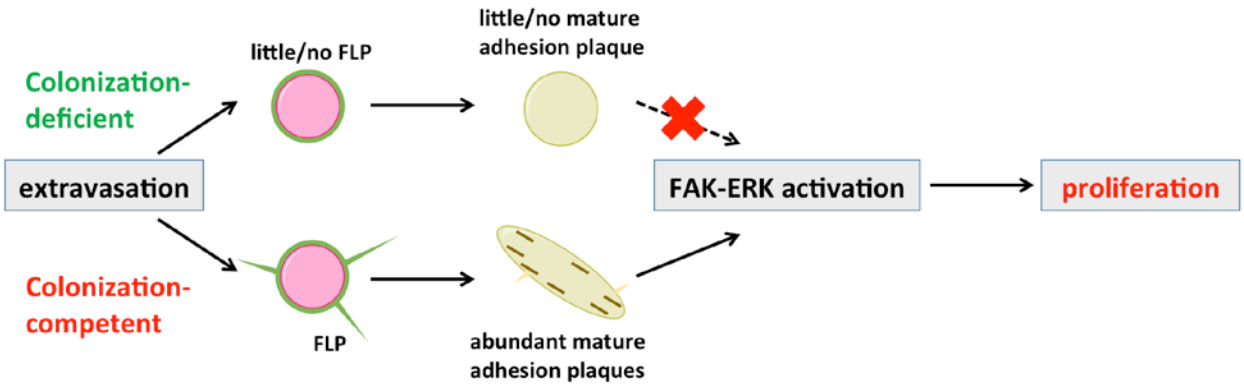


Figure 2. A schematic illustration of signaling events critical to initiate cancer cell proliferation in foreign tissue parenchyma. [Modified from (Shibue et al., 2012)]

Formation of FLPs requires the action of several cytoskeleton-regulatory proteins (CSPs) including Rif, Cdc42 (Rho-GTPases important for activation of actin-nucleating factors), and ABPs, such as mDia2 and Ena/VASP. Loss-of-function of these CSPs and ABPs not only impairs FLP induction and metastatic outgrowth, but also reduces the incidence of primary tumors from experimentally implanted breast cancer cells, suggesting that these CSPs and ABPs that are critical for FLP induction are also fundamentally important for tumorigenic ability of breast cancer cells (102).

Almost all major actin-nucleating and F-elongating proteins including those involved in FLP formation (e.g. mDia2 and Ena/VASP) interact with Pfn1, a ubiquitously expressed actin-binding protein and an important regulator of actin dynamics. Actin polymerizing abilities of both formin and Ena/VASP proteins are greatly enhanced in the presence of Pfn1 (103-105). Pfn1 participates in many actin-dependent cellular functions including cell proliferation and migration, although loss-of-function phenotypes are highly context-dependent (106). The rest of this thesis will focus on Pfn1 and its roles in breast cancer.

1.4 PROFILIN-1 AND BREAST CANCER

1.4.1 Pfn1 biochemistry and physiology

Pfn is a small molecule protein of 12 ~15 kDa. To date, four Pfn genes have been discovered in mammals. Pfn1 is the most ubiquitously expressed member among the four isoforms, which is found in all cell types except skeleton muscle (107). Pfn2 expression is predominantly in brain and is also expressed at low levels in other tissues (107,108). Pfn3 and Pfn4 expressions are

mainly restricted to testis (109,110). It is unlikely that Pfn1 and Pfn2 can complement each other as indicated by the following studies: 1) Mice depleted of Pfn2 have severe neurological problems despite the presence of Pfn1 expression (111); 2) Mice with homozygous Pfn1 knockout died as early as two-cell stage irrespective of the presence of although weakly expressed Pfn2 (112); 3) Pfn1 and Pfn2 have differential effects on cell motility and invasion in both normal (MCF10A) and breast cancer (SUM159) cell lines (113).

Pfn1 can bind to three classes of ligands: G-actin, poly-L-proline (PLP) motif bearing proteins and phosphoinositides (PPIs), including phosphatidylinositol-monophosphate (PI(4)P), phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), phosphatidylinositol-3,4-bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol-3,4,5-triphosphate (PIP_3) at least *in vitro* (114). Pfn1 can bind to G-actin and PLP-rich proteins simultaneously (115); in addition, it has been suggested that some proteins with PLP sequences bind preferentially Pfn1-actin than Pfn1 alone (116). The PPI binding site of Pfn1 overlaps with its G-actin and PLP binding domains, resulting in a competitive interaction between PPI and actin or PLP rich protein binding for Pfn1 (106).

Due to Pfn1's interaction with these binding partners, Pfn1 participates in many cellular processes, among which, the role of Pfn1 on actin polymerization has gained the most research attention and effort. Previously, Pfn1 was considered an actin monomer sequestering protein since 1) Pfn1 and G-actin binding cannot spontaneously induce nucleation of G-actin trimers, actually Pfn1-actin binding inhibits this process (117,118). 2) Pfn1 can only shuttle G-actin to the barbed ends (plus ends) of F-actin, while it cannot add G-actin to the pointed end (minus ends) (57). Therefore, when capping proteins occupy the barbed ends of F-actin, Pfn1 sequesters G-actin from adding to the pointed end. However, with the presence of pre-existing actin filament barbed end available, Pfn1 serves as a promoter of actin polymerization instead. Pfn1

contributes to actin polymerization in the following three ways: 1) Pfn1 and G-actin binding accelerates the exchange of ADP-G-actin to ATP-G-actin, which is the competent form of G-actin for actin polymerization (114). 2) Pfn1 binds to PLP rich protein, such as ENA/VASP and formins, which are actin filament elongators. This interaction facilitates the Pfn1-G-actin transition from cytosol to the free barbed end of F-actin, thereby promoting F-actin elongation (63). Taken together, Pfn1's role in actin polymerization is G-actin dependent and relies on the availability of barbed end of pre-existing F-actin. Most recently, Pfn1 has been discovered to preferentially deliver Pfn1-G-actin to ENA/VASP than formins in mammalian cells and inhibits Arp2/3 complex-based nucleation (119,120), therefore creating a homeostatic balance among different actin polymerization pathways to allow an integrated action such cell protrusion, cell division or cell migration to be concordantly achieved.

Based on Pfn1's critical function on actin polymerization, it plays a pivotal role on cell migration. Gene deletion of Pfn1 induced defects in migration has been reported in Dictyostelium amoebae (121), drosophila (122), mouse brain (123), mouse chondrocyte (124) and human vascular endothelia cells (125). In addition, gene deletion of Pfn1 in drosophila resulted in late embryonic lethality, and Pfn1 knockout (KO) mice died as early as two-cell stage, indicting Pfn1 is essential for development and survival, which has been implicated with its role in cytokinesis (112). Besides, Pfn1 has been reported to be involved with transcription, mRNA splicing, cell proliferation, membrane trafficking and endocytosis processes (106). More recently, Pfn1's role in stem cells has been studied. Mice with Pfn1 deficiency in mesenchymal progenitor cells have sternum defect in development (126). Deletion of Pfn1 in hematopoietic stem cells has been shown to lead to bone marrow failure, as well as metabolism and apoptosis signaling deregulation (127).

1.4.2 Pfn1 and its tumor suppressive effect in breast cancer

Despite the essential role of Pfn1 in migration, proliferation, development and survival, paradoxically, Pfn1 shows a tumor suppressive effect due to its downregulation in multiple invasive carcinomas including breast cancer (95). Two individual research groups including our lab, using two different breast cancer cell lines in either subcutaneous xenograft or orthotopic xenograft model, demonstrated that re-expression of Pfn1 exhibits inhibition of proliferation *in vivo* (95,128), further confirming Pfn1's tumor suppressive effect in breast cancers. In addition, our lab discovered that Pfn1 overexpression in MDA-MB-231 (MDA-231) cells leads to p27 upregulation (129), which partially explains Pfn1's tumor-suppressive action, however, the molecular mechanism underlying Pfn1's regulation towards p27 still needs further investigation.

Besides the inhibitory effect on proliferation, remarkably, Pfn1 also exerts an anti-migratory effect in breast cancer cells, and this work is mainly accomplished by our lab. It has been shown that invasive breast cancer cell lines have reduction of Pfn1 expression compared with non-invasive breast cancer cell lines (unpublished data). In addition, loss of Pfn1 increases the motility and matrigel invasiveness of breast cancer cells (130,131); while a moderate overexpression of Pfn1 inhibits the motility and matrigel invasiveness of breast cancer cells (128,132). Next, our lab have reported the mechanistic model to explain Pfn1's inhibitory effect on cell motility independent of G-actin binding in two-dimensional (2D) surface culturing condition (133). In this model, Pfn1 binds to PPI at the leading edge of cell membrane, which on one hand, prevents its interaction with G-actin; on the other hand, reduces the generation of $PI(3,4)P_2$, leading to limited translocation of lamellipodin (a $PI(3,4)P_2$ binding protein), resulting in the inhibition of ENA/VASP recruitment to the leading edge. In line with this, Pfn1 depletion increases the lamellipodin at the leading edge, promotes ENA/VASP localization, thereby

leading to the hypermotile phenotype of breast cancer cells. The observations based on perturbing Pfn1 levels in breast cancer cells are consistent with these recently observed in mouse fibroblasts, where Pfn1 depletion enhances Arp2/3 activity and promotes lamellipodium formation at the leading edge, while Pfn1 overexpression inhibits Arp2/3 actin nucleation and disrupting lamellipodium generation at the leading edge (119). Although this important finding is in the context of normal mammalian cells, the relationship established here between Arp2/3 complex and Pfn1 could potentially be extended to breast cancer cells. More recently, our lab further demonstrated that lower Pfn1 level is associated with higher disseminative ability of breast cancer cells, however the ability of colonization at the secondary site is inhibited by Pfn1 deficiency (130), suggesting that Pfn1 is crucially required for tumorigenesis in breast cancer. Together, these findings revealed the complexity of Pfn1's role in breast cancer cells.

1.5 HYPOTHYSIS AND SPECIFIC AIMS OF THIS STUDY

Tumor suppressor gene, as defined by Dr. Weinberg Robert A in 'The biology of Cancer', is a gene that protects a cell from one step on the path to cancer. When this gene mutates to cause a loss or reduction in its function, the cell can progress to cancer, usually in combination with other genetic changes. Given that restoration of Pfn1 in breast cancer cells inhibits tumor growth and Pfn1 expression is lower in breast carcinomas compared with their normal counterparts, one envisions that Pfn1 is a tumor suppressor. However, unlike classic tumor suppressor genes, somatic mutations of the Pfn1 gene are extremely rare and Pfn1 deficient breast cancer cells have impaired colonization ability at the secondary site. Although this is consistent with the fact that Pfn1 is playing various essential physiological roles, the underlying mechanistic basis of Pfn1's

tumor suppressive effect as well as its deficiency of colonization are completely unknown. Based on the existing results, it is postulated that, 1) Pfn1 is not a classic tumor suppressor although it exerts tumor suppressive effect; 2) Pfn1 is an important determinant for stemness, outgrowth and tumorigenic potential of breast cancer cells. Related to the hypothesis, the following aims are proposed,

Specific Aim1: To determine how Pfn1 regulates p27 expression in breast cancer cells.

Specific Aim2: To determine the role of Pfn1 in tumor tumorigenesis of breast cancer cells.

2.0 MATERIALS AND METHODS

2.1 ANTIBODY AND REAGENTS

Monoclonal GAPDH and tubulin antibodies are products of Sigma-Aldrich. Monoclonal p27 antibody was obtained from BD biosciences. Polyclonal phospho-AKT (T308), AMPK, phospho-AMPK (T172) and monoclonal skp2 antibodies were purchased from Cell Signaling Technologies. Monoclonal Histone H1 antibody was from Santa Cruz Biotechnology. Polyclonal phospho-p27 (T187) antibody was purchased from Invitrogen. Phospho-p27 (T198) was a product from R&D systems. Monoclonal Pfn1 antibody was purchased from Novus Biological. R-cadherin antibody was a generous gift from Dr. Rachel Hazan (Albert Einstein College of Medicine, NY). LY294002 compound was purchased from Cell Signaling Technologies. MG132 was a product of Merck Millipore. Compound C was a product from Enzo Life Sciences. Akt Inhibitor (Akti-1/2) was a product from Abcam. All cell culture reagents were products of Invitrogen.

2.2 CELL CULTURE AND TRANSFECTION

Generation and culture of MDA-231 cell lines stably expressing GFP and GFP-Pfn1 have been described previously (128). GFP and GFP-Pfn1 were also subcloned into pQCXIP retroviral

vector (Clontech). Retrovirus packaging and subsequent infection of BT474 and MCF-7 cells were carried out according to the manufacturer's instructions. Infected cells were selected for puromycin resistance (250 ng/ml) and finally, stable cells were sorted based on their GFP fluorescence before experimental use. BT474 and MCF-7 cells were cultured in RPMI1640 and DMEM medium supplemented with 10% FBS respectively. The preparation and maintenance of MDA-231 cells stably transfected with either luciferase shRNA or Pfn1 shRNA have been described in detail previously (134). Constitutively active HA-AKT1 plasmid was obtained from Addgene. Plasmid transfection was preformed performed using Lipofectamine LTX/Plus reagent (Invitrogen) according to manufacturer's instruction. In gene silencing experiments, cells were transfected with pooled non-targeting control or gene-targeted (AMPKα, R-Cadherin, and PTEN) siRNAs obtained from Santa Cruz Biotechnology or smartpool Pfn1 siRNAs obtained from Thermo Fisher Scientific using transfection reagent from Dharmacon according to the manufacturer's instructions.

2.3 PROTEIN EXTRACTION AND IMMUNOBLOTTING

Total cell lysate was prepared by extracting cells with modified RIPA buffer (50 mM Tris-HCl-pH=7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) supplemented with 50 mM NaF, 1 mM sodium pervanadate, and protease inhibitors. Subcellular fractionation was performed as described (129). Briefly, cells were previously cultured in 10 cm dish at 80% confluence. Then cells were extracted in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1% NP-40, 1 mM DTE with protease and phosphatase inhibitors) for 20 min and centrifuged at 5,000 rpm for 30 min. The supernatant was then collected and saved as

cytoplasmic fraction. Cell pellet was then washed with buffer A for 3-4 times, re-suspended in buffer B (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 10% glycerol, and 1 mM DTE with protease and phosphatase inhibitors) and vigorously vortexed for 1 h at 4 °C followed by centrifuging at 13,000 rpm for 10 min. The supernatant was collected as the nuclear fraction (the purity of the nuclear fraction was confirmed by positive and negative immunoblots of Histone-H1 and GAPDH antibodies, respectively). The lysates were centrifuged at 13,000 rpm for 30 min at 4 °C, and the protein concentration was then determined by Bio-Rad protein assay dye reagent concentrate (Bio-Rad). For electrophoresis, same amount of protein samples were loaded to the gel and then transferred onto nitrocellulose membrane. Then the membrane was blocked with either 5% non-fat dry milk or 5% BSA in TBST for 1 h at room temperature. Immunoblotting was then performed overnight with primary antibodies. Followed by 30 min washing with TBST, the blots were incubated with corresponding secondary antibodies, followed by 30 min washing with TBST before imaging with chemiluminescence. Immunoblotting concentrations for different antibodies were: 1:500 (Histone H1, phospho-p27 (T187, T198)), 1:1000 (R-cadherin, AMPKα, phospho-AMPKα (T172), phospho-AKT (T308), skp2), 1:2000 (p27) and 1:4000 (Pfn1, GAPDH and tubulin).

2.4 2D GEL ELECTROPHORESIS

Cells were prepared in 10 cm dish at 70%-80% confluence. Cells were washed twice with pre-chilled DPBS and then washed twice with pre-chilled Tris/sucrose buffer (10 mM Tris, 250 mM sucrose (Invitrogen)). Dishes were placed at oblique for 5 minutes to collect residual washing buffer completely. Cells were extracted using 2D lysis buffer (2 M Urea (Fisher Scientific), 7 M

Thiourea (Invitrogen), 4% (w/v) CHAPS (Sigma), 50 mM DTT (Roche)) and collected into a 50 mg Glass Beads (Sigma) containing bead-beater tube (Biospec). Lysates were pulsed for 20 seconds each time, and 4 times in total in a mini beadbeater (Biospec). The lysates were maintained on ice for 2 minutes between pulses. 0.1 U benzoase nuclease (Sigma) and 2 mM $MgCl_2$ (Fisher Scientific) were then added to the solution followed by incubation on ice for 30 minutes. The lysates were then centrifuged at 3,500 rpm for 5 minutes to be separated from glass beads. After that, the supernatant was centrifuged again at 13,000 rpm for 20 minutes. The supernatant was collected and the protein concentration was measured using the RC DC Protein Assay Kit (Bio-Rad). Isoelectric focusing was performed using the Zoom IPGRunner System (Invitrogen) according to the manufacturer's instructions. Briefly, 50–150 μg protein lysis was re-suspended to reach the final concentrations: 2 M Urea, 7 M thiourea, 4% (w/v) CHAPS, 50 mM DTT, 0.5% (v/v) carrier ampholytes (Invitrogen), 0.005% (w/v) bromophenol blue (Fisher Scientific). Then the mixture solution was loaded into a Zoom IPG Runner Cassette (Invitrogen) with ZOOM IPG Strips (Invitrogen) for 1 hour at room temperature. After that, the isoelectric focusing was conducted on the IPG strips using the following program: 175 V for 30 minutes; linear ramp 175–2000 V over 45 minutes; 2000 V for 105 minutes. IPG strips were then incubated with equilibration buffer (6 M urea, 2% (w/v) SDS, 50 mM Tris pH = 8.8, 20% (v/v) glycerol, 2% (w/v) DTT) for 25 minutes at room temperature, followed by briefly washing with running buffer (25 mM Tris pH 8.3, 192 mM Glycine, 0.1% (w/v) SDS) and sealed in Tris-HCl polyacrylimide gels with running buffer containing 0.5% agarose (Invitrogen) and 0.005% (w/v) bromophenol blue. These gels were then subjected to normal electrophoresis and immunoblotting.

2.5 MAMMARY EPITHELIA CELL (MEC) ACINI ASSAY

MECs were isolated by collagenase digestion of mammary gland of $Pfn1^{flox/flox}$ mice, re-suspended in growth media (5% FBS, 5 µg/ml insulin, 1 µg/ml hydrocortisone, 5 ng/ml EGF, 50 µg/ml gentamycin, 1X antibiotic-antimycotic, HamF12), and transduced with either Ad-GFP or Ad-Cre (10^8 virus/2 × 10^5 cells) before plating on top of a 50 µL growth factor-reduced matrigel (Cultrex, Basement Membrane Extract (BME), Trevigen) layer in each well of a 48-well plate. The flow diagram of the experiment is illustrated in Figure 3.

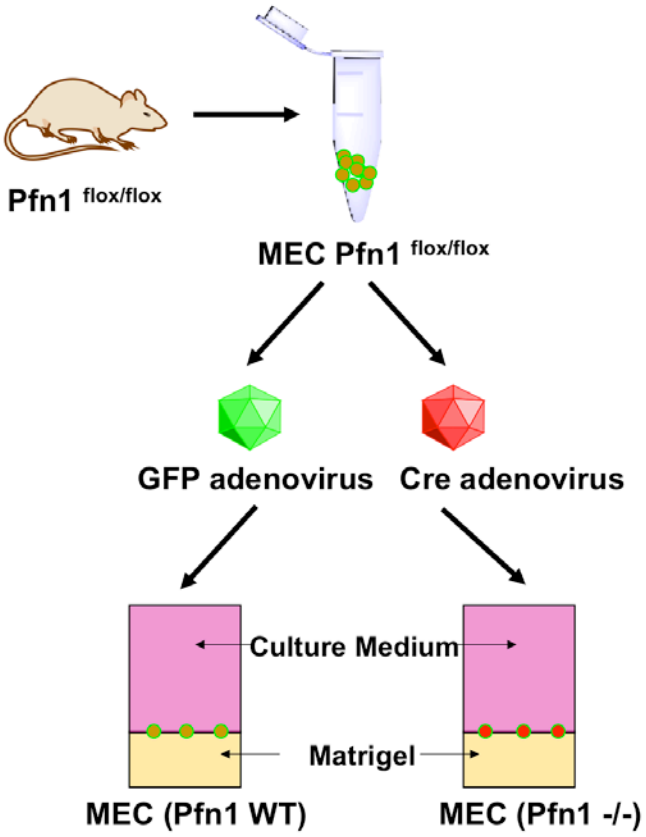


Figure 3. Flow diagram of MEC acini assay.

2.6 3D CELL CULTURE

Cells were seeded in 3D matrix using the 3D 'on-top' assay (135). Briefly, multi well plates were coated with matrigel or matrigel/collagen-I mix (The mix contains a 50/50 volume ratio solution of BME/collagen-I (BD Biosciences) for the final concentrations of 7.9 mg/mL and 2 mg/mL respectively). The 2D-cultured cells were then trypsinized and seeded as single cells on top of the 3D matrix using medium containing 2% fetal bovine serum, 1% antibiotics and 2% matrigel. The 3D culture medium was replaced every 3-4 days. Cells were imaged on an ImageXpress Ultra confocal high content reader using a 4X objective. A total of twenty 50 μm thick planes was acquired and collapsed using a maximum projection algorithm. Objects were enumerated using the MetaXpress multiwavelength cell scoring algorithm.

2.7 ALDH1 ACTIVITY ASSAY

The ALDEFLUOR^{TM} kit (STEM CELL Technologies, Vancouver, BC) was used to identify cells with high levels of ALDH1. ALDH1 activity assay was conducted according to the manufacturer's instructions.

2.8 MAMMOSPHERE FORMATION ASSAY

Mammosphere formation assay was performed as described (136). Briefly, 2D cultured cells were trypsinized and then filtered through a 40 μm cell strainer. The single cell suspensions were

then plated in 12 well ultra-low attachment plates at a density of 1000 cells/well in serum-free mammary epithelium basal medium (Lonza) supplemented with 1% antibiotics, 2% B27 (Linvitrogen-Life Technologies), 5 μg/mL Insulin, 1 μg/mL hydrocortisone (Sigma), 20 ng/mL epidermal growth factor (R&D Systems), 20 ng/mL basic fibroblast growth factor (Stem Cell) and β-mercaptoehtanol (1:25,000). After 8 days incubation, the mammosphere number was counted under an inverted microscope and the mammopshere size was measured.

2.9 RT^2 PROFILER PCR ARRAYS

The total RNA was extracted from 2D cultured MDA-231 cells using RNeasy Mini Kit (Qiagen). The first strand cDNA synthesis was conducted using RT^2 First Strand Kit (SA Biosciences) and the following PCR was performed using in Human Cancer Stem Cells PCR Array (SA Biosciences) according to the manufacturer's instructions. Data analysis was achieved through web based software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). For each experiment, 3 replicate human cancer stem cell arrays were conducted.

2.10 STATISTICAL ANALYSIS

Statistical differences were assessed by t-test or one-way ANOVA followed by Tukey post-hoc test according to experimental strategy and a p-value of less than 0.05 was considered significant.

3.0 THE MOLECULAR MECHANISM UNDERLYING PFN1'S TUMOR SUPPRESSIVE EFFECT THROUGH THE REGULATION OF P27

Breast cancer cells needs to acquire capability to sustain uncontrolled proliferative potential through the regulation of cell cycle (137). Cell cycle progression is tightly regulated by the activation of cyclin/cyclin-dependent kinase (CDK) complexes. Interactions between cyclins and CDKs are inhibited by the action of cyclin kinase inhibitors (CKI). P27 is a prominent member of the CKI family which specifically binds to and inhibits cyclinE/CDK2 complex activity, causing cell-cycle arrest in G1 phase. CyclinE/CDK2 complex serves as a kinase to phosphorylate p27 on threonine 187 (T187) site, which promotes the protein degradation. Downregulation in expression and/or cytoplasmic mislocalization of p27 have been reported in a substantial number of human epithelial cancers (breast, prostate, lung, colon, head and neck) (138). While p27 expression can be controlled at all levels of gene expression including transcription, translation and post-translation, in cancer it is most often deregulated at post-translational level that involves accelerated proteolysis (138). Protein stability as well as sub-cellular (i.e. nuclear vs-cytoplasmic) localization of p27 are critically regulated by its phosphorylation on serine and threonine residues (139). Hyperactivation of PI3K-AKT pathway has been most prominently linked to p27 deregulation in cancer. AKT can directly phosphorylate p27 on multiple residues (S10 and T157) leading to its nuclear exclusion (140,141). AKT can also regulate the activity of Skp2, a key component of the E3 ligase for p27 ubiquitination (142).

P27 can be also phosphorylated on T198 by AMPK (AMP-dependent kinase – a kinase that is activated when AMP:ATP ratio rises in cells) upon nutrient deprivation and this phosphorylation confers increased stability to p27 (143). AMPK-dependent phosphorylation of p27 is a major mechanism that links nutrient deprivation to cell-cycle control.

In this study, a novel mechanistic link between Pfn1 and p27 was established in mesenchymal human breast cancer cells that involves AMPK activation secondary to epithelial morphological reversion.

The contents in this chapter have been published in the following publication:

Chang Jiang, William Veon, Hui Li, Kenneth R Hallows, Partha Roy. Epithelial morphological reversion drives Profilin-1-induced elevation of p27kip1 in mesenchymal triple-negative human breast cancer cells through AMP-activated protein kinase activation. Cell Cycle 2015;14:18:2914-2923.

3.1 RESULTS

3.1.1 Pfn1 causes p27 accumulation in MDA-231 cells primarily through misregulation of p27 proteolysis in the nucleus

It was previously reported that stable overexpression of Pfn1 leads to increased protein stability of p27 in MDA-231 cells (129), suggesting that cellular changes induced by Pfn1 elevation are linked to post-translational regulation of p27. To determine whether differential protein stability of p27 solely accounts for Pfn1-dependent change in p27 expression, the effect of MG132 (a proteasome inhibitor) on the relative levels p27 expression was analyzed in isogenic sublines of

MDA-231 cells stably overexpressing either GFP-Pfn1 or GFP (control). Under DMSO (vehicle control) treated condition, Pfn1 overexpressing cells have markedly higher p27 expression than GFP expressers as expected but p27 differential is nullified when proteasomal pathway is inhibited (Figure 4A). These data demonstrate that elevating Pfn1 expression upregulates p27 in MDA-231 cells through retarding its protein degradation.

P27 is subjected to ubiquitin-proteasome-mediated degradation in both cytoplasmic and nuclear compartments of cells. Subcellular fractionation analyses revealed that Pfn1 overexpression results in p27 elevation only modestly in the cytoplasmic compartment but very dramatically in the nucleus (Figure 4B), further indicating that Pfn1 causes p27 accumulation in MDA-231 cells primarily through misregulation of p27 proteolysis in the nucleus. Poly-ubiquitination and subsequent degradation of p27 in the nucleus is mediated by SCF-skp2 (Skp-Cullin-F-box) E3 ubiquitin ligase complex (144). Subcellular fractionation analyses showed no difference in either cytoplasmic or nuclear content of skp2 between control and Pfn1 overexpressing cells (Figure 4B). Thus, Pfn1 overexpression does not lead to nuclear accumulation of p27 through dysregulation of either the overall expression or subcellular localization of skp2.

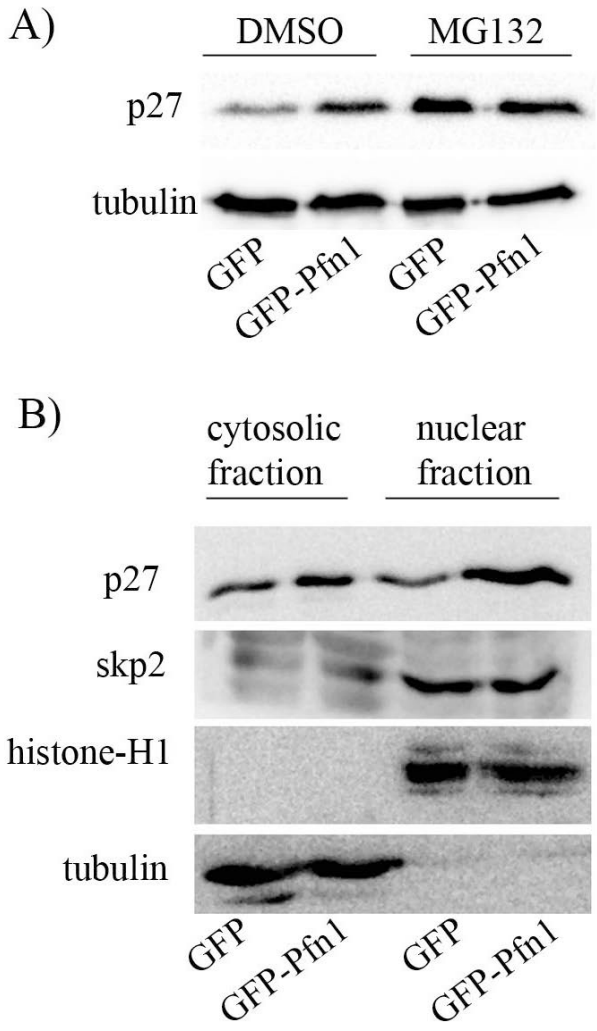


Figure 4. Pfn1 overexpression causes p27 accumulation in MDA-231 cells through inhibiting its protein turnover. A) P27 immunoblot of total cell extracts prepared from sub-confluent cultures of GFP and GFP-Pfn1 expressing MDA-231 cells following treatment with 10 μM of either MG132 (a proteasome inhibitor) or DMSO (vehicle control) for 10 hours. Tubulin blot served as the loading control. B) P27 and skp2 immunoblots of cytosolic and nuclear extracts prepared from GFP and GFP-Pfn1 expressers (tubulin and histone blots served as the loading control for cytosolic and nuclear fractions, respectively).

3.1.2 Pfn1 overexpression has site-specific effects on p27 phosphorylation

Phosphorylation status of p27 is a key determinant of protein stability of p27. For example, phosphorylation of p27 on T187 residue is a key prerequisite for skp2-p27 interaction and skp2-mediated p27 degradation (145). By contrast, phosphorylation of p27 on T198 residue confers increased protein stability to p27 (146). To determine whether Pfn1 overexpression influences the overall phosphorylation pattern of p27, total extracts from control and Pfn1 overexpressing cells were resolved by 2D gel electrophoresis and immunoblotting was performed with anti-p27 antibody. Note that as Pfn1 overexpressers have higher total p27 level, higher amount of total extract was loaded from control cells in order to normalize the total p27 level between the two groups. No discernible difference was found in the overall post-translational modification pattern of p27 between control and Pfn1 overexpressing cells (Figure 5A). These data suggest that p27's ability to become phosphorylated per se is not affected by Pfn1. However, consistent with increased protein stability of p27, Pfn1 overexpressers exhibited reduced and elevated phosphorylation on T187 and T198 residues, respectively (Figure 5B). These data demonstrate that Pfn1 overexpression has site-specific effects on p27 phosphorylation.

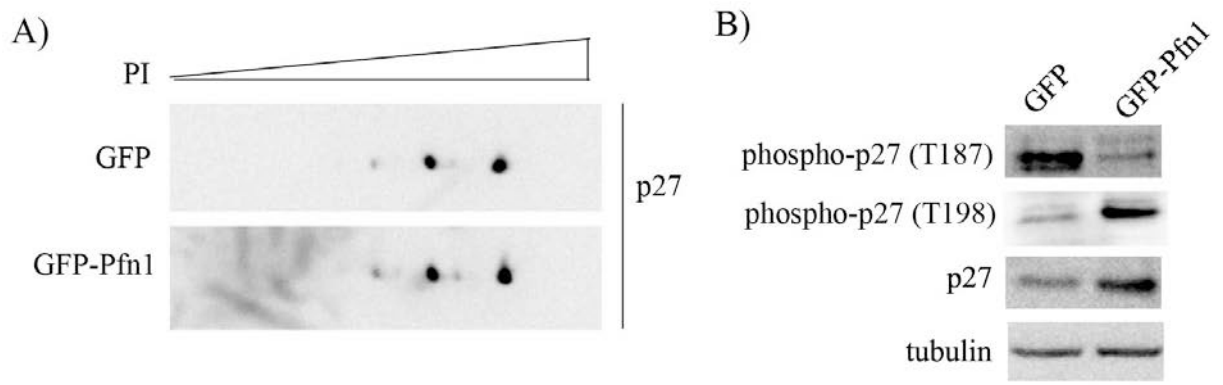


Figure 5. Pfn1 overexpression has site-specific effects on p27 phosphorylation in MDA-231 cells. A) Total lysates prepared from sub-confluent cultures of GFP and GFP-Pfn1 expressing MDA-231 cells were separated by 2D-gel electrophoresis and immunoblotted with p27 antibody. Higher amount of total protein was loaded for the GFP group to normalize the total p27 level between two groups. B) Total cell extracts of GFP and GFP-Pfn1 expressers were separated on an SDS-PAGE and immunoblotted with the indicated antibodies (tubulin blot served as the loading control).

3.1.3 Pfn1-induced elevation of p27 in MDA-231 cells is not due to deficiency in AKT activation

Hyperactivation of PI3K/AKT signaling is a major driving force behind increased proteolysis and/or cytoplasmic mislocalization of p27 in cancer cells (147). For example, AKT can directly phosphorylate p27 on serine 10 (S10) and threonine 157 (T157) residues. Nuclear export of p27 is facilitated by S10 phosphorylation while its nuclear import is inhibited by T157 phosphorylation, and therefore, either of these two phosphorylations can promote cytoplasmic mislocalization of p27. Although p27 cannot be directly phosphorylated by AKT on T187 residue, AKT may indirectly promote T187 phosphorylation through activation of cyclinE-CDK2 (a kinase that is responsible for p27 phosphorylation on T187) complex (148). Loss of

Pfn1 expression has been shown to enhance accumulation of $PI(3,4)P_2$ (a phosphoinositide that is generated downstream of activated PI3K) in MDA-231 cells in response to acute activation of various receptor tyrosine kinases (RTKs) (133). Conversely, elevating Pfn1 expression greatly suppresses RTK-induced generation of $PI(3,4,5)P_3$ (another PI3K-generated phosphoinositide) and the downstream AKT activation (149). Even in a regular culture setting *i.e.* without acute activation of any particular RTK pathway, Pfn1 overexpressers exhibit a marked suppression of AKT activation (as measured by the level of AKT phosphorylation on T308) when compared to control cells (Figure 6A). These data are suggestive of Pfn1's inhibitory effect on PI3K/AKT signaling in MDA-231 cells. Since pharmacological inhibition of PI3K by LY294002 mimics the effect of Pfn1 overexpression in terms of attenuation of T187 phosphorylation with concomitant elevation of p27 expression (Figure 6B), the next inquiry was whether Pfn1-induced p27 accumulation is due to deficient PI3K/AKT signaling. To address this question, two types of rescue experiments were performed. First, the effect of silencing of PTEN (phosphatase and tensin homolog 10 – a dual lipid-protein phosphatase that antagonizes PI3K/AKT signaling by dephosphorylating $PI(3,4,5)P_3$ to $PI(4,5)P_2$) on Pfn1-induced change in p27 expression was studied. PTEN silencing was able to rescue Pfn1 overexpressing cells from defect in AKT activation but surprisingly had no effect on p27 expression (Figure 6C). Likewise, when AKT was directly hyperactivated in Pfn1 overexpressing cells by expressing a constitutively active mutant of AKT (CA-AKT: it harbors phosphomimetic T308D and S473D mutations), p27 accumulation could not be reversed either (Figure 6D). Furthermore, when AKT function was blocked by an inhibitor, p27 differential between control and Pfn1 overexpressers was still maintained (Figure 6E). Collectively, these results demonstrate that Pfn1-induced elevation of p27 in MDA-231 cells is not due to deficiency in AKT activation.

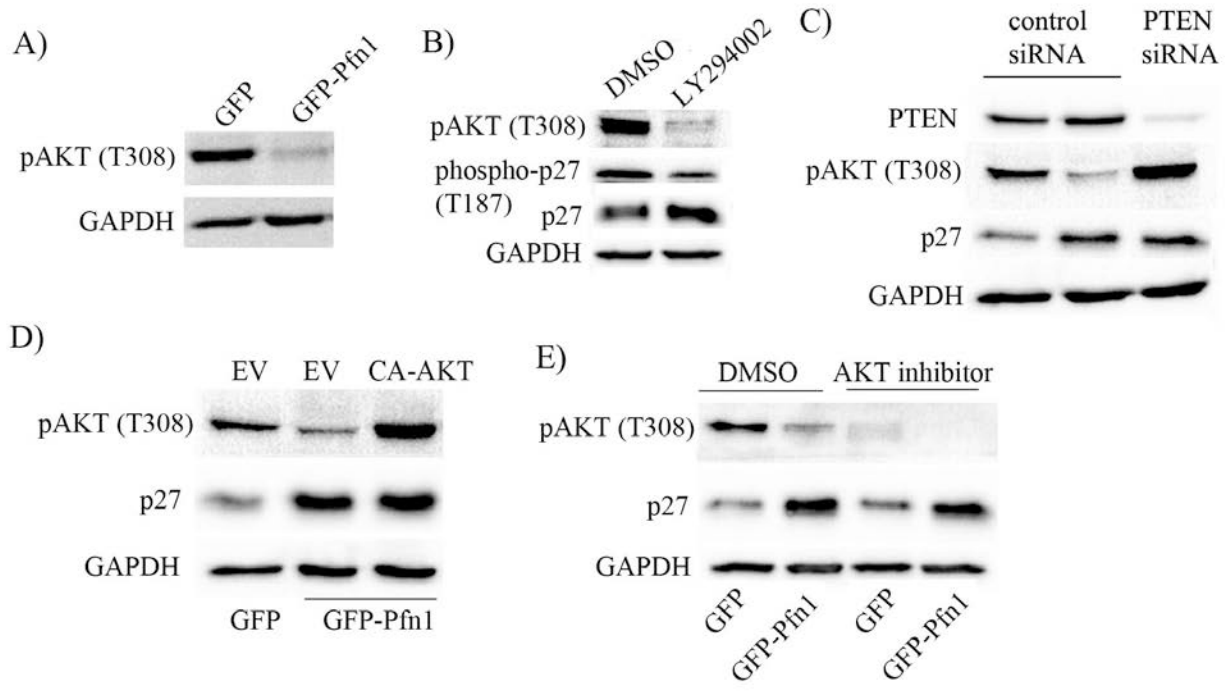


Figure 6. Deficiency in PI3K/AKT signaling does not account for Pfn1-induced accumulation of p27 in MDA-231 cells. A) Total lysates prepared from sub-confluent cultures of GFP and GFP-Pfn1 expressing MDA-231 cells were run on an SDS-PAGE and immunoblotted with phospho-AKT (T308) antibody. B) Total extracts prepared from GFP-expressing cells following treatment with 25 μM of either LY294002 or DMSO (vehicle control) for 24 hours were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. C) Total extracts of GFP and GFP-Pfn1 expressers transfected with 100 nM of either non-targeting control or PTEN-siRNAs were resolved by SDS-PAGE and immunoblotted for PTEN and p27. D) P27 and pAKT (T308) immunoblots of total lysates prepared from GFP and GFP-Pfn1 expressers transfected with either empty vector (EV) or constitutively active AKT (CA-AKT) encoding plasmids. E) Phospho-AKT (T308) and p27 immunoblots of extracts prepared from GFP and GFP-Pfn1 expressers following treatment with 25 μM of either AKT inhibitor or DMSO (vehicle control) for 24 hours. GAPDH blot served as the loading control in all experiments.

3.1.4 Pfn1 overexpression leads to p27 accumulation in MDA-231 cells through stimulating AMPK-dependent phosphorylation on T198 residue

As Pfn1 overexpression causes elevated T198 phosphorylation of p27, a post-translational modification that also increases the protein stability of p27, the next inquiry was whether Pfn1 overexpression causes p27 elevation through affecting AMPK (a candidate kinase for T198 phosphorylation of p27) activity. Phospho-specific immunoblot analyses of total cell extracts showed that Pfn1 overexpression increases T172–phosphorylated form of AMPK (an indicator for activate form of AMPK) while the total expression level of AMPK is unaffected (Figure 7A). Next, rescue experiments involving suppression of AMPK activity were performed using both pharmacological and molecular strategies. Treating cells with AMPK antagonist Compound C dramatically reduced T198 phosphorylation of p27 and completely reversed Pfn1-induced elevation of p27 (Figure 7B-C – in control experiments, DMSO treatment preserved higher levels of T198-phosphorylated form and total p27 in Pfn1 overexpressers relative to control GFP expressing cells as expected). Quantification showed that Compound C treatment led to an average 10-fold reduction in p27 level in Pfn1 expressing cells. As a complementary approach, AMPK function was inhibited through silencing of AMPKα, an essential component of AMPK holoenzyme complex. Similar to the trends of compound C experimental results, silencing of AMPKα also led to downregulation of T198 phosphorylation and 2-fold reduction in the overall expression of p27 expression in Pfn1 overexpressing cells, completely reversing Pfn1-induced p27 accumulation in MDA-231 cells (Figure 7D-E). It is noted that compound C treatment lowered even the basal p27 expression in control GFP-expressing cells which AMPKα knockdown failed to do, and this likely explains the differences in fold-change in p27 expression in Pfn1 overexpressers between the settings of molecular and pharmacological approaches of

AMPK inhibition. This was not totally surprising as AMPKα knockdown was not 100% in these experiments and it has been reported that compound C can have off-target effects on several other kinases besides AMPK (150) which may also impact p27 expression. Nonetheless, the overall similarity between the effects of AMPK silencing and compound C treatment demonstrate that Pfn1 overexpression leads to p27 accumulation in MDA-231 cells through stimulating AMPK-dependent phosphorylation on T198 residue.

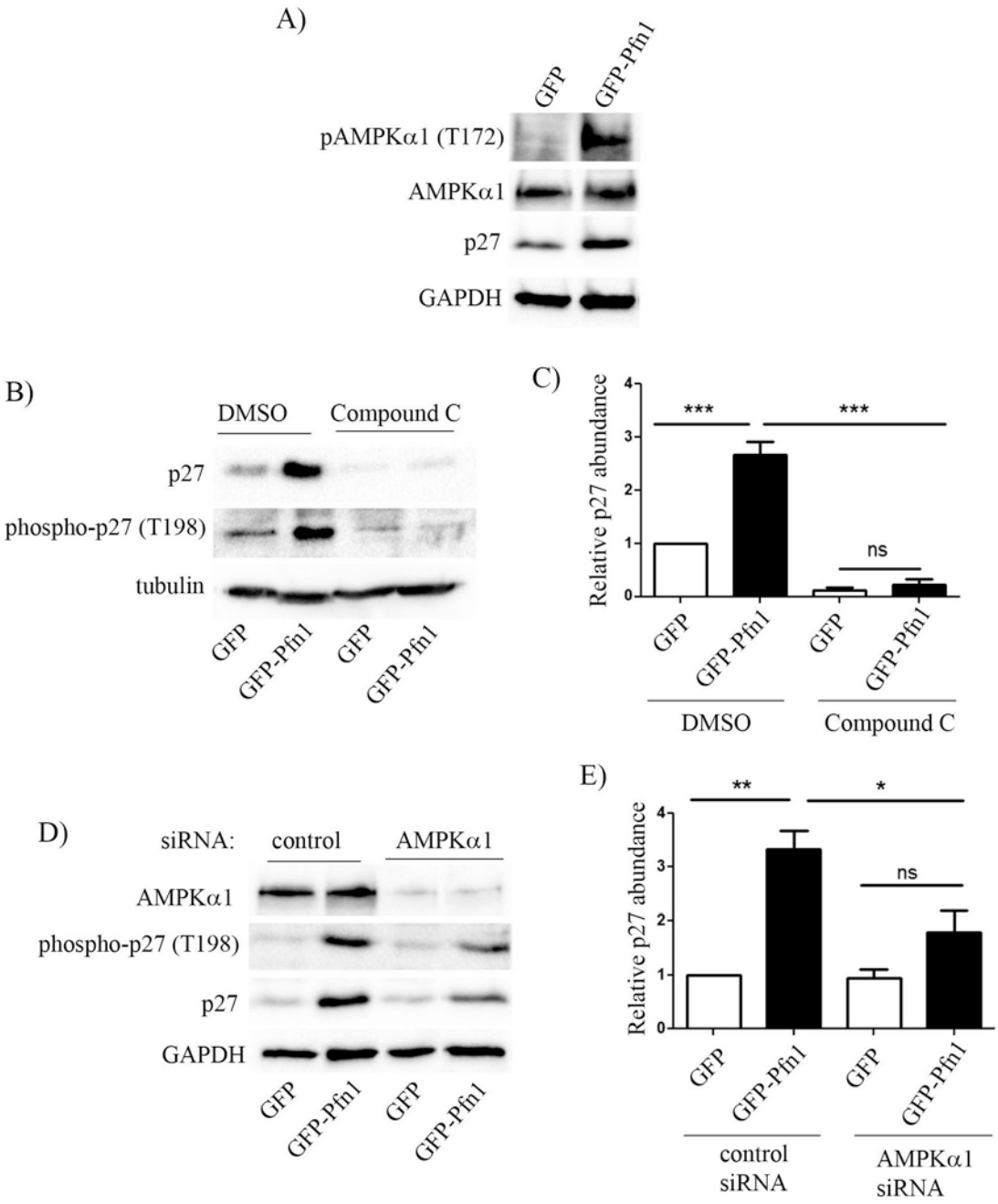


Figure 7. Pfn1 overexpression upregulates p27 in MDA-231 cells through AMPK activation. A) Immunoblots of total extracts show the relative levels of T172-phosphorylated-AMPK, total AMPK and p27 between GFP and GFP-Pfn1 expressers. B-C) Representative immunoblots showing relative levels of T198-phosphorylated and total p27 between GFP and GFP-Pfn1 expressers following treatment with 10 μM of AMPK antagonist Compound C or DMSO (vehicle) for 24 hours. D-E) Representative

immunoblots showing relative levels of AMPKα, T198-phosphorylated and total p27 between GFP and GFP-Pfn1 expressers 72 hours after transfection with 100 nM of either non-targeting control or AMPKα1-specific siRNAs. The bar graph on the right summarizes the data from 3 independent experiments. GAPDH and tubulin blots served as the loading controls (***: $p< 0.001$, **: $p< 0.01$, *: $p< 0.05$; ns: not significant).

3.1.5 Epithelial morphological reversion plays a key role in AMPK-dependent p27 accumulation upon overexpression of Pfn1 in mesenchymal breast cancer cells

Next, whether Pfn1 overexpression induced p27 accumulation is sensitive to culture conditions was investigated. It was found that elevation of T198 phosphorylation and overall accumulation of p27 in Pfn1 overexpressers is more pronounced in confluent than in sub-confluent culture conditions (Figure 8 – note that the degree of confluence has no effect on p27 expression in control cells). Based on this observation, it was speculated that cell-cell interaction may play a role in Pfn1-induced p27 accumulation in MDA-231 cells. MDA-231 cell line is null for expression of most of the major cadherin family of adherens junction (AJ) proteins including E-, P-, and N-cadherin and displays all characteristics of post-epithelial-to-mesenchymal transition. MDA-231 cells however express R-cadherin (retinal cadherin) and it has been reported that stable Pfn1 overexpression is able to morphologically transform this cell line into an epithelial phenotype through upregulating R-cadherin and formation of R-cadherin-based AJs (151). A similar Pfn1-dependent epithelial phenotypic induction was also reported by another group in CAL-51 (152), an otherwise mesenchymal breast cancer cell line that loses its tumorigenic ability upon Pfn1 overexpression (95). Note that at least in the case of MDA-231 cells, it has been confirmed that Pfn1-induced morphological transformation is not a classical mesenchymal-

to-epithelial transition (MET) as it was not accompanied by loss of vimentin (a marker for mesenchymal cells) and induction of E-cadherin (a marker for epithelial cells) expressions (151). Since stable Pfn1 overexpression in ER+/PR+ epithelial-type breast cancer cell lines (BT474, MCF-7) does not lead to p27 elevation (Figure 9), it is likely that Pfn1 does not have a direct mechanism of regulating p27 expression. This prompted a further query on whether epithelial phenotypic transformation plays a role in Pfn1-dependent upregulation of p27 in MDA-231 cells.

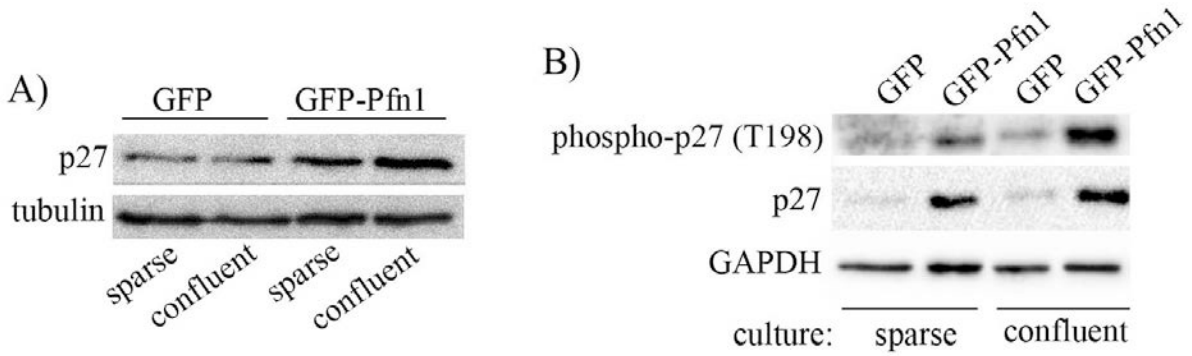


Figure 8. Pfn1-induced p27 upregulation in MDA-231 cells increases with cell confluence. GFP and GFP-Pfn1 expressers were either maintained in sub-confluent condition or grown to confluency. Cell extracts prepared under these conditions were resolved by SDS-PAGE and immunoblotted for the relative levels of p27 (panel A) and T198-phosphorylated p27 (panel B) between the two sublines (GAPDH and tubulin blots served as the loading control).

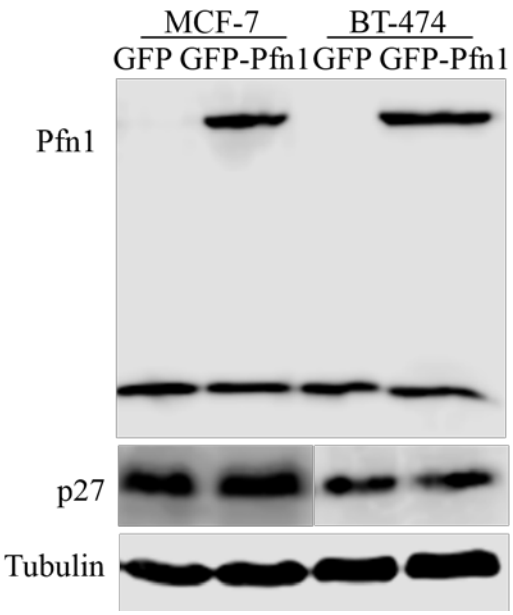


Figure 9. Pfn1 overexpression does not cause p27 elevation in epithelial breast cancer cells. Total extracts of MCF and BT474 cells stably transduced with GFP and GFP-Pfn1 encoding retrovirus were immunoblotted for Pfn1, p27 and GAPDH (loading control) antibodies.

To test this, Pfn1 overexpressing cells were reverted to a mesenchymal phenotype through silencing of R-cadherin expression (Figure 10A). Downregulation of R-cadherin expression did not completely abrogate but reversed Pfn1-induced p27 accumulation by 1.5-fold in MDA-231 cells (Figure 10B-C; note that R-cadherin silencing did not have any effect on p27 expression in control GFP expressers). R-cadherin depletion also resulted in a prominent suppression of AMPK activation and T198 phosphorylation of p27 in Pfn1 overexpressing cells (Figure 10D).

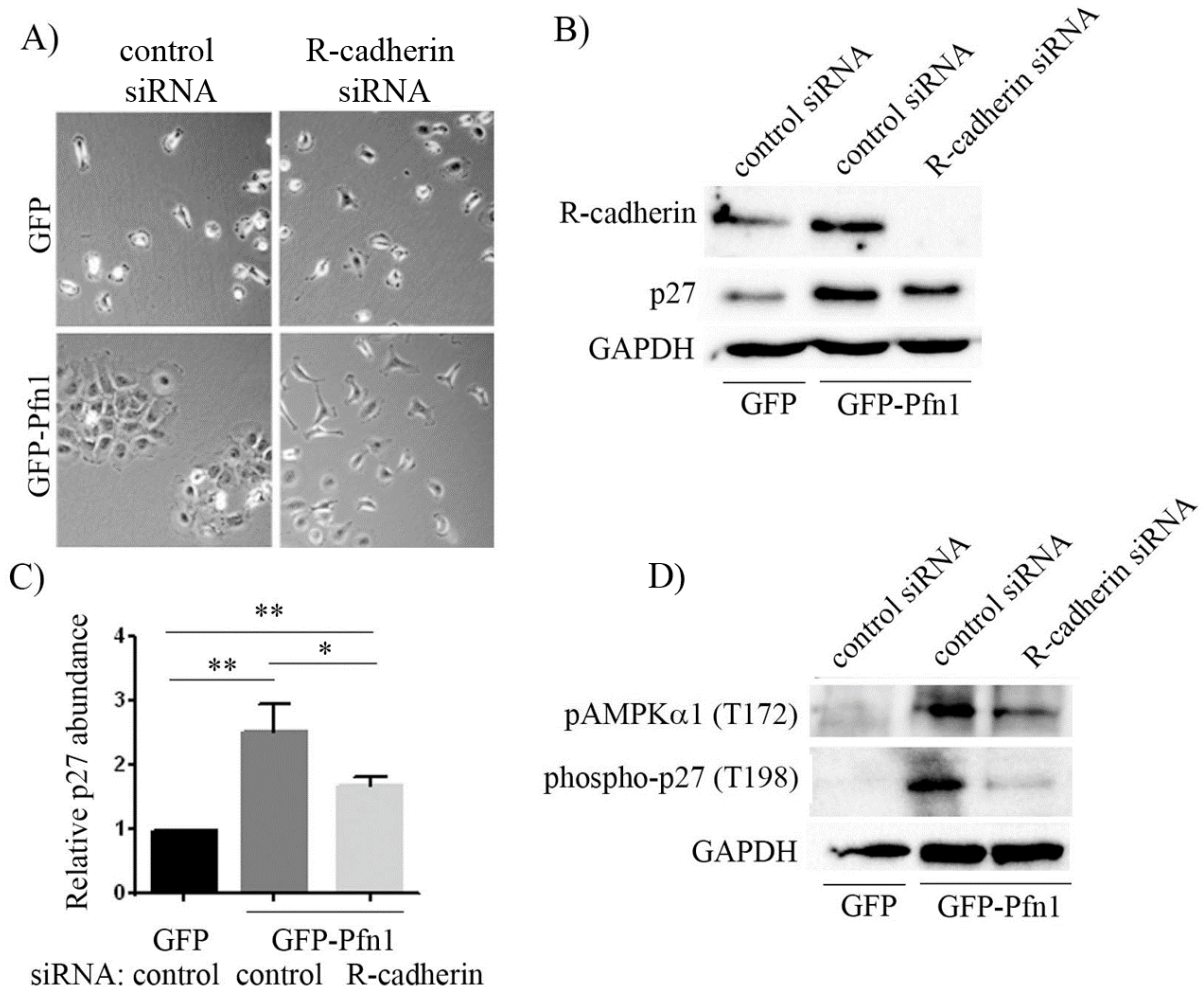


Figure 10. Epithelial reversion plays a role in Pfn1-induced p27 upregulation in MDA-231 cells. A) Phase contrast micrographs of GFP and GFP-Pfn1 expressers following transfection with either control or R-cadherin-specific siRNAs demonstrate that Pfn1 overexpression can induce epithelioid morphological reversion (as revealed by clustering of cells) in mesenchymal MDA-231 cells which can be reversed by silencing of R-cadherin. B-C) Representative immunoblots (panel B) of total extracts from GFP and GFP-Pfn1 expressers transfected with the indicated siRNAs showing changes in p27 expression specifically in Pfn1 overexpressers upon R-cadherin depletion (vimentin blot: loading control). The bar graph on the right (panel C) summarizes the data from 5 independent experiments (***: $p< 0.001$, **: $p< 0.01$; ns: not significant). D) Representative immunoblots of total lysates of GFP and GFP-Pfn1 expressers transfected with either control or R-cadherin siRNAs show downregulation of AMPKα-phosphorylation and p27-

phosphorylation on T172 and T198 residues, respectively, upon R-cadherin depletion (GAPDH blot: loading control).

Overall, these data demonstrate that epithelial morphological reversion plays a key role in AMPK-dependent p27 accumulation upon overexpression of Pfn1 in mesenchymal breast cancer cells (Figure 11).

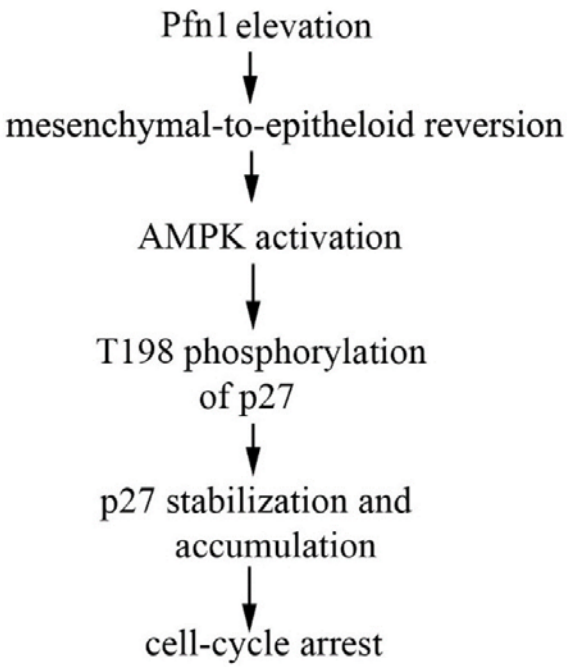


Figure 11. A proposed model depicting that Pfn1 overexpression elevates p27 accumulation in mesenchymal breast cancer cells through impacting AMPK pathway as a consequence of cadherin-dependent epithelioid reversion.

3.2 DISCUSSION

Cell-cell adhesion plays critical role in embryonic development, differentiation and maintenance of tissue architecture. Disruption of cell-cell adhesion which occurs during EMT through downregulation of junctional components promotes cell migration and proliferation. Although EMT was originally described as a morphogenetic process that occurs during normal embryonic development, some aspects of EMT are also recapitulated during metastatic progression of epithelial-derived tumors. Loss of E-cadherin (the central player in the makeup of AJ in epithelial cells) function, a hallmark of EMT, promotes invasiveness of carcinoma cells (153-157). Conversely, experimental restoration of E-cadherin suppresses tumorigenic ability and invasive phenotype of E-cadherin negative cancer cells (158), pointing to a critical role of cell-cell adhesion in the regulation of tumor initiation and malignant progression. E-cadherin engagement causes growth suppression of epithelial tumors at least partly through increasing the cellular level of p27 (159). In this study, it is demonstrated that a parallel mechanism involving R-cadherin, an important but under-studied member of cadherin-family proteins that mediates mesenchymal-to-epithelial morphological transformation (160-162) and plays a key role in Pfn1-induced elevation of p27 expression in mesenchymal breast cancer cells (129). R-cadherin expression is often associated with induction of EMT in epithelial cells most likely owing to its ability to displace E-cadherin from AJs (163) and accordingly several types of carcinoma cells have lower R-cadherin expression than their normal counterparts (164). However, in cancer cells that have already lost E-cadherin expression (such as MDA-231 cells), R-cadherin induces MET-like morphological transformation similar to the action of E-cadherin as shown previously and regulate p27 as demonstrated herein, suggesting some degrees of functional similarity between the two cadherin family of AJ proteins.

How cadherin engagement upregulates p27 is not clearly understood. It has been previously shown that PTEN (a negative regulator of skp2 and AKT) can be recruited to AJs in epithelial cells through its binding to MAGI (an AJ adaptor protein), and this causes stabilization and increase in the overall expression level of PTEN (165). As actions of skp2 and AKT promote downregulation of p27, PTEN-mediated skp2/AKT modulation could certainly serve as the mechanistic basis for cadherin-dependent p27 regulation in cells. While Pfn1 overexpression in MDA-231 cells also causes elevation of PTEN and suppression of AKT activation (Figure 6) (149), surprisingly, no evidence of AKT's involvement in Pfn1-dependent accumulation of p27 was found. Rather, it was demonstrated that Pfn1 overexpression causes p27 accumulation through cadherin-dependent upregulation of AMPK activation and p27 phosphorylation. Therefore, the present findings now reveal a new pathway of cadherin-mediated p27 regulation in cells. LKB1, a tumor suppressor that is linked to Peutz-Jeghers syndrome, phosphorylates and activates AMPK (166). While in some cases activation of LKB1-AMPK pathway causes p27 upregulation (143), there are also examples where hyperactivation of this pathway can cause cell cycle arrest at G1 phase without affecting the level of p27 (167), therefore suggesting context specificity of LKB1-AMPK pathway in p27 regulation. Recently, it has been shown that LKB1 can co-localizes with E-cadherin at AJs, and LKB1-mediated activation of AMPK requires maturation of AJ (168), a finding that is consistent with our observation of reduction of AMPK activation upon disruption of AJ in Pfn1 overexpressing cells. It would be interesting to determine in the future whether Pfn1 regulates AMPK activity through R-cadherin-dependent modulation of LKB1. As LKB1 is also required for maintaining epithelial integrity (169), cadherin-LKB1 could be a mutually-reinforcing molecular link for AMPK-mediated regulation of cell-cycle through p27 modulation.

It has been shown that actin-binding of Pfn1 is critical for its ability to induce R-cadherin mediated epithelial morphological transformation in MDA-231 cells (151). Although it remains to be seen whether Pfn1 causes tumor-suppression partly though p27 upregulation, a causal connection between epithelial reversion and p27 upregulation upon Pfn1 overexpression in the cell line of this study is still consistent with the requirement for actin-binding of Pfn1 for its tumor-suppressive effect in mesenchymal breast cancer cells, as demonstrated previously (128,152). Given that Pfn1 overexpression does not have any effect on p27 expression in epithelial breast cancer cell lines, it is speculated that p27 as a potential intermediary molecule for Pfn1's tumor-suppressive action, if true, it would be applicable to only certain subtypes (e.g. basal triple-negative) of breast cancer cells. It will be interesting to extend this findings to other triple-negative breast cancer cell lines of mesenchymal vs epithelial types in the future for a more definitive answer.

Finally, the present finding that Pfn1 overexpression stimulates AMPK activation has much broader implications in the context of cancer beyond and above p27 regulation. AMPK activation is known to promote autophagy which has opposing effects on cancer progression (170). On one hand, autophagy causes cell death and suppression of primary tumor. On the other hand, autophagy promotes cancer cell survival in the face of metabolic stress/hypoxia ultimately resulting in metastatic progression and therapeutic resistance. Therefore, it will be interesting to explore in the future whether autophagy plays any role in Pfn1's regulation of tumor growth and chemo-sensitivity of breast cancer cells.

4.0 THE ROLE OF PFN1 IN TUMORIGENESIS OF BREAST CANCER CELLS

It has been known for a long time that oncogenic transformation causes alteration in actin cytoskeleton. Through a series of studies in the recent past, it has now become increasingly clear that actin cytoskeleton plays a critical role in governing tumor-initiating capacity of cancer cells. Gene knockout studies have shown that homozygous deletion of Pfn1 arrests division of embryonic stem cells causing early embryonic lethality in mammals (112), suggesting Pfn1 is absolutely essential for embryonic stem cell division. Whether this defect is a result of a possible alteration in actin cytoskeleton is not known. Defect in cell proliferation upon loss-of-function of Pfn1 has also been reported in differentiated cells (125,171). However, phenotypic consequence of loss of Pfn1 function in differentiated cells is not as severe as observed in embryonic stem cells since it did not cause a complete cell cycle arrest but reduced the overall rate of cell proliferation. Pfn1 was shown to be important for the final separation of daughter cells during late cytokinesis (124). Collectively, these findings suggest that Pfn1 is required for cell proliferation at least in a physiological setting. In light of these observations, it is somewhat surprising that Pfn1 expression is downregulated in human breast cancer, and that genetic restoration of Pfn1 expression in breast cancer cell lines can dramatically suppress their tumorigenic ability *in vivo* (95,128). Whether reduced Pfn1 expression favors tumorigenic potential of breast cancer cells is not known. The present study investigated how perturbation of Pfn1 impacts tumorigenic potential of breast cancer cells.

4.1 RESULTS

4.1.1 Pfn1 is essential for growth of normal mammary epithelial cells

First, to determine whether Pfn1 is important for proliferation of normal MECs in a physiological environment, MECs were isolated from $Pfn1^{flox/flox}$ mice ($Pfn1^{flox/flox}$ –promoter and Exon 1 of Pfn1 gene is flanked by LoxP sites (Figure 12A)), and were then transduced with either control GFP-adenovirus (Ad-GFP) or Cre recombinase (Cre)-encoding adenovirus (Ad-Cre). The Cre expression would target the Lox sequences and subsequently lead to Pfn1 gene deletion. The transduced cells were then seeded as single cells on 3D matrigel to allow to grow as multicellular aggregates, ultimately leading to acinar structure formation. This is a well-established 3D culture assay that mimics mammary epithelial morphogenesis into alveolar structures *in vivo* (172,173). At the end of the experiments, control MEC outgrew into large multicellular spheroids and transformed into a hollow acinar structure as expected, while those transduced with Ad-Cre (identified by a reporter GFP expression from Ad-Cre vector) exhibited as severe outgrowth-deficient phenotype (Figure 12B). Note that untransduced (GFP-negative) cells in Ad-Cre group were able to outgrowth normally suggesting that the outgrowth-deficient phenotype is specifically due to Cre-mediated Pfn1 deletion. The average spheroid diameter of control MEC was 3.7 times greater than those transduced with Ad-Cre ($p< 0.001$) (Figure 12C). Overall, these data demonstrated that Pfn1 is essential for MEC outgrowth in 3D ECM environment.

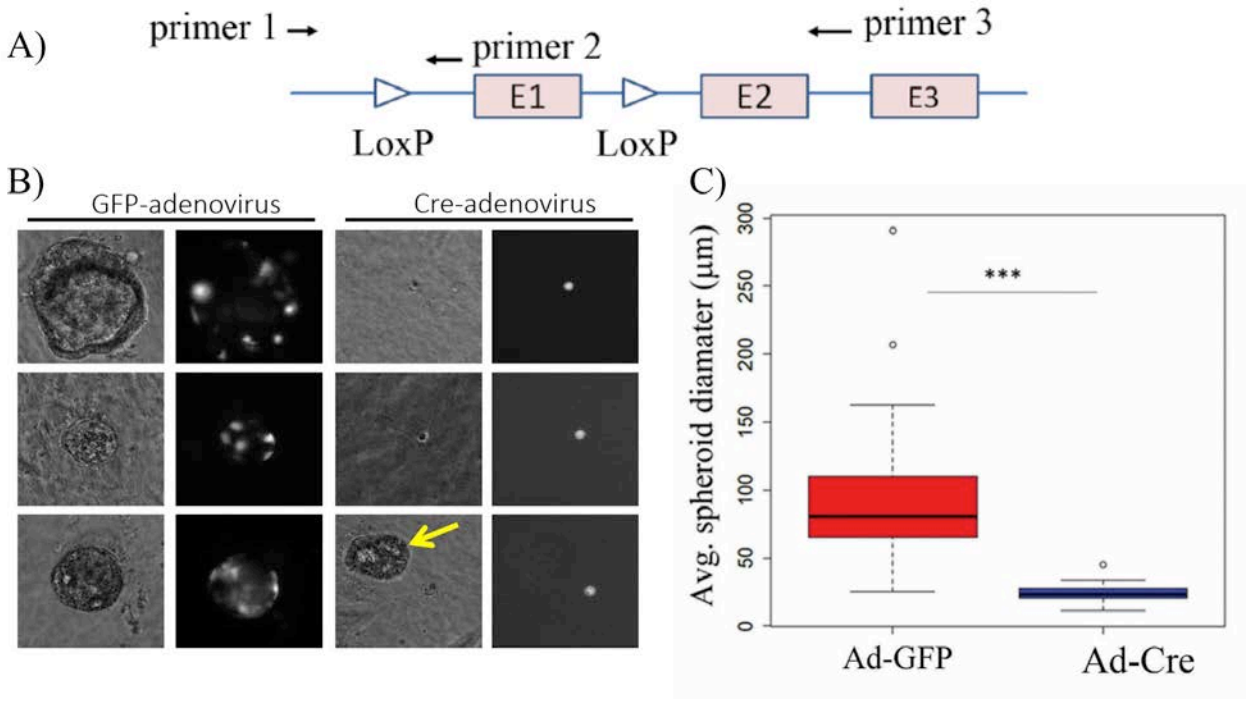


Figure 12. Cre-mediated acute deletion of floxed-Pfn1 alleles dramatically impairs the outgrowth ability of normal MECs on 3D matrigel. A) Pfn1 construct with LoxP sequence insertion. B) Phase-contrast and fluorescence images comparing outgrowth and acini forming ability of control (transduced with Ad-GFP) vs $Pfn1^{-/-}$ (transduced with Ad-Cre with GFP reporter) MEC derived from $Pfn1^{flox/flox}$ mice 12 days after culture on 3D matrigel (arrow indicates acini formation from GFP-negative untransduced MEC in Ad-Cre group). C) A bar graph summarizes the average spheroid diameter for the two groups (data are based on pooled analyses of 3 mice of each genotype; ***: $p < 0.001$).

4.1.2 Loss of P Pfn1 is essential for FLP formation and efficient outgrowth of breast cancer cells on 3D ECM culture

Based on the foregoing data, it was hypothesized that loss of Pfn1 expression would negatively impact tumor-initiating ability of breast cancer cells. To test this hypothesis, MDA-231 sublines stably expressing either Pfn1-shRNA or control luciferase (luc)-shRNA were used to perform the

following experiments (Figure 13A shows near complete knockdown of Pfn1 expression in Pfn1-shRNA transfected cells). Note that in monolayer 2D culture, Pfn1-depleted MDA-231 cells, if at all, proliferated at a slightly faster rate than either control shRNA transfectant or the parental cell line (Figure 13B). These data confirmed that loss of Pfn1 expression does not cause a growth arrest of MDA-231 cells in 2D monolayer culture.

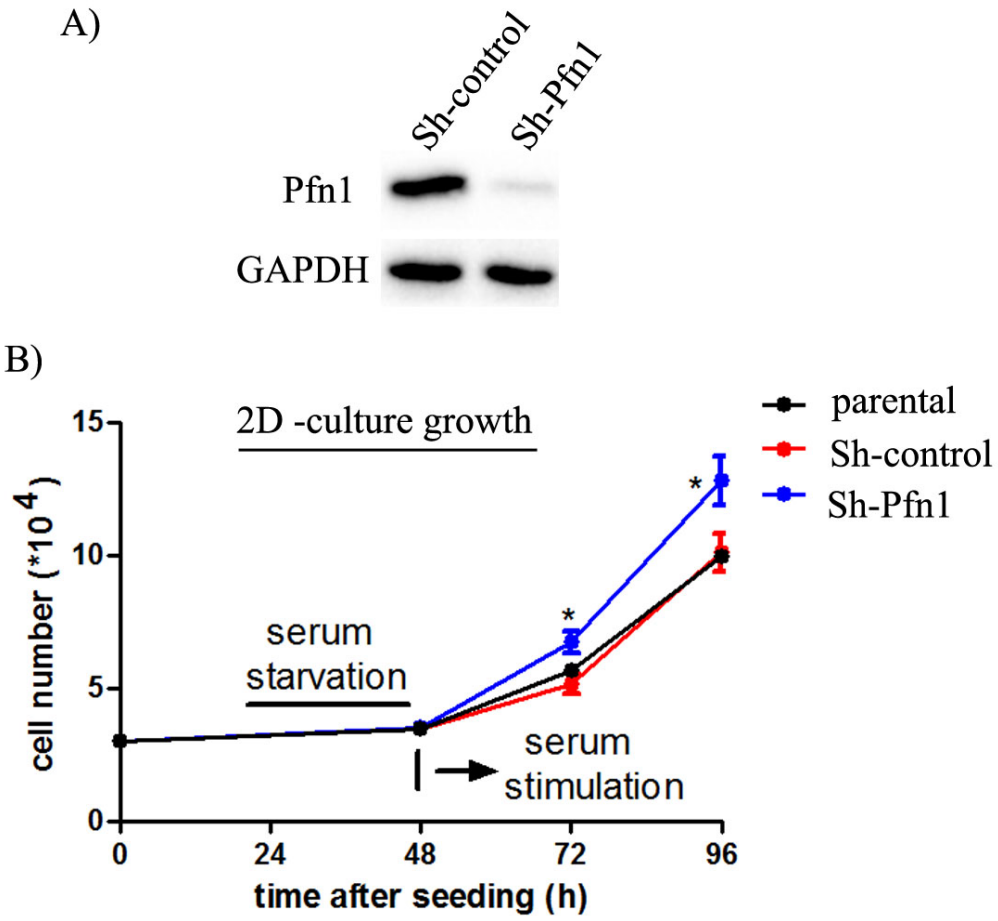


Figure 13. Effect of Pfn1 depletion on MDA-231 growth in 2D monolayer culture. A) Pfn1 immunoblot of total extracts from MDA-231 cells that are stably transfected with either control or Pfn1 shRNA. GAPDH blot served as the loading control. B) Proliferation kinetics of parental, control and Pfn1-KD MDA-231 cells on 2D tissue-culture dish (equal number of cells in each group were plated

overnight in triplicates, serum-starved for 24 hours and then allowed to proliferate in serum-containing media for another 48 hours). These data are summarized from 3 independent experiments (*: $p < 0.05$).

Several recent studies have shown that outgrowth ability of sparsely seeded breast cancer cells on 3D BME matrix successfully predicts their tumor-initiating capacity *in vivo*. Therefore, the relative ability of control vs Pfn1-depleted MDA-231 cells to outgrow from single cells on 3D BME matrix at different seeding densities (250, 500, and 1000 cells/well in a 384-well format) was assessed. In striking contrast to their modest proliferative advantage over control cells in 2D monolayer culture, Pfn1 KD cells showed a dramatic impairment in their ability to outgrow on 3D BME matrix (Figure 14A-B), a result that is consistent with their lower tumor-initiating potential *in vivo*. Outgrowth kinetics revealed that MDA-231 cells began to outgrow after a few days of delay, and this delay period became shorter with increasing initial density of cell seeding (Figure 14C). While Pfn1 KD cells outgrew at a much slower rate than control cells for all seeding densities, the differences in their growth rates were reduced as cell seeding density increased as judged by the relative slopes of the growth curves. These results demonstrate that Pfn1 depletion induces an outgrowth-deficient phenotype in breast cancer cells specifically in 3D-ECM environment that is cell-density dependent.

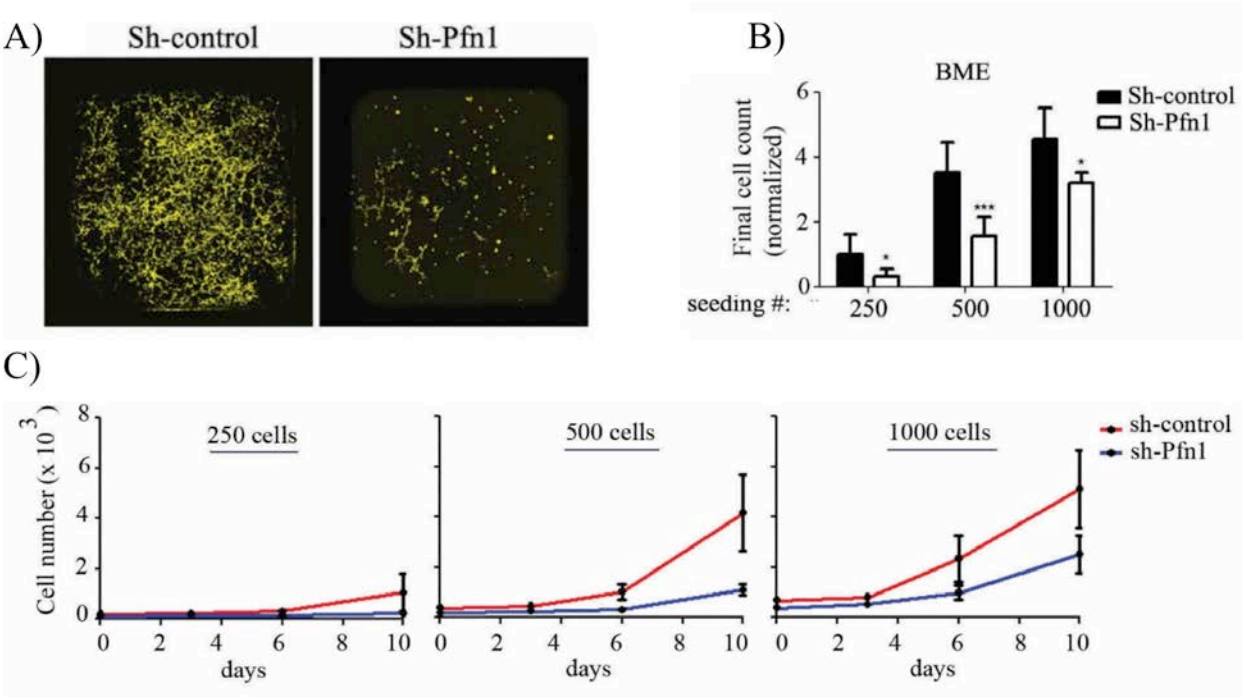


Figure 14. Pfn1 depletion inhibits outgrowth ability of breast cancer cells on 3D BME matrix. A) Representative images of outgrowth of control and Pfn1 KD MDA-231 cells on 3D BME matrix from an initial seeding of 500 cells/well in a 384-well plate. B) A bar graph summarizing the final number of cells (counted on day 10) in control vs Pfn1 KD groups for different seeding densities. All data are normalized to the final number of cells in control group for initial seeding density equal to 250 cells/well based on cell-count analyses of 3 technical replicates per condition from 2 independent experiments. C) Effect of Pfn1 depletion on the kinetics of MDA-231 outgrowth on 3D BME matrix. Outgrowth kinetics of control and Pfn1 KD MDA-231 cells on BME matrix at different cell-seeding densities (250, 500, 1000 cells/well). These data are based on real-time cell count analyses of 3 technical replicates per condition from 2 independent experiments. Values are presented as mean ± SD (*: $p < 0.05$, ***: $p < 0.001$).

Induction of FLPs is an important early event that eventually enables outgrowth-promoting proliferation switch of breast cancer cells on ECM. The next inquire was whether loss of Pfn1 expression has any effect on FLP forming ability of MDA-231 cells. Almost half of the

population of control cells were able to spread and initiate FLPs within 3 days of seeding on BME matrix, whereas a vast majority (> 80%) of Pfn1 KD cells maintained a round morphology and failed to initiate FLPs (Figure 15A-B). These results demonstrate that Pfn1 is an essential molecular player for FLP formation by breast cancer cells.

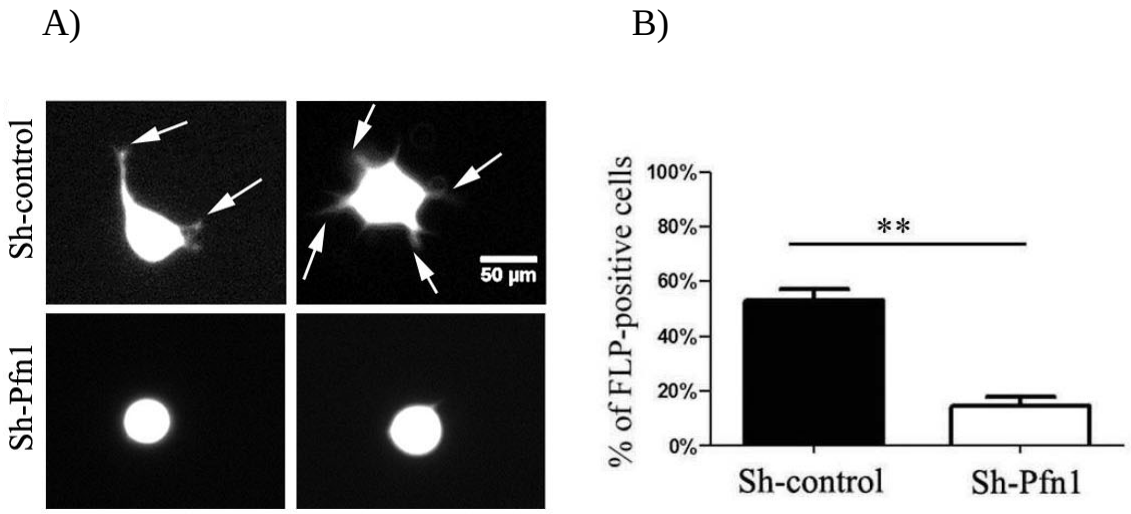


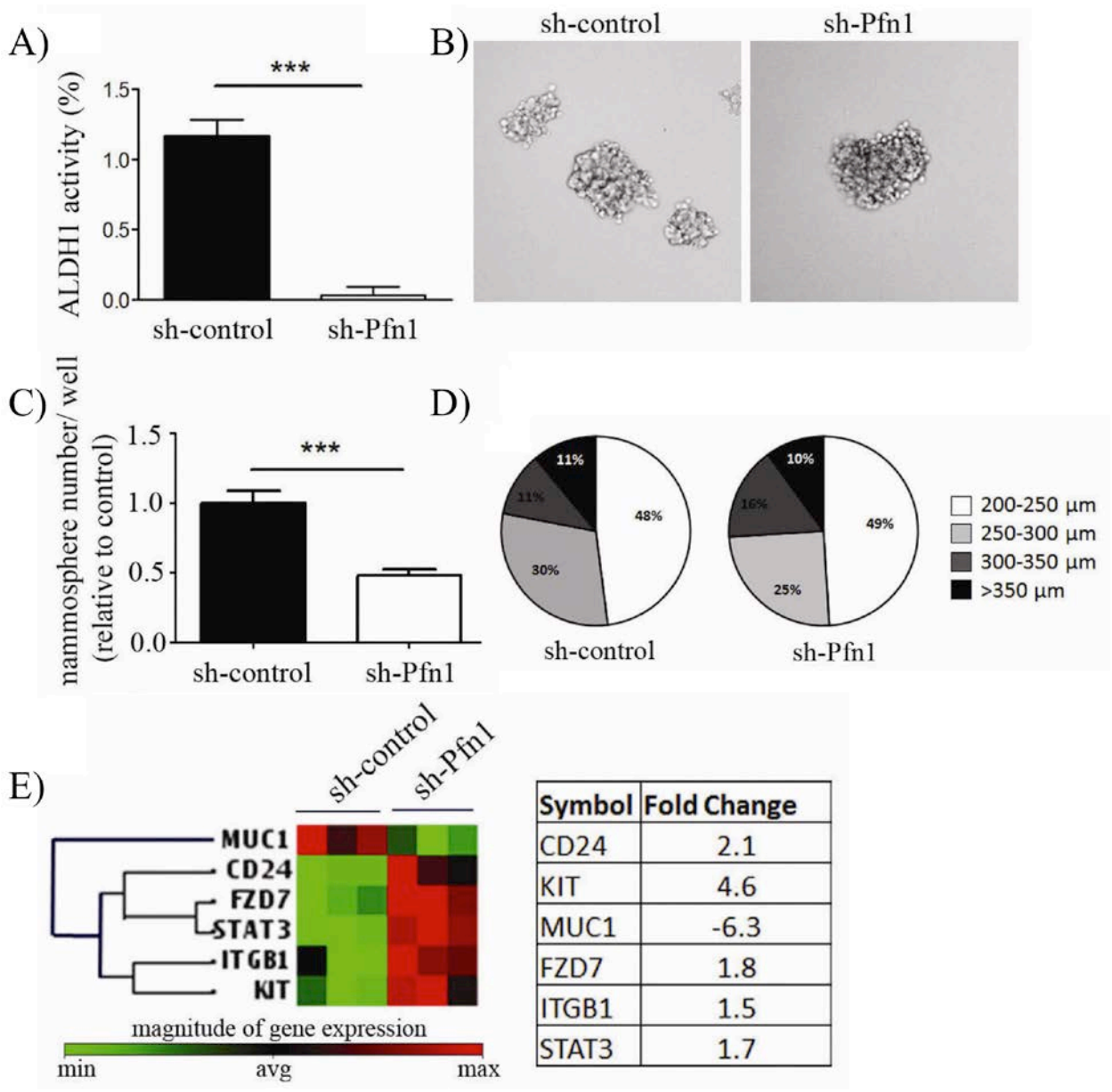
Figure 15. Pfn1 depletion inhibits FLP formation of breast cancer cells on 3D BME matrix. A) Fluorescence images of control and Pfn1 KD MDA-231 cells showing FLP (arrow) induction predominantly by control cells within 3 days of seeding on BME matrix (expression of reporter RFP from stable incorporation of shRNA enabled detection of FLPs by fluorescence imaging). D) A bar graph summarizing % FLP-positive MDA-231 cells in control vs Pfn1 KD group (these data are based on analyses of 99 control and 60 Pfn1 KD cells from 2 independent experiments) Values are presented as mean ± SD (**: $p < 0.01$).

4.1.3 Pfn1 deficiency affects stemness of breast cancer cells

Breast cancer cells that exhibit stem-like characteristics have high tumor-initiating potential. This sub-population of cells is also endowed with greater ability for FLP induction. Since loss of Pfn1

expression causes defect in FLP formation and reduced outgrowth, the next inquiry is whether stemness of breast cancer cells is also impacted by Pfn1 depletion. Breast cancer stem and progenitor cells express high levels of ALDH1 and have the ability to form mammospheres on non-adherent substrates. It was that depleting Pfn1 expression causes a significant reduction in ALDH1 activity (as measured by ALDEFLUOR assay) (Figure 16A) and mammosphere forming ability of MDA-231 cells (Figure 16B-D). In mammosphere assay, it was observed that the difference was only in the number of mammospheres between control and Pfn1 KD groups, but the overall size distribution of mammospheres was very similar between the two groups. Overall, these results demonstrate that loss of Pfn1 expression reduces stemness-enriched pool of breast cancer cells.

The expression of a panel of 84 cancer stem cell (CSC) related genes in control vs Pfn1 KD cells were profiled using a PCR array. Filtering the genes using a 1.5-fold cut-off revealed differential expression of 6 genes (MUC1 (decreased), CD24, Kit, FZD7, ITGB1 and STAT3 (increased)) associated with Pfn1 KD (Figure 16E). Among these three genes, MUC1 (a gene that is overexpressed in breast cancer stem cells (174)) showed the most prominent change (>6 fold downregulation) in expression as a result of Pfn1 KD (Figure 16E). Breast cancer stem cells are also characterized by high CD44 and low CD24 expression. Therefore, a 2-fold increase in CD24 expression also appears to be consistent with reduced stem-like phenotype of breast cancer cells upon Pfn1 depletion (175,176).



Symbol	Fold Change
CD24	2.1
KIT	4.6
MUC1	-6.3
FZD7	1.8
ITGB1	1.5
STAT3	1.7

Figure 16. Pfn1 depletion reduces stemness-enriched pool of breast cancer cells. A) Relative ALDH1 activity of control and Pfn1 KD MDA-231 cells as measured by Aldefluor® assay (data summarized from 3 independent experiments; *: $p < 0.05$). B) Representative images of mammosphere formation by control and Pfn1 KD cultures of MDA-231 cells. C-D) A bar graph (panel C) and pie charts (panel D) summarizing the number of mammospheres and the size distribution of mammospheres, respectively, in control vs Pfn1 KD cultures of MDA-231 cells (data summarized from 3 independent experiments; **: $p < 0.01$). E) A cluster diagram depicting differentially expressed (fold-change cut-off= 1.5; p-value< 0.05)

cancer stem cell-related genes between control and Pfn1 KD MDA-231 cells. The table alongside displays the fold-change of each differentially expressed gene (data summarized from 3 independent experiments).

4.1.4 Elevating Pfn1 expression also inhibits FLP, stemness and outgrowth of breast cancer cells

It has been reported that even modestly elevating Pfn1 expression in triple-negative breast cancer cells (MDA-231 and CAL-51) completely suppresses their ability to induce primary tumors in both orthotopic and subcutaneous xenograft models (95,128). In the present study, it was found that Pfn1 overexpression also markedly inhibited FLP formation (Figures 17A-B) and outgrowth (Figures 17C-D) of MDA-231 cells on BME matrix. Collectively, these results demonstrated that elevating Pfn1 levels can also inhibit tumor-initiating ability of breast cancer cells.

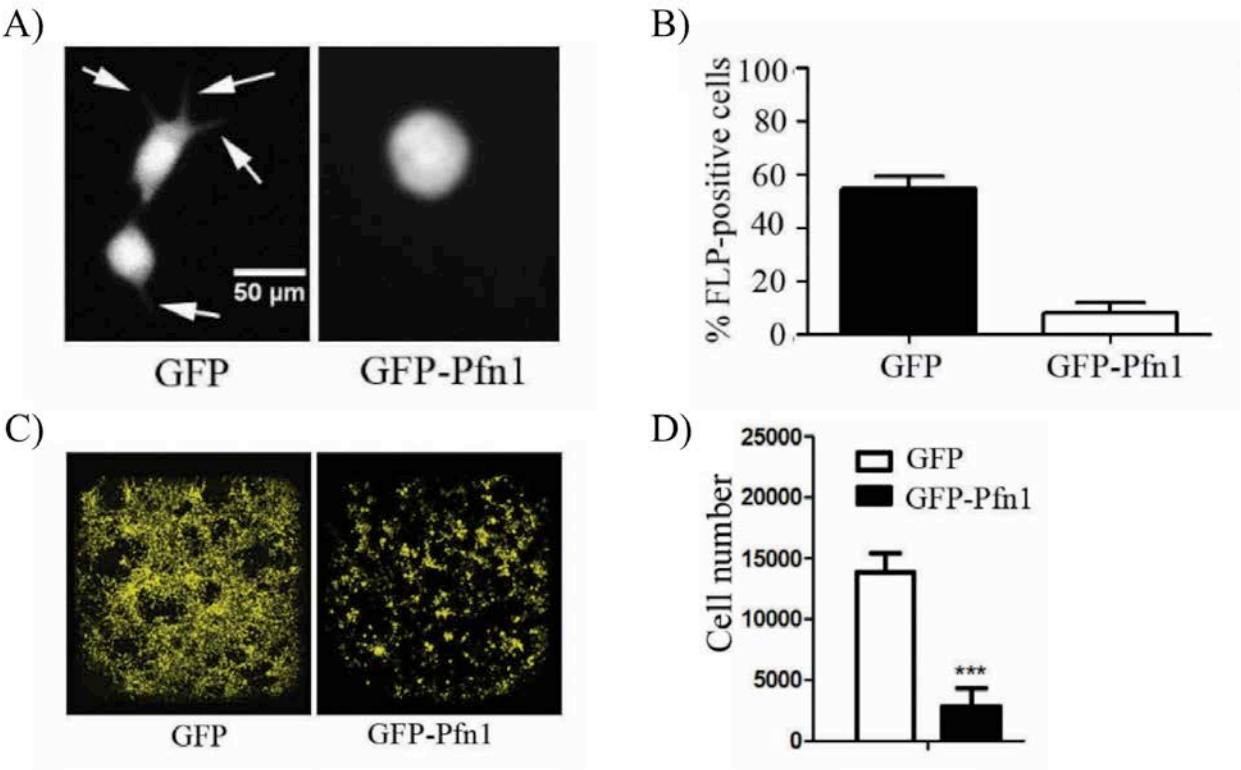
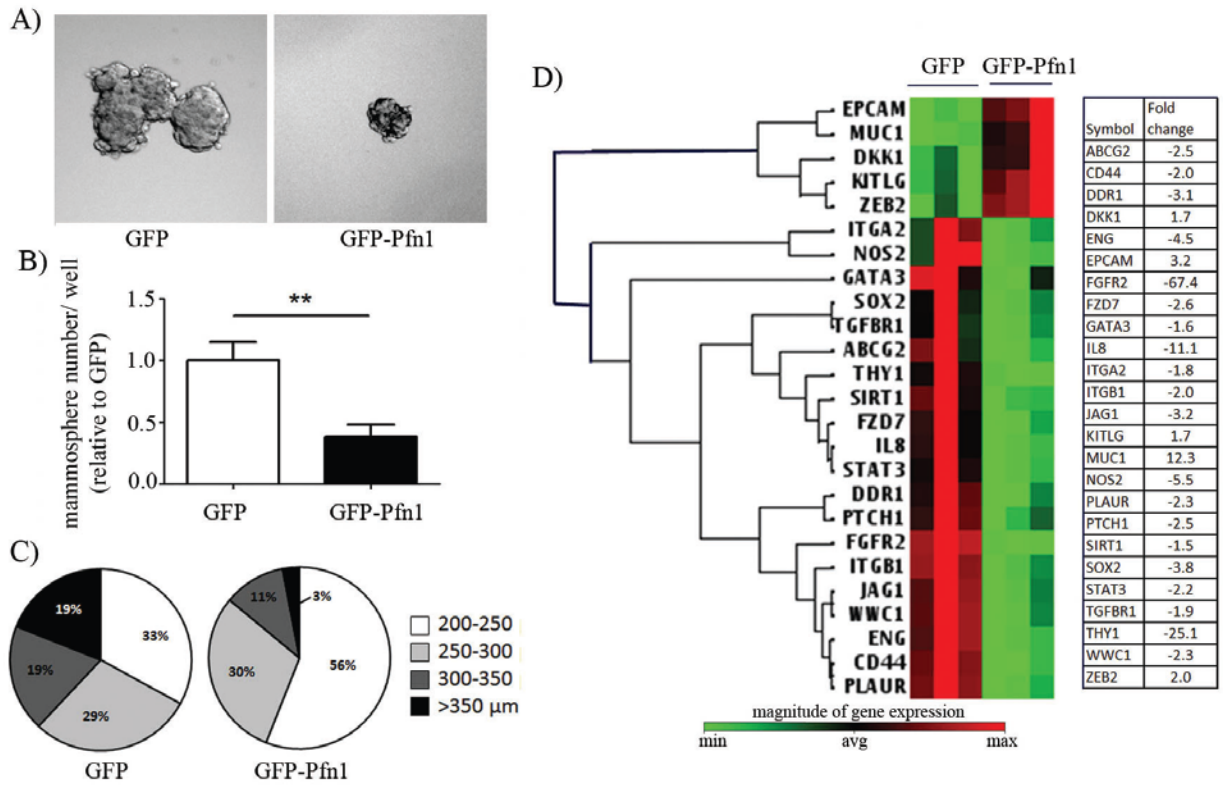


Figure 17. Elevating Pfn1 level inhibits FLP formation and outgrowth of MDA-231 cells on 3D BME matrix. A) Fluorescence images of GFP and GFP-Pfn1 expressing cells showing FLP (arrow) induction predominantly by GFP cells within 3 days of seeding on BME matrix. B) A bar graph summarizing % FLP-positive MDA-231 cells in GFP vs GFP-Pfn1 group (these data are based on analyses of 114 GFP and 49 GFP-Pfn1 expressing cells from 2 independent experiments). C) Representative images of outgrowth of GFP and GFP-Pfn1 expressing cells on 3D BME matrix (these images were acquired on day 10 after an initial seeding of 2000 cells/well in a 384-well plate). D) A bar graph summarizing the final number of cells (counted on day 10) in GFP vs GFP-Pfn1 groups (these data are based on cell-count analyses of 3 technical replicates per condition from 4 independent experiments).

Mammosphere assays were next performed in which Pfn1 overexpressing MDA-231 cells formed significantly fewer mammospheres than control cells, suggesting that Pfn1 elevation also attenuates the stemness-enriched pool of breast cancer cells (Figures 18A-C). In addition to

affecting the number, Pfn1 overexpression also limited the growth of mammospheres as evident from significantly higher fraction of mammospheres in smaller sized groups. This may also mean that self-renewal capacity of breast cancer stem/progenitor cells is also suppressed by Pfn1 overexpression. Compared to Pfn1 depletion, overexpression of Pfn1 caused a much more dramatic alteration in the expression profile of stem-cell associated genes. Twenty-five out of a total of 84 profiled genes were differentially expressed and 80% of those differentially expressed genes were found to be downregulated upon Pfn1 overexpression (Figures 18D). These included genes that a) are required for maintenance and self-renewal of stem cells (e.g. SOX2- reduced by 3.8 fold; FGFR2- reduced by 67-fold; CD44 – reduced by 2-fold; ITGB1 – reduced by 2-fold) (177-180), b) expand the pool of breast CSC and increase their self-renewal (e.g. IL8 - reduced by 11-fold; STAT3 – reduced by 2-fold) (181,182) and c) enhance CSC activities of breast cancer cells indirectly through facilitating their interactions with tumor-associated macrophages (e.g. Thy1 - reduced by 11-fold) (183). Note that 4 genes (MUC1, STAT3, FZD7, ITGB1) also showed a reverse trend in the expression upon depletion of Pfn1 raising a possibility that Pfn1 may be directly involved in regulating the pathways controlling the expression of these genes.

Overall, similarities in the phenotypes (i.e. defects in FLP formation, outgrowth deficiency and reduced stremness) caused by depletion and elevation of Pfn1 expression suggest that a balanced Pfn1 expression is most conducive for FLP formation, stemness, outgrowth and tumorigenic ability of breast cancer cells.



Symbol	Fold change
ABCG2	-2.5
CD44	-2.0
DDR1	-3.1
DKK1	1.7
ENG	-4.5
EPCAM	3.2
FGFR2	-67.4
FZD7	-2.6
GATA3	-1.6
IL8	-11.1
ITGA2	-1.8
ITGB1	-2.0
JAG1	-3.2
KITLG	1.7
MUC1	12.3
NOS2	-5.5
PLAUR	-2.3
PTCH1	-2.5
SIRT1	-1.5
SOX2	-3.8
STAT3	-2.2
TGFBR1	-1.9
THY1	-25.1
WWC1	-2.3
ZEB2	2.0

Figure 18. Pfn1 overexpression reduces stemness-enriched pool of breast cancer cells. A) Representative images of mammosphere formation by GFP and GFP-Pfn1 overexpressing cultures of MDA-231 cells. B-C) A bar graph (panel B) and pie charts (panel C) summarizing the number of mammospheres and the size distribution of mammospheres, respectively, in GFP vs GFP-Pfn1 expressing cultures of MDA-231 cells (data summarized from 3 independent experiments; **: $p< 0.01$). D) A cluster diagram depicting differentially expressed (fold-change cut-off = 1.5; p-value< 0.05) cancer stem cell related genes between GFP and GFP-Pfn1 expressing MDA-231 cells. The table alongside displays the fold-change of each differentially expressed gene. Data was summarized from 3 independent experiments).

4.1.5 Collagen can rescue outgrowth-deficient phenotype of breast cancer cells upon loss but not elevation of Pfn1 expression

Recently it has been shown that fibrotic environment can induce an outgrowth phenotype in otherwise dormant (non-proliferative) breast cancer cells (19). This can be mimicked in 3D outgrowth assays by addition of collagen-I to the BME matrix. It was found that presence of collagen-I stimulated outgrowth of both control and Pfn1 KD cells, and importantly rescued the outgrowth-deficient phenotype of Pfn1 KD cells (Figure 19A). Correlated with these findings, collagen-I also induced FLP formation in almost 100% of cells in either group and rescued Pfn1 KD cells from their defects in spreading and FLP formation (Figure 19B). Note that whether or not collagen was present, Pfn1 KD cells produced shorter FLPs than control cells. These results demonstrate that ECM composition is an important deterministic factor for how outgrowth ability of breast cancer cells is impacted by loss of Pfn1 expression. In a complementary set of experiments, addition of collagen-I also significantly accelerated the growth of control GFP cells as expected but only had a modest effect on the growth characteristics of Pfn1-overexpressing cells. Importantly, collagen-I failed to rescue Pfn1-overexpressors from their defect in FLP induction and ECM-induced outgrowth (Figures 19C-D), and this is in stark contrast with our observations in the KD setting. These data document that breast cancer cell outgrowth is more robustly suppressed upon elevation than depletion of Pfn1, and further suggest that there could be differences in the underlying mechanisms by which Pfn1 depletion and overexpression inhibit outgrowth and tumor initiating ability of breast cancer cells.

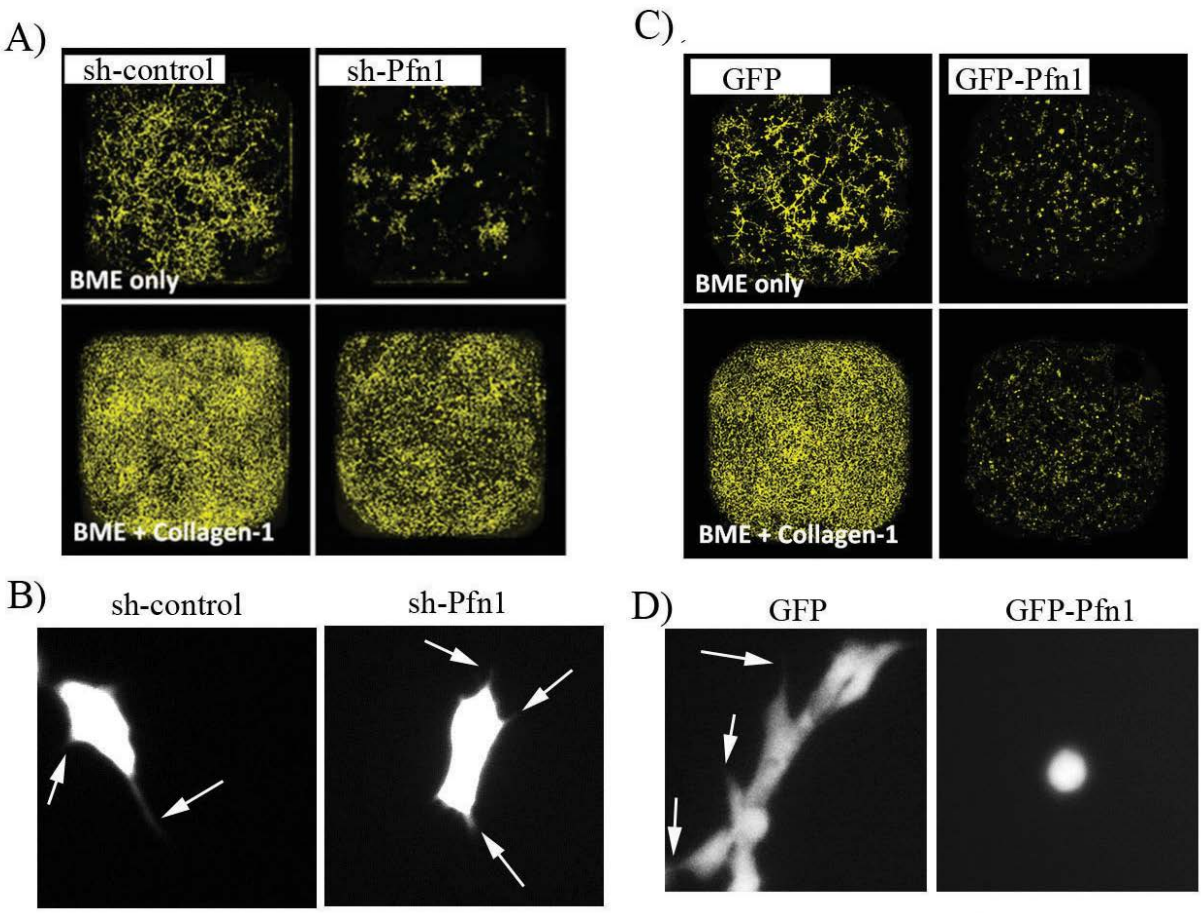


Figure 19. Collagen-I rescues outgrowth-deficiency of breast cancer cells upon depletion but not elevation of Pfn1. A, C) Representative images of outgrowth of the indicated groups of MDA-231 cells on BME matrix with or without addition of collagen-I (images were taken on day 8 after seeding 500 (panel A) and 1000 (panel C) cells/well). B, D) Representative images of FLP-forming abilities of the indicated groups of MDA-231 cells 3 days after seeding on collagen-I supplemented BME matrix (FLPs are indicated by arrows).

4.2 DISCUSSION

In this study, several novel findings have been reported. First, Pfn1 is essential for outgrowth of normal MEC. Second, Pfn1 is a critical determinant of two biological features that are associated with greater tumor-initiating potential (FLP formation and cancer stemness) of cancer cells. Third, reduced outgrowth potential of breast cancer cells upon both gain and loss of function of Pfn1 suggested that a balanced expression of Pfn1 is most conducive for tumorigenic potential of breast cancer cells.

Although Pfn1 expression is downregulated in human breast cancer and that elevating Pfn1 expression impairs tumorigenic ability of breast cancer cells, at least of triple-negative subtype (95,128,130). Based on the present findings that loss of Pfn1 does not provide normal MEC and cancer cells with a selective advantage for growth and/or survival, it is now clear Pfn1 is not a classic tumor-suppressor gene in the context of breast cancer.

Pfn1 is a common denominator of multiple cellular pathways of actin assembly including those involving mDia and Ena/VASP proteins (119). Therefore, a reduction in FLPs, outgrowth and tumor-initiating potential of breast cancer cells upon Pfn1 depletion is consistent with previously established roles of mDia2 and Ena/VASP in these aspects. It may also indirectly suggest that mDia2 and Ena/VASP utilize the Pfn1:actin complex for elongating actin filaments to generate FLPs in breast cancer cells. A somewhat unexpected observation was that FLP formation is also suppressed upon elevation of Pfn1 expression. It is speculate that differential effects of Pfn1 on actin nucleation vs F-actin elongation may explain this phenomenon. On one hand, Pfn1 promotes barbed-end directed F-actin elongation, but on the other hand, it acts as a suppressor of Arp2/3-dependent actin nucleation process (119). Although Arp2/3-dependent actin nucleation promotes branched F-actin structures leading to lamellipodial protrusion,

parallel arrays of actin filaments as found in classical filopodia are, in fact, initiated by convergence of branched F-actin structures requiring Arp2/3 activity (184). Therefore, it is possible that FLP initiation is also dependent on Arp2/3-mediated actin nucleation, and this step is inhibited when Pfn1 expression is elevated beyond a certain level. Other possibilities include sequestration of proteins involved in FLP formation by an excess Pfn1, resulting in altered FLP dynamics.

FAK-Src-ERK signaling triggered by integrin-based focal adhesions (FA) is a key driver of outgrowth of breast cancer cells *in vitro* and *in vivo*. FAK inhibition impairs anchorage-independent outgrowth of breast cancer cells but does not affect their growth in 2D adherent culture (185). These phenotypes are similar to the characteristics of Pfn1-depleted breast cancer cells as they also exhibit outgrowth deficiency only on BME matrix and low-attachment plates (as in the mammosphere assay) but not in 2D adherent culture. It has been shown that at least in 2D monolayer culture, Pfn1 depletion causes a marked reduction in FAs in endothelial cells and normal MEC, and conversely, elevating Pfn1 expression promotes FA and FAK activation in breast cancer cells (125,128,132). Therefore, it is speculated that loss of Pfn1 in breast cancer cells negatively impacts FAK signaling through downregulating either initiation and/or maturation of FAs. It has been shown that FA plaques that eventually enable FAK-Src-ERK-mediated outgrowth of breast cancer originate and mature along the FLPs (102). However, any type of membrane protrusion also requires adhesions to the surrounding ECM for its stable anchoring which otherwise undergoes retraction. Therefore, FLPs and FAs may be mutually sustained through a feed-forward loop. It is found that Pfn1 depletion led to increased expression of ITGB1 (integrin-β1), a receptor for collagen-I. This could explain why collagen-I was able to rescue Pfn1-depleted cells from their defects in FLP induction and outgrowth. Conversely,

reduction in ITGB1 expression upon elevation of Pfn1 expression may also be a part of the reason why outgrowth-deficiency of Pfn1 overexpressers could not be rescued by collagen-I.

The observation that either depletion or elevation of Pfn1 expression suppresses the stem-like phenotype of breast cancer cells is consistent with the requirement for balanced Pfn1 expression for tumor-initiating ability of breast cancer cells. It is found that compared to Pfn1 depletion, elevating Pfn1 expression causes a much more robust suppression of genes associated with maintenance and self-renewal of CSCs (FGFR2, SOX2, STAT3, and IL8). Since CSCs are considered to be the most potent tumor-initiating cells, downregulation of CSC-associated genes could certainly be a plausible mechanism by which Pfn1 overexpression causes tumor suppression in breast cancer cells. This will need to be studied in the future.

Finally, this study provides some interesting insights on the choice of Pfn1-centric strategy one could potentially consider in combating metastatic breast cancer of at least triple-negative subtype. Mortality of breast cancer patients primarily results from overt metastatic outgrowth of disseminated cancer cells in the distant organs. Therefore, molecular strategies that could induce a dormant phenotype of breast cancer cells at the secondary sites may lead to a conceptual framework of new lines of targeted therapy against metastatic breast cancer. It has been shown that loss of Pfn1 expression has contrasting effects on early vs late steps of breast cancer metastasis. Specifically, Pfn1 depletion enhances dissemination-promoting activities (invasion and transendothelial migration) of breast cancer cells, facilitating their escape from the primary tumor. But metastatic outgrowth of extravasated breast cancer cells at the secondary site requires Pfn1, a finding that is consistent with outgrowth-deficient phenotype and lower tumor-initiating capacity of breast cancer cells upon Pfn1 depletion (130). Since outgrowth-deficiency of breast cancer cells upon Pfn1 depletion is cell-density dependent and rescuable by collagen-I,

it is possible that Pfn1 depletion may become an ineffective strategy to halt metastatic outgrowth at an advanced stage of the disease when too many micrometastases have already developed or in a fibrotic condition. By contrast, Pfn1 overexpression blocks outgrowth of breast cancer cells regardless of presence or absence of collagen-I, suggesting that it may be a more robust strategy to inhibit metastatic outgrowth of breast cancer cells than depletion of Pfn1. Furthermore, since CSCs offer resistance to therapy and are thought to cause relapse of breast cancer, a robust suppression of CSC-associated genes in breast cancer cells upon Pfn1 overexpression could also make elevation of Pfn1 as an attractive strategy to sensitize breast cancer cells to chemotherapy *in vivo*. In fact, in cell culture model, there is already evidence for increased susceptibility of breast cancer cells to cytotoxic agents upon overexpression of Pfn1 (129,186). In summary, elevating Pfn1 expression could become an effective strategy against metastatic breast cancer.

5.0 CONCLUSIONS

This work determined that Pfn1 is not a classic tumor suppressor although it has a tumor-suppressive effect in breast cancer cells.

The current study provided one molecular mechanism underlying Pfn1's tumor suppressive effect in breast cancer. It is demonstrated that Pfn1 overexpression leads to accumulation of p27 through promoting AMPK activation and AMPK-dependent phosphorylation of p27 on T198 residue, a post-translational modification that leads to increased protein stabilization of p27. This pathway is mediated by Pfn1-induced epithelial morphological reversion of mesenchymal breast cancer through cadherin-mediated restoration of AJs. These findings not only elucidate a potential mechanism of how Pfn1 may inhibit proliferation of mesenchymal breast cancer cells, but also highlight a novel pathway of cadherin-mediated p27 induction and therefore cell-cycle control in cancer cells.

It was determined in this work that, unlike classical tumor suppressors, loss of Pfn1 does not promote tumorigenesis or tumor growth; instead Pfn1 is critically required for mammary tumor initiation and growth. Acute gene deletion of Pfn1 dramatically impairs the outgrowth of mouse MECs on 3D matrigel. Likewise, stable silencing of Pfn1 expression impairs outgrowth of human breast cancer cells on 3D matrigel *in vitro* but not proliferation on 2D tissue-culture substrate. Correlated with these phenotypic changes, Pfn1 KD suppresses stemness of breast cancer cells. Interestingly, the outgrowth-deficient phenotype of Pfn1-depleted breast cancer

cells on 3D matrigel can be fully rescued by addition of collagen-I which suggests that alteration in cell-matrix adhesion and downstream signaling underlies the growth-related phenotypic changes induced by loss of Pfn1 expression. Finally, consistent with tumor suppressive action of Pfn1, the outgrowth deficient phenotype of breast cancer cells can also be elicited by Pfn1 overexpression, further suggesting that an optimum range of Pfn1 expression is most conducive for mammary tumor growth. Based on these results, it is concluded that Pfn1 expression level is an important determinant for tumorigenic potential of breast cancer cells.

5.1 FUTURE DIRECTIONS

5.1.1 To identify other molecular pathways underlying Pfn1's tumor suppressive action in breast cancer cells

As discussed earlier in this work, Pfn1 induced upregulation of p27 in TNBC cells can only partly explain Pfn1's tumor suppressive effect. Therefore, more work needs to be done regarding global changes brought by Pfn1 overexpression in gene expression profile and pathways relevant for cell proliferation and survival in breast cancer cells. And this work needs to be extended to other kinds of breast cancer cell lines, not limited to TNBC cells.

5.1.2 To characterize the effects of Pfn1 overexpression on breast cancer metastasis *in vivo*

The effects of Pfn1 KD on breast cancer metastasis has been characterized before, including promoted dissemination and poor colonization ability (130). Similar work needs to be done to characterize the effects of Pfn1 overexpression. Although the present studies indicate Pfn1 overexpression leads to reduced cell motility and suppressed tumor growth, these effects needs to be put in the context of metastasis process to be confirmed *in vivo*.

5.1.3 To further explore the molecular pathways underlying Pfn1's regulation of tumorigenic potential of breast cancer cells

In the present work, it has been demonstrated that perturbation of Pfn1 expression has influence on cancer stemness and FLP formation. However, detailed pathway analysis needs to be done to explore the exact mechanism, such as how Pfn1 regulates FLP formation, whether FAK signaling is perturbed by Pfn1 expression levels, and how Pfn1 exerts impact on cancer stemness.

5.1.4 To study the role of other Pfn isoforms, such as Pfn2, in tumorigenesis and metastasis in the context of breast cancer

Besides Pfn1, Pfn2 is the most abundant Pfn in mammalian cells. The two Pfn isoforms are 65% identical at the amino acid level, and share similar biochemical properties except that Pfn1 has higher affinity with actin than that of Pfn2 (114). Despite that, Pfn1 and Pfn2 have been reported to demonstrate different effects on actin cytoskeleton organization, cell migration and invasion at

least in MCF10A (a non-tumorigenic mammary epithelial cell line) and SUM159 (a basal breast cancer cell line) cells (113). More recently, it has been shown that Pfn2 overexpression promotes, whereas Pfn2 knockdown reduces lung cancer growth and metastasis (187); and increased Pfn2 level enhances, whereas decreased Pfn2 level suppresses the migration, invasion, and stemness of HT29 human colorectal cancer stem cells (188). Therefore it would be interesting to determine if Pfn2 plays any role in tumorigenesis and metastasis in the context of breast cancer.

5.1.5 To investigate whether Pfn elevation can prevent further growth of established colonies *in vitro* and *in vivo*

Considering the tumor suppressive effect of Pfn1 presented in this study, and the role that Pfn2 plays in lung cancer growth and metastasis (187), it will be also interesting to study beyond the tumor initiation effects of Pfn, and to investigate whether Pfn elevation can prevent further growth of established colonies *in vitro* and *in vivo*. This would pave the way for development of new therapeutic strategies by modulating Pfn levels to prevent tumor growth and to terminate cancer relapse.

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