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Missing-in-Metastasis regulates cell motility and invasion via PTPδ mediated changes in SRC activity

A Dissertation Presented

by

Fauzia Chaudhary

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Molecular and Cellular Biology

Stony Brook University

May 2014

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Abstract of the Dissertation

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by

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Doctor of Philosophy

in

Molecular and Cellular Biology

Stony Brook University

2014

Missing in Metastasis (MIM), also known as MTSS1, is a scaffold protein that is down-regulated in multiple metastatic cancer cell lines compared to non-metastatic counterparts. MIM regulates cytoskeletal dynamics and actin polymerization, and has been implicated in the control of cell motility and invasion. MIM has also been shown to bind to a receptor PTP, PTPδ, an interaction that may provide a link between tyrosine phosphorylation-dependent signaling and metastasis. We used shRNA-mediated gene silencing to investigate the consequences of loss of MIM on the migration and invasion of the MCF10A mammary epithelial cell model of breast cancer. We observed that suppression of MIM by RNAi enhanced migration and invasion of MCF10A cells, effects that were mediated by enhancing the stability and quantity of PTPδ. Furthermore, analysis of human clinical data indicated that PTPδ was elevated in breast cancer samples when compared to normal tissue. We demonstrated that the SRC protein tyrosine kinase is a direct substrate of PTPδ and, upon suppression of MIM, we observed changes in the phosphorylation status of SRC, in particular the inhibitory site (Tyr 527) was hypophosphorylated, whereas the activating autophosphorylation site (Tyr 416) was hyperphosphorylated. Thus, the absence of MIM led to PTPδ mediated activation of SRC. Finally, the SRC inhibitor SU6656 counteracted the effects of MIM suppression on cell motility and invasion. This study illustrates that both SRC and PTPδ may be considered therapeutic targets for metastatic tumors associated with loss of MIM.

to my parents

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List of Abbreviations

MIM- missing-in-metastasis

RPTP- receptor protein tyrosine phosphatase

EMT- epithelial to mesenchymal transition

Acknowledgments

I want to thank my mentor, Dr. Nicholas K Tonks for his support and motivation. He is an extremely thorough and detail oriented scientist. He has spent incredible amount of time training me. He has not only made me enjoy the process of writing and reading science but also made me an independent researcher.

I want to thank my thesis committee members, Dr. Robert Lucito, Dr. Linda VanAelst, Dr. Nick Carpino and Dr. Todd Miller for providing invaluable guidance and support and insights during the course of my PhD.

I want to thank my labmates especially Gaofeng Fan and Benoit Boivin for their constant motivation and ideas in moving my project in the right direction. I want to thank Mike Feigin from Muthuswamy Lab for proofreading my manuscripts and providing invaluable suggestions. I want to acknowledge the help provided by Lorraine Baldwin who is a part of the administrative staff.

My classmates from the MCB program have been instrumental in making this arduous journey very enjoyable. I would like to thank Nihan Kara, Wangzhi Li and An-yun Chang for being great friends and companions during my PhD.

I would like to thank my wonderful family. My parents, my brothers, my sister-in-laws and my lovely niece and nephew have been a constant source of support and happiness during my PhD days and otherwise. I also want to acknowledge my husband Dr. Satyajeet Sharma. Without his support and encouragement I would not have completed my PhD.

Vita, Publications and/or Fields of Study

1. Missing-in-Metastasis regulates cell motility and invasion via PTPδ mediated changes in SRC activity (manuscript submitted)

   Chaudhary F, Lucito R, Tonks NK

2. Receptor Protein Tyrosine Phosphatase alpha Regulates Focal Adhesion Kinase Phosphorylation and Contributes to ErbB2-Mediated Mammary Epithelial Cell Motility

   Boivin B, **Chaudhary F**, Dickinson BC, Haque A, Pero SC, Chang CJ, Tonks NK.

CHAPTER 1

1.1.1 Protein tyrosine phosphorylation

Protein phosphorylation usually occurs on amino acids that contain a hydroxyl group, such as serine, threonine and tyrosine residues. Phosphorylation of tyrosine plays an important role in the control of protein functions. Protein tyrosine phosphorylation is controlled by two families of enzymes, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). pp60v-SRC, a Rous sarcoma virus (RSV), was the first identified PTK [1]. Subsequently, 90 Tyrosine kinases have been identified in the human genome [2]. During the same time, PTP activity was also discovered [3-5]. PTP1B was the first PTP that was purified [6,7]. The primary sequence of PTP1B was determined, and found to be homologous to the intracellular segment of the transmembrane receptor-like protein CD45, indicating that CD45 itself was a receptor-like protein tyrosine phosphatase [8-11]. This evidence suggests that, like the PTKs, PTPs exist as either non-receptor enzymes or transmembrane receptor-like enzymes that have the potential to regulate signal transduction directly through dephosphorylation of tyrosine residues of proteins. Data from various sources support a role for PTKs in transformation and PTPs are hypothesized to function as tumor suppressor [12,13]. But it is important to look at the PTKs and PTPs together in the right context and not in isolation.

1.1.2 Protein tyrosine kinases

There are two classes of tyrosine kinase, receptor tyrosine kinase and non-receptor tyrosine kinase. Receptor tyrosine kinases are type I transmembrane proteins possessing an N-terminal extracellular domain that binds to ligands, a transmembrane domain and a C-terminal cytoplasmic segment that includes the catalytic domain. Non-receptor tyrosine kinases lack a transmembrane domain, most are soluble intracellular proteins, but a subset associate with membranes via a membrane targeting modification, such as myristoylation. Tyrosine phosphorylation controls a wide range of protein function including enzyme activity, localization and protein-protein interactions. Tyrosine kinases function in several signaling cascades, where extracellular signals are transmitted through cell membrane to the cytoplasm and to the nucleus, modifying gene expression. Mutations in tyrosine kinases can make them constitutively active which might lead to cancer initiation and progression.

Two major PTK families have been linked directly with the development and progression of breast cancer: the human epidermal growth factor receptors (HERs) and the non-receptor SRC-family kinases (SFKs). HERs include 4 receptor-like proteins (HER1/EGFR, HER2/Neu, HER3, and HER4) that form different homo- or hetero-dimers. HER2 does not have a known ligand, but dimerizes with other HER proteins, whereas HER3 lacks catalytic activity. HER1/EGFR and HER2 tyrosine kinase hyperactivity is directly related with breast cancer [14,15]. Upon ligand binding and receptor dimerization, the catalytic activity of HER kinase domain is positively regulated by Tyr phosphorylation, as a result of autophosphorylation and phosphorylation by other

PTKs, including SFKs [15,16]. SFKs include 9 non-receptor TKs, among which SRC is a major player in breast cancer.

1.2.1.1 SRC

SRC is a member of a non-receptor tyrosine kinase family. It has been implicated as a regulator of cell proliferation and survival and plays a complex role in cell adhesion and motility. Evidence for a role for SRC in cancer is compelling [17]. However, only a few translational clinical studies have been undertaken in this field. There is strengthened translational proof for a definitive role of SRC in cancer. Nevertheless, there remains a need to find a robust biomarker to identify patients responsive to SRC inhibitors for clinical trials.

1.2.2 SRC structure

The three dimensional structure of c-SRC is well understood. It has a N-terminal 14-carbon myristoyl sequence, a unique segment, a SH3 and SH2 domain, a protein tyrosine kinase domain and a short C-terminal regulatory tail (Figure 1-1). The N-terminal myristoylation of SRC is required for association with cellular membranes and is crucial for the transformation of oncogenic SRC mutants [18-20]. SFKs can be found at different subcellular locations. They are most abundantly localised in the cell cytoplasm, but are re-localised to the membrane when activated [21]. Subcellular

localisation has also been suggested to regulate SRC activity [22]. The cellular location of this protein seems to play an essential element for cell transformation [17,23]. Activation of SRC has been associated with its localization to the cell membrane. Recently SRC's functions have been extended where cytosolic SRC regulates protein trafficking and nuclear SRC regulates cell cycle. The unique amino-terminal domain varies between SRC kinase family members. The SRC-homology domains are involved in autoregulating SRC kinase activity and interacting with substrates to form intracellular signaling complexes. SRC activation is dependent on the interaction of different SH domains with each other and a carboxy-terminal (C-terminal) domain.

1.2.3 SRC activation and mechanism

The activation pathway for SRC involves dephosphorylation of a negative regulatory site as well as phosphorylation of an activation site [19]. The C-terminus Tyr 527 once phosphorylated binds to SH2 domain. Phosphorylation at Tyr 527 keeps c-SRC in a closed configuration. Dephosphorylation of Tyr 527 causes a configuration change, displaying the substrate-binding pocket, allowing the kinase access to substrates and leading to phosphorylation of Y416, which is required for full catalytic activity. More recently a different activation pathway has been discovered. Platelet-derived growth factor receptor (PDGF) or human epidermal growth factor receptor 2 (HER2) driven phosphorylation of c-SRC at Y215 has been shown to block binding to the C-terminal regulatory sequence and result in a 50-fold activation of SRC [17,24].

The SH2 and SH3 domains can bind to growth factor receptors that contain their own tyrosine kinase activities, including EGFR, HER2, PDGFR, VEGFR and FGFR. The SH2 and SH3 domains can also bind to and activate cytoskeletal proteins such as FAK and p130CAS [25]. This direct interaction with substrates that contain their own tyrosine kinase domain can activate the intrinsic tyrosine kinase activity of SRC, potentially altering localisation of SRC to sites of action.

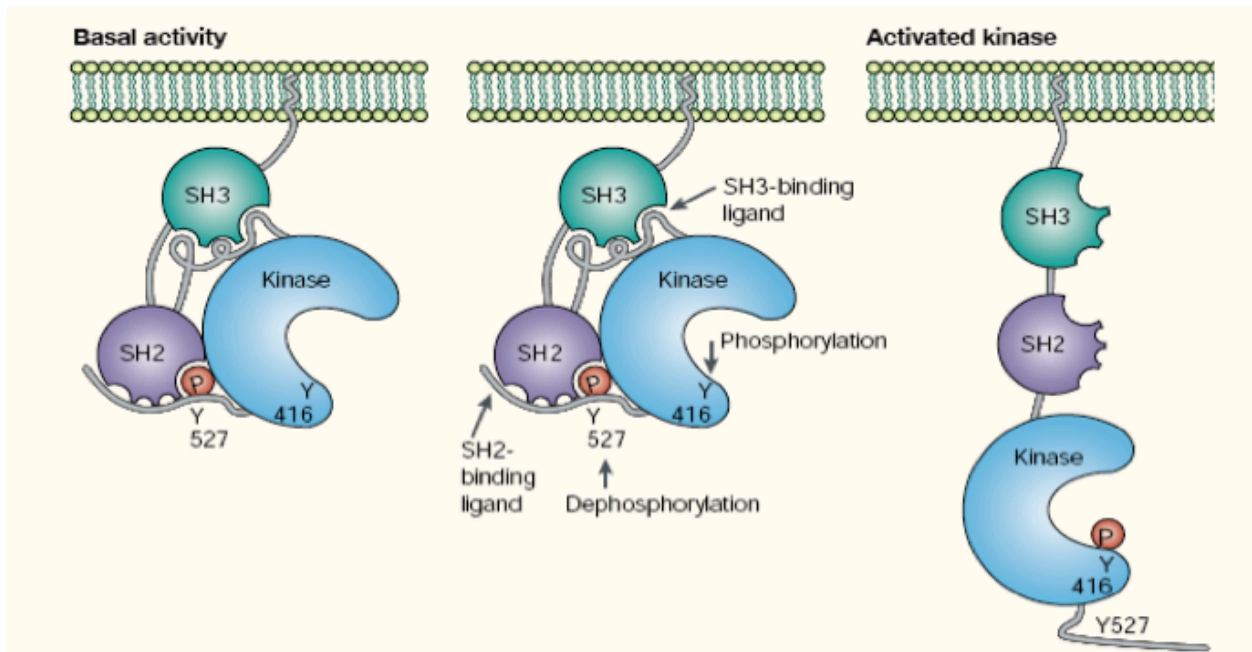


Figure 1-1: SRC structure and function

The left panel represents the inactive conformation of SRC, in which Tyr 527 interacts with the SH2 domain, positioning the SH3 domain to interact with the linker between the SH2 and catalytic domains. The middle panel illustrates different mechanisms involved in the activation of SRC, and the right panel represents the open or active conformation.

1.2.4 SRC and cell signaling pathways

SRC is engaged in multiple cellular processes and takes a central role in cell signaling [26] influencing processes, which are involved in oncogenesis. Tumor progression from early disease to advanced or metastatic disease typically involves both tumor growth (cellular proliferation) and metastasis to distant sites (cellular migration). Metastasis is a complex multistep process involving loss of cellular adhesion, increased motility, intravasation, invasion, extravasation, resistance to anoikis, colonisation of a site distant to the primary tumor, and angiogenesis [27]. Each of these processes is regulated by SRC, suggesting that it plays a role in progression and metastasis of solid tumors [28].

A lot of attention has been in the field of SRC inhibitors and they are currently being assessed as important therapeutic targets [29]. Although these approaches show potential, all conventional therapies have encountered the problems of limited response and acquired resistance [30], and combinatorial approaches are being considered to try and overcome some of these issues. Combinatorial therapies involving SRC inhibitors and chemotherapy are in trials in various settings of metastatic cancer [31]; however, combination strategies that facilitate a more targeted intervention in particular signaling pathways may be of greatest benefit. In addition, it will be extremely important to identify those patient populations that would benefit most from SRC-directed therapies. We show in our study that we can target the population showing resistance to SRC inhibitors using a combinatorial approach.

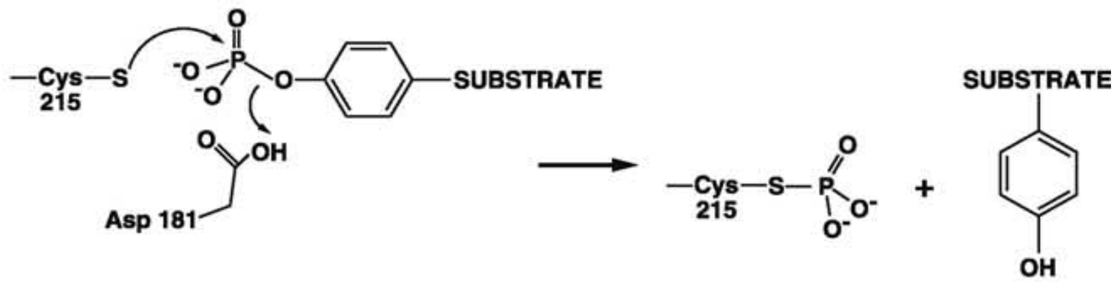
1.3.1 Protein Tyrosine Phosphatases

There are 105 genes that encode PTPs that have been identified in the human genome. These include 21 receptor-like PTPs and 82 non-receptor PTPs [32]. PTPs are defined by the presence of an active site signature motif, $HC(X)_5R$ (X is any amino acid), in which the invariant Cys and Arg residues are essential for catalysis [33]. All members of the PTP family follow a two-step reaction to catalyze protein dephosphorylation (Figure 1-2). The low-pKa cysteine residue (pKa = 4.7-5.4) [34] in the signature motif initiates a nucleophilic attack on the phosphate group of the substrate. The conserved Arg stabilizes the Cys-thiolate, assists substrate binding and maintains the transition-state of the phosphate. Also, an invariant Asp (As 181 in PTP1B) serves as a general acid to protonate the tyrosyl leaving group of the substrate and leads to the formation of a cysteinyl-phosphate intermediate. In the second step, a water molecule is oriented by hydrogen bonding to a conserved glutamine residue (Gln 262 in PTP1B). The negatively charged Asp181 serves as a general base to promote the nucleophilic attack of the water molecule. This leads to the hydrolysis of the phosphocysteine intermediate and the release of the phosphate [33].

Based on their substrate specificity, PTPs are broadly divided into two groups, the classical PTPs and the dual specificity phosphatases (DSPs) [35]. The substrate specificity of the classical PTPs and the DSPs is determined by the architecture of the active site cleft. The catalytic cysteine lies at the bottom of the catalytic cleft. In the classical PTPs, there is an invariant Tyr (Tyr 46 in PTP1B) that defines the depth of the

catalytic cleft, thereby ensuring specificity for phosphotyrosine, which has a longer side chain. In contrast, the catalytic cleft of the DSPs is shallow because the residues that define the depth of the catalytic cleft are shorter and the important residues that interact with the aromatic ring of the substrate pTyr are missing [36-39]. This allows both phosphotyrosine and phosphoserine/threonine to access to the catalytic Cys, which lies at the bottom of the cleft.

STEP 1



STEP 2

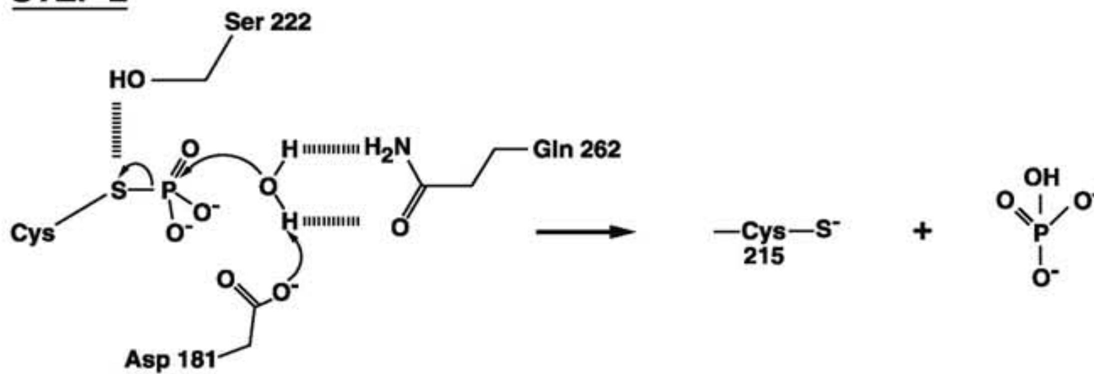


Figure 1-2 : General catalytic mechanism of PTPs

(Tonks NK, *FEBS Lett.* 2003. 546(1):140-8)

1.3.2 PTKs and PTPs as therapeutic targets in cancer

The role of tyrosine kinase in the control of cellular growth and differentiation is central to all organisms and has been found to participate in human neoplastic diseases. Disruption of PTPs and their activity can change the protein phosphorylation, resulting in the propagation of abnormal responses to extracellular stimuli and leading to the development of human diseases, including cancer [35]. Thus, the ability to alter specifically signaling pathways regulated by protein tyrosine phosphorylation holds enormous therapeutic potential. This rationale has recently led to the development of small molecular PTK inhibitors for the treatment of cancers, such as Gleevec, which targets Bcr-Abl, for the treatment of chronic myelogenous leukemia, and Iressa (Gefitinib) and Tarceva (Erlotinib), which target EGFR, for the treatment of non-small cell lung cancer [40]. In addition, antibody-based PTK inhibitors were also developed, such as Herceptin (Trastuzumab), which targets ErbB2, for the treatment of malignant breast cancer [41]. One huge limitation with therapeutic targets is that patients develop resistance against the drug. A better understanding of protein tyrosine phosphorylation has led to development of new therapeutic strategies and the identification of novel therapeutic targets. Several tyrosine kinase inhibitors are undergoing human trials and several are in the pipeline of drug discovery.

Protein tyrosine phosphorylation plays a major role in cellular signaling. The level of tyrosine phosphorylation is controlled by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Disturbance of the normal balance between PTK and PTP activity results in aberrant tyrosine phosphorylation, which has been linked to the etiology of several human diseases, including cancer. A number of PTPs have been

implicated in oncogenesis and tumor progression and therefore are potential drug targets for cancer chemotherapy [35]. These include PTP1B, which may augment signaling downstream of HER2/Neu; SHP2, which is the first oncogene in the PTP superfamily and is essential for growth factor-mediated signaling; the Cdc25 phosphatases, which are positive regulators of cell cycle progression; and the phosphatase of regenerating liver (PRL) phosphatases, which promote tumor metastases [42]. As PTPs have emerged as drug targets for cancer, a number of strategies are currently been explored for the identification of various classes of PTP inhibitors. These efforts have resulted many potent, and in some cases selective, inhibitors for PTP1B, SHP2, Cdc25 and PRL phosphatases. Structural information derived from these compounds serves as a solid foundation upon which novel anti-cancer agents targeted to these PTPs can be developed.

1.3.3 PTEN as a tumor suppressor

Some of PTPs are established or potential tumor suppressors [42]. Mutational analyses have provided links between several PTP genes to various types of human cancer. These include PTEN, PTPRF, PTPRG, PTPRJ, PTPRO, PTPRT, PTPN3, PTPN6, PTPN13, PTPN14, and DUSP6 [35]. Among these PTPs, Pten is established as a tumor suppressor in various types of human cancer through extensive investigation that includes mouse models of tumorigenesis [35,42]. Notably, physiological substrates of Pten are phosphoinositide 3-phosphates, not proteins. There is substantial evidence

that DEP1 (PTPRJ) is a tumor suppressor [42,43]. Roles of other PTPs as tumor suppressors are less well-established.

Phosphatase and tensin homology (PTEN) acts as a tumor suppressor [44]. This phosphatases is involved in the regulation of cell cycle, preventing cells from proliferating rapidly. PTEN was found to be mutated in a large number of cancers. It contains a tensin like domain and a catalytic domain similar to that of dual specificity protein tyrosine phosphatases. Unlike most protein tyrosine phosphatases, this protein preferentially dephosphorylates phosphoinositide substrates. It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-triphosphate in cells and functions as a tumor suppressor by negatively regulating AKT/PKB pathway [44]. PTEN is one of the most commonly lost tumor suppressors in human cancer. Up to 70% of patients with prostate cancer are estimated to have lost a copy of PTEN gene. Frequent inactivation of PTEN has also been shown to occur in glioblastoma, endometrial cancer. In breast and lung cancer reduced expression of PTEN has been reported.

1.3.4 SHP2, an oncogenic PTP

SHP2 is a non-transmembrane PTP that contains two SH2 domains N-terminal to the catalytic domain [45]. There are two tyrosine residues in the C-terminal tail (C-tail) of SHP2, which can be phosphorylated by PTKs, and are critical for the regulation of SHP2 activity. It has been proposed that the N-terminal SH2 domain (N-SH2) associates with the catalytic cleft of the PTP domain and thereby blocks the entry of the

substrate to the catalytic cleft. This maintains SHP2 in an inactive state. SHP2 is activated by two proposed mechanisms. In the first mechanism, the SH2 domains of SHP2 interact with the phosphotyrosine in particular sequence motifs in another protein, which releases and exposes the catalytic cleft of SHP2 to its substrate. In the second mechanism, SHP2 can be activated by phosphorylation of C-terminal tyrosine residues. Germline mutations of SHP2 have been identified in ~50% of Noonan Syndrome (NS) patients, who have a high incidence of leukemia [46,47]. In addition, point mutations on SHP2 gene were detected in 20 to 25% of sporadic juvenile myelomonocytic leukemia (JMML). Mutations identified in JMML and NS cluster at the interface between the N-SH2 and PTP domain in the structure of SHP2. These mutations abolish the association of N-SH2 with the PTP domain, thus generating a constitutively active SHP2 that promotes oncogenic signaling through activating the Ras/MAPK and SRC signaling pathways. SHP2 can activate Ras either by blocking the binding of the negative regulator RasGAP to the RTKs or by inactivating Sprouty, which inhibits Ras activity. In addition, SHP2 could activate SRC family of kinases (SFKs) to enhance oncogenic signal. It has been shown that SHP2 could dephosphorylate the transmembrane protein PAG/CBP and blocks the recruitment and activation of Csk, which phosphorylates the inhibitory tyrosines (Tyr527 on mouse SRC) and inactivate SFKs [45].

1.3.5 PTPs and cancer

PTPs have been thought of as tumor suppressors as they antagonize the effects of kinases which have been shown to have a more oncogenic effect. More recently, a role for specific PTPs to promote cell transformation has been illustrated. Only for a few PTPs, the underlying molecular mechanisms that connect their activity to the development of cancer have been characterized.

Interestingly, PTPs can also potentiate, rather than antagonize, actions of PTKs. This mode of synergy enhances mitogenic signaling, leading to cell transformation. Thus ectopic expression of PTPα results in SRC activation and causes transformation [48]. The mechanism for the PTPα induced SRC activation is dephosphorylation of the inhibitory Tyr 527 in the C-terminal tail of SRC. Conversely, deletion of PTPα in mice leads to diminished kinase activity of SRC which strongly suggests that PTPα is a physiological positive regulator of SRC kinase in vivo. SHP2 has been shown to play a positive role in a number of growth factor mediated signaling pathways and the phosphatase activity of SHP2 is required for the Ras-dependent cellular proliferation and survival. A number of activating (gain of function) mutations in SHP2 have been identified as the cause of the inherited disorder Noonan syndrome and several forms of leukemia and solid tumors. Most recently, the PRL (phosphatase of regenerating liver) phosphatases have been implicated as potential oncogenes that promote cell growth and tumor invasion.

Cellular pathways regulated by tyrosine phosphorylation could offer a rich source of drug targets for developing novel therapeutics. The development of small molecule inhibitors to modulate protein tyrosine phosphorylation has transformed the approach to

cancer therapy and is likely to have significant impact on other therapeutic areas. Indeed, small molecule inhibitors that block the activity of a narrow spectrum of PTKs exhibit much less toxicity than the currently used chemotherapeutic agents. The potential of such targeted therapeutics has been well demonstrated by the successful treatment of chronic myelogenous leukemia with imatinib mesylate and non-small cell lung cancers with gefitinib, which target Bcr/Abl and epidermal growth factor receptor (ErbB1) aberrantly activated in the malignancies, respectively. Given the critical role of PTPs in cellular signaling and the fact that deregulation of PTP activity also contributes to the pathogenesis of a number of cancers, inhibitors of the PTPs are also expected to have therapeutic value. Furthermore, since no formal functional linkage exists between the PTKs and PTPs, i.e. a PTP may catalyze the dephosphorylation of substrate proteins phosphorylated by more than one PTK, inhibitors of the PTPs may also have unique modes of action. Thus, PTPs represent novel and attractive targets for cancer treatment.

1.3.6 PTPs and breast cancer:

Protein tyrosine kinases have been shown to play an important role in breast cancer, suggesting that PTPs may play an equally important role in the development of breast cancer. However, to date, the role of most PTPs in the development of breast cancer is still unknown. Analysis in cellular or animal model has shown that some PTPs, such as PTPRE, DEP1, and PTP-BAS might play a role in the development of breast

cancer [49]. Abnormal expression of several PTPs has been observed in breast tumors e.g. PTPRA is overexpressed in ~30% of breast tumors [50]. Expression of PRL3 [51] and LAR [52] was found to be up-regulated in metastatic breast cancer. On the other hand, expression of certain PTPs such as PTPRG is lower in breast tumors [53]. Genomic mutations in PTPs, such as PTEN [54], PTPN12 (PTP-PEST) [55], and PTPRF (LAR) [56] have also been identified in breast tumors. Breast tumors with depleted PTEN show decreased sensitivity to Herceptin, suggesting that PTEN might be a marker to predict susceptibility of breast tumor to the drug [57,58]. Moreover, expression of some PTPs may also be regulated in response to stimuli or oncogene activation such as estrogen [59] or ErbB2 [60,61], respectively. Therefore, the change in certain PTPs may be useful as a prognostic/diagnostic marker in breast cancer.

1.3.7 LAR family of RPTP

RPTPs are a subfamily of transmembrane proteins that are important in regulating signaling through protein tyrosine dephosphorylation [35] (Figure 1-3). Members of the LAR-like RPTPs subfamily are expressed in neural specific patterns and have been directly implicated in axon growth regulation in invertebrates [62,63]. The LAR-like transmembrane protein tyrosine phosphatases (LAR, PTPσ, PTPδ) contain two intracellular phosphatase domains and an extracellular segment consisting of Ig-like and fibronectin type III like domains, commonly found in cell adhesion molecules [64]. This structural architecture implies that LAR-like phosphatases may

function in integrating tyrosine phosphorylation-dependent cellular signaling with cell-cell and cell-matrix interactions. In fact, LAR-like RPTPs have been implicated in biological processes that rely upon regulated cell-cell, cell-matrix contact, such as control of neuronal path finding [65,66]. LAR localizes to the ends of focal adhesions most proximal to the cell nucleus, implicating LAR-like RPTPs in disassembly of focal adhesions [64]. Signaling events downstream of LAR-like RPTPs that result in alteration in axonal guidance involve Rho-GTPase-dependent cytoskeletal remodeling [67]. It has also been demonstrated using LAR-/- mice that LAR plays a significant role in the signaling events responsible for mammary gland development and function [68]. This corroborates the hypothesis that RPTPs, in particular those of the LAR subfamily, might be important candidates in breast cancer studies. As the LAR-like RPTPs resemble the cell adhesion molecules, RPTPs are assumed to bind to ligands on the surface of other cells. The interactions with these cell adhesion molecules can promote neurite growth [69].

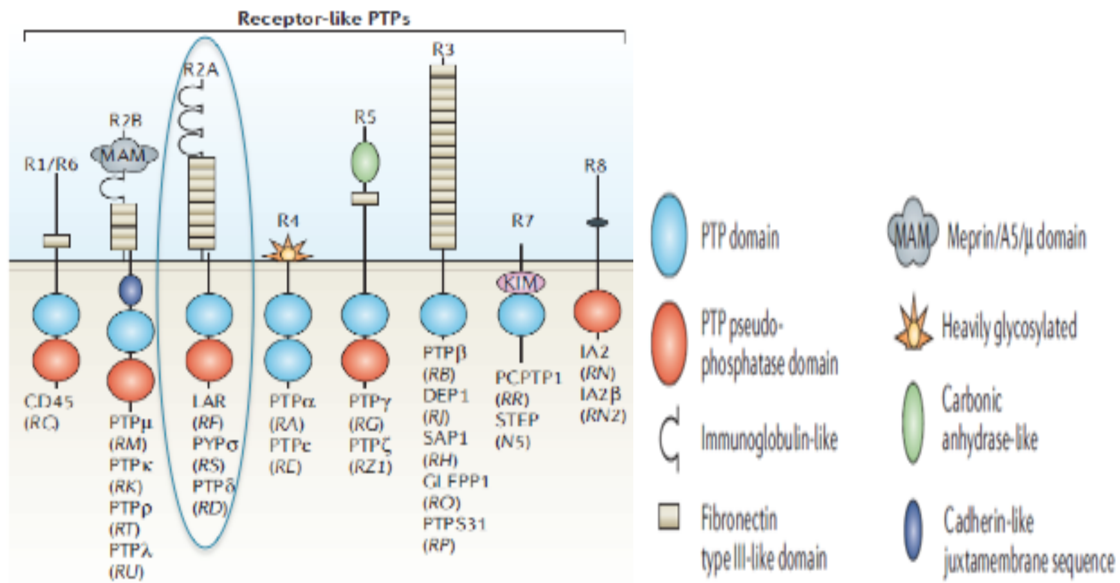


Figure 1-3: Protein Tyrosine Phosphatases

1.3.8 PTPRD/RPTPδ/PTPδ

PTPδ is a LAR like phosphatase. It belongs to the type IIA subfamily of the protein tyrosine phosphatases. It contains an extracellular region composed of eight fibronectin type III domains and three Ig like domains, a single transmembrane segment and two tandem intracytoplasmic tyrosine phosphatase domains.

PTPδ is an important regulator of axon growth and guidance and is highly expressed in the central nervous system and is found to be concentrated in growth cones of elongating processes [70,71]. PTPδ is a homophilic cell adhesion molecule [72]. These homophilic interactions serve to promote neuronal adhesion and neurite outgrowth [72,73].

PTPδ has been suggested to be a tumor suppressor due to its inactivation in a number of human cancers including head and neck, melanomas and lung cancer [74,75]. Its role as a tumor suppressor in neuroblastoma is due to its downregulation in primary tumors and lower mRNA expression [76]. Chromosome 9p, harboring PTPRD gene is a frequent target of microdeletion in primary tumors [77]. Early evidence for the involvement of PTPRD in lung adenocarcinoma came from detection of homozygous deletions in primary tumors and cell lines of small lung carcinoma and non-small lung carcinoma. Somatically acquired mutations were found in 11 out of 188 lung adenocarcinoma samples. In colorectal carcinoma PTPRD was found to be somatically mutated. PTPRD is also subject to chromosome shedding in 6% tumors studied [78]. In contrast to genomic studies, PTPδ loss of function in mice is not reported to increase tumor incidence [79]. This implies there is no clear evidence for a tumor suppressor role of PTPδ in neuroblastomas. Although, PTPδ has been shown to be mutated or

downregulated in cancers such as neuroblastoma, there have been conflicting reports as to its function as a tumor suppressor [80,81], and may simply reflect the complex role of PTPs in cancers.

Resistance to tyrosine kinase inhibitors has remained a challenge among cancer patients. There has been precedence of protein phosphatases playing a role in metastasis. PRL3 (protein tyrosine phosphatase of regenerating liver 3) has been shown to be a critical player in cancer cell metastasis, invasion, migration and angiogenesis. The association of elevated levels of PRL3 in multiple cancers especially CML has been validated. Suppression of PRL-3 could provide potential opportunity for improving anti-CML therapy, especially in tumors with Imatinib or tyrosine kinase inhibitor resistant patients. Similarly, we anticipate through our studies that MIM-/- metastatic tumors can be targeted by inhibitors against SRC and PTPδ. This new outlook of combinatorial therapy might be a way to combat relapse and resistant mechanisms of cancer cells.

1.4.1 Metastasis

Metastasis is a key event in the spread of cancers to remote sites and the major cause of fatality. Metastasis involves intrinsic and extrinsic factors that are genetic and microenvironmental signals. Metastasis requires dissemination of cells from the primary tumor site to a secondary site where they can proliferate [82]. For the most part, normal epithelial cells do not disseminate to other parts of the body and proliferate when compared with metastatic cells. Pathophysiology of metastatic cascade encompasses

release of cells from the primary tumor site, transport of the cells in the blood vessels, attachment of tumor cells to the remote site, invasion and colonization of secondary site by tumor cells [82] (Figure 1-4). This spread into surrounding tissue often leads to eventual death in many cancer patients [83]. Understanding the mechanisms which facilitate the invasive transition in cancer is therefore very important.

1.4.2 The pathogenesis of metastasis

The process of cancer metastasis consists of sequential and interrelated steps [82]. After transformation, neoplastic cell growth is slow and progressive. Vascularization is essential for tumor mass to grow, followed by local invasion of the host stroma by tumor cells. EMT has also been shown to play a crucial role in cell movement and changes in cytoskeletal dynamics [84] . Circulation of tumor cells within the vascular is both hematologic and lymphatic. The next step is survival of tumor cells through circulation and extravasation of the cells and eventually the proliferation into secondary tumor.

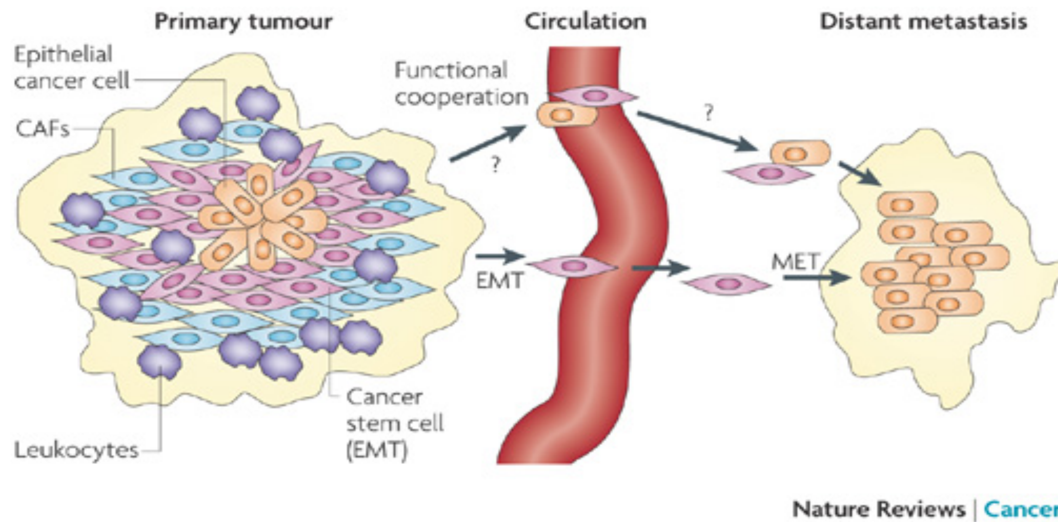


Figure 1-4 The metastatic cascade

Metastasis is a result of four ordered processes: release of cells from the primary tumor site, transport of the cells in the blood vessels, attachment of tumor cells to the remote site, invasion and colonization of secondary site by tumor cells.

1.4.3 Cell migration

Cell migration requires a series of repetitive, integrated processes to produce coordinated cellular movements. The initial event in migration for cultured fibroblasts is the protrusion of a lamellipodium — a broad, thin, actin-rich protrusion of the cell membrane at the leading edge. A lamellipodium can be stabilized or can retract; migrating fibroblasts often produce extra lamellipodia that move backwards on the cell surface as membrane ruffles [85]. Another class of protrusion at the leading edge comprises smaller finger-like, actin-rich extensions that are known as filopodia. The Rho family of GTPases is important in regulating the actin remodelling that is involved in generating these structures [86]. Following protrusion, new, small, punctate sites of cell attachment to extracellular matrix which are known as focal complexes are formed close to the leading edge, and are thought to be precursors of larger more elongated focal complexes. Focal adhesions link the ECM to actin cytoskeleton through integrins and other signalling molecules in multi-molecular complexes [87]. The next event in the migratory process is cell-body translocation, which is brought about by actomyosin contractility. The final two phases in cell migration are the release of cell contacts at the rear of the cell and recycling of membrane receptors from the rear to the front. Phosphatases have a crucial role in regulating the timing, duration and localization of these independent events to facilitate controlled cellular movement. Molecular mechanisms leading to cell migration involves cytoskeletal changes, cell-matrix interactions, proteolysis, actin-myosin contractions, and focal contact disassembly [88]. Regulation includes small GTPases (such as Rho, cdc42, and Rac), integrin-containing focal adhesion assembly and disassembly, secreted and plasma membrane-tethered

proteases, and the actomyosin contractile machinery. Growth-factor signaling, such as that mediated through the Met receptor, EGFR, PDGFR can modulate many of these activities either directly or indirectly. Several recent studies have implicated components of this cell motility machinery in metastatic progression.

Malignant cells in cancers move much faster than similarly transformed cells on tissue-culture plastic or in reconstituted extracellular matrix [89]. Intriguingly, acquisition of this motile property coincides with manifestation of the metastatic phenotype [89]. Therefore the relevance of this prometastatic motile behavior to human cancer and dissecting the pathways that underlie it are important avenues for research.

1.4.4 Cell Invasion

Cell invasion involves cancer cells in the primary tumor to break into the normal tissue and invade into the surrounding stroma. In order to invade the stroma, carcinoma (tumor tissue derived from epithelial cells) must break into the basement membrane (BM) which is a specialized ECM that is crucial in organizing epithelial tissues. In addition to structural roles played by the BM, components of this ECM contain growth factor molecules. The ECM also plays a significant role in distinct signaling pathways such as those initiated by integrin-mediated cell-matrix adhesions culminating into changes in cell polarity, proliferation, invasiveness, and survival [90].

Tissue architecture of normal epithelium serves as an intrinsic barrier to

invasiveness that must be surmounted by invading metastatic cells which leads to developing malignancy. Moreover, modulation of ECM stiffness, achieved by altering collagen crosslinking, affects carcinoma progression via altered integrin signaling [91].

1.4.5 Collective invasion and individual invasion

Most types of carcinomas can invade as multicellular units through a process termed "collective invasion." Alternatively, individual tumor cells may invade via two distinct programs, "mesenchymal invasion" and "amoeboid invasion" program [88]. Mesenchymal invasion is protease-stress fiber- and integrin-dependent and amoeboid invasion is protease, stress fiber and integrin-independent. Differential expression of molecules that enable either mesenchymal or amoeboid invasion can be observed in signatures of local invasiveness derived from mammary carcinoma models [92].

Tumor cells can switch between these various invasion strategies in response to changing microenvironmental conditions. This has led to the idea that suppression of single-cell invasion requires inhibition of both the mesenchymal and amoeboid invasion programs [88]. Certain regulators of invasion function as pleiotropically acting factors that simultaneously modulate components of both pathways.

In order to overcome various obstacles to invasion, carcinoma cells orchestrate a biological program known as epithelial-mesenchymal transition (EMT), which is important for multiple aspects of normal embryonic morphogenesis. The EMT program,

which involves loss of cell polarity, dissociates the cells within epithelial cell sheets into individual cells that exhibit multiple mesenchymal properties, including invasiveness [93].

1.5.1 Epithelial to Mesenchymal transitions

During the course of cancer progression to malignancy, neoplastic cells undergo reversible transitions between evolving phenotypic states including epithelial and mesenchymal phenotypes. This plasticity is enabled by underlying shifts in epigenetic regulation. Some of the pleiotropic transcription factors are known to effect these alterations by controlling the expression of key target genes [94]. These regulators depend on complex epigenetic mechanisms such as induction of changes in the modifications of chromatin-associated histones, in order to achieve the changes in gene expression observed during epithelial-mesenchymal transitions (EMTs). These associations indicate that an understanding of the functional interactions between EMT will provide crucial insights into the fundamental mechanisms underlying cancer progression and may, in the longer term, generate new diagnostic and therapeutic modalities for treating high-grade malignancies

The cells within human carcinomas reside within a spectrum of phenotypic states, ranging from a fully differentiated epithelial state to a dedifferentiated mesenchymal state, each of which is associated with distinct functional characteristics. The bulk of carcinoma cells exhibit predominantly epithelial characteristics but in order

to invade, disseminate to distant tissues and subsequently form metastatic colonies, epithelial cells must change at least transiently, into a more mesenchymal phenotype. This shift is achieved by the activation of the complex cell-biological program termed the EMT. During an EMT, carcinoma cells shed their epithelial characteristics, including cell-cell adhesion, polarity and lack of motility, and acquire mesenchymal traits, including motility, invasiveness and, some of the attributes of stem cells.

In normal and cancerous epithelial tissues, it seems that the physiologic activation of EMT programs depends on multiple signals that a cell receives from its nearby microenvironment. Various signaling factors can trigger the induction of an EMT program, doing so by activating a diverse array of intracellular signaling cascades. In response, a bunch of EMT-inducing transcription factors (EMT-TFs) becomes expressed and functionally activated. The forced expression of individual EMT-TFs, such as TWIST, SNAIL, SLUG or ZEB1, has been found to activate EMT programs in epithelial cells, and their elevated expression has been well documented in invasive tumors [94]. Although the activation of an EMT program by individual extracellular signaling factors is possible in principle, it seems more likely that these signals work in various combinations towards expression of EMT-TFs and in turn the activation of EMT programs.

In carcinoma cells, the newly acquired mesenchymal traits resulting from activation of an EMT program are required to execute most steps of the invasion-metastasis cascade. This cascade includes the ability of carcinoma cells to invade locally in the vicinity of primary tumors, intravasate, travel through the circulation, extravasate, survive in a distant organ and form micrometastatic deposits, some of

which may eventually form macroscopic metastases. This step of colonization involves the adaptation of carcinoma cells to foreign tissue microenvironments.

Importantly, this acquisition of mesenchymal attributes by carcinoma cells need not be permanent, as cells that have passed through an EMT while in the primary tumor may later revert to an epithelial state through a mesenchymal-epithelial transition (MET), highlighting the reversible nature of these changes. Indeed, within sites of dissemination, newly arrived carcinoma cells are unlikely to encounter the contextual signals that induced their precursors in the primary tumor to activate EMT programs; this may permit them to lapse back into an epithelial state. The reversibility of the EMT process involves reprogramming of gene expression and implies that epigenetic regulators have important roles in this process.

1.5.2 Phenotypic plasticity of cancer cells

The contributions of the EMT program in promoting cancer cell invasion and metastasis have been well documented in many types of carcinoma, including those arising in the breast, prostate, colon, head and neck, ovary and lung. In addition, such a program has been found to generate cells that either exhibit stem-like properties or are poised to enter into the stem cell state. This acquisition of stem-like characteristics holds important implications for the successful completion of the invasion-metastasis cascade by disseminated cancer cells. Passage through an EMT often imparts tumor-initiating properties to carcinoma cells, and these traits would seem to be crucial for the

ability of disseminated cancer cells to serve as initiators of secondary tumors in distant sites. Of additional interest, diverse lines of evidence have increasingly indicated that the stem cell programs operating in carcinomas are quite similar to those that function in the normal cells of origin, that is, those residing in the tissues in which carcinomas initially arise.

1.5.3 EMT markers

A hallmark of EMT is the downregulation of E-cadherin to reinforce the destabilization of adherens junctions. Additionally, the repression of genes encoding claudins and occludin, and desmoplakin and plakophilin, stabilizes the dissolution of apical tight junctions and desmosomes, respectively. These changes in gene expression prevent the formation of epithelial cell-cell junctions and result in the loss of the epithelial barrier function. The repression of genes encoding epithelial cell junction proteins is accompanied by the activation of genes, the protein products of which promote mesenchymal adhesion [95]. Specifically, the downregulation of E-cadherin is followed by the increased expression of mesenchymal neural cadherin (N-cadherin), which results in a cadherin switch that alters cell adhesion [96]. Through this switch, the transitioning cells lose their association with epithelial cells and acquire an affinity for mesenchymal cells through homotypic N-cadherin interactions; these interactions are weaker than homotypic E-cadherin interactions and facilitate cell migration and invasion. N-cadherin connects to the cytoskeleton through alpha-catenin and beta-

catenin and also interacts with p120 catenin, signaling mediators and receptor tyrosine kinases (RTKs) such as platelet derived growth factor (PDGF) and fibroblast growth factor receptors (FGFRs). EMT also activates the expression of neural cell adhesion molecule (NCAM), another adhesion molecule that interacts with N-cadherin to modulate the activity of RTKs that are associated with it. NCAM interacts with the SRC family tyrosine kinase FYN to facilitate the assembly of focal adhesions, migration and invasion.

Alterations in the expression of genes encoding cytoskeletal and polarity complex proteins also contribute to EMT. The intermediate filament composition changes with the repression of cytokeratin and the activation of vimentin expression [97]. Keratin and vimentin filaments regulate the trafficking of organelles and membrane-associated proteins, but they show differences in the proteins that they target to the membrane. Changes in intermediate filament composition also enable cell motility, possibly owing to the interaction of vimentin with motor proteins.

1.5.4 Cytoskeletal changes and motility

Cells that undergo EMT reorganize their actin cytoskeleton into one that enables dynamic cell elongation and directional motility. New actin-rich membrane projections facilitate cell movement and act as sensory extensions of the cytoskeleton. These projections include sheet-like membrane protrusions called lamellipodia and spike-like extensions called filopodia at the edge of lamellipodia. Actin-rich invadopodia exert a

proteolytic function in ECM degradation, thus facilitating cell invasion [98]. Finally, EMT is characterized by increased cell contractility and actin stress fiber formation. These dynamic changes in actin organization are probably mediated by regulatory proteins such as myosin, but the molecular mechanisms controlling F-actin dynamics during EMT remain to be elucidated. RHO GTPases regulate actin dynamics and control actin rearrangement during EMT. Among these, RHOA promotes actin stress fiber formation, whereas RAC1 and CDC42 mainly promote the formation of lamellipodia and filopodia. Following activation by RAC1 or CDC42, the kinase p21 activated kinase 1 (PAK1) activates targets that are involved in cell spreading and motility. RHO GTPases also regulate the formation of cell–cell junction complexes and the stability of adherens junctions. Cytoplasmic p120 catenin, which is generated during EMT, represses RHO activity. This may facilitate the dissociation of cell junctions and activates RAC and CDC42 to induce the formation of membrane protrusions and cell motility. RAC1 activation at the leading edge stimulates PI3K, which recruits RAC GEFs. RHO GTPases also control the reorganization of the microtubule cytoskeleton. RAC1 promotes microtubule polymerization and is itself activated by it at the leading edge of cells, which sets up a positive feedback loop that participates in the reorganization of the microtubule cytoskeleton. The reorganization of the cytoskeletal architecture and polarity complexes, which result in cell shape changes, cell elongation, membrane protrusions and front–rear polarity, are essential in EMT and facilitate cell migration.

One of the important consequences of EMT is that invading cells break through the BM, and enter the stroma. As tumor progresses, the stroma becomes increasingly active and acquires many of the attributes of the stroma of tissues [99]. Accordingly,

tumor cells invading into stroma encounter fibroblasts and myofibroblasts, endothelial cells, adipocytes, and various bone marrow-derived cells such as mesenchymal stem cells, as well as macrophages and other immune cells [99,100].

Characterizations of stromal cells provide evidence of their critical roles in facilitating the malignant behavior of carcinoma cells. One of the studies involving microarray profiling of the tumor-associated stroma derived from breast cancer patients reveals characteristic expression signatures associated with metastatic outcome [101]. Additionally, an expression signature that shows the transcriptional response of cultured fibroblasts to serum and thus reflects one component of wound-healing responses correlates with increased risk of metastatic recurrence in human breast, gastric, and lung carcinomas. These observations are consistent with the role of an increasingly activated stroma in driving malignant behavior of carcinoma cells.

1.6.1 Intravasation

Intravasation is the entering of the invasive cells into the lymphatic or blood vessels. The lymphatic spread of carcinoma cells is observed in human tumors and represents an important prognostic marker for disease progression [102]. Intravasation requires changes that can promote the ability of carcinoma cells to cross the endothelial cell barriers. Additionally, the intravasation of breast carcinoma cells can be enhanced by secretion of epidermal growth factor (EGF) and colony-stimulating factor-1 (CSF-1) by TAMs and carcinoma cells [103]. Intravasation is affected by the characteristics of

tumor associated blood vessels.

Through a variety of mechanisms–many of which converge on the actions of vascular endothelial growth factors (VEGFs)—tumor cells stimulate the formation of new blood vessels within their local microenvironment via the process termed neoangiogenesis. The interactions between adjacent endothelial cells that form the tumor-associated microvasculature and the absence of extensive pericyte coverage are likely to facilitate intravasation. In support of this notion, the capacity of cyclooxygenase-2 (COX-2), epiregulin (EREG), MMP-1, and MMP-2 to synergistically promote carcinoma intravasation was tied to their ability to stimulate neoangiogenesis and the formation of leaky blood vessels [102].

1.6.2 Extravasation

Extravasation is the movement of carcinoma cells crossing the lumina into the tissue parenchyma by penetrating the endothelial cell that separate vessel lumina from the stromal microenvironment. Disseminated carcinoma cells attempting to reach the brain must traverse the blood-brain barrier; similarly, endothelial cells lining pulmonary microvessels normally create an impermeable barrier. In contrast, carcinoma cells arriving in the bone or liver encounter perforated blood vessels that are highly permeable even in their normal state and consequently would seem to pose only minor obstacles to extravasating tumor cells [104].

In order to overcome physical barriers to extravasation that operate in tissues with low intrinsic microvessel permeability, primary tumors are capable of secreting

factors that perturb these distant microenvironments and induce vascular hyperpermeability. Some of these factors such as angiopoietin2 (Angpt2), MMP-3, MMP-10, placental growth factor, and VEGF secreted by various types of primary tumors are capable of inducing permeability prior to the arrival of carcinoma cells in the lungs, thereby facilitating the subsequent extravasation of CTCs [105].

These findings provide evidence for a model in which extravasation at certain distant organ sites necessitates cell-biological programs that are not required either for intravasation or for extravasation at alternative sites of dissemination, again highlighting the critical role of the specific tissue microenvironments present at possible sites of metastasis formation.

1.6.3 Metastatic Colonization

Proliferation of disseminated carcinoma cells in the microenvironment of a foreign tissue and formation of large macroscopic metastases is called metastatic colonization. It seems that the vast majority of disseminated tumor cells are dormant [106]. Human breast carcinomas instigate the growth of otherwise-indolent tumor cells, micrometastases. This instigation is accompanied by incorporation of bone-marrow cells (BMCs) into the stroma of the distant, once-indolent tumors. The activated BMCs mimic the systemic effects imparted by instigating tumors. The ability of disseminated tumor cells to escape dormancy and to begin active proliferation may depend on several mechanisms that are needed to convert foreign microenvironment into a more

adaptable one. Indolent disseminated tumor cells may depend on the activation and mobilization into the circulation of bone marrow-derived cells and the subsequent recruitment of these cells to a metastatic site [107]. The distinct tissue microenvironments impose dramatically different organ-specific requirements for metastatic colonization is illustrated by the minimal overlap between genes identified as candidate mediators of the metastatic colonization of breast cancer cells in bone, lung, brain, or liver [108-110].

These findings hold important implications for our understanding of the molecular mechanisms underlying this final step of the invasion-metastasis cascade. This implies that the distinct adaptive programs governing metastatic colonization is determined by the organ site at which metastatic colonization occurs and the tissue of origin of the disseminating primary tumor cells. Stated differently, it is known, for example, that (1) breast carcinoma cells colonizing the lungs utilize different genetic and/or epigenetic programs than do the same breast carcinoma cells colonizing the bone, brain, or liver and (2) breast carcinoma cells colonizing the bone utilize different molecular programs than do prostate carcinoma cells colonizing the same bone tissue.

The complex processes in metastasis, cell migration and cell invasion, are regulated by a repertoire of cellular proteins. Presumably, a class of molecules termed metastasis suppressors are involved in this homeostatic control. Metastasis suppressors are by definition, cellular factors that, when re-expressed in metastatic cells, functionally inhibit metastasis without significantly inhibiting tumor growth.

1.7.1 Metastasis-Suppressor Genes

There has been evolving literature on a class of genes that function, positively or negatively, in the regulation of metastasis [102,111], the complex process through which malignant cancer cells leave a primary organ site, invade through basement membranes and connective tissue structures, journey to a distant site through the lymphatic or haematogenous circulation and finally establish as secondary tumor in a distant organ. Genes that regulate these steps in the metastatic cascade are conceptually similar to those that regulate transformation and tumorigenesis in the sense that they can be either promoters or suppressors of the phenotype. Genes that inhibit metastasis but do not affect the ability of the transformed cells to produce a tumor at the primary site (which would define them as tumor suppressors) are known as metastasis suppressor genes. Just as tumor promoters such as oncogenic Ras or SRC and tumor suppressors such as PTEN or p53 regulate tumorigenesis, there are proteins that act as promoters and suppressors of metastasis.

Until recently, few metastasis suppressor genes had been characterized. These genes regulate key cell signaling pathways, including both G-protein-coupled and tyrosine kinase receptor signaling, and small GTPase and MAPK signal transduction and other functions as diverse as adhesion, migration, cell death and angiogenesis [112]. One reason that there have been relatively few metastasis suppressor genes described until recently is that their identification and characterization involves a convergence of several types of data and requires an *in vivo* metastasis model for testing suppressor function. This has been greatly supported by the availability of several immunocompromised mouse strains for xenograft models [113]. Experiments to

identify metastasis suppressors often include screens to identify candidate genes by comparing cells or tissues of different metastatic competence, examining the expression or mutation status of such candidates in human tumor tissues and, indispensably, showing in an *in vivo* metastasis assay that reconstitution of the suppressor in fact does suppress metastasis formation without abrogating proliferation or tumorigenesis.

The basic rationale for development of therapies targeting metastasis suppressor genes is the same as that for targeting metastasis in general [114,115]. However, mechanistic insights obtained from studying these proteins have led to the identification of vulnerability of metastatic cancer and perhaps the most relevant target for therapeutic intervention, metastatic colonization in the second tissue site. Interestingly, circulating cancer cells or even those found as single or small clusters in distant organs are not necessarily the indicator of clinically overt metastatic disease [116], although they are surprisingly pervasive and seem to occur early in localized disease in both patient series and animal models [117]. By contrast, colonization occurs when these disseminated cells grow from single or clusters of ectopic cancer cells (micrometastases) into clinically apparent macrometastases, a step that is extremely inefficient [118,119]. It is noteworthy that among cancers analyzed, 29% to 37% of cases without metastasis at diagnosis exhibit lymph nodes that are positive for tumor cells [120]. In short, many tumors may have already disseminated widely on a cellular basis before the time of diagnosis [106]. Thus, in patients whose primary tumors are successfully treated, subsequent suppression of metastatic colonization is of utmost importance therapeutically [106]. In the case of signaling pathways regulated by metastasis suppressor, targeting the function of metastasis suppressor genes that work

specifically at a key junction would be highly specific rather than treatment through conventional cytotoxic agents. Either way, this kind of suppression attempts to delay relapse, which may equate to a functional cure in patients who die from non-cancer-related causes.

Though not all metastasis suppressor genes have been demonstrated to suppress this rate-limiting colonization step, a growing number of studies have demonstrated that several of them function at this point [121]. Using experimental assays modeling a setting of widely disseminated cells, their expression has been shown specifically to suppress single and small clusters of viable cells in a dormant state in the second parenchyma, whereas control cells progress to form gross macrometastatic nodules. The genes identified to date include KISS1 which is a G-protein coupled receptor ligand for GPR54, KAI1 which is a membrane glycoprotein, NM23 which is a nucleoside diphosphate kinase (NDPK) and MAP2K4, which have been reviewed recently [122]. These and other metastasis suppressor genes may also function at other steps in the metastatic cascade. Indeed, many have been shown experimentally to suppress processes such as cellular motility, invasion and survival during dissemination. However, given the early, wide dissemination of cancer cells, these properties are likely to prove less adaptable to the colonization therapeutic niche. For a few of these metastasis genes, loss of expression or function is requisite for the development of distant metastases, because they suppress one of the key steps of invasion, dissemination, arrest, survival and growth in a second parenchyma. The role of other genes, such as CD44 and maspin/PI5 (a serine protease inhibitor), in metastasis suppression is less well defined. The potential mechanism of action of all of

these genes has been inferred by analogy to other family members and observations in model systems. How these genes and their protein products function to suppress metastasis in vivo is the subject of enthusiastic study.

1.8.1 Missing-in-Metastasis (MIM/MTSS1)

Missing-in-Metastasis (MIM), also referred to as MTSS1, is one such metastasis suppressor, with potential roles in the control of cellular migration and invasion. MIM is expressed in a diverse range of tissues and is down-regulated in several cancers, including breast [123-127]. Thus it has been proposed that loss of MIM function may promote the metastatic potential of cancer cells.

MIM was first identified as a metastasis suppressor in bladder carcinoma cell lines with decreased metastatic potential. Subsequently, MIM was found to be downregulated in prostate, hepatocellular carcinoma and other cancers.

MIM is a multi-domain protein, the structure of which suggests a scaffolding function [128]. The presence of WH2 and IMD domains implies a functional link with the actin cytoskeleton and recruitment of MIM to specific cytoskeletal networks. Such interactions implicate MIM in cytoskeletal changes that underlie regulation of metastasis. The central segment of MIM is rich in proline, serine, and threonine residues, and plays an important regulatory role in MIM function. Importantly, this central segment of MIM binds to several key cellular proteins, including association with

the receptor protein tyrosine phosphatase (RPTP) family protein, PTPRD [129]. Interaction studies using the yeast two-hybrid approach and analysis of MIM interaction with recombinant PTPδ illustrate that MIM binds to the cytoplasmic domain of PTPδ [129]. Since RPTPs are known to regulate tyrosine phosphorylation-dependent signaling, this MIM-PTPδelta interaction may provide a functional link that influences the signal transduction events that underlie the establishment of an invasive state.

MIM has been reported to play a role as a metastasis suppressor in both prostate cancer and bladder cancer [124]. This evidence suggests that MIM acts as a scaffold protein that interacts with actin-associated proteins to modulate lamellipodia formation [130,131].

Recently, evidence suggests that MIM is a regulator of carcinogenesis in hepatocellular carcinoma [127], and is a member of the sonic hedgehog (SHH) signaling pathway that modulates Gli responses during growth and carcinogenesis [132]. A functional role for MIM in tumorigenesis and metastasis is yet to be established. The study of MIM has been restricted to a limited of cancer types, with little support from the clinical aspect and lack of availability of reagents. Studies suggest that further analysis of MIM expression or inactivation in tissue samples and its association with different human malignancies will define a novel candidate to be used as a marker of primary tumors or metastasis.

1.8.2 MIM and binding partners

MIM has also been reported to bind to the membrane tyrosine phosphatase PTPδ through its central domain (Figure 1-5). PTPδ binding was discovered using yeast two-hybrid analysis and confirmed biochemically. Subsequently, it was shown [129,133] that PTPδ was involved in the cytoskeletal remodeling effect of MIM using a blocking peptide derived from PTPδ. MIM had a role in the localisation of PTPδ to specific regions of the plasma membrane, but that the phosphatase activity of PTPδ did not appear to be required for this localisation. MIM co-localises with the actin-binding protein cortactin and apparently interacts with cortactin via a proline-rich sequence adjacent to the MIM C-terminal WH2 motif [134].

In *in vitro* assays, MIM modestly enhanced cortactin mediated actin polymerization. MIM could play a role in regulation of N-WASP and cortactin-mediated actin assembly. MIM co-localizes with cortactin and N-WASP in dorsal ruffles. MIM was tyrosine-phosphorylated in response to PDGF stimulation [134]. SRC was shown to phosphorylate MIM implicating MIM's role in signaling pathway involving the PDGF receptor, SRC and actin cytoskeleton.

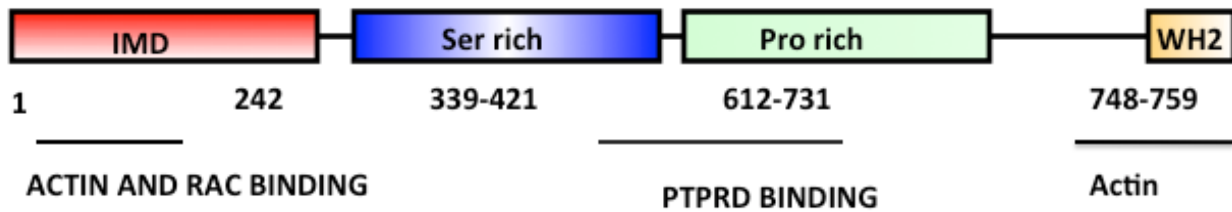


Figure 1-5 MIM and its binding partners

MIM's association with actin is attributed to two actin-binding domains: the amino-terminal 250 amino acid IRSp53/ MIM homology domain (IMD) and the carboxyl-terminal 30 amino acid WASP homology domain 2 (WH2). The central region of MIM is rich in proline, serine, and threonine residues, and plays an important regulatory role in MIM function. MIM binds to several key cellular proteins including the transcription factor Gli, the tumor suppressor Sufu, and the receptor protein tyrosine phosphatase (RPTP) family protein, PTPRD.

CHAPTER 2

Results

Breast cancer is one of the leading cancers for women in terms of occurrence and fatality. Around 232,670 American women will be diagnosed with invasive breast cancer in 2014 which is predicted to lead to 40,000 related deaths (American Cancer Society. *Breast Cancer Facts and Figures 2013-2014*). Breast cancer is a heterogeneous disease forming within the breast ducts or lobules. Breast cancer invasion requires that cancer cells breach the epithelial cell basement membrane and migrate into the surrounding tissue.

There are multiple pathways of progression to an invasive cancer. Progression of ductal breast cancer, which accounts for 80% of all breast cancer is from normal pathology through atypical ductal hyperplasia to ductal carcinoma in situ (DCIS), then invasive ductal carcinoma (IDC), and culminating into metastatic disease. DCIS can be defined as a proliferation of cancer cells situated within confines of the breast duct. Ductal breast cancer invasion, therefore, requires that cancer cells breach the epithelial cell basement membrane and migrate out of the duct into the surrounding tissue. Metastatic gene signatures may ultimately be important in the clinical management of breast cancer. However, metastasis of ductal breast cancer requires the transition from an in situ breast tumor to an invasive tumor. It is therefore important to study cell invasion and the proteins regulating this process.

In this study, we show that MIM regulates cell invasion. We further provide evidence that PTPδ plays a role in the regulation of tyrosine phosphorylation signaling and cellular invasion in the context of MIM negative tumors.

PTPδ might be a crucial regulator of physiological processes such as cell motility and cell invasion in the context of MIM. We present evidence that loss of MIM promotes cell invasion in breast epithelial cells. In addition, suppression of MIM increases expression of PTPδ and invasion of breast epithelial cells through activation of SRC. Hence, our data suggest that MIM is a metastasis suppressor protein functioning via its interacting partner PTPδ in breast epithelial cells.

2.1.1 Loss of MIM induced migratory behavior in mammary epithelial cells

The genetic background to breast cancer invasion is complex and multifactorial. Acquisition of a motile phenotype is essential for tumor cells to become invasive. Cell motility is fundamentally important for a myriad of physiological functions. Cell migration is essential for organogenesis and the generation of polarity. Cell motility plays a key role in neuronal communication and the trafficking of leukocytes. However, cell motility is also extremely important in pathophysiological settings such as wound closure and cancer. In the context of breast carcinoma, the ability of epithelial cells at the edge of a histologically confined tumor to migrate away from the primary site is an early determinant of the transition from an in situ into an invasive phenotype. Also, since metastasis cannot happen without the initial invasion, a better understanding of the

migratory mechanisms used by cells is important for our understanding of breast cancer.

It has been previously reported that loss of MIM transcript correlates with the invasive state of breast cancer cell lines. However, the role of MIM during cell invasion and cell migration has not been elucidated. To understand the role of MIM during the invasion and migration of mammary epithelial cells, MIM was suppressed using RNAi and stable MCF10A populations were generated. Depletion of MIM by two distinct shRNAs was validated by immunoblotting (Fig 2-1A). The effect of MIM suppression was investigated using a transwell migration assay. Cells with two distinct MIM shRNAs showed upto a 4-fold increase in migratory potential as compared to those stably expressing a control shRNA (Fig 2-1B). Hence, attenuation of MIM expression contributed to increased cell motility in MCF10A cells. In addition, MCF10A cells normally form clusters when grown as sub-confluent cultures, however, we observed that suppression of MIM led to scattering of single cells that do not form clusters (Figure 2-1C).

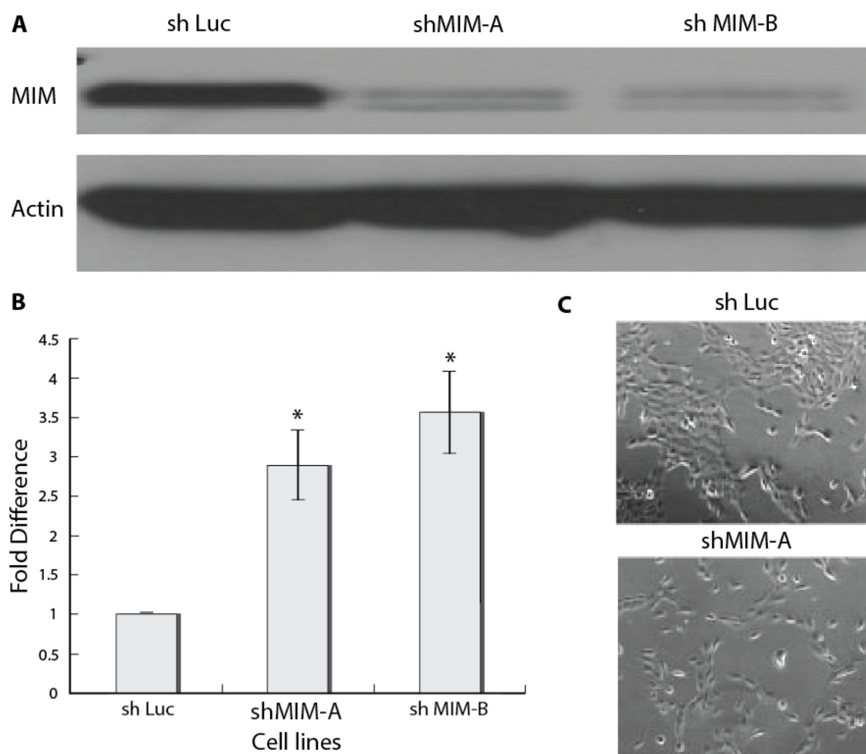


Figure 2-1. Suppression of MIM induced an increase in mammary epithelial cell motility

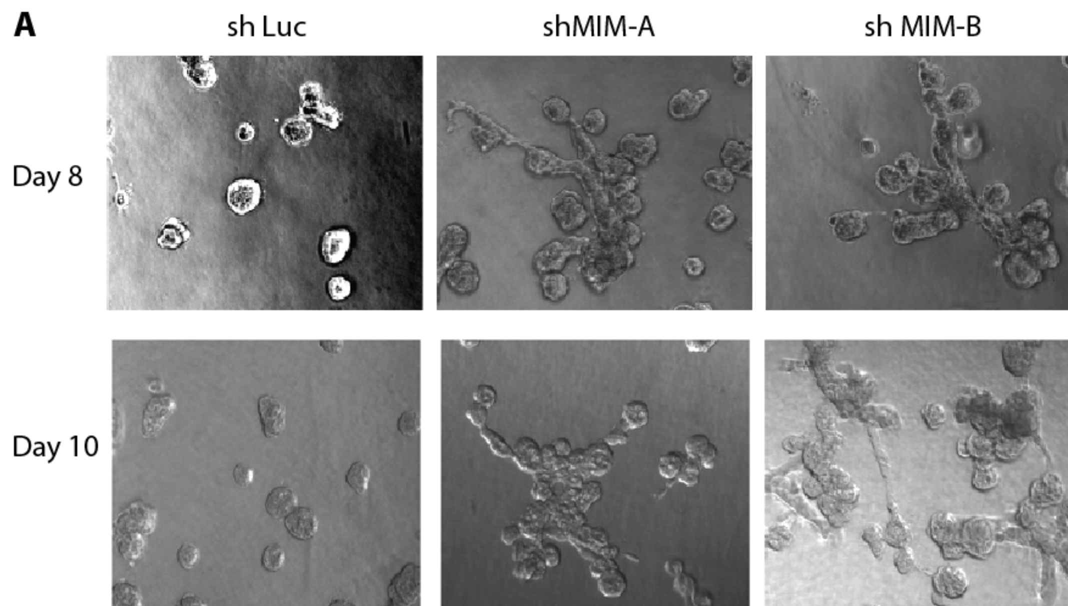
(A) RNAi-mediated suppression of MIM by shRNA, measured by immunoblotting cell lysates with antibodies to MIM. **(B)** MCF10A cells infected with control or two distinct MIM-directed shRNAs were seeded in transwell migration chambers, incubated for 48 h, and cells that had migrated were counted. The motility of the control cells was normalized to 1. Error bars represent S.E.M. (n=3). * denotes p value<0.05 **(C)** Scattering of MCF10A cells induced by suppression of MIM was visualized in sub-confluent culture.

2.1.2 Loss of MIM induced an invasive behavior in mammary epithelial cells

To study the role of MIM in cell invasion, three dimensional culture of MCF10A cells were performed as described earlier [135]. 3D tissue culture, which involves growing breast epithelial cells on synthetic extracellular matrix called matrigel, recapitulates numerous features of glandular epithelium. Under these conditions normal epithelial cells undergo a series of events including proliferation and apoptosis to form acini, which are characterized by the presence of a central hollow lumen and polarized cells surrounding the lumen. Various processes in cellular transformation can be dissected such as filling of the lumen, loss of polarization, escape from proliferative suppression and loss of cell adhesion using 3D model systems. In addition to all these mechanistic changes involved in cancer, the 3D epithelial cell system can also be used to study cell invasion. The heterogeneity in cell population is not only influenced by cancer cells themselves but by the surrounding microenvironment of the tumors. Given the intrinsic complexity of the invasion process, 2D culture systems are limited in their ability to model invasion. However with the advances in 3D culture techniques, using modified Matrigel/Collagen matrix, certain processes associated with invasion can be modeled. Consistent with the migration assay, attenuation of MIM function in MCF10A cells led to disorganized acini structures and formation of cellular protrusions in the invasion assay (Fig 2-2A). Quantitative Invasion assays were performed in transwell filters using 8μm pore size, Growth Factor Reduced Matrigel Invasion Chambers (BD Biosciences, Cat# 354483). Cells where MIM was depleted showed a significant increase in number of invasive cells when compared with the cells with luciferase short

hairpin (Fig 2-2B). In summary, these results are consistent with the role for MIM in regulating cell-migration and tissue invasion—both important features of metastasis.

A



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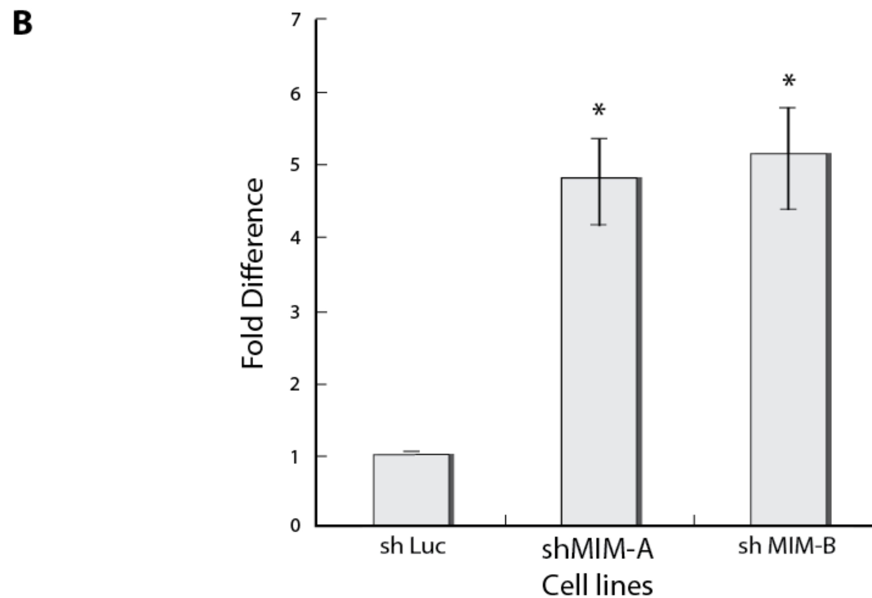


Figure 2-2. Suppression of MIM promoted mammary epithelial cell invasion.

(A) Induction of invasive structures in MCF10A cells in which MIM was suppressed by two distinct shRNAs. The micrographs were taken at Day 8 and Day 10 of 3D culture in Matrigel-collagen. Scale bar represents 100 μm. **(B)** MCF10A cells infected with control or two distinct MIM-directed shRNAs were seeded in transwell migration chambers in which the membrane was coated with Matrigel, incubated for 48 h, and cells that had migrated through the Matrigel coating were counted. Invasion by the control cells was normalized to 1. Error bars represent S.E.M. (n=3). * denotes p value<0.05

2.1.3 Loss of MIM induced an increase in levels and stability of PTPδ

As MIM is involved in regulating cell invasion, and MIM and PTPδ are binding partners [129], this interaction possibly underlies one of the functional links between MIM and tyrosine phosphorylation dependent signaling pathways. Interestingly, the level of PTPδ was elevated in cells in which MIM was suppressed compared to the control cells (Fig 2-3A). To explore further why MIM suppression led to increased PTPδ, we measured PTPδ protein stability by inhibiting protein synthesis using cycloheximide. PTPδ was found to be more stable upon cycloheximide treatment in cells with MIM knockdown when compared to the control cells (Fig 2-3B). To show that the effect of MIM on the stability of PTPδ was specific, we also tested the stability of LAR. Although the protein level of LAR was altered upon loss of MIM, there was no change in the stability of LAR upon MIM suppression.

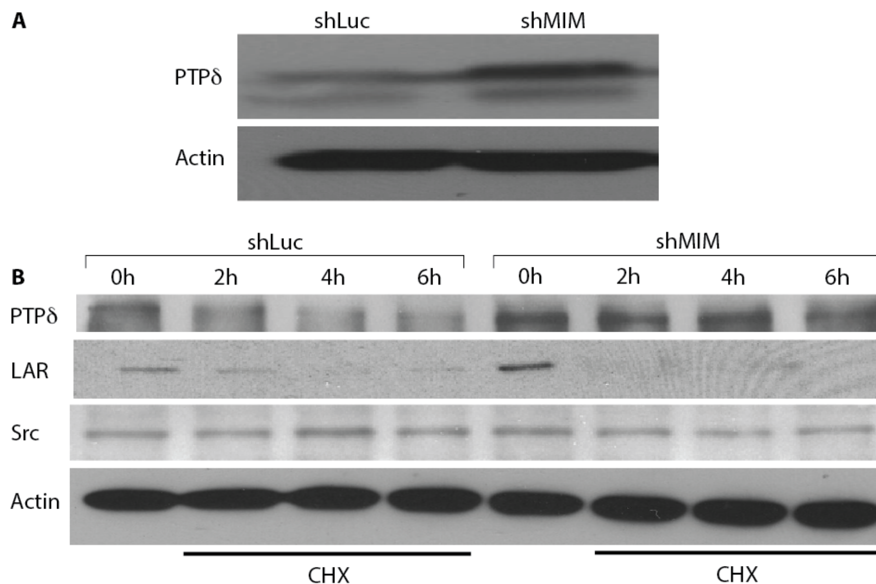
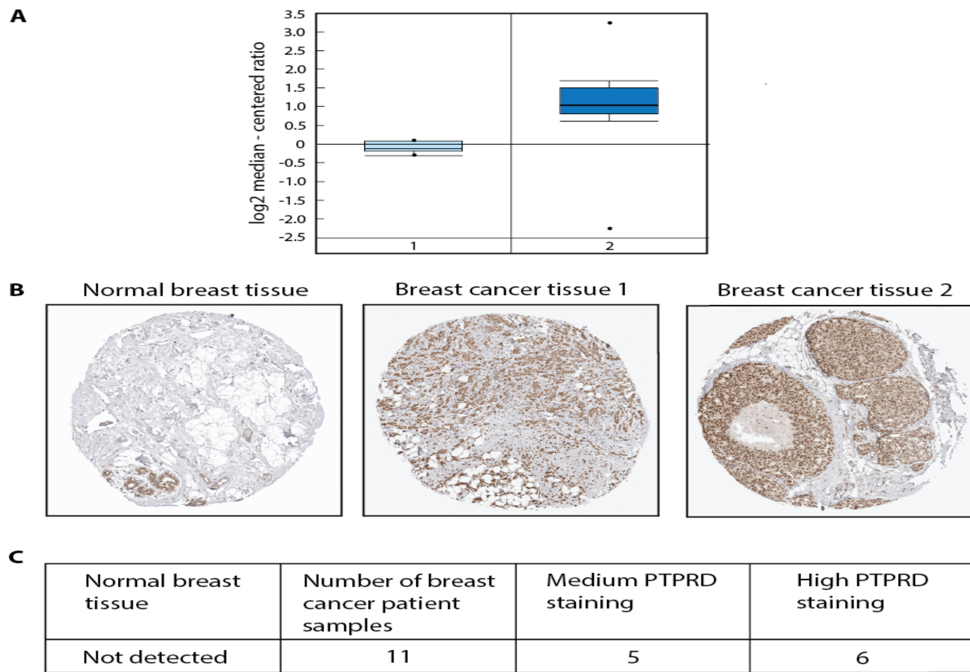


Figure 2-3. Suppression of MIM increased the levels and stability of PTPδ protein. **(A)** Immunoblot of lysates expressing indicated shRNA to illustrate that treatment of MCF10A cells with MIM-directed shRNA is accompanied by increased levels of PTPδ. **(B)** Control and shMIM-expressing MCF10A cells were treated with cyclohexamide, and lysates were prepared at the indicated time points. The levels of PTPδ and LAR were assessed by immunoblotting (20 μg of total lysate protein). The levels of SRC were measured as a control.

2.1.4 PTPδ expression in human breast cancer

To investigate if PTPδ was altered in human breast cancer, we analyzed the data from Oncomine [101]. In this study, 53 breast tumor stroma samples and 6 normal breast stroma samples were analyzed on agilent 44K microarrays (Fig 2-4A). The study showed that PTPδ mRNA levels are elevated in the tumor samples when compared with the normal samples. We also analyzed PTPδ immunohistochemistry which gave an approximate indication of protein expression in which 11 patient samples were stained for PTPδ expression from the Human Protein Atlas. The normal breast tissue showed staining for PTPδ in glandular and myoepithelial cells (Fig 2-4B). Although there was some basal staining detected in normal breast tissue, 6 out of 11 patient samples showed relatively high PTPδ staining when compared with the normal tissues.



Normal breast tissue	Number of breast cancer patient samples	Medium PTPRD staining	High PTPRD staining
Not detected	11	5	6

Figure 2-4. Expression of PTPδ in human tumor samples.

(A) PTPδ expression was analyzed in datasets from the Oncomine (Compendia Bioscience) database (http://www.oncomine.org). The Finak Breast dataset, which constituted 53 breast tumor stroma samples and 6 normal breast stroma samples that were analyzed on Agilent 44K microarrays, showed increased mRNA levels of PTPδ in the tumor samples (P value is 7.94E-15). **(B)** PTPd protein expression data from the Human Protein Atlas (www.proteinatlas.org). Representative images of Immunohistochemistry staining for one sample of normal breast tissue and two breast cancer tissue specimens from patients are shown. **(C)** Table indicates PTPd protein expression levels analyzed by immunohistochemistry in 11 patient samples, of which 5

patient samples showed medium and 6 patient samples showed high staining relative to the normal breast tissue.

2.1.5 Invasion phenotype induced by loss of MIM is mediated by altered levels of PTPδ

To test whether these changes in PTPδ levels and stability in the MIM-knockdown cells had an effect on cell invasion and motility caused by MIM suppression, cell lines co-expressing hairpins against both MIM and PTPδ were generated. The suppression of PTPδ alone did not have any effect on cell invasion but the co-expression of both the short hairpins resulted in abrogation of the invasion phenotype caused by MIM suppression (Fig 2-5A). This suggests that attenuation of PTPδ expression blocked the invasion phenotype induced by MIM-knockdown alone. These results support the model that MIM regulates PTPδ cellular function. This effect of PTPδ on MIM signaling was further confirmed by quantitative invasion assays performed on transwell invasion chambers (Fig 2-5B). Suppression of MIM resulted in an increase in cell invasion that was partially blocked upon co-suppression of MIM and PTPδ. To determine if the enzymatic activity of PTPδ was required for regulation of invasion, we generated stable cells in which we overexpressed wild type PTPδ and the catalytically inactive CS mutant and tested these cells using the invasion assay. The overexpression of wild type PTPδ, but not the CS mutant, resulted in an increase in cell invasion, suggesting that MIM functions through increasing the levels of PTPδ (Fig 2-5C).

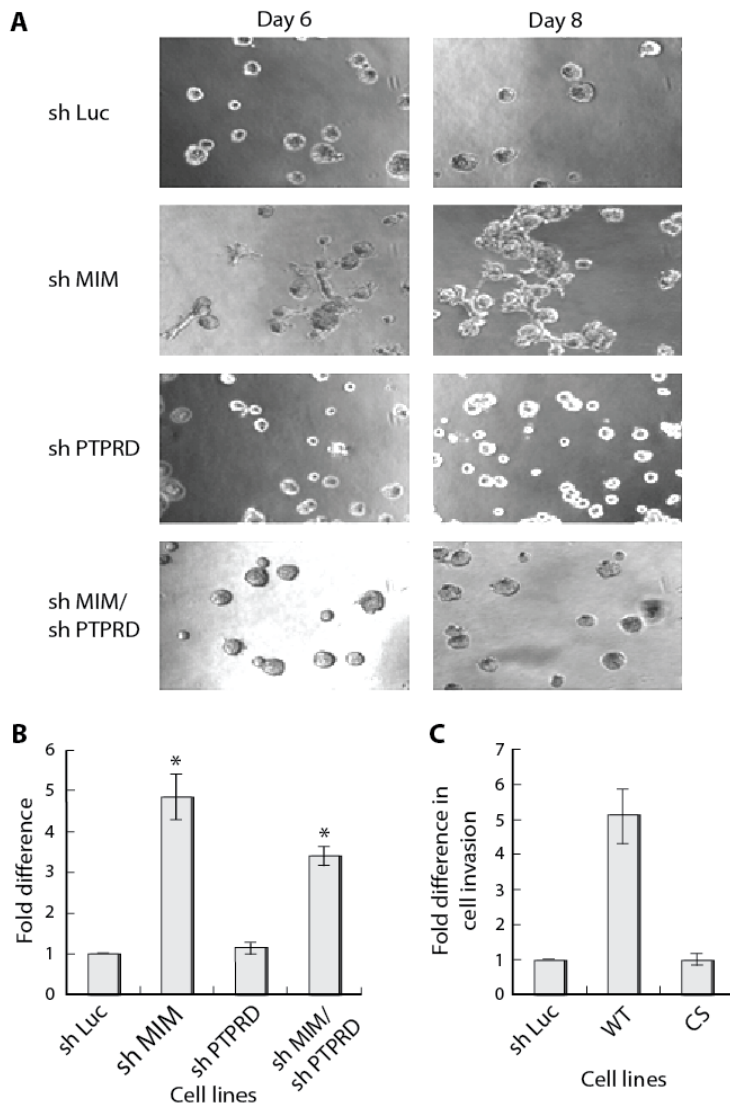


Figure 2-5. The formation of invasive structures that accompanied suppression of MIM was abrogated by co-suppression of PTPδ.

(A) Phase images of the MCF10A acini expressing the indicated shRNA. **(B)** Co-suppression of PTPδ impaired cell invasion induced by suppression of MIM. **(C)** Expression of wild type active PTPδ, but not the catalytically inactive mutant form of the enzyme, was sufficient to induce cell invasion. In both **B** and **C**, cell invasion was quantitated using transwell chambers coated with Matrigel. Data are presented as change in cell invasion relative to the luciferase control. Error bars represent S.E.M. (n=3). * denotes p-value<0.05.

2.1.6 Invasion phenotype induced by loss of MIM was mediated by activation of SRC

MIM regulates sonic hedgehog signaling and promotes ciliogenesis by antagonizing SRC-dependent phosphorylation of cortactin [136]. This regulation has been shown to increase the activity of SRC by phosphorylation at the Tyr 416 residue. SRC family of protein kinases plays a key role in cell differentiation, motility, proliferation, and survival. SRC contains an N-terminal 14 carbon myristoyl group, an SH3 domain, an SH2 domain, a protein tyrosine kinase domain, and a C-terminal regulatory tail [137]. Under basal conditions, 90% of SRC is phosphorylated at Tyr 527, which binds the internal SH2 domain, blocking the substrate binding and thereby rendering SRC inactive [138]. SRC also undergoes an intermolecular autophosphorylation at Tyr 416; this residue is present in the activation loop, and its phosphorylation promotes kinase activity.

An important mechanism for SRC activation involves pTyr 527 dephosphorylation. Depending on the cell type and the context, different phosphatases have been shown to dephosphorylate SRC at Tyr 527. When we examined the phosphorylation status of SRC upon loss of MIM, it was observed that autophosphorylation on Tyr 416 occurred to a greater extent in MIM depleted cells (Fig 2-6B) whereas the phosphorylation at the C-terminal residue on Tyr 527 was decreased significantly in MIM depleted cells (2-6A). This is consistent with an activating role for PTPδ in the regulation of SRC.

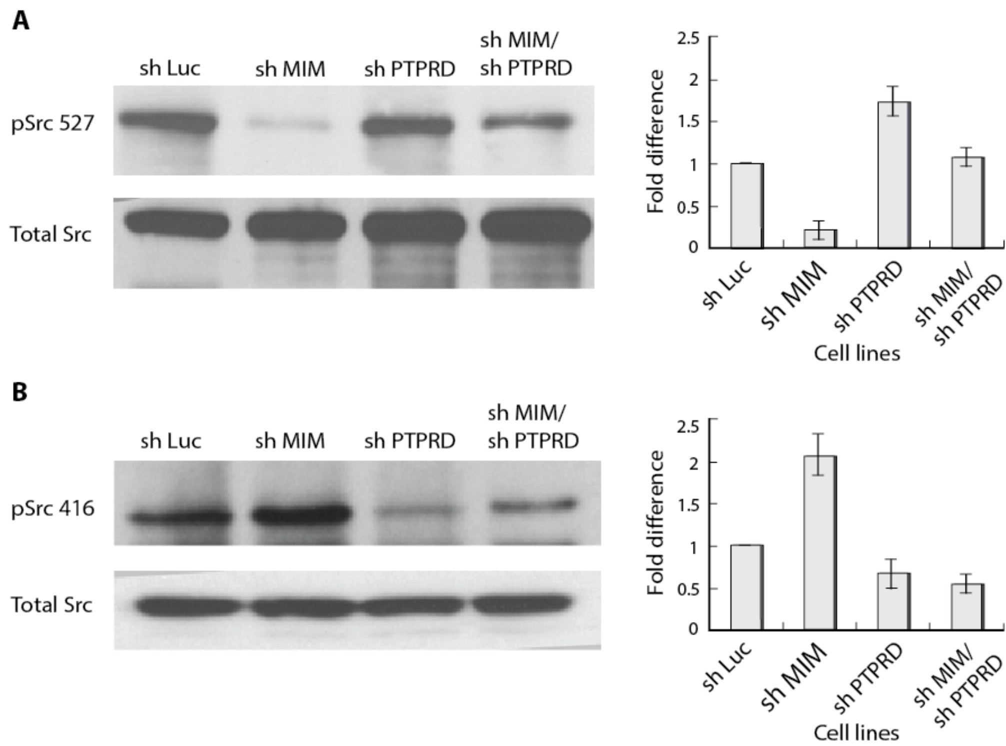


Figure 2-6. Suppression of MIM led to activation of the protein tyrosine kinase SRC.

(A) SRC was immunoprecipitated from lysates of MCF10A cells expressing the indicated shRNA and blotted for the presence of phosphate on Tyr 527. Right panel: immunoblots from three independent experiments were quantitated. Error bars represent S.E.M (n=3). **(B)** SRC was immunoprecipitated from lysates of MCF10A cells expressing the indicated shRNA and blotted for the presence of phosphate on Tyr 416. Right panel: immunoblots from three independent experiments were quantitated. Error bars represent S.E.M (n=3).

2.1.7 MIM functioned through SRC family kinases to regulate cell motility and invasion

We showed that SRC autophosphorylation was elevated in MCF10A cells in which MIM was suppressed. To test whether SRC activity is essential for the function of MIM, we analyzed the effect of the inhibitor, SU6656 [139] on 3D cell invasion. Interestingly, the SRC inhibitor attenuated cell invasion that was induced by suppression of MIM (Fig 2-7A). We further substantiated the effect of SU6656 with the quantitative invasion assay, where inhibition of SRC was shown to be sufficient to abrogate the effect of MIM suppression on cell invasion (Fig 2-7B). These observations indicate that SRC plays an important role in MIM-induced suppression of cell invasion.

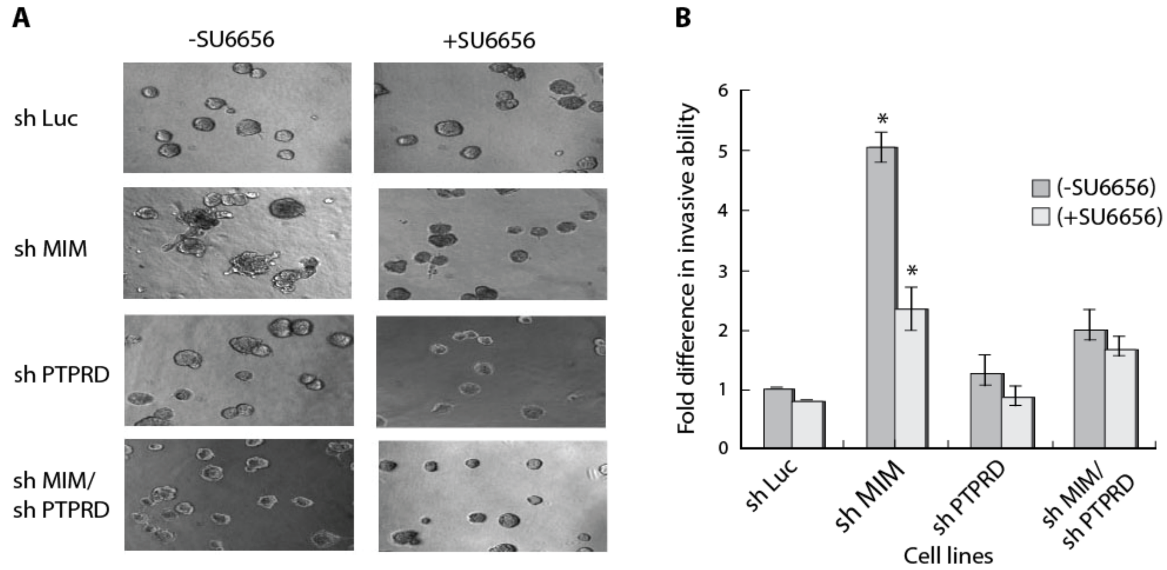


Figure 2-7. Invasion induced by suppression of MIM was blocked by SU6656, an inhibitor of SRC.

(A) Phase images of cells treated in the presence or absence of SU6656 (5μM) taken at Day 6. **(B)** The invasion of the MCF10A cells that expressed the indicated shRNAs was quantitated in coated transwell chambers in the absence or presence of SU6656 (5μM). Data are presented as change in cell invasion relative to the luciferase control in the absence of SU6656. Error bars represent S.E.M (n=3). *denotes p value<0.005

2.1.8 SRC was a direct substrate of PTPδ

To verify whether SRC was a potential substrate of PTPδ, we investigated if PTPδ directly dephosphorylates SRC in vitro. SRC was immunoprecipitated from lysates of pervanadate treated MCF10A cells expressing PTPδ short hairpin. Under these conditions SRC was phosphorylated at Tyr 527. The immunoprecipitated SRC was then incubated with wild type active PTPδ and the catalytically dead mutant and dephosphorylation was measured using specific antibodies. Immunoblots revealed that PTPδ specifically dephosphorylate SRC at Tyr 527 and not Tyr 416 (Fig 2-8A).

Earlier work from our laboratory has shown that mutation of Asp181 to Ala abolishes the catalytic activity of PTP1B but maintains its binding affinity for the substrates, thus making a substrate trapping mutant form of the enzyme [140,141]. Using this strategy we generated the substrate trapping mutant of PTPδ, which would be catalytically inactive but would retain its affinity for substrates (termed DA).

The complex generated by the binding of the trapping mutant to its substrate can be isolated by immunoprecipitation. We expressed wild type PTPδ and the substrate trapping (DA) mutant in MCF10A cells. Cell lysates from wild type and DA mutants were immunoprecipitated with PTPδ antibody and probed with an anti-SRC antibody. We observed a stable interaction between the trapping mutant and SRC but not between wild type and SRC (Fig 2-8B). To examine whether the interaction of SRC with the trapping mutant of PTPδ occurred through its active site, the trapping mutant experiment was performed in the presence of sodium orthovanadate. Sodium orthovanadate is a competitive inhibitor and will prevent the substrate from binding to

the enzyme. The interaction of SRC with PTPδ was inhibited by vanadate, indicating that SRC might represent a direct substrate of PTPδ (Fig 2-8C).

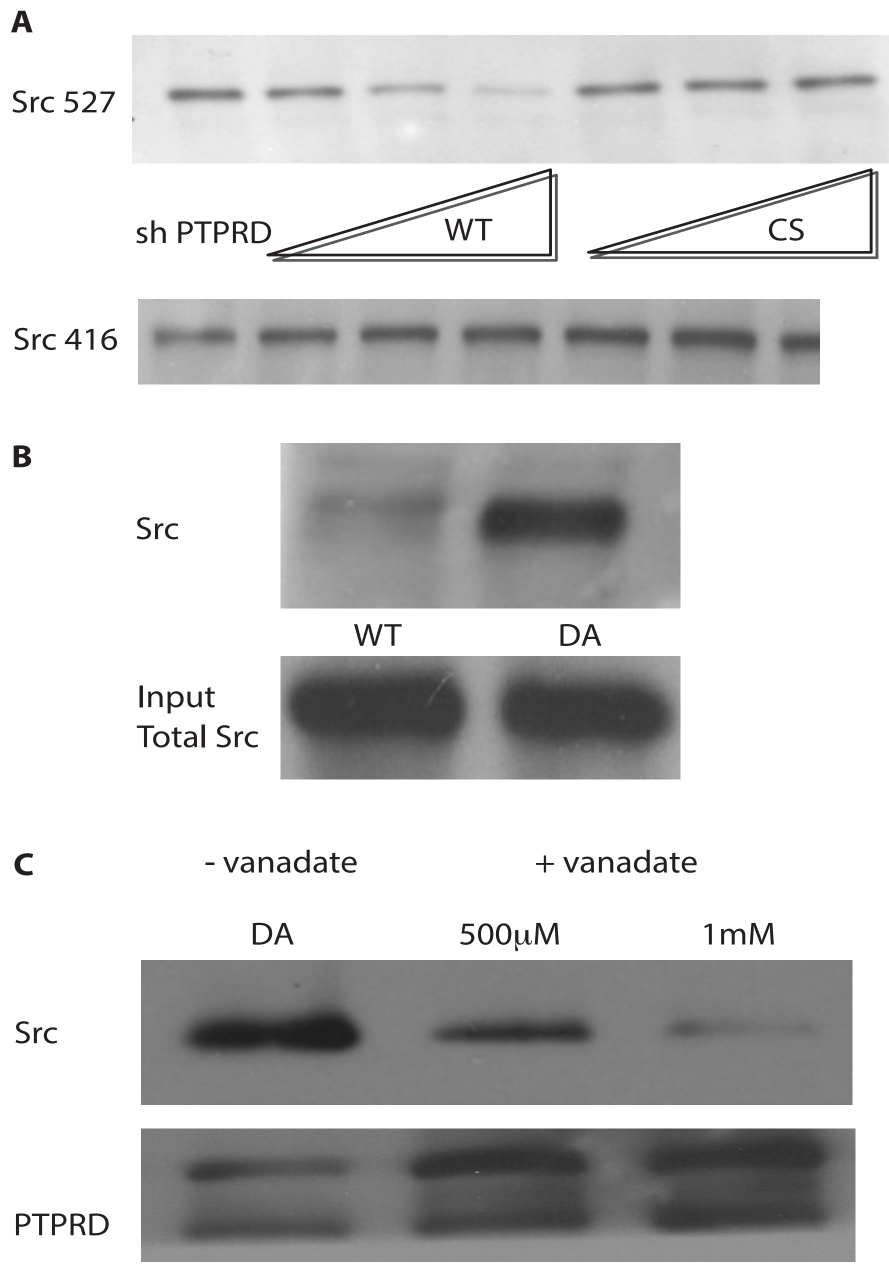


Figure 2-8. Identification of SRC as a direct substrate of PTPδ

(**Figure 2-8**. Identification of SRC as a direct substrate of PTPδ)

(A) SRC was immunoprecipitated from lysates from MCF10A cells treated either with shPTPRD (for enhanced phosphorylation of Tyr 527) or shMIM (for enhanced phosphorylation of Tyr 416). The immunoprecipitates were treated with recombinant PTPδ, either active wild type (WT) or inactive (CS) mutant proteins, as indicated. Proteins were resolved by SDS-PAGE and immunoblotted using phospho-specific antibodies to Tyr 527 and Tyr 416. **(B)** MCF10A cells expressing WT and DA mutant were treated with 50 µM pervanadate for 30 minutes. PTPδ was immunoprecipitated, then protein complexes were analyzed by SDS-PAGE and immunoblotted with anti-SRC antibody. **(C)** Immunoblot analysis of the association of SRC with PTPδ substrate trapping mutant from cell lysates prepared in the absence and presence of vanadate (500µM and 1mM).

CHAPTER 3

3.1 MIM and actin cytoskeleton

3.1.1 Introduction

The actin cytoskeleton is a key regulator of cell morphology and migration [142]. Consequently, it is crucial for embryonic development and many physiological functions in adult animals. Aberrations in the function of the actin cytoskeleton are also central in cancer, and many actin-binding proteins have been directly linked to the metastatic behavior of cancer cells [142-144].

Processes mediated by the actin cytoskeleton, such as the formation of plasma membrane protrusions during cell migration, are often closely linked to direct membrane remodeling [145]. Recent studies demonstrated that the members of an extended protein family, characterized by the presence of a membrane binding and deforming BAR (Bin–Amphiphysin–Rvs) domain, function at the interface between the actin cytoskeleton and plasma membrane during the formation of membrane protrusions or invaginations [146,147]. These proteins can either generate positive membrane curvature to facilitate the formation of plasma membrane invaginations (e.g. BAR, N-BAR and F-BAR domain proteins) or induce negative membrane curvature to promote the formation of plasma membrane protrusions (I-BAR and IF-BAR domain proteins) [148,149].

Missing-in-metastasis (MIM/MTSS1), an actin and membrane binding protein, was first identified as a tumor suppressor, which is downregulated in bladder carcinoma cell lines with increased metastatic potential [124]. Subsequent studies also demonstrated decreased expression of MIM in other cancer types [125,150]. In mice, MIM is expressed in various tissues and its expression varies according to the developmental stage. MIM is composed of a C-terminal actin-monomer-binding WH2 (WASP homology 2) domain and an N-terminal I-BAR (inverse BAR) domain, also known as an IRSp53/MIM homology domain (IMD) [129,130,151]. The I-BAR domain was originally proposed to bundle actin filaments and thus induces the formation of filopodia-like plasma membrane protrusions when expressed in cells [130]. It was later discovered that I-BAR domain does not possess actin filament bundling activity but rather binds to membranes rich in phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2], which it then deforms into tubular structures in vitro [131,148,152]. MIM was recently also implicated in the Sonic hedgehog (Shh) signaling pathway [132]. Shh is a morphogen that regulates many developmental processes. Aberrations in Shh signaling has been linked to many cancers [153]. MIM was characterized as a Shh-responsive gene in both normal tissues and basal cell carcinomas, and was initially proposed to increase Gli-mediated transcription through direct interactions with Gli1 and Gli2 transcription factors. MIM was also shown to regulate ciliogenesis by antagonizing SRC-dependent phosphorylation of cortactin [132,133,154]. This provided a link between MIM expression levels and metastatic potential of certain cancer types.

3.1.2 WH2 domain in MIM

The WH2 (WASP homology domain-2) is a small actin monomer-binding motif and is found in many proteins that regulate the actin cytoskeleton, including the β-thymosins, ciboulot, WASP, and verprolin/WIP (WASP-interacting protein). Recombinant MIM protein interacts with actin monomers and inhibits actin filament nucleation in vitro. Overexpression of mouse MIM in NIH 3T3 cells results in the decrease of actin stress fibers and formation of abnormal actin filament structures. This suggests that MIM is an actin binding protein that regulates cytoskeletal dynamics.

3.1.3 IMD domain

Missing-in-metastasis (MIM) and insulin receptor tyrosine kinase substrate p53 (IRSp53) form part of a new family of actin cytoskeleton adaptor proteins [155]. Like most actin binding proteins, MIM and IRSp53 are multidomain proteins, containing protein-protein interaction modules, involved in signaling and localization, and structurally conserved actin binding motifs.

MIM is a modular protein. Its actin binding function can be attributed to two spatially separated actin-binding domains: an N-terminal 250 aa IRSp53/MIM homology domain (IMD) and a C-terminal 30 aa WASP-homology domain 2 (WH2) [148]. The 475 aa central region sandwiched in between these two actin-binding domains is rich in Pro, Ser, and Thr residues. This region appears to play regulatory/scaffolding roles; it binds

receptor protein tyrosine phosphatase δ (RPTPδ) [156], the transcription factor Gli and the tumor suppressor Sufu [154], and the SH3 domain of cortactin [134], a protein implicated in the nucleation and stabilization of Arp2/3-mediated filament branches [157,158].

Despite low sequence similarity, the IMDs of MIM and IRSp53 are structurally similar and, therefore, may bind actin and Rac in a similar manner. The structure of the IMD is generally related to that of the BAR (Bin-Amphiphysin-Rvs) domain, a fold involved in membrane binding [159]. However, the overall shape of the two domains is markedly different, which probably explains their different roles in membrane curvature sensing. The WH2 domain of MIM is unusual, both because of its localization in the protein and the way in which it interacts with actin.

3.1.4 MIM, actin and Rac

Overexpression of MIM inhibits cell motility in a manner depending on interaction with cortactin and G-actin. MIM may be implicated in cell motility by modulating different actin binding proteins such as cortactin.

MIM expression is regulated by Sonic hedgehog (Shh) and MIM expression increases during development in the hair follicles of skin as well as in basal cell carcinomas induced by Shh expression [154]. This regulation implies that levels of MIM are likely to be controlled during developmental program in various tissues and also that

MIM expression may be altered in cancer cells, leading to changes in the signaling and architecture of the cytoskeleton.

MIM’s interaction with the actin cytoskeleton and with Rac of the Rho GTPase family is its potential link to cancer metastasis. MIM-B is widely expressed and its expression maintained in various metastatic cell lines. MIM activity towards the actin cytoskeleton requires its IMD and Rac interaction. MIM induces actin-rich membrane protrusions through the activation of Rac, potentially acting as a scaffold protein to recruit Rac effectors and organise actin assembly. When MIM is overexpressed in serum-starved tissue culture cells, it induces a ruffling response, and it induces Rac activation.

3.2 MIM and EMT

The reversible transitions between two phenotypic states including epithelial and mesenchymal is EMT. This change or plasticity is enabled by underlying shifts in epigenetic regulation, pleiotropic transcription factors that culminate into expression of key target genes. These regulators depend on complex epigenetic mechanisms such as induction of changes in the modifications of chromatin-associated histones, in order to achieve the changes in gene expression observed during epithelial-mesenchymal transitions (EMTs). These associations indicate that an understanding of the functional interactions between EMT will provide crucial insights into the fundamental mechanisms

underlying cancer progression and may, in the longer term, generate new diagnostic and therapeutic modalities for treating high-grade malignancies.

As discussed in the previous chapters, metastasis suppressors might provide us with targets for combinatorial therapies. These suppressors, like tumor suppressors might provide new avenues for treatment in case of metastatic cancers. Hence we decided to check the effect of MIM on epithelial to mesenchymal transition. These effects might provide us with insights as to the significance of MIM in the process of metastasis and the therapeutic niche that can be targeted.

3.3 RESULTS

3.3.1 Loss of MIM promotes aberrant actin dynamics

The decrease in cell adhesion or cell scattering induced by loss of MIM may be due to aberrant actin dynamics at cell-cell junction. To investigate the effect of loss of MIM on actin, we performed staining using rhodamine phalloidin. In a confluent layer of shluc, F-actin was organized at cell-cell junction to form cortical actin, whereas shMIM cells had loosely organized F-actin (Figure 3-1)

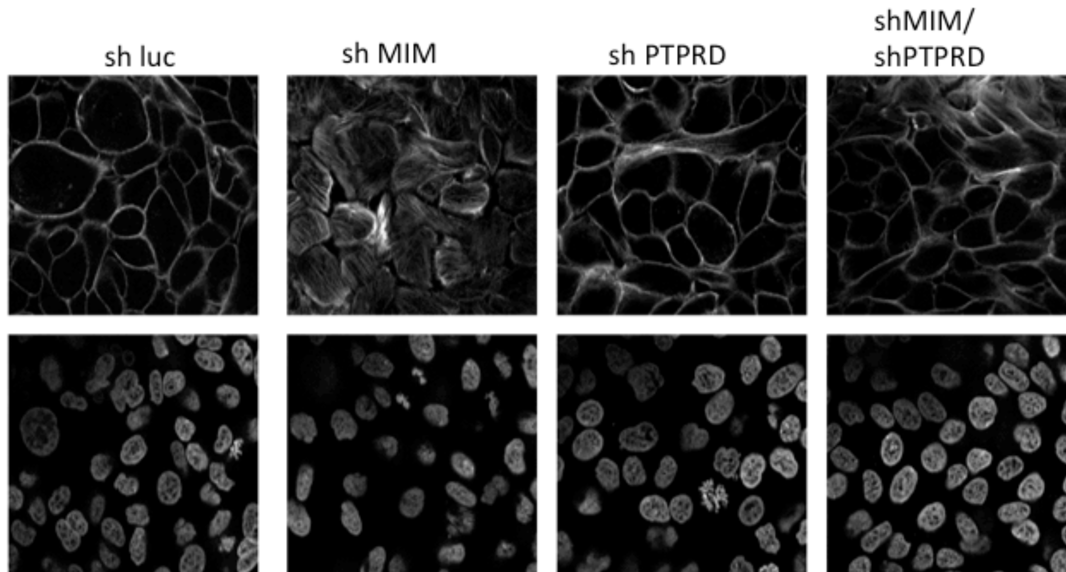


Figure 3-1 MIM suppression induced actin stress fiber formation.

MCF10A cells were stained for actin using Rhodamine-Phalloidin antibody. The top panel shows actin staining while the bottom panel shows staining for nucleus (DAPI)

3.3.2 Loss of MIM activates Rac signaling

MIM is known to interact with Rac GTPase. constitutive activation of Rac induces disruption of E-cadherin junctions by promoting aberrant actin remodeling [160]. To monitor changes in Rac-GTP levels in MIM shRNA cells we used p21-activated kinase (PAK)-binding domain (PAK-PBD)-pulldown assays [161]. Cells lacking MIM had a marked increase in the levels of Rac–GTP levels under when compared with the levels present in shluc cells (Figure 3-2)

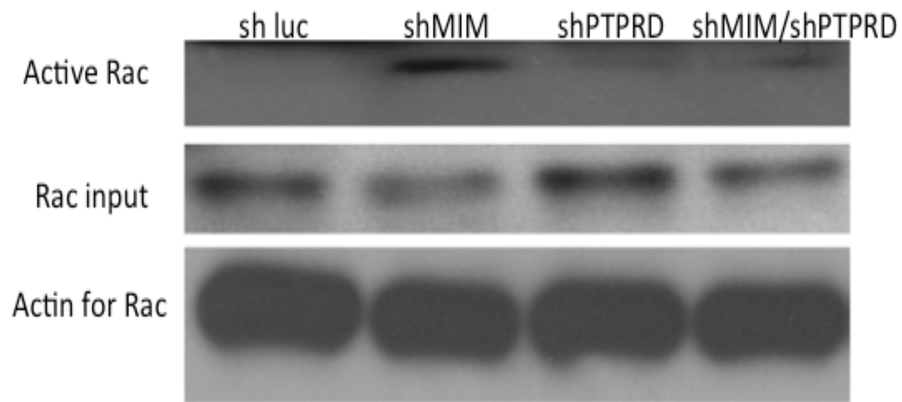


Figure 3-2 MIM suppression induced Rac activation

Cell lysates were made from MCF10A cells expressing the indicated short hairpins. PAK binding domain was used to monitor the Rac-GTP levels and probed with anti-Rac antibody. Equal amounts of protein was loaded. Actin was used as a loading control.

3.3.3 Loss of MIM induced a decrease in E-cadherin levels

Epithelial-to-mesenchymal transition is a definitive feature of aggressive metastatic breast cancer. Initiation of metastasis requires cellular invasion which is enabled by EMT. E-cadherin repression helps carcinoma cells lose cell-cell adhesion and it facilitates breaking through the basement membrane. We analyzed the expression levels and localization of E-cadherin upon suppression of MIM. Our immunofluorescence and QPCR analysis indicates that E-cadherin levels are significantly reduced in MIM knockdown cells (Figure 3-4 and 3-6).

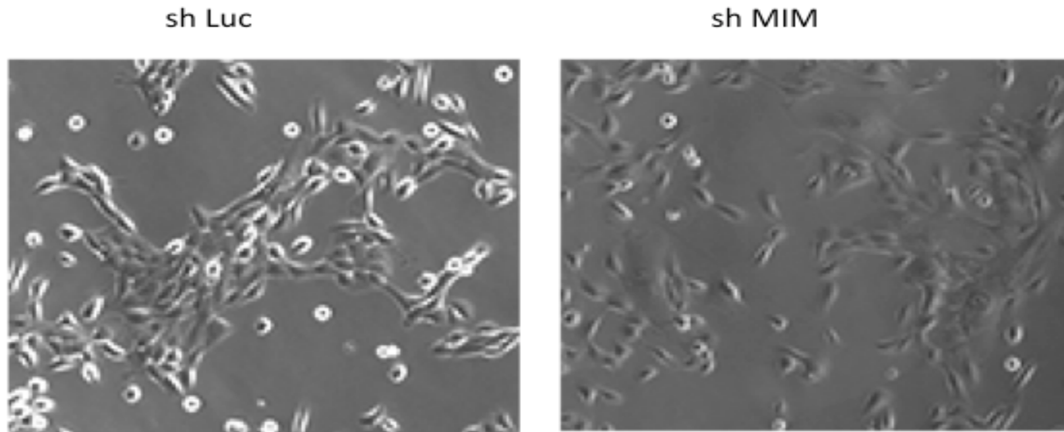
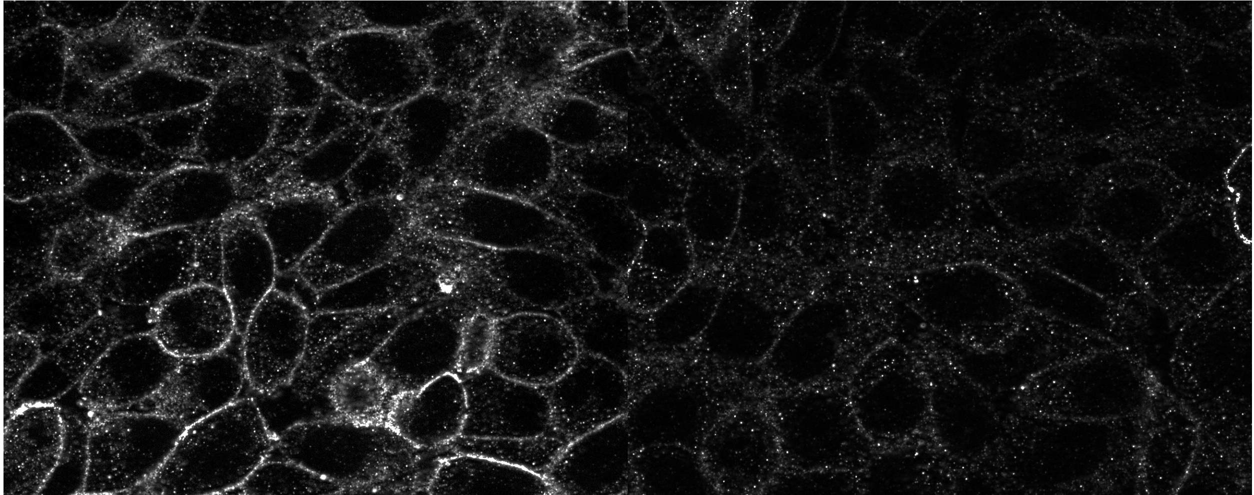


Figure 3-3 Loss of MIM induces EMT in MCF10A cells.

Morphology of MCF10A cells stably expressing the luciferase short hairpin and MIM short hairpin.



sh luc **shMIM**

Figure 3-4 Immunoflourescence staining using E-cadherin antibody.

shluc cells show an intense staining of E-cadherin which is localized to the membrane whereas MIM suppression leads to a diffused staining pattern.

3.3.4 Loss of MIM induced an increase in vimentin levels

Vimentin, a major constituent of the intermediate filament family of proteins, is ubiquitously expressed in normal mesenchymal cells and is known to maintain cellular integrity and provide resistance against stress. Vimentin is overexpressed in various epithelial cancers, including prostate cancer, gastrointestinal tumors, tumors of the central nervous system, breast cancer, malignant melanoma, and lung cancer. Vimentin's overexpression in cancer correlates well with accelerated tumor growth, invasion, and poor prognosis. Vimentin has been recognized as a marker for epithelial-mesenchymal transition (EMT). Although EMT is associated with several tumorigenic events, vimentin's role in the underlying events mediating these processes remains unknown. We analyzed the expression levels of Vimentin in MCF10A cells upon loss of MIM. Vimentin levels were significantly increased when analyzed using QPCR and Immunofluorescence (Figure 3-5, 3-6). This provides an evidence that absence of MIM leads to an EMT phenotype in MCF10A cells.

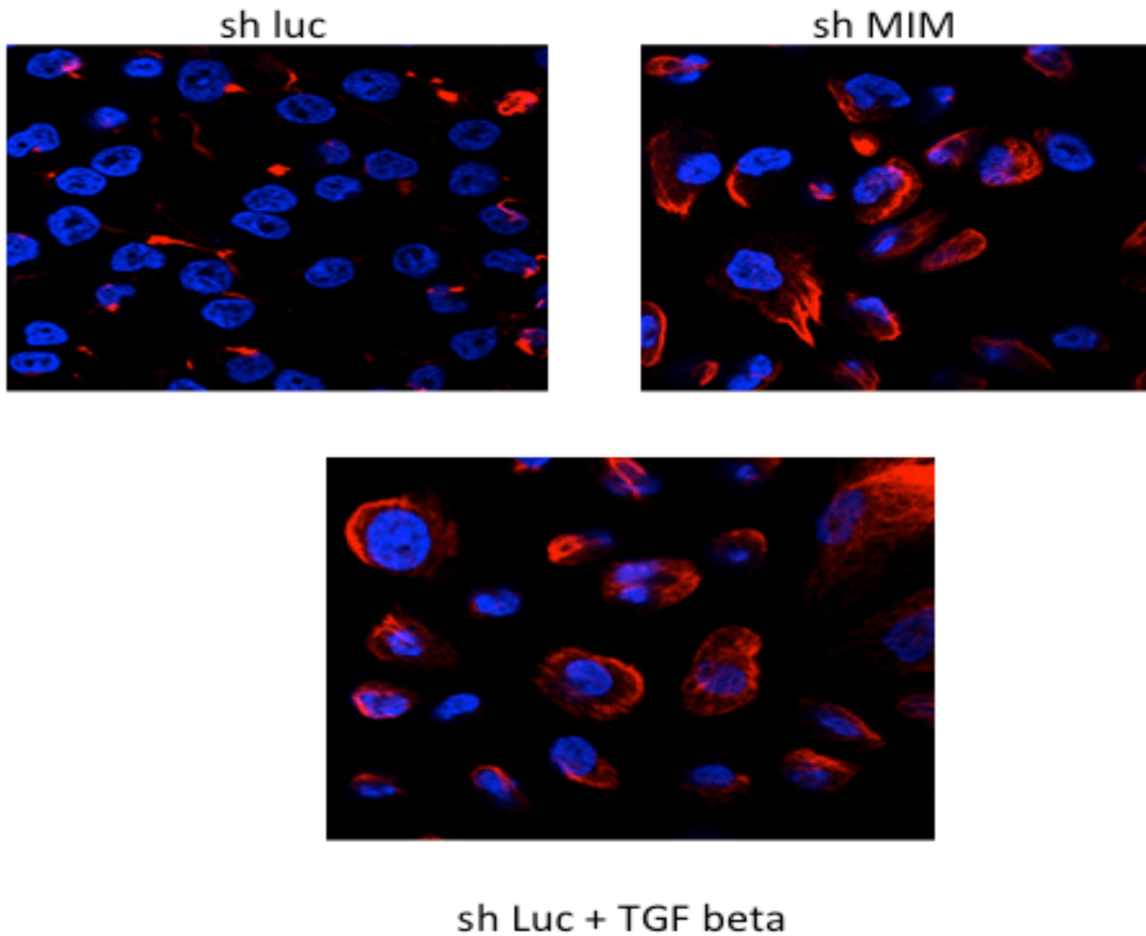


Figure 3-5 Immunofluorescence images using Vimentin antibody (Red) is shown.

sh MIM shows an increased vimentin staining when compared to shluc cells. Control cells where TGF beta induces an EMT phenotype were stained after 48 hours of treatment. Nuclei are stained with DAPI (Blue).

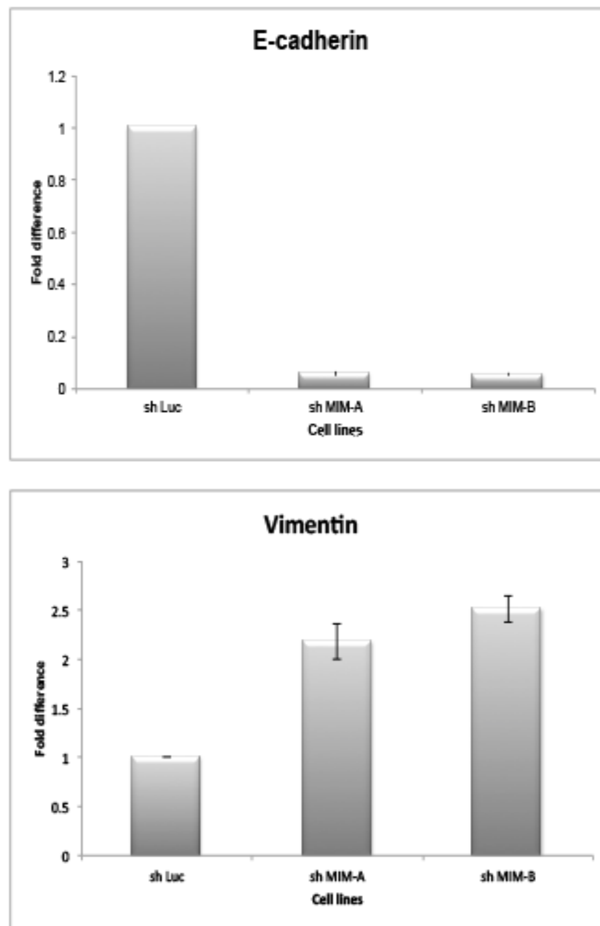


Figure 3-6 Quantitative PCR analysis indicating mRNA expression levels of E-cadherin and Vimentin.

RNA was extracted from MCF10A shluc and shMIM cells and used to prepare cDNA. Specific primers were used to analyze the levels of E-cadherin and Vimentin and the expression levels were normalized to actin.

3.3.5 Loss of MIM increased the levels of SNAIL transcription factors

Of the three vertebrate SNAIL proteins, SNAIL1 (also known as SNAIL) and SNAIL2 (also known as SLUG) activate the EMT programme during development and cancer. They repress epithelial genes by binding to E-box DNA sequences through their carboxy-terminal zinc-finger domains. Multiple signaling pathways cooperate in the initiation and progression of EMT and they often activate SNAIL1 expression. TGFβ, Notch, and growth factors that act through RTKs, all activate SNAIL1 expression depending on the physiological context. It also cooperates with the SMAD3–SMAD4 complex to cause the TGFβ-mediated repression of E-cadherin. We analyzed the expression of SNAIL1 in MCF10A cells and found through mRNA expression data that SNAIL is elevated in expression in MIM knockdown cells (Figure 3-7). This further corroborates our understanding that MIM induces Epithelial to mesenchymal transition in MCF10A cells

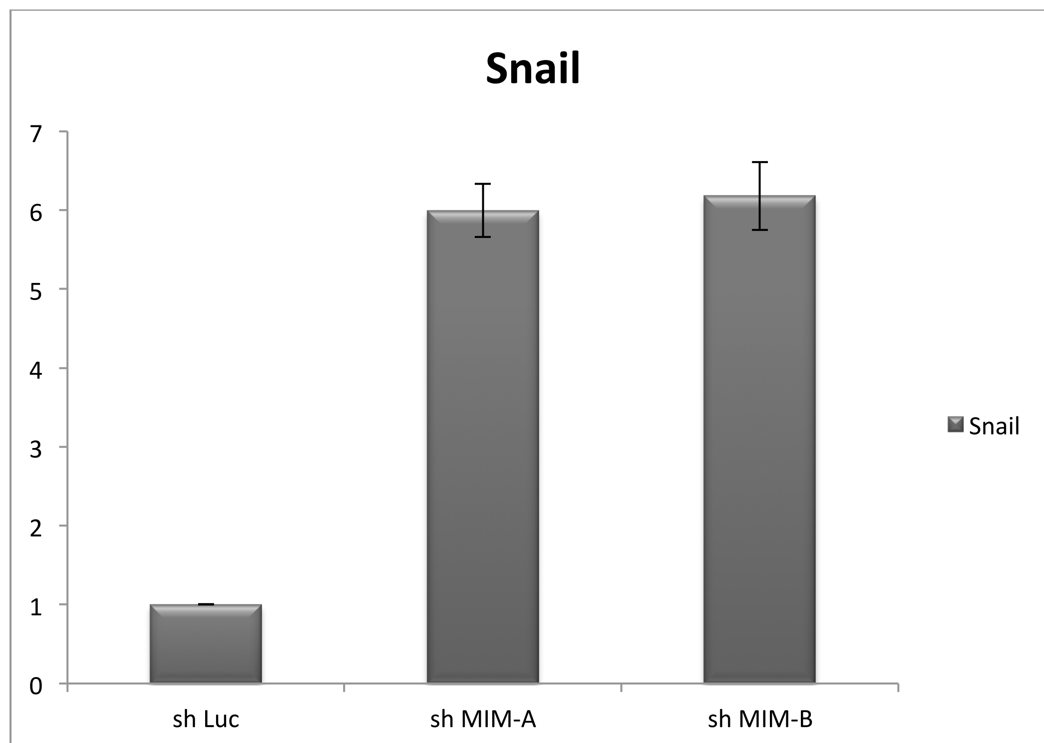


Figure 3-7 Quantitative PCR analysis indicating mRNA expression levels of SNAIL1

RNA was extracted from MCF10A shluc and shMIM cells and used to prepare cDNA. Specific primers were used to analyze the levels of SNAIL1 and the expression levels were normalized to actin.

CHAPTER 4

Discussion

Based on the data presented here, we propose a model that illustrates a novel mechanism by which MIM and PTPδ regulate breast epithelial cell motility and invasion (Figure 4-1). Our data indicate that in the absence of MIM, the stability of PTPδ was enhanced and the phosphatase accumulated, which in turn promoted dephosphorylation of Tyr 527, the inhibitory site in the C-terminal tail of SRC. This was associated with autophosphorylation at Tyr 416 and activation of SRC. This suggests that under normal conditions, MIM functions to suppress the levels of PTPδ and thereby to suppress SRC activation, motility and invasion of breast epithelial cells.

4.1 MIM as a metastasis suppressor

Metastasis is a major cause of morbidity and mortality in developed countries. Mortality associated with breast cancer has declined considerably due to early diagnosis and improvements in current therapies. Nevertheless, disease recurrence and the transition to metastasis still remain a challenge. MIM is widely expressed in human and mouse tissues, suggesting a potential to exert effects broadly in multiple cancer contexts. It was originally defined by the fact that its transcript was missing in metastatic cells, but not in the non-metastatic counterparts, with methylation of CpG islands thought to play an important role in silencing. Consequently, a biomarker such as MIM may prove to be a valuable tool for detection of metastasis. Furthermore, loss of MIM,

and the activation of PTPδ/SRC-dependent signaling, may define a signature that would permit specific targeting of that population of metastatic cell. However, expression analyses have revealed situations in which MIM may be down-regulated more generally in tumor cells, rather than exclusively in metastasis [128]. Nevertheless, the absence of MIM would still provide a defining signature that would facilitate specific targeting of such tumor cells.

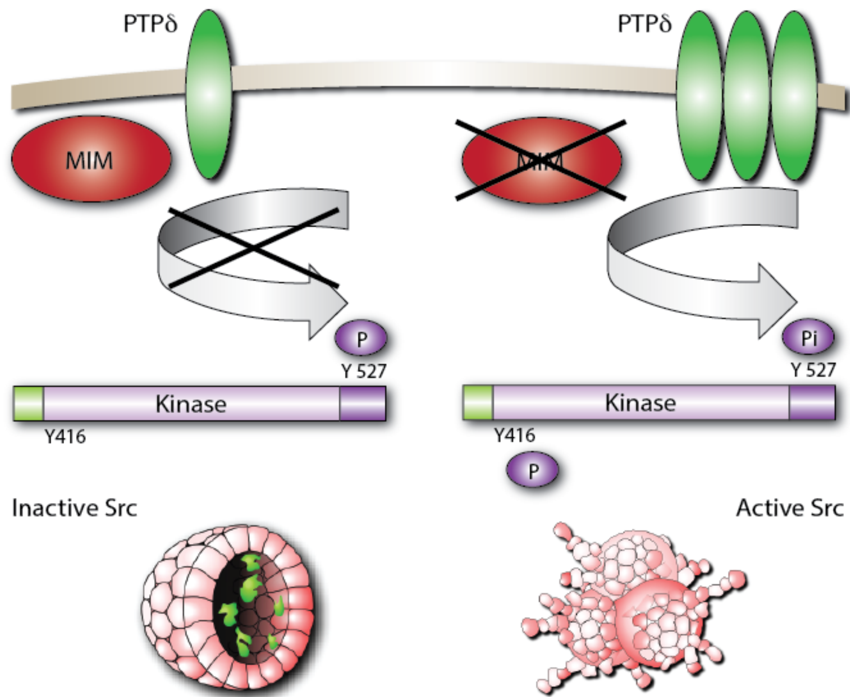


Figure 4-1 Model to explain the relationship between expression of MIM and PTPδ-induced activation of SRC

4.2 MIM regulates the stability of PTPδ

Although the mechanism by which the absence of MIM led to stabilization of PTPδ remains to be defined, a mechanistic precedent is provided by the regulation of MYC by AXIN1 [162]. MYC is a pleiotropic transcription factor that regulates proliferation, cell cycle and metabolism [163]. AXIN1 is a tumor suppressor and a scaffold protein that regulates protein complexes involved in multiple signaling pathways. It was recently reported that AXIN1 also promotes the degradation of MYC and thus regulates the expression of this oncoprotein [162,164]. Control of protein stability is a major determinant of the level of MYC expression, which is in turn controlled by its phosphorylation status in various malignancies. The stability of MYC correlates with increased phosphorylation at Ser62 and decreased phosphorylation at Thr58 [164]. The switch between phosphorylation of these residues is regulated by AXIN1. Whether the effects of MIM on PTPδ stability are associated with changes in phosphorylation status remains to be established, but the increased levels of PTPδ in MIM-depleted cells provide a direct link to tyrosine phosphorylation-dependent signaling and the switch to a more invasive state.

4.3 PTPδ as a SRC phosphatase

SRC is a critical regulator of signaling pathways that affect cell migration, adhesion and invasion. We have demonstrated that the enhanced cell migration and cell invasion that is a consequence of suppression of MIM resulted from stabilization of PTPδ which catalyzed the dephosphorylation and activation of SRC. As such, PTPδ

joins those members of the PTP family with the ability to regulate the activation status of SRC. PTP-mediated regulation of SRC is complex, due to their ability to control phosphorylation of both activating and inhibitory sites. Initially, expression of another receptor PTP, PTP-alpha, was shown to transform rat embryo fibroblasts through dephosphorylation and activation of SRC [48] . The prototypic receptor PTP, CD45, was also shown to act positively to promote antigen receptor signaling through dephosphorylation and activation of SRC family PTKs [35,165]. Nontransmembrane PTPs, including PTP1B [166] have also been reported to function as activators of SRC by dephosphorylating the C-terminal Tyr 527 residue. Other phosphatases, such as PTPN23 [167] and PTP-BAS [168], have been shown to dephosphorylate SRC at its autophosphorylation site, thus antagonizing SRC activity. Therefore, the members of the PTP family offer a mechanism for fine control over the signaling function of SRC. Overexpression of SRC is observed in many cancers, including breast cancer [169]; however, overexpression or hyperactivation of SRC in transgenic mouse models is not sufficient to induce a higher grade of breast tumor and metastasis [170]. In contrast, transgenic overexpression of SRC in a p21-/- background dramatically induces tumor growth and metastasis, suggesting that the combination with a second hit may augment the transforming activity of SRC [171] . Considering the positive effect of PTPδ on SRC activity, loss of MIM may promote tumor growth and metastasis particularly in breast tumors in which SRC is overexpressed.

4.4 PTPδ as a tumor suppressor or oncogene

When one considers the prevalence of PTKs as oncoproteins, it was anticipated that PTPs, through their catalysis of the complementary dephosphorylation step, would serve as tumor suppressors. Now we know that several PTPs exert a tumor suppressor function. In fact, detailed sequence analyses of members of the PTP family have revealed a wide variety of mutations in PTP genes in various cancers[172]. The *PTPRD* gene, encoding PTPδ, is one such example [74,75]. In this case, in addition to mutations that would be predicted to inactivate catalytic function, the clustering of mutations in portion of the gene encoding the extracellular segment of the protein highlights the potential importance of interactions with ligands in the regulation of activity. Nevertheless, it is also now apparent that PTPs have the capacity to function positively to promote signaling. Furthermore, aberrant upregulation of PTP genes has been detected in multiple cancers, indicative of an oncogenic function [35,165]. An excellent example is the SH2 domain-containing PTP SHP2, which is encoded by the *PTPN11* gene. SHP2, which normally facilitates RAS activation, exists in a low activity state under basal conditions and is activated following interaction of its SH2 domains with pTyr residues on proteins that target the PTP to signaling complexes. Activating somatic mutations in *PTPN11*/SHP2 allow the enzyme to adopt the active conformation in the absence of the normal stimulus and have been associated with hyperactivation of MAPK and other signaling pathways and increased risk of sporadic childhood malignancies, such as juvenile myelomonocytic leukemia and acute myeloid leukemia [46]. Another example is the *PTP1B* gene, which is located at chromosome 20q13, a region that is frequently amplified in breast cancer and associated with poor prognosis.

It has also been reported that PTP1B is over-expressed in breast tumors together with the oncoprotein tyrosine kinase HER2. Mice expressing activated alleles of HER2 in mammary glands develop multiple mammary tumors and frequent metastases to the lung; however, when such mice were crossed with *PTP1B*-knockout mice, tumor development was delayed and the incidence of lung metastases was decreased [173,174]. Conversely, targeted overexpression of PTP1B alone was sufficient to drive mammary tumorigenesis in mice [173], illustrating that it can play a positive role in promoting signaling events associated with breast tumorigenesis. A similar tumor promoting role was suggested in prostate cancer [175]. Nevertheless, SHP2 functions as a tumor suppressor in cartilage [176] and PTP1B has also been shown to exhibit tumor suppressive effects, for example in lymphomagenesis in a p53-deficient background [177]. Consequently, there is precedent for members of the PTP family to function as a tumor suppressor, a tumor promoter or both, depending upon context.

To date, somatic mutations in the *PTPRD* gene have been reported in diverse tumors [74,178] and germline mutations of *PTPRD* have been found in metastatic Ewing sarcoma [179]. Also, PTPδ has been reported to exhibit tumor suppressor activity through inhibition of STAT3 activation [178]. Nevertheless, there have been conflicting reports of both tumor suppressor and tumor promoter functions of PTPδ in neuroblastoma [178,180]. Our study is consistent with a tumor promoting function of PTPδ through dephosphorylation and activation of SRC. Data from the Human Protein Atlas, which indicated elevated expression of PTPδ in tumor samples from patients, compared to normal breast tissue, are also consistent with such a positive role in the

regulation of signaling in breast cancer cells. This raises the exciting possibility that such positively acting PTPs as PTPδ may prove to be important therapeutic targets for new ways to intervene in cancer.

4.5 Therapeutic implications and conclusions

SRC has been established as a critical regulator of multiple signaling pathways involved in cell proliferation, survival, angiogenesis and metastasis [181]. Elevated levels and activity of SRC protein have been reported in several cancers, including breast cancer, and the extent of overexpression and hyperactivation correlates with metastatic potential, particularly in colon and breast cancer [182]. In addition, SRC activation has been associated with increased signaling through growth factor receptors, GPCRs or hormone receptors and influences multiple stages of tumor growth and progression [183]. Consequently, multiple SRC inhibitors are currently being assessed as therapeutics [29]. Although these approaches show potential, all PTK-directed therapies have encountered the problems of limited response and acquired resistance [30], and combinatorial approaches are being considered to try and overcome this. Combinatorial therapies involving SRC inhibitors and chemotherapy are in trials in various settings of metastatic cancer [31]; however, combination strategies that facilitate a more targeted intervention in particular signaling pathways may be of greatest benefit. In addition, it will be extremely important to identify those patient populations that would benefit most from SRC-directed therapies. Overall, this study suggests that patients with tumor or metastatic lesions defined by loss of MIM may be

one such population. Furthermore, our work suggests that targeting such metastases with combinations of inhibitors of SRC and PTPδ may be more effective in abrogating the signals that underlie aberrant cell invasion than targeting either the PTK or the PTP alone. Although several members of the PTP family have been validated as therapeutic targets, they remain underexploited in large part due to the challenge of developing active site-directed inhibitors with drug development potential. Nevertheless, recent studies have highlighted the potential importance of dimerization in the inactivation of receptor PTPs [184,185]. Consequently it may be possible to design agents that target the extracellular segment of RPTPs and antagonize RPTP function indirectly via regulation of dimerization. This raises the possibility of producing therapeutic agents that act via the extracellular segment of PTPδ, which may offer a new approach, together with inhibitors of SRC, to targeting specifically the MIM-depleted population of metastatic cells.

4.6 MIM and actin cytoskeleton

The aberrant increase in Rac–GTP activity was required for MIM-loss-induced effects on cortical actin. Thus, loss of MIM promotes Rac activation, which leads to reorganization of actin dynamics. This aberrant actin dynamics probably decreases cell adhesion between epithelial cells and promotes invasion.

We speculate that, in addition to loss of MIM, unregulated activation of Rac can promote aberrant actin remodelling and compromise cell–cell cohesion to promote invasive behavior of epithelial cells. Elevated expression levels of Rac have been

observed in invasive cells [186,187]. Understanding the mechanisms of actin regulation during tumor progression will provide new insights into tumor cell behavior.

CHAPTER 5

Future Directions and Concluding Remarks

5.1 MIM-/- tumors

The most deadly aspect of cancer is its ability to metastasize from a primary tumor to secondary sites. Metastasis suppressors have provided us with new tools for investigating the multiple points at which cancer can be attacked [112]. Therefore, it is crucial to identify the mechanisms and functional consequences of MIM's action and understand the implications of MIM expression in cancers including breast cancer.

There remains several questions about function of MIM. Our study is amongst a few which shows an invasion phenotype in cells where MIM is depleted. Loss of MIM has been shown to be an important marker for metastatic cancer both using cell lines and tumor samples. Deciphering the signaling changes that occur upon MIM depletion might provide us with targets for therapeutic intervention. In our study, we show that suppression of MIM led to increase in PTPδ expression and stability. This change in PTPδ led to activation of SRC which is already an important target for cancer therapy.

5.2 SRC inhibitors

Despite improvement of overall survival and better control of early stage disease of patients with breast cancer, the incidence of metastasis continues to increase. We propose that MIM regulates invasion through PTPδ and SRC. SRC inhibitors have been

studied in the context of metastatic cancers [188]. Unfortunately there is no effective therapy for metastasis and the occurrence is essentially due to relapse or lack of diagnosis of metastasis. SRC activation has been shown to be critical in promoting brain metastasis in breast cancer. Combinatorial regimens targeting SRC is proven to treat HER2 positive brain metastasis. SRC activation promoted tumor cell extravasation into brain via permeabilization of blood brain barrier. In combination with EGFR/ Her2 dual targeting drug lapatinib, combinatorial regimen prevented outgrowth of disseminated breast cancer cells leading to longer survival of mice with metastasis. With these evidences and activation of SRC in our model system, we think that combinatorial inhibition of SRC and PTPδ in breast cancers which are MIM-/- might be a more effective therapy for metastatic setting.

Experimental data with small-molecule inhibitors of SRC in breast cancer are encouraging, demonstrating effective suppression in vitro of tumor growth and invasion and inhibition of disease spread in preclinical models in vivo. Recently several SRC inhibitors have been tested in Phase I clinical trials where they are well tolerated and demonstrate low toxicity. Overall, these findings suggest that SRC inhibitors present a future therapeutic option in the treatment of breast cancer growth and progression.

Overexpression of human epidermal growth factor receptor-2 (HER2 or ERBB2) is associated with poor clinical prognosis and survival in breast cancer. Treatment with Herceptin (trastuzumab), a humanized antibody that targets HER2, in patients with HER2-positive breast cancer shows clinical benefit; however, many patients do not respond to treatment due to either de novo or acquired resistance [189]. Since cancer cells developing trastuzumab resistance are heterogenous, management of patients

with HER2-positive breast cancer remains clinically challenging. Activation of the non-receptor tyrosine kinase SRC seems to be a common element into both the de novo and acquired mechanisms of trastuzumab resistance [190]. Trastuzumab-resistant, HER2-overexpressing breast cancer cells show SRC activation which is mediated by increased phosphorylation at Tyr 416 and occurs downstream of multiple receptor tyrosine kinase pathways and plays a critical role in acquired trastuzumab resistance. SRC is also shown to interact directly with the protein phosphatase PTEN, and PTEN loss in breast cancer cells results in SRC phosphorylation and development of de novo trastuzumab resistance [191]. Importantly, in both in vitro and in vivo models, treatment with saracatinib, an orally available small molecule inhibitor of SRC, sensitized trastuzumab-resistant cells and tumors to trastuzumab treatment. Analysis of primary breast tumors further correlated relative increases in SRC activity with clinical resistance to trastuzumab. These results suggest that combined treatment with both trastuzumab and SRC inhibitor can overcome resistance and provide more effective therapy for patients with HER2-overexpressing breast cancer.

As there is precedence to developing drug resistance in cancer cells due to SRC activation, our study also implies that combining inhibition of SRC and another activating factor (in our case PTPδ) might provide a more robust way to hit metastatic cells in breast cancer.

5.3 PTPs as anticancer targets

Aberration of the normal balance between PTK and PTPs has been linked to cancer. Abnormal expression and activation of PTKs such as EGFR and Her2, SRC and Abl have been shown to be pro-oncogenic. On the other hand, PTPs are often viewed to reverse the PTK action and therefore sometime considered as tumor suppressor genes. PTEN was the first tumor suppressor gene encoding a phosphatase in the PTP superfamily, is mutated in a wide variety of cancers [192]. Subsequently other PTPs, including RPTPρ, RPTPγ, LAR, PTPH1, PTP-BAS, and PTPD2 were found to be mutated and shown to have tumor suppressor functions. Interestingly, PTPs can also potentiate and amplify actions of protein tyrosine kinases. This mode of synergy enhances mitogenic signaling, leading to cell transformation.

As discussed in the Introduction, SHP2 has been shown to play a positive role in a number of growth factor mediated signaling pathways and the phosphatase activity of SHP2 is required for the Ras-dependent cellular proliferation and survival [193]. A number of activating mutations in SHP2 have been identified as the cause of the inherited disorder Noonan syndrome, leukemia and solid tumors. PRL (phosphatase of regenerating liver) phosphatase have been implicated as potential oncogene that promote cell growth and tumor invasion [194].

It appears that cellular pathways regulated by tyrosine phosphorylation could offer a source of drug targets for developing novel therapeutics. Development of small molecule inhibitors has revolutionized cancer therapy and the way we approach this problem. Small molecule inhibitors that block the activity of a narrow spectrum of PTKs

exhibit much less toxicity than the currently used chemotherapeutic agents. The potential of such targeted therapeutics has been well demonstrated by the successful treatment of chronic myelogenous leukemia with imatinib mesylate and non-small cell lung cancers with gefitinib, which target Bcr/Abl and epidermal growth factor receptor (ErbB1) aberrantly activated in the malignancies, respectively.

Given the critical role of PTPs in cellular signaling and the fact that deregulation of PTP activity also contributes to the pathogenesis of a number of cancers, inhibitors of the PTPs are also expected to have therapeutic value. Furthermore, since no functional overlap exists between the PTKs and PTPs, i.e. a PTP may catalyze the dephosphorylation of substrate proteins phosphorylated by more than one PTK, inhibitors of the PTPs may also have unique modes of action. Thus, PTPs represent novel and attractive targets for cancer treatment.

PTP1B is a ubiquitously expressed phosphatase that appears to be involved in the regulation of several growth factor signaling pathways. Besides a role in insulin and leptin signaling, PTP1B may also be involved in several other physiological and pathological processes. For example, PTP1B is capable of catalyzing the dephosphorylation of activated epidermal growth factor receptor and platelet-derived growth factor receptor in fibroblasts, although PTP1B–/– mice show no obvious evidence of increased epidermal growth factor receptor or platelet-derived growth factor receptor activity. In addition to its effects on receptor tyrosine kinase itself, PTP1B may play positive (signal enhancing) roles downstream of growth factor receptors and integrins.

PTP1B can remove the inhibitory tyrosyl phosphorylation site on SRC family kinases in human breast cancer cell lines or in response to integrin signaling in fibroblasts, thereby promoting SRC family kinase activation. Since PTP1B can promote both the SRC kinase and the Ras/Erk pathways, which constitute major components of HER2/*Neu* signaling, PTP1B may represent a new therapeutic target in breast cancer.

Interestingly, PTP1B is up-regulated in HER2/*Neu*-transformed cell lines and 90% breast tumors overexpress Her2 and PTP1B. PTP1B is required for HER2/*Neu*-induced breast cancer and that PTP1B deficiency delays HER2/*Neu*-induced mammary tumorigenesis and protects from lung metastasis. Moreover, PTP1B has also been shown to contribute to the oncogenic properties of colon cancer cells through SRC activation [195]. These findings imply that inhibition of PTP1B may be beneficial for the treatment of breast and colon cancer [196].

Protein phosphatases represent 4% of the druggable genome. It is now realized that specific PTPs can function as oncogenes that contribute positively to the initiation and maintenance of human cancer. Potential PTP targets for novel anticancer drug discovery are identified in all four classes of PTPs. While catalytic pockets of many PTPs are considered challenging for drug discovery efforts, appropriate surface properties surrounding PTP catalytic sites do exist that allow development of potent and selective inhibitors for many PTPs. This notion has been supported by the identification of a number of highly potent and selective inhibitors to specific PTPs.

Although progress has been made, the field of PTP inhibitor development is in its infancy. Target identification and validation in vitro and in vivo, lead discovery and

optimization, identification of specific substrates in vivo and surrogate markers for assessment of target inhibition. Pharmacologic profiling and selective toxicity analysis all require great efforts. The realization that PTPs do not simply counter the function of PTKs, but could cooperate with PTKs to promote oncogenesis should attract great interests in developing PTP inhibitors for cancer therapy in the coming years. Given the precedence and significance of inhibition of phosphatases, our study suggests inhibition of PTPδ for metastatic cancer would be worth looking into.

5.4 Inhibition of RPTPs

Researchers have developed multiple strategies for interfering with RTK signaling. These include a small molecule called lapatinib and monoclonal antibody called Herceptin that binds to Her2. Trastuzumab binds to domain IV of the [197] extracellular segment of the HER2/neu receptor[197]. Cells treated with trastuzumab undergo arrest during the G1 phase which effects to reduced proliferation. It has been suggested that trastuzumab induces some of its effect by downregulation of HER2/neu leading to disruption of receptor dimerization and signaling through the downstream PI3K cascade. Similarly dimerization of RPTPs provides a potential mode of regulating their activity. RPTPs have traditionally been considered to be inactive when dimerized and active when monomers [185]. Studies revealed that RPTPa exists as dimers on the cell surface and that this dimerization inhibits the activity of the phosphatase via the interaction of the catalytic domains which prevents the binding of substrates. This suggests a model where RPTPs exists as inactive dimers on the cell surface in the

absence of a ligand and binding of ligand dissociates the dimers and activates the PTP. So one way to inhibit PTPδ might be through inducing dimerization. At least it's a possibility worth exploring.

CHAPTER 6

Materials and Methods

Antibodies

Anti-PTPd antibody was from Novus Biologicals. Stained tissue sections in the Human Protein Atlas were generated using the same antibody. Antibodies to SRC-pTyr 527, SRC-pTyr 416 and total SRC protein, as well as antibodies to MIM, were from Cell Signaling Technology.

Cell culture

MCF-10A cells were obtained from ATCC (Manassas, VA) and cultured in Dulbecco's modified Eagle medium (DMEM)–F-12 (Invitrogen) supplemented with 5% donor horse serum, 20 ng/ml epidermal growth factor (EGF), 10 µg/ml insulin, 100 ng/ml hydrocortisone, 100 ng/ml cholera toxin, 100 U/ml penicillin, and 100 µg/ml streptomycin. Growth factor-reduced Matrigel was purchased from BD Biosciences.

Generation of cells expressing shRNA targeting MIM and PTPδ

For stable suppression of MIM in MCF10A cells, we expressed a pMLP retroviral vector (in a pMSCV backbone) using the targeting sequences TCTTCTGCAGCTTCAGCGT and TCTTTTTGATCTCATGCCG incorporated into the sequence of the human microRNA-30 (miR30). The infected cells were selected using puromycin (1-2 μg). For double selection, *PTPRD* shRNA, using the targeting sequence TGCATACATCTTAGACTCT, was subcloned in pMSCV hygro and selected using

hygromycin (100 µg/ml). pcDF1-PTPRD (plasmid 25642) was ordered from Addgene. Infections were carried out as previously described [74]. The GST- PTPd fusion construct in pGEX vector was a kind gift from Dr. Timothy Chan. Inactive (CS) and substrate-trapping (DA) mutations were engineered into pcDF1-PTPRD and pGEX-PTPRD constructs using site-directed mutagenesis (Quickchange II XL kit from Stratagene) as directed by the manufacturer. The coding sequences were verified by DNA sequencing.

Cell migration and invasion assays

Cell motility was measured using Cell Culture Inserts (8.0-µm pore size) for six-well plates (BD Falcon). To visualize cell invasion, we used eight-well chamber slides (BD Biosciences) precoated with 70 µL of 1:1 mixture of Matrigel and Collagen I (BD Biosciences). On day 1, 4000 cells were grown per well in the presence of 5 ng/mL EGF [198]. Cell morphology was photographed on days 8 and 10. The phase images were taken by a Zeiss Axiovert 200M using AxioVison 4.4 software. To quantitate cell invasion, we used BD BioCoat Matrigel Invasion Chambers, 8.0-µm pore size. MCF10A cells (1×10^6) were grown in the insert. After 48 h, the cells retained inside the insert were removed, and those that migrated to the other side of the insert were fixed and stained with DAPI and counted.

Cycloheximide study

MCF10A cells expressing the appropriate shRNA were serum-starved overnight, followed by treatment with cycloheximide (100 mg/ml). Cell lysates were collected at the indicated times and protein concentrations were determined by Bradford.

Immunoprecipitation and immunoblotting

Cells were grown to 90% confluency in 10 cm plates, washed with cold 1 X phosphate buffered saline (PBS) and extracted using 800μl of lysis buffer consisting of 50mM Tris-HCl (pH 7.5), 150mM NaCl, 5mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 20mM b-glycerophosphate, 1mM sodium vanadate, 1mM sodium fluoride and protease inhibitor cocktail. All steps were carried out on ice or at 4°C. Cells were lysed for 1h, centrifuged at 12,000 x g for 10 min and protein concentrations were determined using the Bradford assay. Lysates (Total protein 1 mg) were pre-cleared for 60 min with protein A/G-Sepharose. The supernatants were first incubated for 60 min with appropriate antibodies and 10 μl of protein A/G Sepharose was then added for another 60 min. The immune complexes were pelleted at 3000 x g for 5 min and washed three times with cold lysis buffer. The beads were resuspended in 20 μl of 5X Laemmli sample buffer and heated at 95°C for 1 min. Proteins were resolved by SDS-PAGE and detected by immunoblotting.

Substrate trapping assay

Serum-starved MCF10A cells expressing wild type or substrate-trapping DA mutant of PTPδ were pretreated with 50 μM pervanadate for 30 minutes. Cells were rinsed with ice-cold PBS and lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitor tablet (EDTA-free, Roche). Dithiothreitol (DTT) was added to a final concentration of 10 mM and cells were lysed for 30 min on a rotating wheel at 4°C. Cell debris was centrifuged at 12,000 x g for 10 minutes and protein concentrations were determined by the method of Bradford. An equal amount of lysate (100 μg) was diluted in cold lysis buffer and pre-cleared for 30 min with anti-Flag-agarose beads. To test for PTPδ substrate-trapping capacity, lysates expressing either wild type or PTPδ trapping mutant were immunoprecipitated with PTPδ antibody. The PTPδ-substrate complexes were pelleted at 3000 x g for 5 min, washed 3 times with lysis buffer, resuspended in Laemmli sample buffer and binding of SRC was detected by immunoblotting.

In vitro phosphatase assay

GST-tagged wild type PTPδ and CS mutant were purified on GS-agarose [199]. The reduced enzyme was then incubated with immunoprecipitated SRC at 30°C for 30 min. The reaction was terminated with Laemmli sample buffer, proteins were resolved by SDS-PAGE and substrate dephosphorylation was visualized by immunoblotting.

RNA extraction

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and 2 µg was reversed transcribed using random hexamers (Applied Biosystems) and 20U reverse transcriptase (Invitrogen).

SYBR-Green real-time RT–PCR

Quantitative real time PCR (qRT-PCR) amplification of cDNA was carried out using the following. The resulting complementary DNAs were used for PCR using SYBR-Green Master PCR mix (Applied Biosystems) in triplicates. Expression of mRNA was measured for Twist1, E-cadherin, vimentin, Snail and N-cadherin on the ABI PRISM 7900 HT Sequence Detection System with SYBR Gene Expression Master Mix. Target gene expression was normalized to actin levels. Results are representative of at least 3 independent experiments, with each sample being run in triplicate.

Appendix

Receptor Protein Tyrosine Phosphatase α Regulates Focal Adhesion Kinase Phosphorylation and Contributes to ErbB2-Mediated Mammary Epithelial Cell Motility*

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*Running title: PTPα regulation of ErbB2 signaling in mammary epithelial cells

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Keywords: PTP alpha, FAK, GRB7, ErbB2, cellular migration, breast cancer

Background: PTPα has been implicated in breast cancer but its function remains to be defined.

Results: Suppression of PTPα led to a GRB7-dependent, ErbB2-mediated increase in mammary epithelial cell migration. PTPα dephosphorylated FAK on Tyr407.

Conclusion: PTPα functions to suppress ErbB2 signaling events that lead to migration of breast cancer cells.

ABSTRACT

We have investigated the role of PTPα in regulating signaling by the ErbB2 oncoprotein in mammary epithelial cells. Using this model we have demonstrated that activation of ErbB2 led to the transient inactivation of PTPα, suggesting that attenuation of PTPα activity may contribute to enhanced ErbB2 signaling. Furthermore, RNAi-induced suppression of PTPα led to increased cell migration in an ErbB2-dependent manner. The ability of ErbB2 to increase cell motility in the absence of PTPα was characterized by prolonged interaction of GRB7 with ErbB2, and increased association of ErbB2 with a β1-integrin-rich complex, which depended on GRB7-SH2 domain interactions. Finally, suppression of PTPα resulted in increased phosphorylation of FAK on Tyr^{407}, which induced the recruitment of vinculin and the formation of a novel FAK complex in response to ErbB2 activation in mammary epithelial cells. Collectively, these results reveal a new role for PTPα in the regulation of motility of mammary epithelial cells in response to ErbB2 activation.

Reversible tyrosine phosphorylation, catalyzed by the synchronized and complementary activity of protein tyrosine kinases (PTKs) and protein tyrosine

phosphatases (PTPs), is primarily utilized in multicellular eukaryotes to communicate between and within cells [200]. The coordinated activity of the large families of PTKs and PTPs regulates the function of proteins involved in a plethora of cellular processes and the disruption of this PTK-PTP balance has been linked to the etiology of several diseases, including cancer. Initial studies revealing the critical role of PTKs in promoting oncogenesis [201] led naturally to the concept that PTPs may function as tumor suppressors [202]; however, the situation is more complex. Interestingly, PTPs that function as the products of oncogenes have been discovered, such as the SRC-homology-2 domain containing phosphatase (SHP2) [202,203], as well as PTKs with tumor suppressor activity, such as spleen tyrosine kinase (SYK) [204].

Protein Tyrosine Phosphatase alpha (PTPα, encoded by the PTPRA gene) is a receptor-like, transmembrane member of the PTP family that catalyzes phosphoryl hydrolysis on proteins through a well-defined mechanism [205]. These enzymes are characterized by the active-site signature motif HCX_5R, in which the cysteine residue is involved in nucleophile attack on the phosphotyrosyl residue of the substrate. PTPα is broadly expressed [206-209] and has been implicated in a variety of biological and pathological processes including cell cycle arrest [210], neuronal differentiation [211], and tumorigenesis (reviewed in [212]). Of particular significance, PTPα has been implicated in the positive regulation of signaling pathways and is among a small group of receptor-like PTPs, which includes PTPβ (*PTPRB*), PTPξ (*PTPRZ*), PTP-LAR (*PTPRF*), PTPγ (*PTPRG*) and SAP1 (*PTPRH*), showing oncogenic potential [202].

The catalytic activity of PTPα resides within a tandem arrangement of cytosolic phosphatase domains [205]. The membrane-proximal D1 domain of PTPα is essential and contains most of the catalytic activity. Uniquely to PTPα, the membrane-distal D2 domain is also active, but with lower specific activity than D1. Furthermore, D2 appears to play a role in sensing reactive oxygen species (ROS) [213,214] and, following oxidation, may participate in “inside-out” signaling by altering the rotational coupling of PTPα molecules within a receptor dimer [215]. There is considerable evidence supporting a role for PTPα in activating SRC and other SRC family kinases (SFKs) [48,212,216,217]; however, the biological activity of PTPs is highly context dependent

[218] and it is possible that PTPα may recognize other physiological substrates. In fact, $p130^{cas}$ [219], Kv1.2 [220] and the insulin receptor [221] have also been proposed to be substrates of PTPα.

The ability of PTPα to activate SFKs is the mechanism by which this receptor-like phosphatase transforms rat embryo fibroblasts [48]. On this basis, it has been assumed that PTPα functions positively to promote tumorigenesis. Consistent with this, PTPα is overexpressed in late stage colon carcinoma [222], in oral squamous carcinoma [223] and gastric carcinoma [224]. Nevertheless, the situation is actually more complex. Expression of PTPα varies widely in breast cancer. In some cases, high levels of PTPα are associated with low tumor grade and reduced aggressiveness [50]. In addition, metastasizing breast tumors (stage 3) were reported to express low levels of PTPα [50]. Consistent with this, ectopic expression of PTPα in ErbB2-positive human mammary tumor cells reduces tumor growth and delays lung metastasis [50]. In contrast, experiments in MMTV-ErbB2/$PTPα^{-/-}$ mice suggest that ablation of PTPα does not contribute to ErbB2-induced mammary tumor initiation or metastasis [225]. In light of these apparently conflicting observations, the present study was designed to address the function of PTPα in ErbB2-induced signaling in human mammary epithelial cells.

EXPERIMENTAL PROCEDURES

Materials – Anti-PTPα and 4G10 antibodies were from Millipore. Anti-PTPα pY789 and anti-FAK antibodies were from Cell Signaling Technology. Anti-GRB7 antibodies were from Abnova, HRP-conjugated anti-HA antibodies were from Roche and anti-β1-integrin antibodies were from BD transduction. Anti-FLAG, anti-β-actin, PT-66 agarose-conjugated beads, anti-FLAG M2 beads and anti-HA beads were purchased from Sigma. HRP-conjugated secondary antibodies were from Jackson Laboratories. Protease inhibitor cocktail tablets were from Roche. Catalase and superoxide dismutase were from Calbiochem. Surfact-Amps Nonidet P-40, zeba desalt spin columns, EZ-Link iodoacetyl-PEG2 (IAP)-biotin and iodoacetic acid (IAA) were from Thermo Scientific.

G7-18NATE peptide (sequence: WFEGYDNTFPC cyclized via a thioether bond) was prepared by S. Pero [226]. Peroxyfluor-6 acetoxymethyl ester (PF6-AM) was prepared by B. Dickinson [227]. AP1510 was purchased from ARIAD Pharmaceuticals.

*Generation of FLAG-tagged PTP*α *fusion proteins* – Full-length Human PTPα was cloned into p3XFLAG-CMV-13 mammalian Expression Vector (Sigma # E4776), in which the N-terminal preprotrypsin leader sequence preceding the multiple cloning region was deleted. Using p3XFlag-CMV13-PTPα (WT) as the template, expression constructs for trapping mutants [PTPα ($D1^{DA}$) and PTPα ($D2^{EA}$)] were generated by Quick Change mutagenesis. All these constructs have C-terminal 3XFLAG tag.

Hydrogen peroxide molecular imaging – Molecular imaging of ErbB2-induced hydrogen peroxide production in 10A.B2 cells was studied using a Perkin-Elmer Ultraview Spinning Disk confocal operating on a Nikon Ti microscope with the In Vivo Scientific Chamber, heater and gas regulator as previously described [227]. Images were analyzed using ImageJ (Wayne Rasband, US National Institutes of Health).

Assay of PTP oxidation - In PTPs, the catalytic cysteinyl residue is present as a thiolate anion in resting cells. After ErbB2 activation by AP1510, the cells were lysed in a degassed buffer at pH 5.5 containing IAA. The active-site cysteinyl residue of PTPs that remained in a reduced state were terminally inactivated by alkylation. Conversely, the active-site cysteines of PTPs that were oxidized by second-messenger ROS molecules were protected from irreversible alkylation. IAA was then removed from the lysate by size exclusion chromatography, and the reversibly oxidized active-site cysteinyl residues were reduced back to the thiolate ion with TCEP. PTPs were maintained in pH5.5 buffers and incubated with a biotinylated sulfhydryl-reactive IAP probe. After purification by streptavidin pull-down, those PTPs that were oxidized in response to ErbB2 signaling were identified by immunoblotting [228].

*Generation of cells expressing shRNA PTP*α– For stable PTPα knockdown in 10A.B2 cells, we expressed a pMLP retroviral vector (in a pMSCV backbone [229]) using the targeting sequence CAGATGGTGCAAACCGATA incorporated into the sequence of the human microRNA-30 (miR30). The infected cells were selected in

medium containing 1.0-2.0 μg puromycin and EGFP co-expression was verified using a Zeiss Axiovert 200M microscope.

Immunoprecipitation and immunoblotting – HA-ErbB2, tyrosine-phosphorylated proteins and FAK were immunoprecipitated as follows. Cells were grown to 90% confluence in 10 cm plates, serum-starved for 16h and stimulated with AP1510 to induce ErbB2 dimerization and activation for the indicated times. After treatment, the cells were washed with cold phosphate buffered saline (PBS) and extracted in 800 μl of a lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 20 mM NaF, 1 mM phenylmethylsulphonyl fluoride (PMSF) and protease inhibitor cocktail. All subsequent steps were carried out on ice or at 4°C. Cells were lysed on a rotating wheel at 4°C for 30 min. Cell debris were centrifuged at 12,000 x g for 10 min and protein concentrations were determined. An equal amount of protein was diluted in cold lysis buffer and precleared for 60 min with protein A/G-Sepharose. The supernatants were first incubated for 60 min on a rotating wheel with appropriate antibodies and 10 μl of protein A/G-Sepharose was then added for another 60 min. The immune complexes were pelleted at 3000 x g for 5 min and washed 3 times with lysis buffer. The beads were resuspended in 20 μl of 4X Laemmli sample buffer and heated at 95°C for 1 min. Proteins were separated by SDS-PAGE and detected by immunoblotting.

Cell migration assays – Cell motility was studied using a Boyden chamber-based migration assay [230], using Cell Culture Inserts (8.0-μm pore size) for six-well plates (BD Falcon). For siRNA studies, knockdowns were performed with specified siRNAs (sia-1: 5'-CAGAUGGUGCAAACCGAUA dTdT-3, sia-2: 5'-AAGCUGGGAGCCAUUCCAAUU dTdT-3') using Lipofectamine as described [231]. To quantitate cell motility, 100,000 cells were seeded on the inserts. After 48 hours, the cells were washed with 1XPBS and fixed with 5% buffered Formalin solution, stained and counted. The cells retained inside the insert were removed, and those that had migrated through the pores to the bottom surface of the transwell were counted. For each condition, the number of migrating cells was counted in 8 random microscopic

fields. The number of migrating cells in the control 10A.B2 cells without stimulation was normalized to 1. Where indicated, AP1510 (1 μM), G7-18NATE-Penetratin (G7-18NATE-P) peptide (GRB7 inhibitory peptide; WFEGYDNTFPC-RQIKIWFQNRRMKWKK) or Penetratin (RQIKIWFQNRRMKWKK) were added to the culture medium at the beginning of the assay. Cell motility was quantitated after 48 h.

In vitro phosphatase assay – HA-tagged PTPα was expressed in HEK293T cells, purified and washed several times with ice-cold reducing buffer (50 mM HEPES pH7.4, 100 mM NaCl, 0.1% Triton X-100, 2 mM DTT, protease inhibitors tablet) for 10 min on ice to complete reduction of PTPα. The reduced enzyme was then incubated with phosphorylated FAK at 30°C for 30 min. The reaction was stopped by addition of 20 μl of 4X Laemmli sample buffer and heated at 95°C for 1 min. Proteins were separated by SDS-PAGE and substrate dephosphorylation was visualized by immunoblotting.

RESULTS

Cooperation between PTPα and ErbB2 signaling in mammary epithelial cells – We tested the effects of suppressing the expression of PTPα on ErbB2-induced signaling in mammary epithelial cells using two independent siRNA sequences. The effect of PTPα suppression on ErbB2-induced cell motility was examined using a Boyden chamber-based migration assay. Dimerization and activation of ErbB2 was induced in MCF10A cells that express a well-characterized chimeric form of the kinase (10A.B2) the activity of which can be induced by addition of a small molecule dimerizer, AP1510, as described previously [232]. Activation of ErbB2 in parental 10A.B2 cells, or cells treated with scrambled siRNA resulted in an ~3.5 fold stimulation of migration. Following treatment with an siRNA sequence previously shown to suppress PTPα [231] and a second siRNA designed using the RNAi Codex program at Cold Spring Harbor Laboratory, we observed that ErbB2 activation resulted in an 6-8-fold increase in cell motility compared to the basal migration observed in unstimulated 10A.B2 cells (Fig. 1A). Consistent with this observation, both siRNAs efficiently suppressed PTPa expression, whereas the non-specific siRNA did not (Fig. 1B)

A stable cell line expressing the most effective short hairpin RNA (shRNA) targeting PTPα was then established and cells were selected. Following the selection, the depletion of PTPα by shRNA in 10A.B2 cells was estimated to be ~ 90%. Specificity in the effect of the shRNA was illustrated by the fact that expression of the closely related PTP family member, PTPε, was not affected (Fig. 1C). Using these shRNA-expressing 10A.B2 cells, the effect of PTPα suppression on ErbB2-induced cell motility was then re-examined. In presence of AP1510, migration of parental 10A.B2 cells was increased 3-fold. In contrast, following shRNA-depletion of PTPα, ErbB2 activation resulted in a 5-fold increase in cell motility compared to the basal migration of unstimulated 10A.B2 cells (Fig. 1D). Hence, attenuation of PTPα contributed to increased ErbB2 signaling.

Transient oxidation and inactivation of PTPα in response to ErbB2 signaling in mammary epithelial cells – Hydrogen peroxide (H_2O_2) has been shown to inactivate protein tyrosine phosphatases and thereby to promote protein tyrosine phosphorylation-

dependent signal transduction [233]. Suppressing the expression of a particular PTP effectively reproduces oxidation-mediated inactivation and increases the phosphorylation of sites that are targeted by that PTP, to promote downstream events in the signaling cascade [234]. To determine whether PTPα was reversibly oxidized in the context of ErbB2 signaling, first we measured H_2O_2 production using molecular imaging with a specific fluorescence indicator, Peroxyfluor-6 acetoxymethyl ester (PF6-AM) [227]. This probe features an aryl boronate chemical switch that allows for selective detection of H_2O_2 over other ROS molecules [235-238]. Following serum withdrawal, 10A.B2 cells were loaded with PF6-AM and treated with AP1510 (Fig. 2A). ErbB2 activation caused a rapid and time-dependent increase in intracellular fluorescence. Considering the known selectivity of PF6-AM, our data indicate that following addition of AP1510 and ErbB2 dimerization in 10A.B2 mammary epithelial cells endogenous generation of H_2O_2 occurred in less than 2 minutes and peaked at 5 minutes. In addition, pre-incubation of 10A.B2 cells with a chemical inhibitor of NADPH oxidases, diphenyleneiodonium (DPI), prevented ErbB2-mediated H_2O_2 production and intracellular fluorescence of PF6-AM.

We tested whether DPI compromised signaling downstream of ErbB2, as previously reported for other receptor tyrosine kinases [228,239]. Serum-starved cells were stimulated with AP1510 in the presence or absence of DPI. Cellular extracts were prepared and tyrosine phosphorylated proteins were visualized by anti-pTyr immunoprecipitation and immunoblotting. Interestingly, inhibition of H_2O_2 production by DPI led to decreased tyrosine phosphorylation of proteins of ~180 kDa, 75 kDa, 55 kDa and 45 kDa (Fig. 2B). In addition, blotting with anti-HA revealed that the ~180 kDa band co-migrated with ErbB2. Hence, decreasing the acute generation of H_2O_2, which would be expected to increase the level of active PTPs, compromised tyrosine phosphorylation, activation of ErbB2 and its downstream targets in mammary epithelial cells.

We have previously shown that PTPα is reversibly oxidized upon EGF receptor activation in IMR90 fibroblasts [240]. Hence, we used a modified cysteinyl-labeling assay [240] to ascertain whether PTPα activity was also regulated by H_2O_2 generated

upon ErbB2 activation in 10A.B2 cells. This is a three-step method in which the reversibly oxidized invariant catalytic cysteine residue is specifically biotinylated by a thiolate anion-directed probe (Fig. 2C). Serum-starved 10A.B2 cells were incubated with AP1510 and lysed in an anaerobic workstation, which was purged, and constantly supplied with ultrapure argon gas to prevent post-lysis oxidation of PTPs. We detected only minimal biotin labeling of PTPα in untreated cells; however, the biotinylation pattern from the cysteinyl-labeling assay revealed that the reversible oxidation of PTPα occurred in a biphasic manner upon ErbB2 activation (Fig. 2D). Thus, ErbB2 activation attenuated PTPα activity by reversible oxidation in mammary epithelial cells.

*Identification of Focal Adhesion Kinase as a substrate of PTP*α – To identify substrates of PTPα that were components of ErbB2-induced signaling pathways, we treated 10A.B2 cells with AP1510, to activate ErbB2, and pervanadate, to amplify the signal of potential phosphotyrosine-containing substrates for analysis. Substrate trapping mutant forms of PTPα, PTPαD1^{DA} and PTPαD2^{EA} (Fig. 3A), were then utilized to identify potential physiological substrates of the PTP, as described previously (34). Proteins involved in ErbB2 signaling and cell migration were tested as potential interacting partners of PTPα(WT), PTPα(D1^{DA}) or PTPα(D2^{EA}). Interestingly, the PTK focal adhesion kinase (FAK) was enriched with PTPα trapping mutants (Fig. 3B). Considering previous studies implicating FAK in ErbB2-induced cell migration, invasion and focal adhesion turnover [241,242], we investigated this novel PTPα-FAK interaction further.

To determine whether the interaction of FAK with the PTPα trapping mutant occurred via the PTP active site, the trapping experiment was performed in presence of sodium orthovanadate, a competitive inhibitor and transition state analog of phosphate that prevents substrate binding [243]. The interaction of FAK with PTPα(D1^{DA}) was inhibited by vanadate, indicating the involvement of the active site and suggesting that FAK may represent a direct substrate of PTPα in cells (Fig. 3C). Under the conditions of our substrate trapping experiments, it is possible that multi-protein complexes, containing the mutant PTP, may be isolated. Therefore, we investigated whether PTPα could dephosphorylate FAK directly *in vitro*. FAK was immunoprecipitated from lysates

of 10A.B2 cells treated with AP1510 and pervanadate. In these conditions, FAK was phosphorylated on Tyr-397, Tyr-407, Tyr-576, Tyr-861 and Tyr-925 (Fig. 3D). The immunoprecipitates of phosphorylated FAK were then incubated with wild type active PTPα and dephosphorylation was measured using phospho-specific anti-pTyr antibodies. Immunoblots revealed that PTPα could dephosphorylate phosphoTyr-407 of FAK specifically *in vitro*, whereas none of the other Tyr residues was dephosphorylated following incubation with the phosphatase. A role of PTPα in regulating the phosphorylation status of FAK Tyr-407 was verified in parental 10A.B2 cells and in 10A.B2 cells stably expressing shRNA for PTPα. By immunobloting using a phosphosite-specific antibody, we observed transient phosphorylation of Tyr-407 of FAK 30-45 minutes following ErbB2 activation with AP1510 (Fig. 3E). In contrast, FAK Tyr-407 phosphorylation was sustained from 15 to 60 minutes following ErbB2 activation when PTPα expression was compromised. Collectively, these results suggest a novel role for PTPα in regulating phosphorylation of FAK on Tyr-407.

Interaction of FAK pTyr-407 with vinculin – Tyrosine phosphorylation of FAK by SRC and PYK2 (Proline-rich Tyrosine Kinase-2) leads to its activation and association with several SH2 domain-containing proteins, as well as with focal adhesion proteins such as paxillin and vinculin. Previous studies in human ventricular endothelial cells have shown that phosphorylation of FAK on tyrosine 407 by PYK2 led to the recruitment of FAK to vinculin and paxillin [244,245]. Hence, in order to understand the role of PTPα in dephosphorylating FAK, we tested whether FAK pTyr-407 behaved similarly in mammary epithelial cells. FAK was immunoprecipitated from 10A.B2 cells that were incubated with AP1510 for 30 minutes. We observed FAK interaction with paxillin and vinculin in 10A.B2 cells when PTPα was suppressed; however, vinculin was not co-immunoprecipitated with FAK in parental 10A.B2 cells (Fig. 4). In addition, since FAK interacts directly with GRB7 (Growth factor receptor bound 7) to promote cell migration [246], we also tested for the presence of GRB7 and its interacting partner ErbB2 [247] in FAK complexes. Both GRB7 and ErbB2 were detected in FAK complexes upon ErbB2 activation, independently of PTPα expression. This shows that the hyperphosphorylation of FAK Tyr-407 observed in the absence of PTPα contributed to the recruitment of vinculin to FAK in a multiprotein complex.

Increased association of β1-integrin and GRB7 with ErbB2 upon suppression of PTPα – A significant body of evidence indicates that the presence of vinculin in focal adhesions is critical for integrin-mediated cell adhesion and migration (reviewed in [248]). β1-integrin is required for proliferation, survival and invasiveness of human breast cancer cell lines [249]. Integrins associate with the EGF receptor [250,251] and ErbB2 transactivation is impaired in β1-integrin-deficient breast tumors [252]. Considering that an ErbB2-vinculin complex was detected in absence of PTPα, and that vinculin is recruited to the cytoplasmic tails of β-integrins [253], we tested whether the increased ErbB2-mediated migration observed in the absence of PTPα coincided with a change in the formation of an ErbB2-β1-integrin complex. We monitored the association of β1-integrin and GRB7 with ErbB2. ErbB2 was immunoprecipitated following activation in intact 10A.B2 cells, or in cells in which PTPα expression was compromised, and immunoblotted for interacting proteins (Fig. 5). The interaction of PTPα, GRB7 and β1-integrin with ErbB2 was transient and peaked between 30-45 min in parental 10A.B2 cells whereas suppression of PTPα levels resulted in a rapid and sustained association of β1-integrin with ErbB2. Hence ErbB2-dependent migration in the absence of PTPα is likely to coincide with enhanced signaling of the receptor at β1-integrin-rich focal adhesions.

Decreased PTPα expression led to enhanced ErbB2-GRB7 interaction and GRB7-dependent cell migration – The *GRB7* gene, encoding the SH2-containing adaptor protein GRB7, is part of the *ERBB2* amplicon, an ~86kbp region that includes six genes (*TCAP*, *PNMT*, *PERLD1*, *HER2*, *C17orf37/C35* and *GRB7*) that is amplified in breast cancer [254]. It has been reported that GRB7 is present at focal adhesions [255] regulating motility and tumorigenesis in cancer cells [256]. GRB7 was present in FAK and ErbB2 immunoprecipitates following the activation of ErbB2 by AP1510. Hence, the presence of GRB7 in the ErbB2-FAK-β1-integrin complexes prompted us to investigate the relationship between PTPα, GRB7 and ErbB2 as part of a potential mechanism leading to increased motility of 10A.B2 cells. To this effect, ErbB2 was activated, then immunoprecipitated from 10A.B2 cells expressing shRNA for PTPα or from parental 10A.B2 cells, and the immunoprecipitates were probed for the presence of the GRB7 adaptor. Consistent with previous observations [247], GRB7 associated with ErbB2 (Fig.

6A). In 10A.B2 cells, there was a low basal level of association, with gradual GRB7 recruitment to ErbB2 upon receptor activation; in contrast, following PTPα knockdown, the basal level of interaction was increased and ErbB2-induced association of GRB7 to ErbB2 occurred more rapidly (Fig. 6A).

In order to examine the importance of the ErbB2-GRB7 interaction on cell motility observed in the absence of PTPα, we tested the effects of a GRB7 inhibitor on ErbB2-induced migration. We have used a non-phosphorylated inhibitor peptide specific for the GRB7 SH2 domain, G7-18NATE (GRB7-peptide18-No Arms Thioether; Fig. 6B) bound to a penetratin peptide (G7-18NATE-P) [226], which has previously been shown to attenuate the migration of pancreatic cancer cells [257]. The effect of inhibiting GRB7 on ErbB2-induced cell motility in 10A.B2 cells stably expressing shRNA for PTPα was examined using a Boyden chamber-based migration assay. ErbB2 activation resulted in a 5-fold increase in cell migration compared to the basal migration observed in unstimulated 10A.B2 cells; however, treatment of these PTPα-depleted cells with AP1510 in the presence of the GRB7 inhibitor G7-18NATE-P abolished ErbB2-induced cell motility, whereas incubation with the penetratin peptide alone had no effect (Fig. 6C). This suggests that the increased interaction between GRB7 and ErbB2 observed in absence of PTPα led to a GRB7-dependent increase in 10A.B2 cell migration.

DISCUSSION

Although PTPα has the capacity to display oncogenic properties, its biological role in mammary epithelial cells and breast cancer is unclear [50,231,258] reviewed in [203]. It has been shown that PTPα expression levels vary widely among breast tumors; furthermore, it is unclear whether PTPα plays a positive or negative role in signaling in breast cancer [50]. In this study, we examined the role of PTPα in ErbB2 signaling, using a chimeric form of the kinase that could be induced by addition of a small molecule dimerizing agent, AP1510, in human mammary epithelial 10A.B2 cells. We found that PTPα was a negative regulator of ErbB2-dependent 10A.B2 cell motility. In addressing the function of PTPα in ErbB2 signaling, we uncovered a novel function of the phosphatase in regulating the phosphorylation of FAK on tyrosine 407, regulating FAK binding to vinculin and prolonging the association of ErbB2 with GRB7 and β1-integrins. In addition, PTPα-mediated, ErbB2-dependent cell motility was also dependent upon GRB7 acting as an ErbB2-interacting protein. The consequences of RNAi-induced suppression of PTPα suggest an important role for this receptor protein tyrosine phosphatase in controlling ErbB2 signal transduction leading to migration of human mammary cancer cells.

We have previously shown that PTPα is reversibly oxidized following EGF receptor activation [240]. Others have also observed a role of PTPα in EGF receptor signaling in diverse mechanisms such as aging [259], as well as in cell-substratum adhesion [260]; however, this is the first study implicating PTPα in the regulation of ErbB2-mediated cell motility. We utilized siRNA targeting human PTPα, designed using the RNAi Codex program at CSHL, and confirmed the migration phenotype with another siRNA sequence previously shown to be a potent suppressor of PTPα expression in the Shalloway lab [231]. By repressing PTPα expression, which would mimic the oxidation-mediated reversible inactivation of the enzyme that occurs in signaling in cells, we increased the phosphorylation of sites targeted by PTPα. This suggested that the transient inactivation of PTPα may control the phosphorylation of FAK and the formation of GRB7-complexes involved in the migratory phenotype. Reversible oxidation of the catalytic cysteine of the D1 domain would be expected to inactivate its function directly.

Furthermore, reversible oxidation of the D2 domain of PTPα has been shown to cause the formation of a disulfide bond with the catalytic cysteine of the counterpart D2 domain in the dimer, thereby inducing a conformational change and inhibition of the D1 domain (reviewed in [205]). Hence, the reversible oxidation of either D1 or D2 cysteines of PTPα, as detected by the cysteinyl-labeling assay, is a measure of the inactivation of PTPα occurring following the rapid increase in intracellular hydrogen peroxide that takes place upon acute ErbB2 activation.

The highly dynamic process of cell migration, regulated by tyrosine phosphorylation within focal complexes, involves modulation of cell-substrate adhesion and recruitment of over 50 structural proteins to the cytoplasmic segments of α- and β-integrins [261]. FAK is a central regulator of focal complexes. It has been implicated in cancer cell motility *in vitro*, in addition to being an important contributor to tumor invasion, metastasis, and malignancy [262-264]. There have been reports indicating that in certain circumstances PTPα, acting via stimulation of SRC, may promote phosphorylation of Tyr 397 in FAK [265]. In this study, analysis by RNAi-induced suppression of PTPα, application of substrate-trapping mutant forms of the enzyme and a direct phosphatase activity assay all illustrate dephosphorylation of Tyr407 of FAK by PTPα. We did not observe significant changes in any other sites of tyrosine phosphorylation in FAK, highlighting the specificity of the phosphatase. Moreover, we have found that FAK Tyr407 phosphorylation was prolonged upon ErbB2 activation when PTPα expression was compromised. FAK Tyr-407 has previously been shown to be phosphorylated by PYK2 in response to Vascular Endothelial Growth Factor (VEGF) stimulation [245] as well as by SRC [266]. In addition to its function as a kinase, phosphorylation of FAK is known to promote its function as a scaffold protein [246]. We have observed that FAKpTyr407 displayed preferential recruitment to vinculin, in addition to being associated with ErbB2, GRB7 and paxillin. This pTyr407-dependent interaction between FAK and vinculin has also been observed by others [244]. However, the significance of pTyr407 phosphorylation is still unclear. Previous groups have shown that FAK Tyr407 phosphorylation occurs at focal adhesions and at the periphery of migrating cells [245,267], in tumor cell differentiation [268] and in epithelial mesenchymal transdifferentiation [267]. Interestingly, it has also been proposed to be a

FAK regulatory site [269]. Thus, since ErbB2-induced cell migration, invasion and focal adhesion turnover is dependent on FAK signaling [241,242], identifying the SH2-containing signaling protein bound to phosphoTyr407 in this context may yield further insight into the role of PTPα in ErbB2-dependent migration of human mammary epithelial cells.

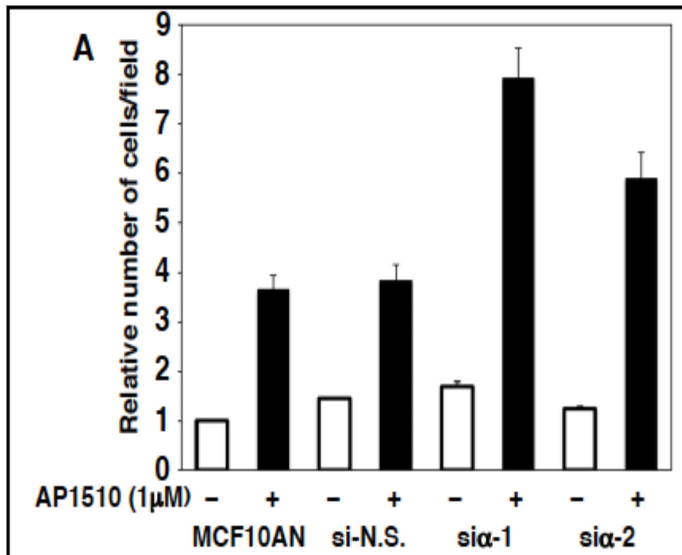
Our study, demonstrating that the SH2 domain peptide inhibitor of GRB7 (G7-18NATE-P) completely abolished ErbB2-mediated 10A.B2 cell migration following suppression of PTPα, stressed the important scaffolding role of GRB7. GRB7 was initially characterized as an interacting partner of ErbB2 at the tyrosine 1139 site [247] and has been implicated in the regulation of focal adhesion function and cell migration [255]. Our studies also illustrate that disruption of PTPα expression regulates the interaction between GRB7 and ErbB2, and suggest a potential role for the GRB7 adaptor protein in the effects of PTPα. It has been reported that GRB7 can form dimers [270], suggesting the possibility that these may provide anchorage points for proteins at focal adhesion complexes. It has been shown that phosphorylation of PTPα at Tyr789, previously identified as a binding site for GRB2 [271], was critical in targeting PTPα to focal adhesions [272,273]. It would be interesting to investigate whether GRB7 is a candidate SH2-containing protein that mediates the recruitment of PTPα to focal adhesions. A phosphotyrosine displacement mechanism has been proposed to facilitate the activation of SRC by PTPα in which pTyr 789 of PTPα engages the SH2 domain of SRC thereby exposing the C-terminal pTyr for dephosphorylation and activation of the kinase [138]. Perhaps pTyr789, functioning as a GRB7-docking site on PTPα, could provide a competing phospho-site to tyrosine 1139 on ErbB2. Therefore, the transient localization of ErbB2 at β1-integrin complexes may be regulated in a similar manner to that observed for the activation of SRC by PTPα, in that engagement of pTyr789 on the phosphatase by GRB7 may expose other sites for dephosphorylation. The presence of GRB7 together with β1-integrins and FAK following ErbB2 activation may also be significant since it has previously been reported that the β1-integrin-FAK axis controls the initial proliferation of micrometastases of mammary carcinoma cells in the lung [274].

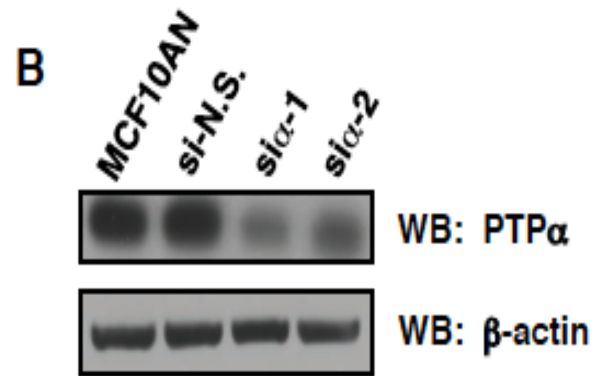
Overall, we have shown for the first time that the suppression of PTPα expression led to a GRB7-dependent increase in migration of human mammary epithelial cells in response to ErbB2 activation. Our data support a novel role for PTPα in regulating the phosphorylation of FAK at tyrosine 407, thereby promoting its association with vinculin at β1-integrin focal adhesion complexes. These novel aspects of PTPα signaling reveal an important role of the phosphatase in the regulation of a key mediator of focal adhesions and cell migration and of ErbB2-mediated mammary cancer cell migration.

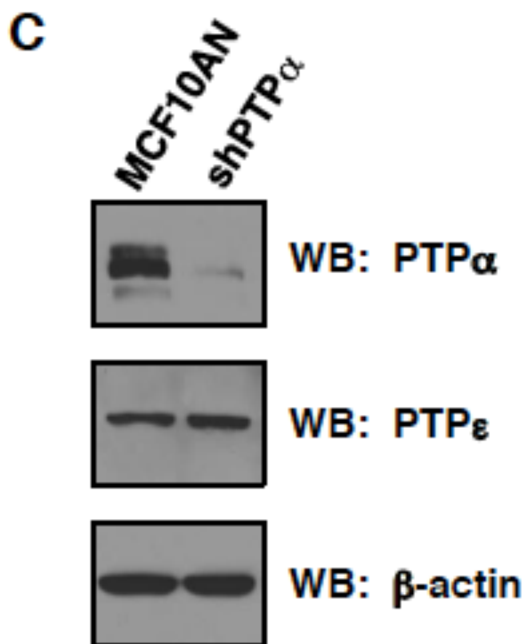
FOOTNOTES

Abbreviations –DPI, diphenyleneiodonium; EGF, epidermal growth factor; FAK, focal adhesion kinase; GRB7, growth factor receptor bound 7; G7-18NATE-P, GRB7-peptide18-no arms thioether-penetratin; H_2O_2, hydrogen peroxide; PF6-AM, peroxyfluor acetoxymethyl ester; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; PYK2, proline-rich tyrosine kinase-2; ROS, reactive oxygen species; SFK, SRC family kinases.

FIGURE LEGENDS







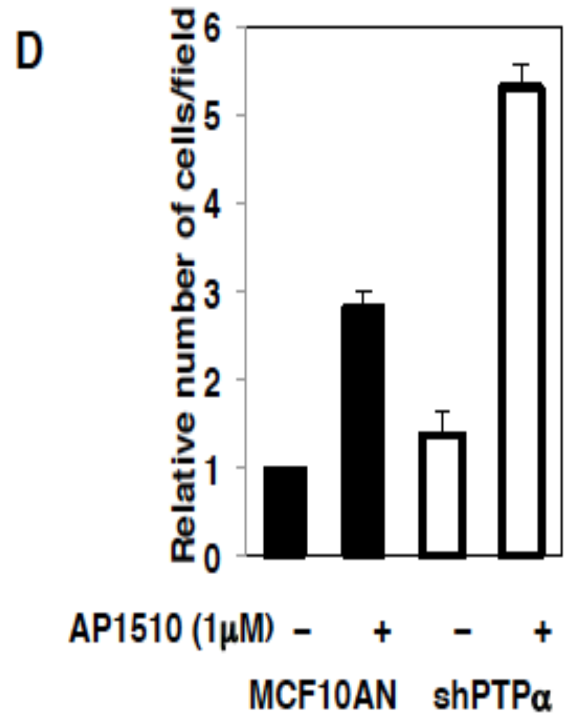
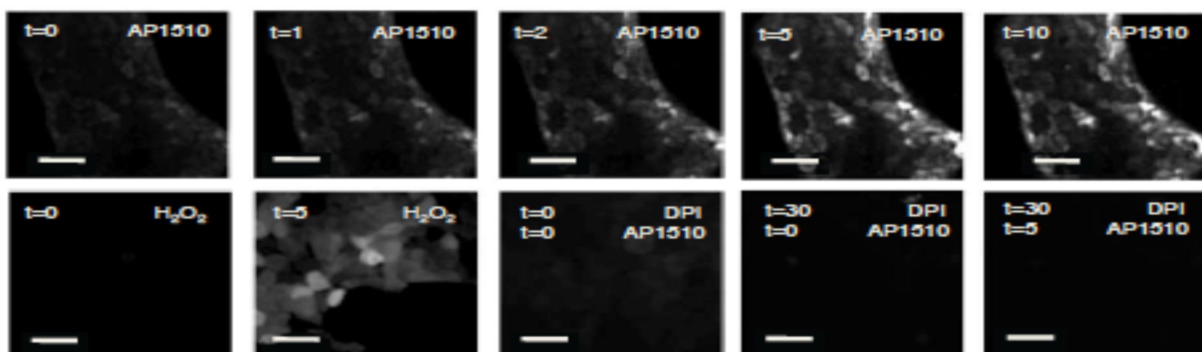
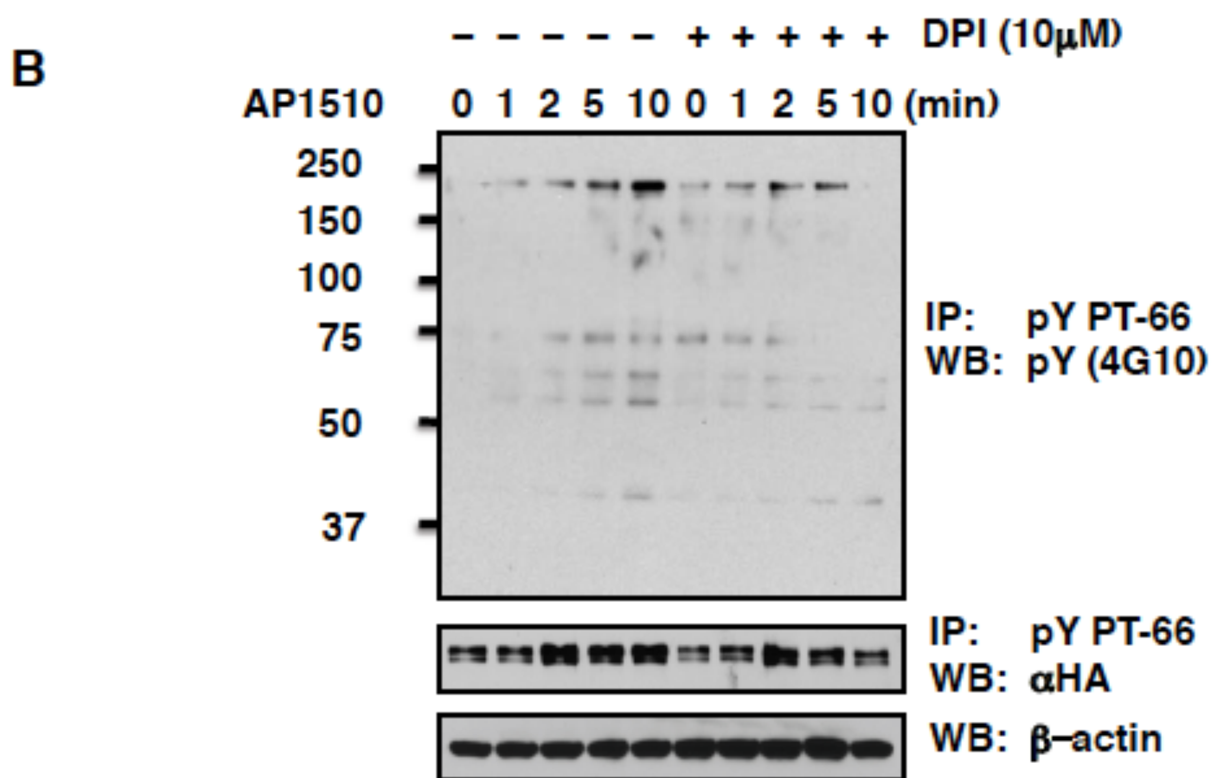
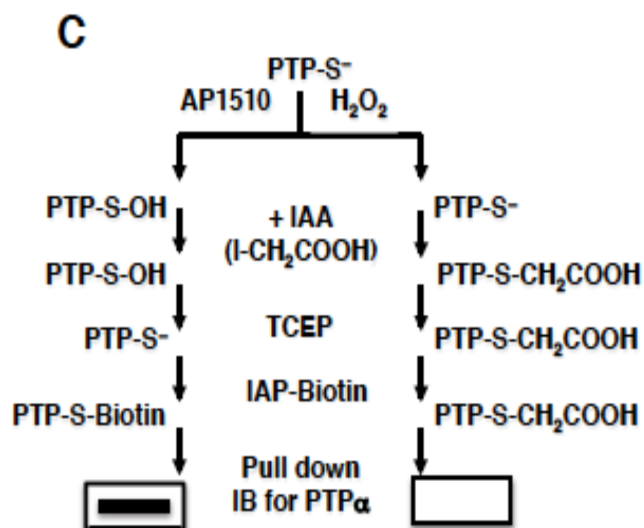


Figure 1. Suppression of PTPα induced ErbB2-mediated 10A.B2 cell motility. (A) 10A.B2 cells either untransfected, or transfected with a non-specific control siRNA (si-N.S.) or two distinct siRNAs targeting PTPα (siα-1 and siα-2) were seeded in transwell migration chambers. After incubation in the absence (-) or presence (+) of 1 μM AP1510 for 48 h, migration was quantitated as described in Experimental Procedures. (B) Effect of siRNA on the expression of PTPα assessed by immunoblotting of cell lysates, using actin as a loading control. (C) 10A.B2 cells were infected with a retroviral vector encoding shRNA targeting PTPα (shPTPα) and selected to create a stable cell line. Lysates from 10A.B2 and 10A.B2-shPTPα cells were immunoblotted with either anti-PTPα rabbit polyclonal antibody or with anti-PTPε goat polyclonal antibody. Loading controls were performed by immunoblotting for actin. (D) Cells (10A.B2 and 10A.B2-shPTPα) were seeded in transwell migration chambers in the absence (-) or presence (+) of 1 μM AP1510 for 48 h, and migration was quantitated as described in Experimental Procedures.




B
- - - - - + + + + + DPI (10μM)
AP1510 0 1 2 5 10 0 1 2 5 10 (min)
250
150
100
75
50
37
IP: pY PT-66
WB: pY (4G10)
IP: pY PT-66
WB: αHA
WB: β–actin


C
PTP-S⁻
AP1510
H2O2
PTP-S-OH
+ IAA
(I-CH2COOH)
PTP-S⁻
PTP-S-OH
PTP-S-CH2COOH
PTP-S⁻
TCEP
PTP-S-CH2COOH
PTP-S-Biotin
IAP-Biotin
PTP-S-CH2COOH
Pull down
IB for PTPα

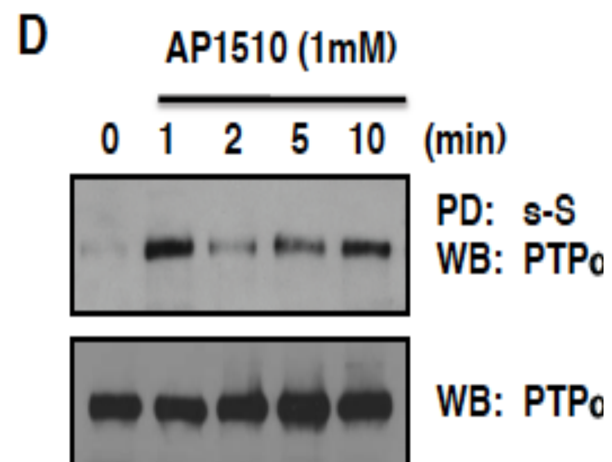
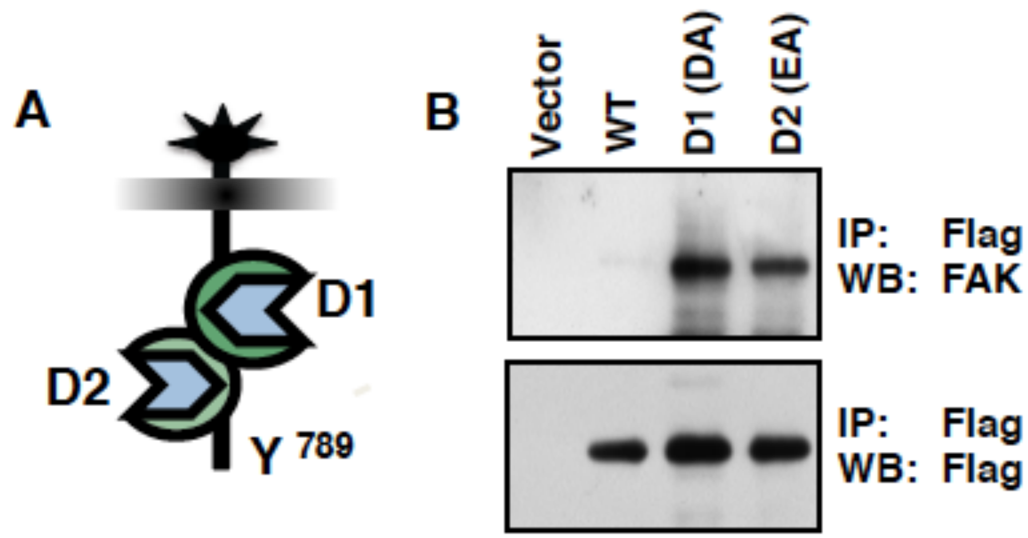
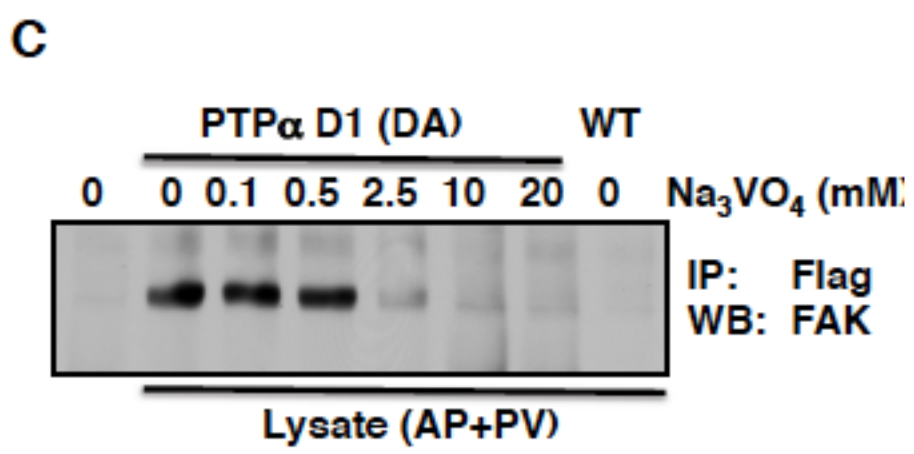

D
AP1510 (1mM)
0 1 2 5 10 (min)
PD: s-S
WB: PTPα
WB: PTPα

Figure 2. ErbB2-mediated H_2O_2 production enhanced phospho-tyrosine signaling and PTPα reversible oxidation in 10A.B2 cells. (A) ErbB2-induced H_2O_2 production was assessed by molecular imaging using PF6-AM. Serum-starved 10A.B2 cells were loaded with 5 µM PF6-AM for 20 min and stimulated with 1 µM AP1510 for the indicated times (min.) and then imaged. Alternatively, 100 µM H_2O_2 was added to the medium for 5 minutes. For DPI treatment, cells were preincubated in medium containing 10 µM DPI for 30 minutes prior to AP1510 stimulation for 5 minutes. The scale bar represents 50 µm. (B) DPI-inhibition of ErbB2-induced tyrosine phosphorylation. Serum-starved 10A.B2 cells were stimulated with 1 µM AP1510 in presence or absence of DPI (10 µM, 30 min) for the indicated times. Tyrosine phosphorylated proteins were immunoprecipitated from 200 µg of cell lysate using PT-66 and immunoblotted using 4G10 anti-pTyr antibodies. ErbB2 was detected using anti-HA antibodies. Loading controls were performed by immunoblotting lysates for actin. (C) Schematic outline of the cysteinyl-labeling assay [228]. (D) Serum-deprived 10A.B2 cells were incubated with AP1510 (1 µM) for the indicated times and subjected to the cysteinyl-labeling assay. Biotinylated proteins were purified on streptavidin-Sepharose beads, resolved on SDS-PAGE and immunoblotted for PTPα. Lysates were also probed for PTPα as controls.


A
D1
D2
Y 789
B
Vector
WT
D1 (DA)
D2 (EA)
IP: Flag
WB: FAK
IP: Flag
WB: Flag


C
PTPα D1 (DA)
WT
0 0 0.1 0.5 2.5 10 20 0
Na_3VO_4 (mM)
IP: Flag
WB: FAK
Lysate (AP+PV)

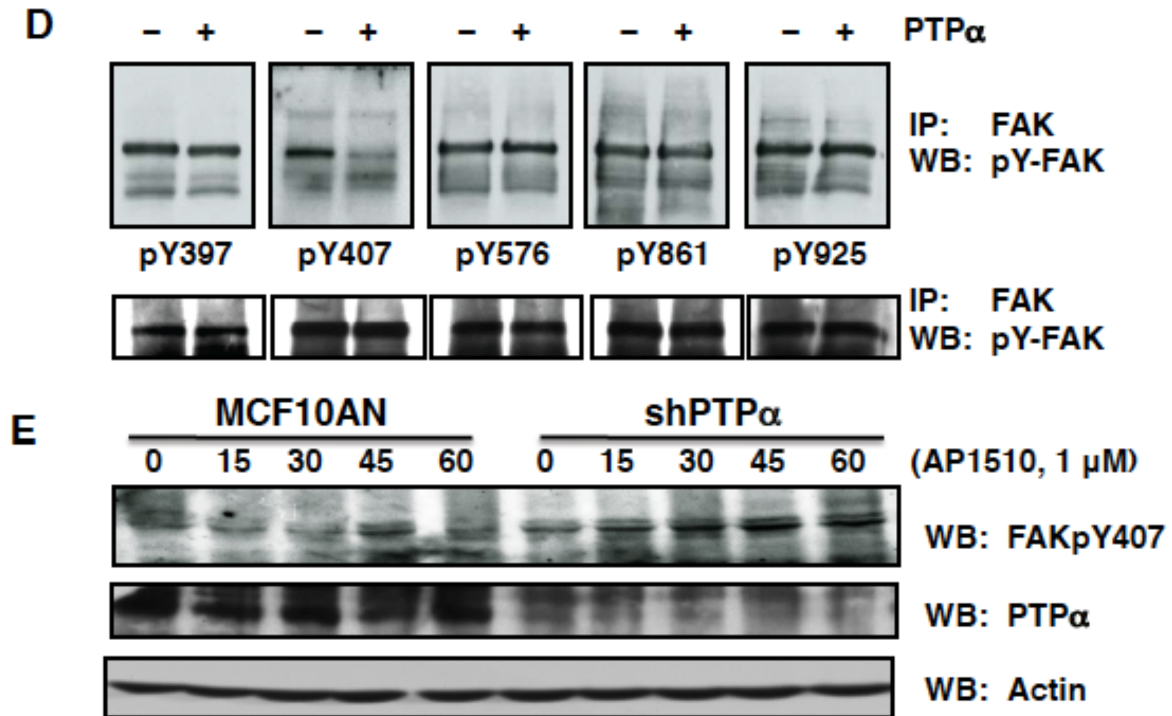


Figure 3. Identification of focal adhesion kinase as a PTPα substrate. (A) Schematic view of the domain composition of PTPα. (B) Immunoblot analysis of FAK associated with substrate trapping mutants of PTPα. 10A.B2 cells were treated with 1 μM AP1510 for 5 min and with 50 mM pervanadate for an additional 30 min prior to lysis. FLAG-PTPα(WT), FLAG-PTPα($D1^{DA}$) or FLAG-PTPα($D2^{EA}$) was incubated with cell lysate, immunoprecipitated with anti-FLAG and protein complexes were analyzed by SDS-PAGE and immunoblotting with anti-FAK or anti-FLAG antibodies. (C) Effects of sodium orthovanadate on the FAK-PTPα$D1^{DA}$ interaction. Purified PTPα($D1^{DA}$) and PTPα(WT) were incubated with cell lysates as in A, with or without the indicated concentration of Na_3VO_4. Protein complexes were immunoprecipitated using anti-FLAG antibodies, analyzed by SDS-PAGE and immunoblotted with anti-FAK. (D) Immunoblot analysis of the catalytic activity of PTPα on FAK *in vitro*. 10A.B2 cellular lysates were

prepared as described in B. Phospho-FAK was immunoprecipitated from 100 μM lysate using 1μg anti-FAK antibody and incubated, or not, with PTPα for 30 min. Proteins were separated by SDS-PAGE and immunoblotted using specific anti-phospho FAK antibodies for pY397, pY407, pY576, pY861, pY965. Blots were stripped and reprobed for total FAK. (E) 10A.B2 and shPTPα cells were incubated with AP1510 for the indicated times, lysates were prepared, separated by SDS-PAGE and immunoblotted with either anti-phospho FAK Tyr-407, anti-PTPα or anti-actin antibodies.

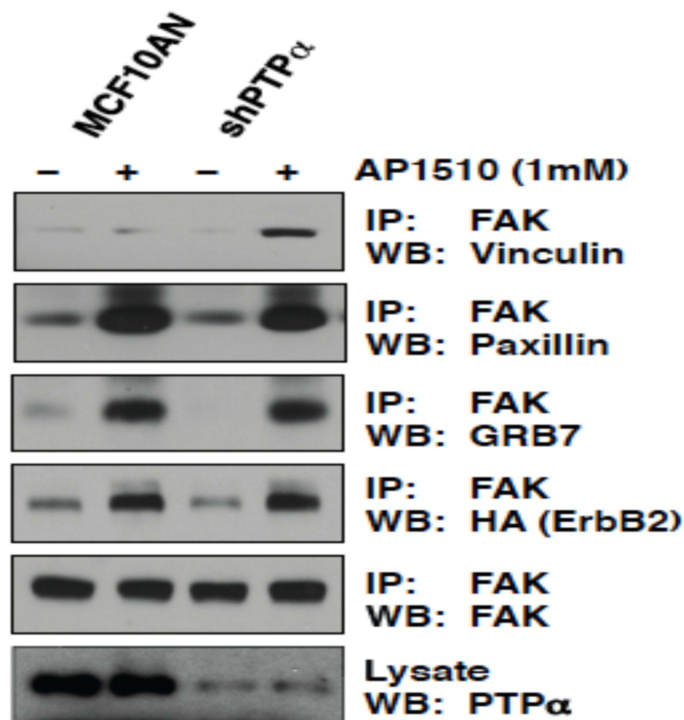


Figure 4. Effect of suppression of PTPα on the recruitment of focal adhesion proteins to FAK. FAK was immunoprecipitated from serum-deprived 10A.B2 or 10A.B2-shPTPα cells plated on fibronectin (25 μg/ml), incubated, or not, with AP1510 (1 μM) for 30 min and lysed*. Proteins were separated by SDS-PAGE, and immunoblotted using anti-vinculin, anti-paxillin, anti-GRB7, anti-HA or anti-FAK antibodies. Lysates were probed for PTP*α *expression.*

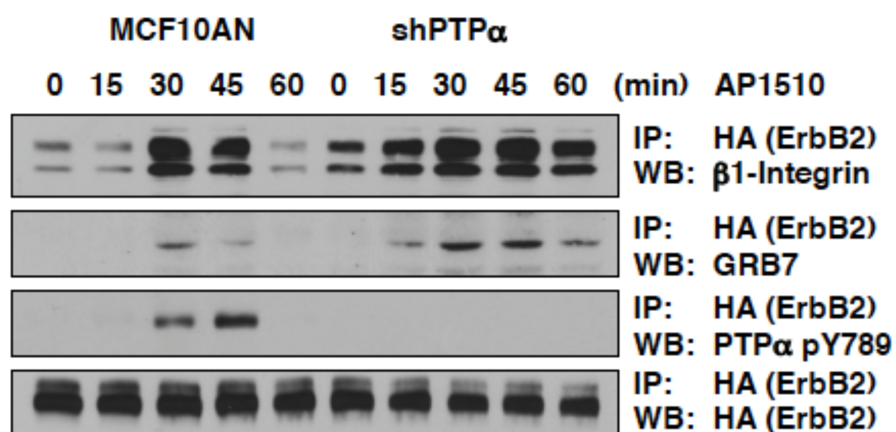


Figure 5. Effect of PTP*α* suppression on ErbB2 interaction with a *β*1-integrin complex*.* *Serum*-deprived 10A.B2 and *10A.B2-shPTPα* cells, *plated on fibronectin (25 μg/ml),* were treated with 1 *μ*M AP1510 for indicated times. HA-ErbB2 was immunoprecipitated from 100 *μ*g of cell lysate using anti-HA and immunoblotted using anti-*β*1-integrin, anti-HA or anti-GRB7 antibodies. ErbB2 was detected using anti-HA antibody.

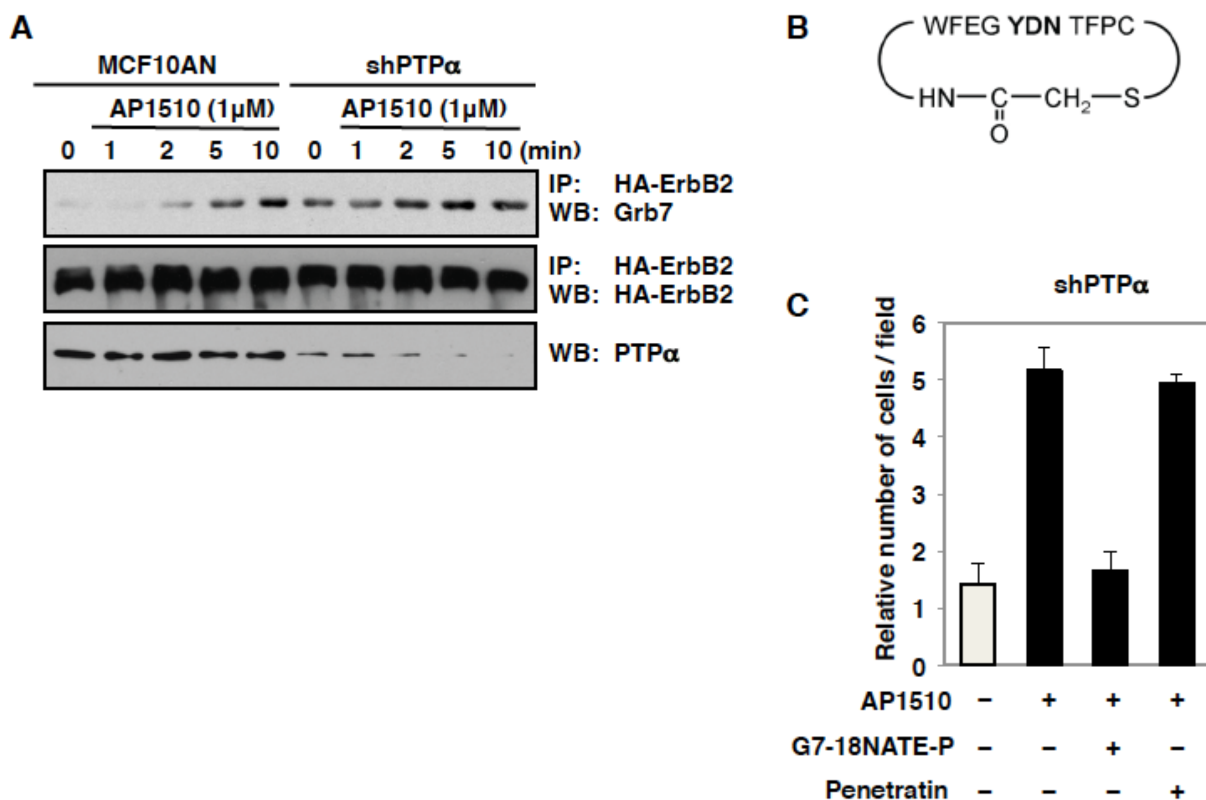


Figure 6. Effects of suppressing PTP*α* on GRB7-ErbB2 signaling in 10A.B2 cells. (A) Association of GRB7 with ErbB2. Serum-deprived 10A.B2 and 10A.B2-*shPTPα* cells were treated with AP1510 (1 *μ*M) for the indicated times. HA-ErbB2 was immunoprecipitated from 100 *μ*g of cell lysate using anti-HA, and immunoblotted using anti-GRB7 antibody. ErbB2 was detected using anti-HA antibody, and lysates were probed for actin and PTP*α* for loading and PTP*α* expression. *(*B) Sequence of GRB7 SH2-domain inhibitor peptide G7-18NATE. (C) Effects of GRB7 inhibitor peptide on ErbB2-induced migration in *10A.B2*-shPTPα cells. 10A.B2 and *10A.B2*-shPTPα cells were seeded in transwell migration chambers in the absence (–) or presence (+) of 1 μM AP1510, 10 μM G7-18NATE-Penetratin (WFEGYDNTFPC-RQIKIWFQNRRMKWKK) or 10 μM penetratin peptide (RQIKIWFQNRRMKWKK), incubated for 48 h, and migration was quantitated as described in Experimental Procedures.

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