UNCOVERING THE BIOLOGICAL FUNCTIONS OF PHOSPHATASE OF REGENERATING LIVER -2

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The Phosphatase of Regenerating Liver (PRL) family, consisting of PRL-1, PRL-2, and PRL-3, is a group of prenylated phosphatases that are candidate cancer biomarkers and therapeutic targets. Individual PRLs are over-expressed in a variety of cancer cell lines and tissues, and elevated PRL expression has been associated with tumorigenesis and metastasis. Although several studies have documented that altered expression of PRL-1 or PRL-3 can influence cell proliferation, migration and invasion, there is an absence of knowledge about the biological functions of PRL-2. Thus, the current study was designed to evaluate the role of PRL-2 in cell migration and invasion in human cancer cells. I found that four lung cancer cells, including A549, over-expressed PRL-2 when compared with normal lung cells. PRL-2 suppression by siRNA or shRNA markedly inhibited cell migration and invasion. PRL-2 suppression by siRNA decreased p130Cas and vinculin expression, increased phosphorylation of Ezrin on tyrosine 146, and decreased ERK phosphorylation upon serum stimulation. There were no significant changes in total p53, Akt and c-Src expression levels or their phosphorylation status, suggesting PRL-2 suppression could inhibit tumor cell migration and invasion through a Src-independent p130Cas signaling pathway. Ectopic expression of wild type PRL-2, a catalytic inactive C101S mutant, and a C-terminal CAAX deletion revealed a requirement for both the PRL-2 catalytic functionality and prenylation site. Expression of wild type but not the mutant forms of PRL-2 caused ERK phosphorylation and nuclear translocation, and promoted tumor cell

migration and invasion. These results support a model in which PRL-2 promotes cell migration and invasion through an ERK-dependent signaling pathway. In addition, thienopyridone, a previously reported PRL inhibitor, showed antiproliferative activity in a concentration-dependent manner, and decreased cell migration and invasion. In summary, these studies demonstrate for the first time that PRL-2 regulates cell migration and invasion in non-small cell lung cancer, and I propose that PRL-2 stimulates cell migration and invasion through an ERK signaling pathway.

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DMSO: Dimethyl sulfoxide

ECM: Extracellular matrix

ERK: Extracellular signal-regulated kinase

ERM: Ezrin/radixin/moesin

FAC: Focal adhesion complex

FBS: Fetal bovine serum

FN: Fibronectin

FT: Farnesyltransferase

GST: Glutathione S-transferase

HA: Hemaglutinin epitope

KD: Knockdown

M: mol/L

MAPK: Mitogen activated protein kinase

MLCK: Myosin light-chain kinase

MLCP: Myosin light-chain phosphatase

MMP: Matrix metalloproteinase

PBS: Phosphate buffered saline

PCR: Polymerase Chain Reaction

PI-3K: Phosphatidylinositol 3 kinase

RIPA: Radioimmunoprecipitation assay

PMA: Phorbol 12-myristate 13 acetate

PRL: Phosphatase of regenerating liver

PTEN: Phosphatase and tensin homologue

PTP: Protein tyrosine phosphatase

ROCK: Rho kinase

RT: Reverse Transcription

SDS: Sodium dodecyl sulfate

Ser: Serine

shRNA: Short hairpin RNAs

siRNA: Short interference RNAs

TBST: Tris-buffered saline Tween 20

Thr: Threonine

TPD: Thienopyridone

TPA: 12-O-tetradecanoylphorbol-13-acetate

Tyr: Tyrosine

WT: wild-type

PREFACE

It has been the most fulfilling five years of my life since I started my Ph.D training in University of Pittsburgh with all the expectations of a new beginning along with a bit of fear from having to stand on my own in a completely different place. This dissertation is an important witness of my experiences in academia, overcoming the difficulties to reach goals that I did not always know I could achieve. I would not be able to make it without the people who walked me through all the good and bad times. Therefore, I would like to take this opportunity to express my deep thankfulness to them.

I would first and foremost like to thank my advisor, **Dr. John S. Lazo** who has been my mentor not only in my dissertation project, but also in my life. He led me into this fascinating world of scientific discovery in the Drug Discovery Institute. He always supported and encouraged me whenever my experiments hit an obstacle while never showing any impatience or disappointment. His wisdom, enthusiasm, optimistic attitude, and bright vision toward science have motivated me to conquer any difficulties I encountered. Any success I achieve is a reflection of his mentorship.

I would also like to thank my committee members: **Drs. Donald B. DeFranco**, **Cary Wu, Daniel Altschuler,** and **Thomas Smithgall** for their generous help, valuable advice and constructive criticism to shape this dissertation.

I also want to thank the former and current Lazo Laboratory members, especially **Pallavi Bansal, Robert J. Tomko**, and **Hiroyuki Achiwa** who are kind enough to answer numerous questions when I began my research. I thank all the laboratory managers: **John Skoko, Catherine Corey, and Celeste Reese** for assistance in protocols, equipments and orderings. I also acknowledge all the other graduate students and fellow colleagues in the **Lazo Lab** for their assistance and help. It was such wonderful pleasure to work with all of them and to share all the sweet memories.

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Most importantly, I want to express my thankfulness and my love to **my parents**, whose unconditional love and trust will sustain me through any hurdles and challenges that I may face. In addition, I want to thank my beloved husband, **Ping Zhang**. We met, dated and happily married in Pittsburgh, all of which seemed so perfectly predestined. Always being supportive and considerate, he is the man to depend on through all my hard times, and the remainder of our lifetime. Last but not least, I am grateful to my baby girl, **Nina Zhang**, who has brought me the happiness that I have never had before.

With sincere respect and appreciation,

Yan Wang

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1.0 INTRODUCTION

1.1 PHOSPHATASES OF REGENERATVING LIVER

1.1.1 Phosphatases of Regenerating Liver (PRLs)

The Phosphatase of Regenerating Liver (PRLs) family represents a novel subfamily of protein tyrosine phosphatases (PTPs), comprising three family members. PRL-1 is originally identified as an immediate early gene in regenerating liver [1]. Subsequently, two other PRLs, PRL-2 and PRL-3, are identified and they are closely related in sequence and structure to PRL-1 [2-4]. The three human PRLs share approximately 80% identity in amino acid sequence and conserved signature PTP active site sequence CX5R [5]. Figure 1 is the amino acid sequence alignment of human PRL-1, PRL-2, and PRL-3. The amino acids common to all three PRLs are in the same color marked with *. The $C(X)_5R$ motif and the CAAX prenylation motif are boxed. Human PRL-1 (also known as PTP4A1 and PTPCAAX1) and PRL-2 (also known as PTP4A2 and PTPCAAX2) share the closest amino acid identity at 87%. PRL-1 and PRL-3 (also known as PTP4A3 and PTPCAAX2) exhibit 79% sequence identity, and PRL-2 and PRL-3 have 76%

sequence identity. The genes encoding PRL-1, PRL-2, and PRL-3 are located on chromosomes 6q12, 1p35, and 8q24.3, respectively. Full-length transcripts of PRLs, 173 amino acids for both PRL-1 and PRL-3 and 167 amino acids for PRL-2, code for protein products of approximately 20 kDa [5].

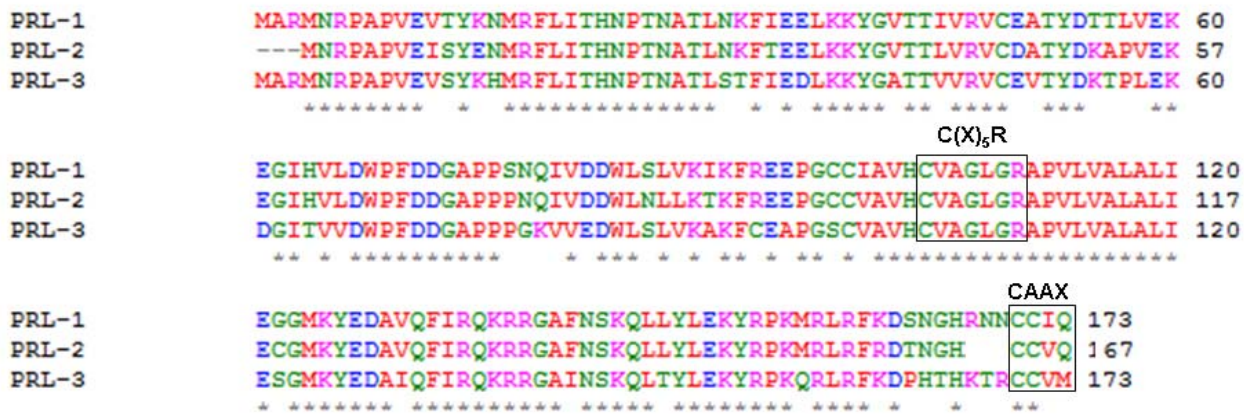

Figure 1. Amino Acid Sequence Alignment of Human PRL-1, PRL-2, and PRL-3 using ClustalW2.

Amino acids common to all three PRLs are shaded. The $C(X)_5R$ motif and the CAAX prenylation motif are boxed.

The PRLs belong to the PTP superfamily. They have an N-terminal catalytic domain containing the signature PTP active site sequence CX_5R. The catalytic or PTP domain is responsible for enzymatic activity, requiring the CX_5R active site or P-loop residues, and the WPD loop residues for phosphate transfer. Figure 2 is a schematic diagram of the PRL proteins.

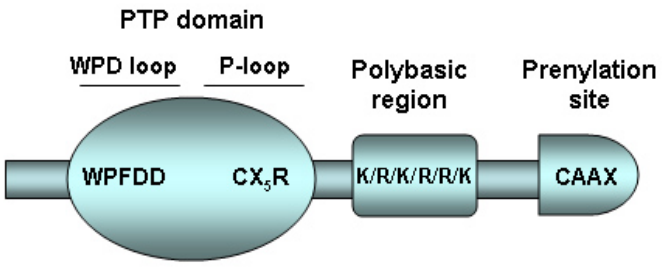

Figure 2. Schematic Diagram of the PRL proteins.

PRL-1, -2, and -3 share a homologous domain structure. The catalytic or PTP domain is responsible for enzymatic activity, requiring the CX5R active site or P-loop residues, and the WPD loop residues for phosphate transfer. A polybasic domain and a prenylation motif (PRL-1, CCIQ; PRL-2, CCVQ; PRL-3, CCVM) are important for determining the intracellular localization of PRL proteins. Modified from reference [6].

A polybasic domain and a prenylation motif (PRL-1, CCIQ; PRL-2, CCVQ; PRL-3, CCVM) are important for determining the intracellular localization of PRL proteins. The detailed introduction to the catalytic domain of PRLs will be discussed further in the following section entitled "PRLs Substrates". The PRLs are unique among the PTPs because they possess a conserved C-terminal CAAX domain for prenylation, where C is cysteine, A is an aliphatic amino acid, and X is any amino acid. The CAAX domain and its prenylation are thought to be important for targeting proteins to intracellular membranes. Therefore, PRLs are typically localized at the plasma membrane and in the intracellular punctuate structures [3, 7]. These punctuate structure resemble those of the secretary and/or endocytotic pathway. Three types of prenyltransferases carry out the addition of farnesyl (C15) or geranylgeranyl (C20) isoprenoids to the cellular proteins: farnesyltransferase (FT), geranylgeranyl transferase I (GGT I) and geranylgeranyltransferase II (GGT II). FT and GGT I are heterodimeric α/β enzymes that mediate prenylation of the CAAX sequence [8, 9], with FT preferring Met, Ser, or Gln in the X

position and GGT I preferring Leu in the X position [10]. GGT II is a distinct α/β dimer that prenylates XXCC, XCXC, or CCXX C-terminal sequences when the substrate protein is bound to a carrier protein called REP (Rab escort protein) [11, 12]. Protein prenylation is important in targeting proteins to intracellular membranes and in protein-protein interactions [10, 13]. For example, PRL-1, PRL-2 and PRL-3 are associated with the plasma membrane and the early endosome and are dependent on the prenylation of the C-terminal CAAX domain [3, 7].

In normal tissue, PRL-3 is expressed in a tissue-specific manner, while PRL-1 and PRL-2 are more ubiquitously expressed. In adult rodents PRL-1, PRL-2, and PRL-3 are predominantly expressed in skeletal muscle, with PRL-1 also being expressed at high levels in the brain and PRL-3 being expressed at moderate levels in the heart [1, 4]. The humans PRL-3 is primarily expressed in heart and skeletal muscle, although low levels of PRL-3 expression are detectable in a number of other tissues as well [4, 14]. PRL expression in humans was not well characterized until an exhaustive examination of the expression pattern and levels of PRL-1 and -2 in various human tissues and cell types was conducted by Dumaual et al. using *in situ* hybridization [15]. From that study, PRL-2 was shown to be nearly ubiquitous expressed. It is undetectable only in a few highly specialized tissues, such as the taste buds and fibrocartilage. PRL-1 is also present in most tissues, albeit at a lower level than PRL-2 and with a somewhat more restricted pattern of expression [15]. The widespread nature of PRL-1 and PRL-2 expression may indicate that they function in basic processes that are common to many tissues and cell types. Although the PRLs are highly homologous, their expression pattern differs among tissues, suggesting PRLs could exhibit functional divergence.

1.1.2 PRLs Expression in Cancer

Individual PRLs are over-expressed in a variety of cancer cell lines and tissues. For example, PRL-3 is dramatically up-regulated in metastatic colorectal carcinomas [16-18], breast carcinoma [19, 20] and gastric carcinomas [21, 22]. Elevated PRL-3 is associated with the progression of these types of human carcinomas [16, 17, 19, 20, 22], making PRL-3 a promising biomarker with predictive significance. The role of PRL-1 and PRL-2 in cancer is less well studied. Recent evidence demonstrates elevated expression of PRL-1 in melanoma, pancreatic cancer, lung cancer cell lines and esophageal squamous cell carcinoma [23-25]. In addition, elevated levels of PRL-2 have been detected in pancreatic, prostate cancer cell lines, pediatric acute myeloid leukemia, and prostate and breast tumor tissues [26-29].

PRL-3 over-expression and its role in cancer have gained widespread interest since Saha et al. reported the initial finding on colorectal carcinomas (CRCs) [30]. PRL-3 is consistently elevated in colonic epithelial cells microdissected from liver metastases compared to non-metastatic colorectal tumor or normal colon epithelium [30]. Other studies, using *in situ* hybridization and/or immunohistochemistry to detect PRL-3 mRNA and protein, have confirmed elevated PRL-3 expression in CRCs. PRL-3 expression is elevated in 11–45% of primary CRC tumors, and also elevated in CRC metastases, not only in the liver, but also in secondary CRC lesions found in the lung, brain, ovary, peritoneum, and lymph nodes [16-18, 31]. In addition, high expression of PRL-3 in primary CRC significantly correlates with venous invasion [16]. Thus, PRL-3 is thought to be a potentially useful predictive marker of colorectal cancer aggression and outcome.

Table 1. PRL Expression in Tumor Tissues and Cell Lines.

Modified from reference [5].

	Cell lines or tissues		Observation	Methods
PRL-1	Melanoma cell lines		5 of 6 show elevated expression	Quantitative RT-PCR
	Pancreatic cancer cell		High expression relative to normal	cDNA microarray
PRL-2	Prostate cell lines		Overexpression in LNCaP, PC-3, and DU-145 cells	RT-PCR
	Prostate cancer patient		2 of 3 samples showed over-expression	RT-PCR
	Breast cancer cell line		Highest PRL-2 level in MDA-MB-231	Quantitative RT-PCR
	Breast cancer tumor samples		Increased PRL-2 level during breast cancer progression	Quantitative RT-PCR
PRL-3	Invasive breast tumor		Expression in vasculature but not in tumor	Serial analysis of gene expression
	Colon	cancer metastasis	Expressed at high levels in 18 of 18 samples	Serial analysis of gene expression
		Normal epithelia	Not expressed	*In situ* hybridization
		Nonmetastatic carcinoma	Not expressed	*In situ* hybridization
		Primary tumors	Expressed at high levels in 44.6% of samples	*In situ* hybridization
		Primary tumors with metastases	Expressed at high levels in 51.8–88.9% of samples	*In situ* hybridization
		Carcinoma metastases	Expressed in 86–100% of samples (depending on site)	*In situ* hybridization
		Normal epithelia	Expressed in 7.1% of samples	Immunohistochemistry
		Primary tumors	Expressed in 23.9% of samples	Immunohistochemistry
		Carcinoma metastases	Expressed in 53.7–66.7% of samples	Immunohistochemistry
	Gastric	Cell lines	Expressed in 87.5% of cells	RT-PCR
		Primary carcinoma without metastasis	Expressed in 50% of samples	RT-PCR
		Primary carcinoma without metastasis	Expressed in 81.5% of samples	RT-PCR

Following the initial findings made by Saha et al. in CRCs, PRL-3 expression has been investigated in other common cancer types, including breast, gastric and liver carcinomas. For example, Radke et al. [19] reported high PRL-3 expression in 42% of 135 ductal carcinomas *in situ,* and in 25% and 16% of two sets (147 and 99 samples) of invasive breast carcinoma. Wang et al. also [20] reported that 35% of 382 operable primary breast cancers expressed a high level of PRL-3. In the context of gastric carcinoma, 68% and 70% of primary tumors are positive for PRL-3 expression [21, 22] using different PRL-3 antibodies for immunohistochemical analyses of the two sample sets, and about half of the positive samples express PRL-3 protein at a high level [21]. Furthermore, each study shows significantly increased frequencies of PRL-3-positive metastases: lymph node (92.6%) [21] or peritoneal metastases (92.1%) [22]. In addition, PRL-3 is up-regulated in liver carcinoma, as Northern blot analysis of PRL-3 RNA levels in 23 liver carcinomas and 5 normal liver samples show about two-fold higher PRL-3 mRNA levels in the carcinomas when compared to the normal tissues [32]. In sum, all the studies described above indicate that elevated PRL-3 expression is associated with the progression of several types of human carcinoma, and the increased PRL-3 level correlates with reduced survival time of patients. Thus PRL-3 shows promise as a biomarker with predictive significance.

The initial evidence accumulated so far supports a link of elevated PRL-1 or PRL-2 to cancer. For example, Wang et al. [24] examined the PRL-1 levels in 76 different cell lines, including 67 cancer cell lines and 9 normal/untransformed cell lines. They reported that PRL-1 mRNA levels are elevated in several tumor cell lines compared with the untransformed cell lines. Melanoma cell lines show the most consistent high levels of expression, specifically five of six melanoma

cell lines express high levels of PRL-1 [24]. Elevated PRL-1 was also reported in pancreatic cancer cell lines by Han et al. [25]. PRL-2 over-expression was reported in the LNCaP prostate cancer cell line, and was also detected in two small sets of prostate carcinoma. Recently, Hardy et al. found that PRL-2 was elevated in primary breast tumors relative to matched normal tissue, and is also elevated in metastatic lymph nodes compared to primary tumors [28].

1.1.3 PRLs Function and Pathways

Despite all the accumulated literature on PRLs expression levels in cancer, the biological function and mechanism of the PRLs have remained elusive for a long time. Many earlier articles describing PRLs focused on their involvement in proliferation and ability to transform nontumorigenic immortalized cell lines. For example, ectopic expression of PRL-1, PRL-2, and PRL-3 can cause increased levels of cellular proliferation [1, 3]. In contrast, in the colorectal adenocarcinoma DLD-1 cell line, transient down-regulation of PRL-3 or PRL-1 by small interfering RNA seems to have no effect on proliferation [16]. There are also some early studies examining PRLs as cell cycle regulators [33, 34], but it has not been confirmed to be common effects for the other cell lines.

Recent findings have focused on the roles that PRLs play in promoting the tumorigenic properties and metastatic ability of cells. The first study to demonstrate that PRL-3 over-expression promoted the tumorigenic properties and metastatic ability of cells was carried out by Zeng et al. using a Chinese hamster ovary (CHO) cell line [35]. The stable expression of myc-

tagged PRL-3 increases cell migration in wound healing and Transwell assays, and also increases cell invasion in Matrigel Transwell assay when compared with β-gal-transfected control cells. Moreover, upon injection of myc-PRL-3-expressing cells into the tail vein of mice induce lung metastases and some liver metastases, whereas control cells do not give rise to metastatic lesions. Subsequently, accumulating evidence supports that PRL phosphatases, especially PRL-3 and PRL-1, are involved in tumor development and metastasis [3, 32, 35], although the molecular mechanism remains largely unclear. Briefly, ectopic expression of PRL-1 or PRL-3 in non-tumorigenic cells can enhance cell proliferation and the migratory and invasive properties of cells [35, 36]. RNA interference-mediated knockdown of the endogenous PRL-1 or PRL-3 in cancer cells can abrogate cell motility and ability to metastasize in a mouse model [16, 23].

Although the molecular mechanism of PRLs-mediated actions is poorly understood, recent findings have revealed that Rho family GTPases [37], Src [23, 36, 38], PI3K [39], p53 [39, 40] and ERK [41, 42] are important for PRL-1 and/or PRL-3 mediated changes on cell invasion and metastasis. In SW480 colorectal carcinoma cells, Fiordalisi et al. found that PRL-1 and PRL-3 activate Rho A and Rho C to promote cell invasion and motility [37]. Independent studies from different groups demonstrate the involvement of Src in PRL-1 or PRL-3 signaling pathways [23, 36, 38]. PRL-3 down-regulates PTEN expression and promotes Epithelial-Mescenchymal Transition (EMT) through PI3K signaling pathways [39]. PRL-3 is reported to be a p53 target involved in cell cycle regulation [39], while PRL-1 is thought to be a new oncogenic p53 target [34, 40]. PRL-3 promotes LoVo colon cancer cell invasion through PRL-3-intergrin β1-ERK1/2 signaling [42], while PRL-3 also facilitates angiogenesis and metastasis by increasing ERK

phosphorylation in lung cancer [41]. Table 2 lists the molecular effects of altered PRL expression, which are investigated in various cultured cell lines, including A549 lung carcinoma cells, CHO Chinese hamster ovary cells, D27 hamster pancreatic ductal epithelial cells, DLD-1 colorectal adenocarcinoma cells, HEK 293 human embryonic kidney cells, HEK 293T human embryonic kidney cells transfected with SV40 T-antigen, HeLa cervical adenocarcinoma cells, MCF10A mammary gland epithelial cells, SW480 colorectal adenocarcinoma cells [6].

Table 2. Molecular Effects of Altered PRL Expression.

The molecular effects of PRL overexpression or ablation were investigated in various cultured cell lines. N.D. not determined. Modified from reference [6].

pathways	Targets	PRL-1	PRL-2	PRL-3
Integrin/Src signaling	Integrin α1	N.D.	N.D.	Binds *in vivo* in HEK 293T
	Integrin β1	N.D.	N.D.	Tyrosine phosphorylation reduced in HEK 293T
	Integrin β3	N.D.	N.D.	Reduced expression in DLD-1
	Src	Decreased expression in PRL-1-depleted A549	N.D.	Increased Src activity in HEK 293
	Csk	Reduced expression in PRL-1-depleted A549	N.D.	Reduced expression in HEK 293
	FAK	Persistent Tyr-397 phosphorylation in PRL-1-depleted A549	N.D.	No effect in HEK 293
	$p130^{Cas}$	Decreased expression in PRL-1-depleted A549	N.D.	Enhanced tyrosine phosphorylation in HEK 293
	paxillin	Reduced expression in PRL-1-depleted A549	N.D.	Reduced expression and reduced Tyr-31 phosphorylation in HeLa
	vinculin	N.D.	N.D.	Decreased localization to focal adhesions in HeLa and CHO, enhanced binding to $p130^{Cas}$ in HEK 293
	$p130^{Cas}$	N.D.	N.D.	Inhibited tyrosine phosphorylation in HEK 293
	ERK1/2	N.D.	N.D.	Increased activity in HEK 293 and HEK 293T
PI3K/Akt signaling	PTEN	N.D.	N.D.	Reduced expression in DLD-1
	Akt	N.D.	N.D.	Increased activity in DLD-1
	GSK-3β	N.D.	N.D.	Decreased activity in DLD-1
EMT	E-cad, γ-cat	N.D.	N.D.	Reduced expression in DLD-1
	Snail	N.D.	N.D.	Upregulated expression in DLD-1
Rho family GTPases	Rho	Increased activity in SW480	N.D.	Increased activity in SW480, reduced in CHO
	Rac	Reduced activity in SW480, A549	N.D.	Reduced activity in SW480, CHO
	Cdc42	Unaffected in SW480, reduced in A549	N.D.	Activity unaffected in SW480
	SRE	Activated in SW480	N.D.	Activated in SW480

1.1.4 PRLs Substrate and Inhibitors

Although PRLs have a clear phosphatase domain, no substrate has been convincingly identified for the PRLs. PRL phosphatases are close to PTPs in sequence but also share close structural homology to dual specific phosphatases (DSPs). Specifically, PRLs have <30% sequence identity to DSPs, but they showed a shallow catalytic pocket, which is typical for DSPs. The catalytic pockets of PRLs are unusually wide and shallow, indicating that PRLs could accommodate a broad range of phosphorylated substrates [6]. Several differences between PRLs and classical PTPs may account for their low catalytic activity *in vitro* and the difficulty in substrate trapping.

Interestingly, Ezrin was recently reported to be a specific and direct target of protein PRL-3 [43]. Ezrin is a linker between the plasma membrane and the actin cytoskeleton. It is regulated by changes in its conformation, and is involved in cellular functions including cell morphogenesis, adhesion and migration. Forte et al. [43] reported that ectopic over-expression of wild type PRL-3 caused a decrease in phosphorylation at both tyrosine residues and Thr567 in the HCT116 colon cancer cell line. Although PRL-3 over-expression in HCT116 cells appeared to affect Ezrin phosphorylation status at Tyr146, Tyr 353, and Thr567, suppression of the endogenous PRL-3 by RNA interference primarily affected the residue Thr567 of Ezrin. In addition, *in vitro* dephosphorylation assays using Flag-PRL-3 as enzyme and immunoprecipitated Ezrin as substrate also suggested that Ezrin-Thr567 was a direct substrate of PRL-3. These observations

challenge the current believe that PRLs belongs to PTPs. Whether or not Ezrin is a substrate for the other PRL family members is still awaited for further study.

Although there is a lack of knowledge on the substrates for the PRLs, evidence suggests that the catalytic activity is important for PRL-3 functionality. For example, a catalytically inactive PRL-3 mutant has significantly reduced migration-promoting activity [35]. EGF-PRL-3 expressing CHO cells rapidly induced metastatic tumor formation in lung while the catalytically inactive mutant expressing ones lost this metastatic activity [44]. Forte et al. [43] reported that ectopic over-expression of wild type PRL-3 but not the catalytically inactive mutant caused decrease in phosphorylation at both tyrosine residues and Thr567 of Ezrin in HCT116 colon cancer cell line. In addition, phosphatase activity, the C-terminal polybasic region and prenylation site are all required for PRL-1 mediated cell growth and cell migration [45].

Considering the importance of the catalytic activity to the biological functions of PRLs, the PRL family has gained widespread attention from the cancer researchers since it is an attractive target for small molecular inhibitors. There has been a long history in targeting PTPs with small molecule inhibitors in cancer treatment [46]. A number of PTPs have been implicated in oncogenesis and tumor progression, and therefore are potential anti-cancer drug targets for cancer chemotherapy. These phosphatases include SHP2, which is the first identified oncogene in the PTP superfamily and is essential for growth factor-mediated signaling; PTP1B, which may augment signaling downstream of HER2/*Neu*; the Cdc25 phosphatases, which are positive regulators of cell cycle progression; and PRL-3 phosphatase, which is thought to promote tumor metastases [47].

As stated previously, PRLs make an attractive target for small molecule inhibitors designed to prevent and/or treat metastases. Potent and selective PRL inhibitors may ultimately constitute a novel and effective family of anti-cancer agents. Unfortunately, very limited information for PRL inhibitor design is available in the literature. Work from Pathak et al. showed that the anti-leishmaniasis drug pentamidine (1,5-di(4-amidinophenoxy)pentane; panel A in Figure. 3) inhibited all three recombinant PRLs *in vitro* and caused tumor shrinkage in a melanoma mouse xenograft model [48]. Pentamidine inhibits the activity of all three PRLs as well as other PTPs like PTP1B, SHP2, and MKP1, so it is unclear if the inhibition of tumor growth is caused by the specific inhibition of a PRL, a combination of the PRLs, or combinations of other phosphatases. Some natural products derived from extracts, such as rhodanine derivatives [49] (panel B in Figure. 3) and biflavonoids [50] (panel C in Figure. 3) have been reported as PRL-3 inhibitors with IC 50 values in the low μM range. Two biflavonoids, ginkgetin and sciadopitysin are isolated from the MeOH extract of the young branches of *Taxus cuspidata*, which inhibit PRL-3 with IC 50 values of 25.8 and 46.2 μM, respectively [50]. This is the first report on PRL-3 inhibitors, isolated from natural sources. Benzylidene rhodanine derivative shows good biological activity, and is the most active compound in this series exhibiting an IC 50 value of 0.9 μM *in vitro* and reduces invasion in cell-based assay [49].

Thienopyridone is the first potent selective PRL inhibitor that suppresses tumor cell three-dimensional growth by a novel mechanism involving p130Cas cleavage [51]. Daouti et al. have identified the small molecule, 7-amino-2-phenyl-5H-thieno(3,2-c)pyridin-4-one (thienopyridone) from a bead-based immobilized metal ion affinity-based fluorescence polarization (IMAP-FP)

assay. Thienopyridone potently and selectively inhibits all three PRLs but not the other phosphatases *in vitro*. It exhibited similar IC 50 values of 0.173, 0.277, and 0.128 μM in PRL-1, PRL-2, and PRL-3 phosphatase assays, respectively. When using DiFMUP as a substrate for all protein phosphatase (PTPase) assays, thienopyridone displays excellent selectivity against PRL-3 phosphatase with an IC 50 of 0.457 μM, but shows minimal effects on other phosphatases. In addition, thienopyridone shows significant inhibition of tumor cell anchorage-independent growth in soft agar, induction of the p130Cas cleavage, and anoikis, a type of apoptosis that can be induced by anticancer agents via disruption of cell-matrix interaction [51].

More recently, Park et al. [52] identified 12 novel PRL-3 inhibitors by means of the virtual screening with docking simulations under the consideration of the effects of ligand solvation in the scoring function. Of the 85,000 compounds library subject to the virtual screening with docking simulations, 200 top-scored compounds were selected as virtual hits. One hundred and ninety-one of them were available from the compound supplier and were evaluated for in vitro inhibitory activity against recombinant human PRL-3. Thirty eight compounds were identified that inhibited the catalytic activity of PRL-3 by more than 50% at the concentration of 50 μM. Among them, 12 compounds shown in panel E of Figure 3 have a high potency with more than 70% inhibition at the same concentration and are selected to determine IC 50 values [52]. The newly identified inhibitors are structurally diverse and reveal a significant potency with IC 50 values ranging from 10 to 50 μM, all of them can be considered for further development by structure-activity relationship or *de novo* design methods.

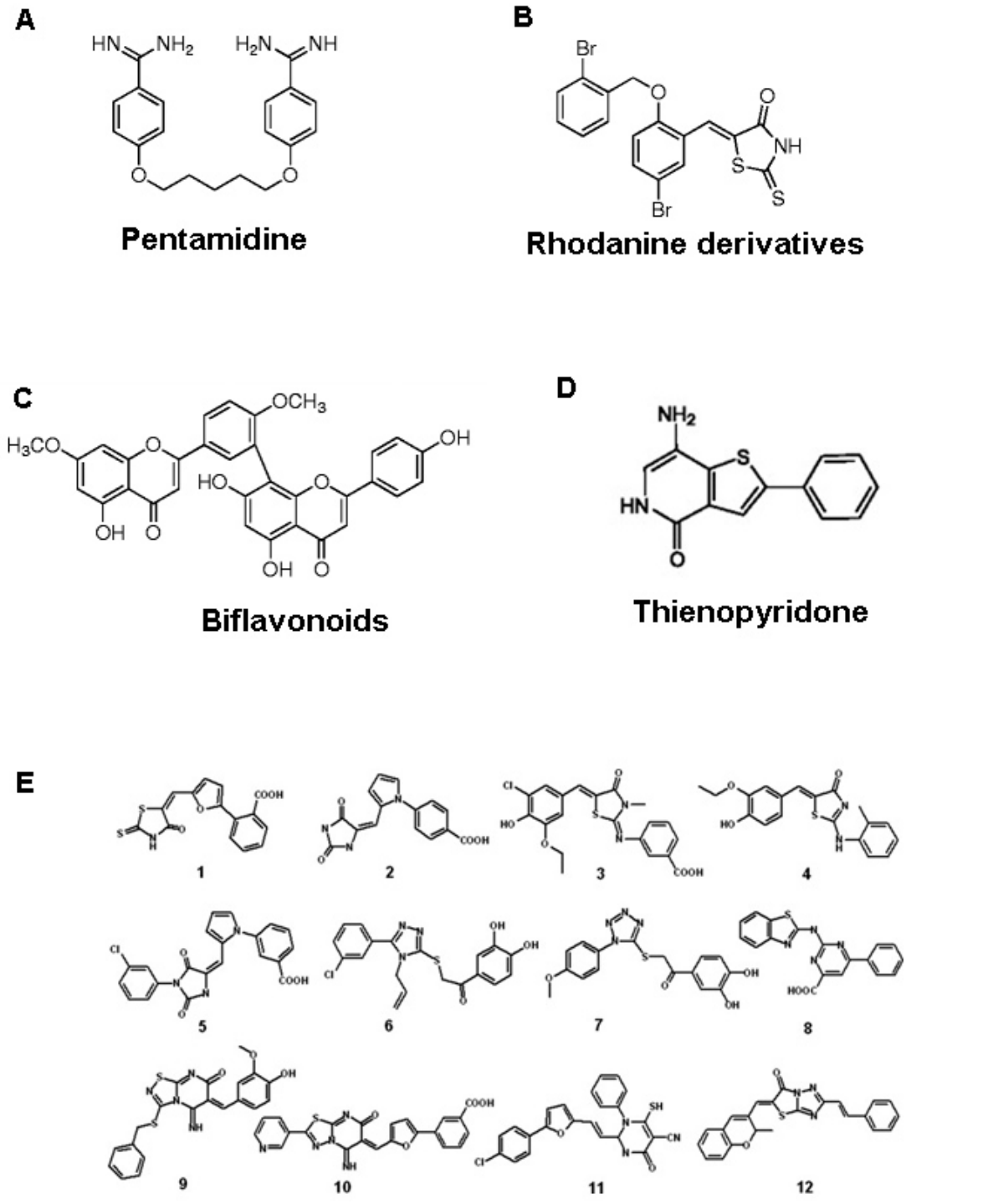

Figure 3. The Structure of PRL Inhibitors.

Collectively, PRL phosphatases represent a novel subfamily of PTPs that are implicated in a number of tumorigenic and metastatic processes. The PRL family is gaining widespread attention because potent PRL inhibitors may ultimately constitute a novel family of anti-cancer agents. Although efforts have been made to identify novel PRL inhibitors, the information is still limited. Further studies are required to establish the selectivity profiles and the modes of action (i.e., competitive/noncompetitive and irreversible) for the compounds. In addition, improving the specificity, stability and solubility of the compounds are also required for making them better PRL inhibitors, and better candidates for anti-cancer agents.

1.2 TUMOR CELL INVASION AND METASTASIS

Cancer is the disease in which a group of cells display uncontrolled growth, invasion, and sometimes metastasis. In 2005, the American Cancer Society reported that cancer has surpassed heart disease as the leading cause of death in the United States in people under the age of 85 [53]. Metastasis occurs when genetically unstable cancer cells adapt to a tissue microenvironment that is distant from the primary tumor. As we know, the majority of deaths (about 90%) associated with cancer are due to the metastasis of the original tumor cells to sites distant from the initial or primary tumor [54]. Recent conceptual and technological advances promote our understanding of the origins and nature of cancer metastasis.

1.2.1 Tumor Metastasis

Localized tumors are easier to treat. For most types of cancer, the acquisition of metastatic ability leads to clinically incurable disease. Tumor metastasis remains a significant contributor to death in cancer patients. Despite the obvious importance of metastasis, the process remains incompletely characterized at the molecular and biochemical levels [55].

To metastasize, cells must develop a number of new properties and proceed through several steps, including: loss of cellular adhesion [56, 57], increased motility and invasiveness [58, 59], entry and survival in the circulation [60], exit into new tissue [61], and eventual colonization of a distant site [62, 63]. Seminal work using experimental assays for metastasis demonstrate that rare clones within malignant cell populations are endowed with several of these metastasis-promoting functions [63]. The implication is that cells that comprise a metastatic lesion are descendants of an exceedingly rare cell from the primary tumor that stochastically expressed many, if not all, of the genes necessary for successful execution of the metastatic cascade [63]. Figure 4 is a schematic of the metastatic process [55, 63].

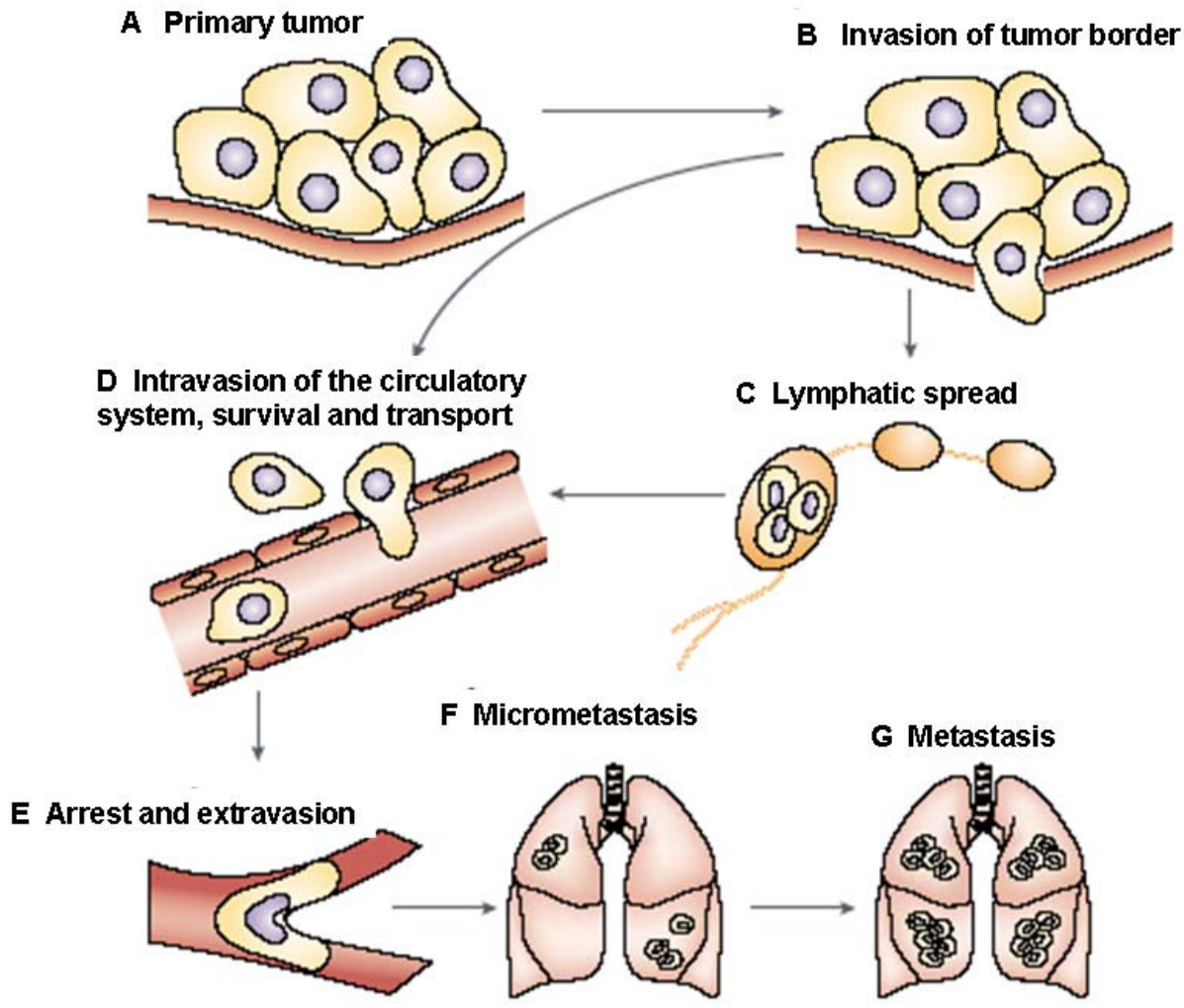

Figure 4. A Schematic of the Metastatic Process.

A schematic of the metastatic process, beginning with **A** | an *in situ* cancer surrounded by an intact basement membrane. **B** | Invasion requires reversible changes in cell–cell and cell–extracellular-matrix adherence, destruction of proteins in the matrix and stroma, and motility. Metastasizing cells can **C** | enter via the lymphatics, or **D** |directly enter the circulation. **E** | Survival and arrest of tumor cells, and extravasation of the circulatory system follows. **F** | Metastatic colonization of the distant site progresses through single cells, which might remain dormant for years, to occult micrometastases and **G** | progressively growing, angiogenic metastases. Modified from reference [55].

The metastatic process is described bellow**.** Cellular transformation or tumorigenesis is the first step in which growth of neoplastic cells must be progressive, with nutrients for the expanding tumor mass initially supplied by simple diffusion [63]. Extensive vascularization must occur if a tumor mass is to exceed 1–2 mm in diameter [64]. The synthesis and secretion of angiogenic factors establish a capillary network from the surrounding host tissue [64]. Tumor progression requires permanent or transient alterations in gene expression or function causing enhanced mobility and invasiveness. Utilizing the properties acquired as a result of progression will facilitate localized invasion, intravasation.

Intravasation is defined as local invasion of the host stroma by some tumor cells and occurs by several parallel mechanisms [65]. Thin-walled venules, such as lymphatic channels, offer very little resistance to penetration by tumor cells and provide the most common route for tumor cell entry into the circulation [66]. Some tumor cells could survive in an anchorage independent manner and are capable to overcome apoptotic signals and immune surveillance. Detachment and embolization of single tumor cells or aggregates occurs next, most circulating tumor cells being rapidly destroyed. After the tumor cells have survived the circulation, they become trapped in the capillary beds of distant organs by adhering either to capillary endothelial cells or to subendothelial basement membrane that might be exposed [63, 67]. The adhesion or arrest could be either physical size entraps them in vessels or receptor mediated attachment. The next step is extravasation, which is the invasion into surrounding tissue facilitated by acquisition of additional invasive and survival properties. It probably is operated by mechanisms similar to those that operate during invasion.

Metastatic colonization is the outgrowth of tumor cells after they have arrived at a distant site. Proliferation within the organ parenchyma completes the metastatic process. To continue growing, the micrometastasis must develop a vascular network [64] and evade destruction by host defenses. The contribution of angiogenesis to the outgrowth of micrometastases, so that they form tumors unlimited by the diffusion range of oxygen, is well described, and inhibitors are in clinical trial [68, 69]. Once the metastatic process at this site is complete, the colonized cells can then re-invade blood vessels, enter the circulation and produce additional metastases [70].

1.2.2 Cell Migration and Invasion

Cell migration and invasion are important because metastasis is dependent on tumor cells acquiring increased motility and invasiveness. Invasion is defined as the penetration of tissue barriers, such as basement membrane and interstitial stroma by cells [71]. Invasion requires adhesion, proteolysis of extracellular matrix components and migration. It occurs during normal cell morphogenesis and wound healing, and also in malignant cells.

Cell migration can be viewed as a series of discrete processes that result in net cell-body movement [72]. Initially, cells take on a polarized phenotype with a distinct cell front and rear. At the leading edge of the cell, actin polymerization and localized decreases in cell-membrane tension lead to the projection of a lamellipodium. This polarized form reflects the asymmetric distribution of the intracellular molecules and forces that are necessary for migration. Receptors for chemotactic molecules, integrins and cytoskeletal proteins that interact with integrins localize

at the leading edge of the cell, thereby loading the region of the cell that is extended in the direction of migration with the receptors and signalling molecules that are necessary to form and react to new adhesive contacts. For the cell to complete its translocation, adhesive contacts at the rear of the cell are released, either by cleaving of the integrins from the cell body using enzymes such as calpain, or by reduction of integrin affinity for the extracellular matrix (ECM). After forming new cell–ECM contacts, activation of intracellular actin–myosin motor units generates contractile forces that lead to the cell body advancing forward [72].

Many proteins that have been implicated in cell–matrix adhesion and cell migration are phosphorylated, which regulates their folding, enzymatic activities and protein–protein interactions. Increasing evidence confirms that phosphatases are essential at each stage of the migration process [73]. Phosphatases can regulate the formation and maintenance of the actin cytoskeleton, control small GTPase molecular switches, and modulate the dynamics of matrix–adhesion interaction, actin contraction, rear release and migratory directionality [74].

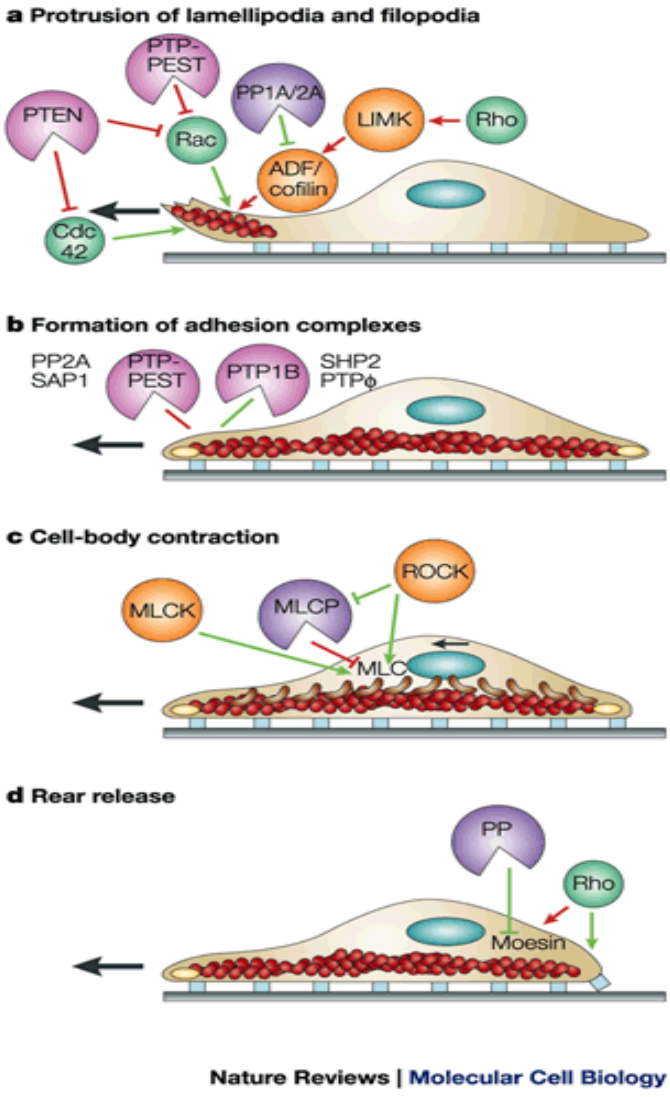

Figure 5. Influence of Phosphatases on Cell Migration.

Colored lines indicate stimulation (green), or inhibition (red), of fibroblast migration.

Reproduced from reference [73].

Figure 5 shows the influence of phosphatases on cell migration [73]. a) Migration is initiated by protrusion of the leading edge and formation of new actin filaments. Rac induces lamellipodia and Cdc42 stimulates filopodia. Their actions are opposed by phosphatase and tensin homologue (PTEN). Rac activation is also opposed indirectly by protein tyrosine phosphatase (PTP)-PEST. The actin-severing protein, actin depolymerizing factor (ADF)/cofilin, can be dephosphorylated by protein phosphatases (PP)1A and PP2A to stimulate migration. LIM kinase (LIMK), which is activated by Rho, phosphorylates ADF/cofilin. b) Attachment at the leading edge occurs first with focal complexes, which develop into focal adhesions. Formation/turnover of focal adhesions is regulated by many phosphatases, some of which include: PP2A, SAP1 and PTP-PEST, all of which are generally inhibitory to migration; and PTP1B, SHP2 and PTP, all of which are generally stimulatory to migration. c) Cell-body contraction results from forces generated through actomyosin interactions. Myosin light-chain (MLC) phosphatase (MLCP) dephosphorylates MLC to inhibit migration and oppose the action of MLC kinase (MLCK). Rho kinase (ROCK) phosphorylates MLCK and inhibits its activity. d) Rho stimulates tail retraction. Rho also phosphorylates the actin-binding-protein moesin, which is dephosphorylated at the rear of the cell, before rear release. Moesin is dephosphorylated by the PP1 and PP2 phosphatases [73].

1.2.3 Focal Adhesion in Cancer

Cell migration is a coordinated process that involves rapid changes in the dynamics of actin filaments, together with the formation and disassembly of cell adhesion sites [74]. The alternation of the components of focal adhesion complex (FAC) is very common in cancer. This leads to disruption of focal adhesion contacts, and has an important effect on tumor cell migration and invasion.

A central question in cell biology is how the extracellular signals are transmitted inside cells to modulate cell adhesion and motility. A complex interplay between the actin cytoskeleton and cell adhesion sites leads to the generation of membrane protrusions and traction forces [75]. External stimuli that control cell migration are transduced into intracellular biochemical signals through the interactions of transmembrane integrins that bind to extracellular matrix (ECM) proteins, growth factors that bind to their cognate cell-surface receptors, or mechanical stimuli such as shear stress that promote deformation of the actin cytoskeleton [57]. Focal contacts are dynamic groups of structural and regulatory proteins that transduce external signals to the cell interior [76]. Focal adhesion kinase (FAK), the most crucial component at focal contacts, is activated by numerous stimuli and functions as a biosensor or integrator to control cell adhesion and motility [77]. The other focal contact proteins, such as paxillin, talin, vinculin, p130Cas, are recruited to the focal contacts to form a large focal adhesion complex (FAC), which transmits the signals to the downstream targets to control cell motility.

Among these components, p130Cas is a critical scaffolding protein, which directly interacts with a number of proteins in the FAC. It contains: (i) an N-terminal SH3 domain; (ii) a proline-rich region; (iii) a large substrate-binding domain containing 15 repeats of YxxP sequence; (iv) a serine-rich region; and (v) a C-terminal domain. The N-terminal SH3 domain binds to several effectors, such as FAK and DOCK180 [78]. The tyrosine residues in the YxxP sequences are a substrate of protein tyrosine kinase and, when phosphorylated, provide a binding site for the SH2 or PTB domains of effector proteins. The C-terminal domain is characterized by a bipartite binding site that includes a proline-rich region (RPLPSPP), which binds to the Src SH3 domain,

and a tyrosine-containing sequence (YDYV), which binds to the Src SH2 domain when phosphorylated [78].

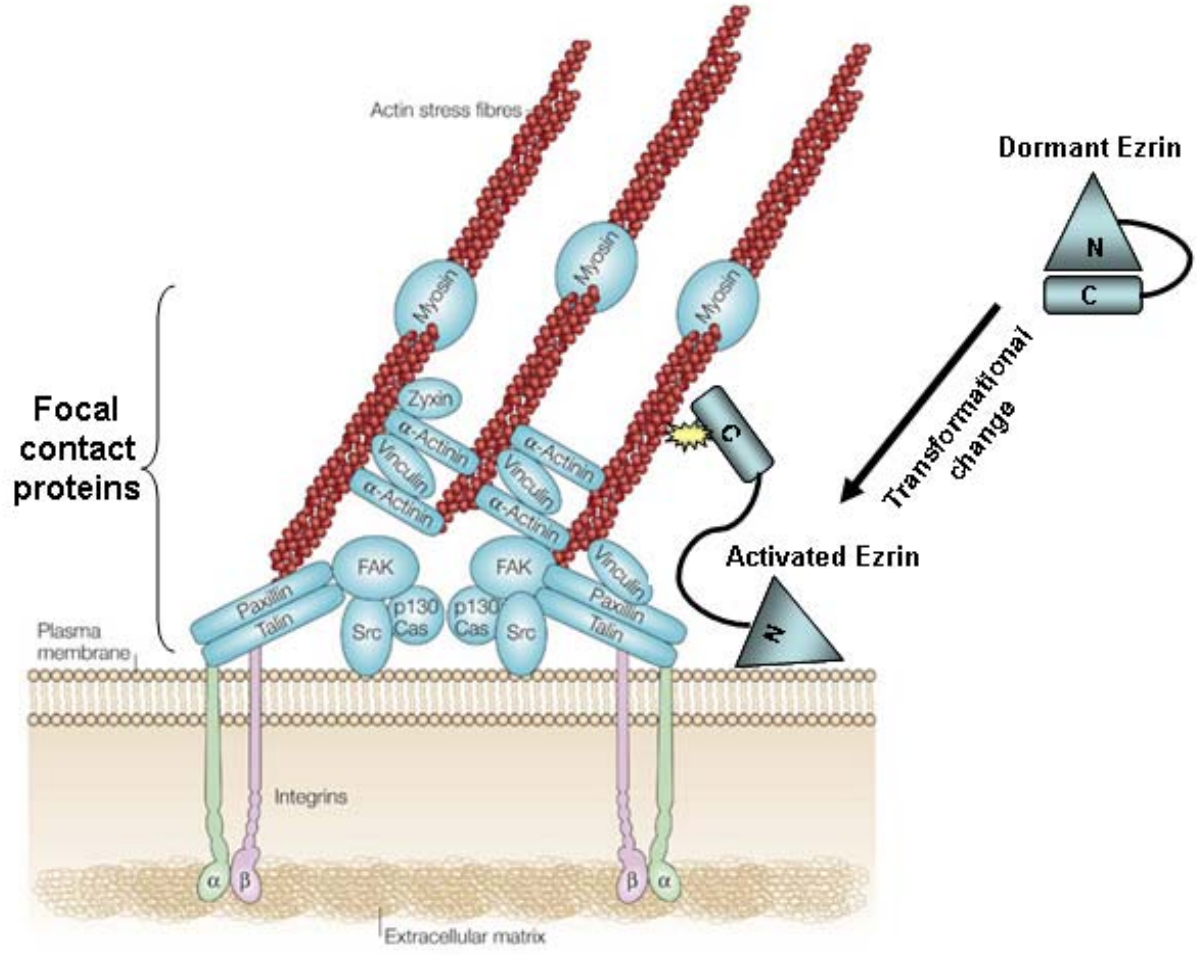

Figure 6. Molecular Architecture of Focal Contacts.

Modified from reference [77].

Figure 6 shows the molecular architecture of focal contacts [77]. The integrin-binding proteins paxillin and talin recruit FAK and vinculin to focal contacts. α-Actinin is a cytoskeletal protein that is phosphorylated by FAK, binds to vinculin and crosslinks actomyosin stress fibres and tethers them to focal contacts. Zyxin is an α-actinin and stress-fiber-binding protein that is present in mature contacts [76]. These proteins are commonly found in most focal contacts. Some other proteins have also been found in focal adhesion complexes. For example, the membrane-associated protein tyrosine kinase Src and the adapter protein p130Cas [79] associate with focal contacts following integrin clustering. Several other proteins such as extracellular signal-regulated kinase 2 (ERK2) and calpain are known to be transiently present at focal contacts [77]. The composition of a focal contact is therefore constantly varying depending on external cues and cellular responses [77].

Ezrin, a major member of the ezrin/radixin/moesin (ERM) family, is a linker between plasma membrane and cytoskeleton. The functions of Ezrin are regulated by conformational changes. The intramolecular interaction between N- and C- terminal domains of Ezrin charges masks several binding sites, leading to a dormant protein. Activation signals regulate Ezrin function by altering the phosphorylation status of Ezrin which modulate these intramolecular interactions [79]. The activated Ezrin crosses-link actin filaments with the plasma membrane directly or indirectly with help of other adaptor proteins, such as EBP-50.

1.2.4 ERK Signaling in Cancer

Mitogen-activated protein kinase (MAPK) cascades are key signaling pathways involved in the regulation of normal cell proliferation, survival and differentiation. Aberrant regulation of MAPK cascades contribute to cancer and other human diseases. There are four major mammalian MAPKKK–MAPKK–MAPK protein kinase cascades: c-Jun N-terminal protein kinase (JNK)/stress-activated protein kinase (SAPK), the p38 MAPK, and the extracellular signal-related kinase (ERK) [80]. The JNK, p38 and ERK pathways are activated by environmental stress, including osmotic shock, ionizing radiation, whereas the ERK pathway is commonly activated by growth factors [81]. Of the four major MAPK pathways, the ERK1/2 MAPKs have attracted intense research interest because of their critical involvement in the regulation of cell proliferation and survival.

ERK is a downstream component of an evolutionarily conserved signaling module that is activated by the Raf serine/threonine kinases. Raf activates the MAPK/ERK kinase (MEK)1/2 dual-specificity protein kinases, which then activate ERK1/2. The activation of Raf and MEK in human cancers supports the critical role of this pathway in human oncogenesis [82, 83]. Moreover, Ras family small GTPases, the most frequently mutated oncogene in human cancers, are key upstream activators of Raf. In normal cells, Ras is GDP-bound and inactive. Ras is mutationally activated in 30% of all cancers, with pancreas (90%), colon (50%), thyroid (50%), lung (30%) and melanoma (25%) [84]. The Ras mutant is constitutively GTP-bound and active, leading to Ras mediated oncogenesis [85, 86].

Once the extracellular signal-regulated kinases ERK1/2 are activated through the Ras-Raf-MEK-ERK signaling pathway they mediate a range of cellular effects, including survival signaling, cell cycle regulation and cell migration [87]. They activate several subsequent kinases, including mitogen-activated protein kinase (MAPK)-interacting kinases (MNK1 and MNK2), mitogen- and stress-activated kinases (MSK1 and MSK2), and 90 kD ribosomal S6 protein kinase (RSK) [87]. In addition, the activated ERK1/2 translocates into the nucleus and activates transcription factors such as the peroxisome-proliferator-activated receptor (PPAR), ELK1, ETS, signal transducer and activator of transcription 1 (STAT1) and STAT3 [87]. The resulting changes in gene regulation affect cell proliferation and/or cell motility in a cell-dependent manner [87].

Considering the importance of the Ras-Raf-MEK-ERK signaling network in cancer, there have been attempts to identify novel target-based approaches for cancer treatment [81]. To date, farnesyltransferase inhibitors (FTIs), R115777 (Zarnestra) and SCH66336 (Sarasar), have been developed to inhibit the prenylation of Ras and are currently in Phase III/II clinical trials for hematologic and other cancers [88, 89]. Sorafenib (BAY 43-9006) is most successful anti-Raf inhibitor in Phase III clinical trials, and received FDA approval in 2005 for the use in advanced renal cell carcinoma (RCC) [90]. In addition, CI-1040 (PD184352) and AZD6244 (ARRY-142886) are two highly selective MEK inhibitors, and are being evaluated in Phase I/II clinical trials with a focus on tumors expected to have activated ERK MAPK signaling [87, 91, 92].

1.3 HYPOTHESIS AND SPECIFIC AIMS

The existing body of evidence clearly demonstrates that PRL-1 and PRL-3 are involved in promoting tumor cell invasion and metastasis in mouse models, although the exact mechanism(s) by which this occurs remain unclear. There is a lack of knowledge of the biological functions of PRL-2.

Elevated PRL-1 and PRL-3 were reported in several cancer cell lines and tissues, and the excess of PRL-1 and PRL-3 play an important role in promoting tumor cell invasion and metastasis [16, 17, 19, 20, 22]. Recently, high PRL-2 expression was found in prostate cancer cell lines and tissues, and breast cancer tissues. In light of that and because the three PRL phosphatases are highly homogenous and have conserved active sites-$C(X)_5R$ motif, I hypothesized that **PRL-2, like PRL-1 and PRL-3, has an essential role in maintaining the malignant phenotype of human cancer.** To test the hypothesis, I studied the roles of PRL-2 in tumor cell migration and invasion using A549 lung cancer cell line as a model system. The specific aims of this dissertation were to 1) address the role of endogenous PRL-2 on tumor cell migration and invasion; 2) investigate the role of ectopic PRL-2 and the functional domains of PRL-2; and 3) investigate a potent PRL inhibitor. The work described in this dissertation will contribute to the understanding of the biological functions of PRL-2 and the molecular mechanisms by which PRL-2 promotes tumor cell migration and invasion. In addition, the investigation of the potent PRL inhibitor will help to develop potent PRL inhibitors and better candidates for anti-cancer agents.

2.0 INVESTIGATION OF THE ROLE OF ENDOGENOUS PRL-2 IN TUMOR CELL MIGRATION AND INVASION

2.1 INTRODUCTION

The three PRL family members (PRL-1, PRL-2, and PRL-3) share a high degree of amino acid sequence identity and conserved active sites, indicating that they might function similarly. PRL-3 and PRL-1, have been implicated in tumor development and metastasis [3, 32, 35], but there is a lack of knowledge of the biological functions of PRL-2. Preliminary experiments from a colleague in my laboratory have demonstrated that the endogenous PRL-1 plays an important role in maintaining the malignant phenotype using A549 lung cancer as the model [23]. As PRL-1 and PRL-2 share approximately 87% amino acid sequence identity and conserved active sites [5, 93], I hypothesize that PRL-2, like PRL-1, has an essential role in maintaining the malignant phenotype of human cancer.

To test the hypothesis, I suppressed PRL-2 expression in A549 lung cancer cells by RNA interference. Then I examined cell migration and cell invasion, using the PRL-2 knockdown cells. I found that PRL-2 suppression by RNA interference markedly inhibited cell migration and invasion. To identify the molecular mechanism of PRL-2 modulated cell migration and invasion, I examined the known biochemical regulators of adhesion and invasion by Western blot using the PRL-2 knockdown cells. Interestingly, I found that PRL-2 suppression inhibited tumor cell migration and invasion via a Src-independent p130Cas signaling pathway. Moreover, ERK phosphorylation was dramatically decreased in response to serum stimulation, indicating that ERK signaling might be a downstream target involved in the PRL-2 mediated alternation on cell migration and invasion. In addition, Ezrin was hyper-phosphorylated at Tyr 146, indicating that Ezrin might be a substrate for PRL-2.

2.2 MATERIALS AND METHODS

2.2.1 Antibodies and Chemical Reagents

Antibodies and reagents were obtained from the following sources: rabbit anti-PRL-2 polyclonal antibody (Bethyl, Montgomery, TX); Pan-PRL antibody (R&D Systems, Minneapolis, MN); recombinant GST-tagged PRLs (BIOMOL International, Plymouth Meeting, PA); anti-p130Cas

(clone 21), anti-paxillin (clone 165), and anti-Csk (clone 52) monoclonal antibodies (BD Transduction Laboratories, San Diego, CA); anti-Ezrin antibody (Sigma-Aldrich Corp, St. Louis, MO) anti-c-Src (SRC2), anti-c-Src (H12), vinculin (H-300), and anti-phospho Tyr^{146} Ezrin (Santa Cruz, CA); anti-GAPDH (14C10), rabbit polyclonal anti-ERK1/2 (p44/42 MAP kinase) and phospho-Erk (Thr202/Tyr204), Tyr^{353} Ezrin and Thr^{567} Ezrin, Akt and pAkt, p53 and p53-Ser^{15}, and Tyr^{418} Src and Tyr^{529} Src (Cell Signaling Technology, Beverly, MA); anti-HA.11 (16B12) from Covance (Emeryville, CA); Anti-β-tubulin antibody from Cedarlane Laboratories (Burlington, ON, Canada); and anti-GST (Upstate Biotechnology, Lake Placid, NY); Phorbol 12-myristate 13 acetate (PMA) from Sigma (Sigma-Aldrich Corp, St. Louis, MO).

2.2.2 Cell Lines and Cell Culture

Cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained in a humidified atmosphere of 5% CO_2 at 37°C. A549 cells were authenticated by RADIL (Columbia, MO) and maintained in BME (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Gemini). CCL202 and SK-LU-1 cells were maintained in MEM (Invitrogen) with 10% fetal bovine serum; H1299 and H460 cells were maintained in RPMI 1640 medium (Mediatech, Manassas, VA) with 10% fetal bovine serum.

2.2.3 RNA Isolation and Semi-Quantitative RT-PCR

Total RNA was extracted with the RNeasy Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Reverse transcription-PCR (RT-PCR) for PRL-1, PRL-2, PRL-3, and actin as an internal control was carried out in a volume of 50 µL by SuperScript III One-step RT-PCR System (Invitrogen) as per manufacturer's instruction. Following primer pairs were used for each reaction: PRL-1, 5'-ACCTGGTTGTTGTATTGCTGTT-3' (forward) and 5'-GTTGTTTCTATGACCGTTGGAA-3' (reverse); PRL-2, 5'-AGCCAGGTTGCTGTGTTGCAG-3' (forward) and 5'-CACAGCAATGCCCATTGGTA-3' (reverse); PRL-3, 5'-AAGGTAGTGGAAGACTGGCT-3' (forward) and 5'-GGTGAGCTGCTTGCTGTTGAT-3' (reverse); β-actin, 5'- AAGAGAGGCATCCTCACCCT-3' (forward) and 5'-TACATGGCTGGGGTGTTGAA-3' (reverse).

2.2.4 Protein Extraction and Western Blotting

Cells were lysed in modified radioimmunoprecipitation (RIPA) assay buffer containing 0.1% SDS, 1% Triton X-100, protease inhibitors (10 µg/mL leupeptin, 10 µg/mL apoprotein, 100 µg/mL AEBSF, 10 µg/mL soybean trypsin inhibitor, 1 mmol/L phenylmethylsulfonyl fluoride), and phosphatase inhibitors (2 mM Na_3VO_4, 12 mM ß-glycerol phosphate, and 10 mM NaF). The lysates were incubated on ice for 30 min with frequent vortexing, and were cleared by centrifugation at 13,000 x *g* for 15 min. Protein content was determined by the method of Bradford protein assay. Total cell lysates (30–40 µg protein) were resolved by SDS-PAGE using

Tris-glycine gels, and incubated with primary antibodies at 4°C overnight or room temperature for 2 h. Bound primary antibodies were detected using horseradish peroxidase–conjugated secondary antibodies (Jackson Immunoresearch) and proteins were visualized using Pierce enhanced chemiluminescence Western blotting substrate (Pierce Biotechnology). For quantitation of protein expression levels, luminescence band intensities were measured on Multi Gauge V3.1 (Fujifilm).

2.2.5 Knocking Down PRL-2 by shRNAs or siRNAs

To deplete endogenous PRL-2, I selected two different 21-nucleotide sequences according to the manufacturer's instructions and software: TGCAGTTCAGTTTATAAGACA (PRL-2 silencing site 376), AAATACCGACCTAAGATGCGA (PRL-2 silencing site 441). The numbers 376 and 441 indicate the starting nucleotide number of shRNA-targeting sequences on the coding PRL-2 mRNA based on the published sequence data from Genbank (accession no. NM_080391). The specificity of each sequence was verified by a BLAST search of the public databases. p*Silencer* 4.1-CMV puro expression vectors (Ambion, Austin, TX) that produce shRNAs targeted against PRL-2 were also prepared according to the manufacturer's instructions. In brief, three sets of oligonucleotides were chemically synthesized: PRL2-376 sense, 5'-GATCC CAGTTCAGTTTATAAGACACTCAAGAGATGTCTTATAAACTGAACTGCAA-3'

; PRL2-376 antisense, 5'-AGCTTTGCAGTTCAGTTTATAAGACATCTCTTGAG TGTCTTATAAACTGAACTGG-3'; PRL2-441 sense, 5'-GATCC ATACCGACCTAAGATGCGACTCAAGAGA TCGCATCTTAGGTCGGTATTTA-3'

; PRL2-441 antisense, 5'-AGCTTAAATACCGACCTAAGATGCGATCTCTTGAG TCGCATCTTAGGTCGGTATG-3' (the underlined sequences contribute to forming shRNAs). The annealed oligonucleotides encoding shRNAs were then subcloned into the *Bam*HI-*Hin*dIII site of the p*Silencer* 4.1-CMV puro vector. For transfection, 1 x 10^5 cells were plated in six-well plates 24h before transfection in normal growth medium. 4 μg of plasmid DNA and 10 μL LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) were combined with 500 μL Opti-MEM I reduced-serum media (Invitrogen), incubated for 20 min at room temperature, and added to each well. After 24 h, the medium was replaced with basal medium Eagle (Invitrogen) with 2 μg/mL puromycin and 10% fetal bovine serum. After 2 weeks, stable round colonies were harvested and cloned by limiting dilution method, named 376i and 441i. The two siRNAs, which were designed to target the identical PRL-2 sites as the two shRNAs, were synthesized by Invitrogen, and named si 376 and si 441. For siRNA transfection, 100 pmol of siRNA and 5 μL LipofectAMINE 2000 were combined with 500 μL Opti-MEM I reduced-serum media and incubated for 20 min at room temperature prior to use.

2.2.6 Cell Adhesion Assay

For cell adhesion, 6 x 10^4 cells were plated on noncoated, collagen I–, fibronectin-, or laminin-precoated 96-well culture plates (BD Biosciences) at 37°C with 5% CO_2. After 60-min incubation in BME medium with serum, nonadherent cells were removed by gently washing twice with PBS. Adherent cells were stained using Hoechst staining solution (Roche Applied Science, Indianapolis, IN). The plates were analyzed with an ArrayScan II using a 10x objective

and a Hoechst 33342 compatible filter set. For this adhesion assays, nine fields in three wells were counted, and each experiment was repeated thrice.

2.2.7 Cell Migration Assay and Cell Invasion Assay

Cell migration assay was performed using Transwell (6.5-mm diameter, 8-μm pore size polycarbonate membrane) migration chambers obtained from Corning (Cambridge, MA). Cells (1×10^5) in 0.5 mL serum-free medium were placed in the upper chamber, and the lower chamber was loaded with 0.8 mL medium containing 10% fetal bovine serum. Cells that migrated to the lower surface of filters were stained with Wright Giemsa solution (Sigma-Aldrich Corp, St. Louis, MO), and five fields of each well were counted after 4 to 24 h of incubation at 37°C with 5% CO_2. Three wells were examined for each condition and cell type, and the experiments were repeated thrice. The cell invasion assay was conducted using BD Biocoat Matrigel 24-well invasion chambers with filters coated with extracellular matrix on the upper surface (BD Biosciences, Bedford, MA). Control inserts were used for migration control. The experiments were done according to the manufacturer's protocol. I added 1×10^5 cells in 0.5 mL of serum-free culture medium to the upper chamber, and after incubation at 37°C for 24 h, I stained cells and determined total cell invasion and migration as described above. Invasiveness was expressed as the percent invasion for each cell type through the Matrigel matrix and membrane relative to the migration through the control membrane.

2.2.8 Statistics

Results were expressed as means ± SE of at least three independent experiments. ANOVA and Student's *t* tests were performed using Graphpad Prism 5 software (Graphpad Software). Differences were considered statistically different if $P < 0.05$. Western blots and autoradiograms were representative of at least three independent experiments.

2.3 RESULTS

2.3.1 Lung Cancer Cell Lines Had High Expression Levels of PRL-2

PRL-3, the well-studied PRL phosphatase family, is elevated in a variety of human carcinomas, with exception of lung cancer [94]. Our previous study [23] indicated that at least one lung cancer cell line, A549, expressed high levels of PRL-1 and PRL-2. This indicates that lung cancer cells might be a good model to study PRL-1 and/or PRL-2. I extended the examination to three other lung cancer cell lines. As indicated in Figure 7, I found significant amounts of PRL-2 mRNA in H1299, H460, SK-LU-1 and A549 cells. All four human lung cancer cell lines had higher PRL-2 mRNA levels relative to those found in the normal human lung fibroblast cell line CCL202. A549, H1299 and H460 but not SK-LU-1 cells had detectable PRL-1 mRNA and none of the cells had detectable PRL-3 mRNA. The A549 lung cancer cells were selected for further study because of our previously interrogation of PRL-1 with this cell line [23].

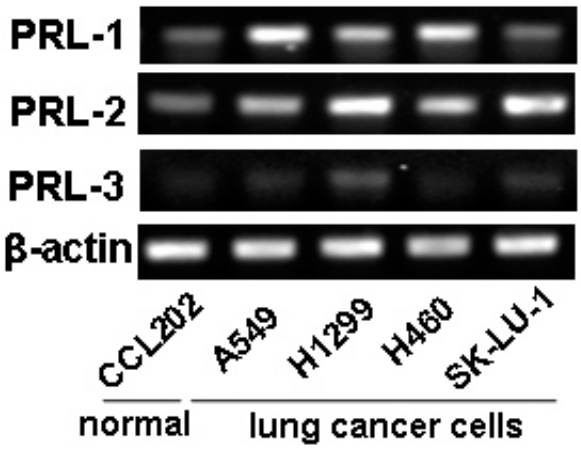

Figure 7. Four Lung Cancer Cells Over-expressed PRL-2.

RT-PCR demonstrated that PRL-2 was over-expressed in several lung cancer cells including A549 cells when compared with normal lung cells. β-actin was used as internal control.

2.3.2 PRL-2 Was Selectively Down-regulated by RNA Interference

To examine the biological functions of endogenous PRL-2, I established A549 cells that stably expressed shRNA for PRL-2. Four different 21-nucleotide sequences were designed for shRNA and the two that provided the best suppression were selected and named according to the starting nucleotide number of the targeting sequence: sh376 and sh441. Clones made with each of the two targeting sequences that persistently exhibited suppressed PRL-2 expression were selected and renamed 376i and 441i; one of the clones obtained with scrambled shRNA (SCR) was also used as a control. The sh376 and sh441 cells chronically expressing PRL-2 shRNA had 50% and

40% less PRL-2 mRNA, respectively (Figure 8A). PRL-1 and PRL-3 mRNA levels were unaffected in the PRL-2 knockdown cells (Figure 8A).

To improve mRNA suppression, I also transiently transfected A549 cells with siRNA targeting the identical shRNA sequences. PRL-2 levels were selectively decreased 75% by two siRNA (Figure 8A). The cell clone numbers 376 and 441 indicate the starting nucleotide number of siRNA- or shRNA-targeting sequences of PRL-2 mRNA. I elected to use the transient knockdown cells for some experiments because they gave superior PRL-2 silencing and amplified the effects on the signaling pathways. I also confirmed that PRL-2 was selectively down-regulated at the protein level by Western blotting using two different antibodies (Figure 8B). The upper blot was obtained with an antibody from R & D Systems (MAB32191) that recognizes all three PRL proteins and the middle blot was produced with a PRL-2 specific polyclonal antibody from Bethyl (BL1205). As shown in Figure 8B, PRL-2 protein was selectively down-regulated in cells transiently transfected with siRNA directed against PRL-2.

The specificity of PRL antibodies were confirmed by Western blotting with recombinant GST-PRL protein (Figure 8C). GST-tagged PRL-1, PRL-2 or PRL-3 was used to test the specificity of the PRL antibodies: Pan antibody (MAB32191) from R & D Systems, (Figure 8C, upper blot) and a PRL-2 specific polyclonal antibody (BL1205) from Bethyl (Figure 8C, middle blot). GST was used as a loading control (Figure 8C, bottom blot).

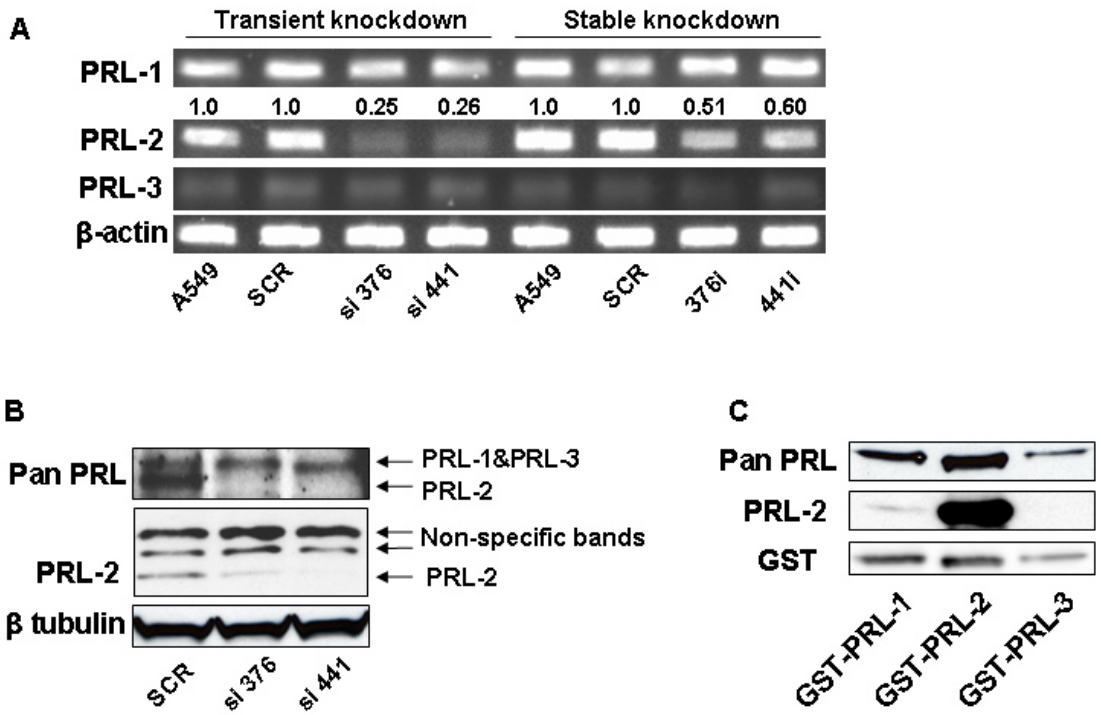

Figure 8. PRL-2 Expression at mRNA and Protein Level Were Significantly Suppressed in the PRL-2 Knockdown Cells.

RT-PCR and Western blotting images were processed and quantified with Fuji Multi Gauge software (Fujifilm). A) PRL-2 was selectively down-regulated at the mRNA level in cells with transient and stable PRL-2 knockdown. The siRNA transiently transfected cells displayed ~80% decrease in PRL-2 mRNA level, while there was a ~50% decrease in the shRNA transfected stable knockdown cells. β-actin was used as the internal control to determine expression levels. The average fold changes, as shown above the blot, were calculated from three independent experiments and normalized to relative PRL-2 levels in the parental A549 cells. B) PRL-2 protein level was selectively down-regulated. β tubulin was used as an internal control. C) The specificity of PRL antibodies were confirmed by Western blotting with recombinant GST-PRL proteins.

2.3.3 PRL-2 Suppression Inhibited Tumor Cell Migration and Invasion

To investigate the role of endogenous PRL-2 in cancer cell metastatic processes, I interrogated cell migration and invasion using Transwell migration and Matrigel invasion chambers, respectively. For migration assay, cells that migrated to the lower chamber at the indicated times were fixed, stained, and counted with a microscope. As shown in Figure 9A and 9B, PRL-2-silenced cells migrated slower than the control cells in the cell migration assay. After 24 h when cell migration was saturated, control cells achieved the same number of migrated cells per field as did the PRL-2-silenced clones (data not shown).

Cell invasion was measured at 24 h when the migration control wells saturated. For Matrigel invasion assay, cells that invaded to the lower chamber were fixed, stained, and counted with a microscope. Control inserts, which were not coated with Matrigel, were used as control for migrated cells. Invasive activity was determined as the percent invasion of the wild type A549 cells through the Matrigel matrix and membrane relative to the migration through the control membrane. With the invasion assay, PRL-2-silenced clones invaded significantly less than the parental cells or scramble cells, $p<0.001$ for both (Figure 9C and 9D).

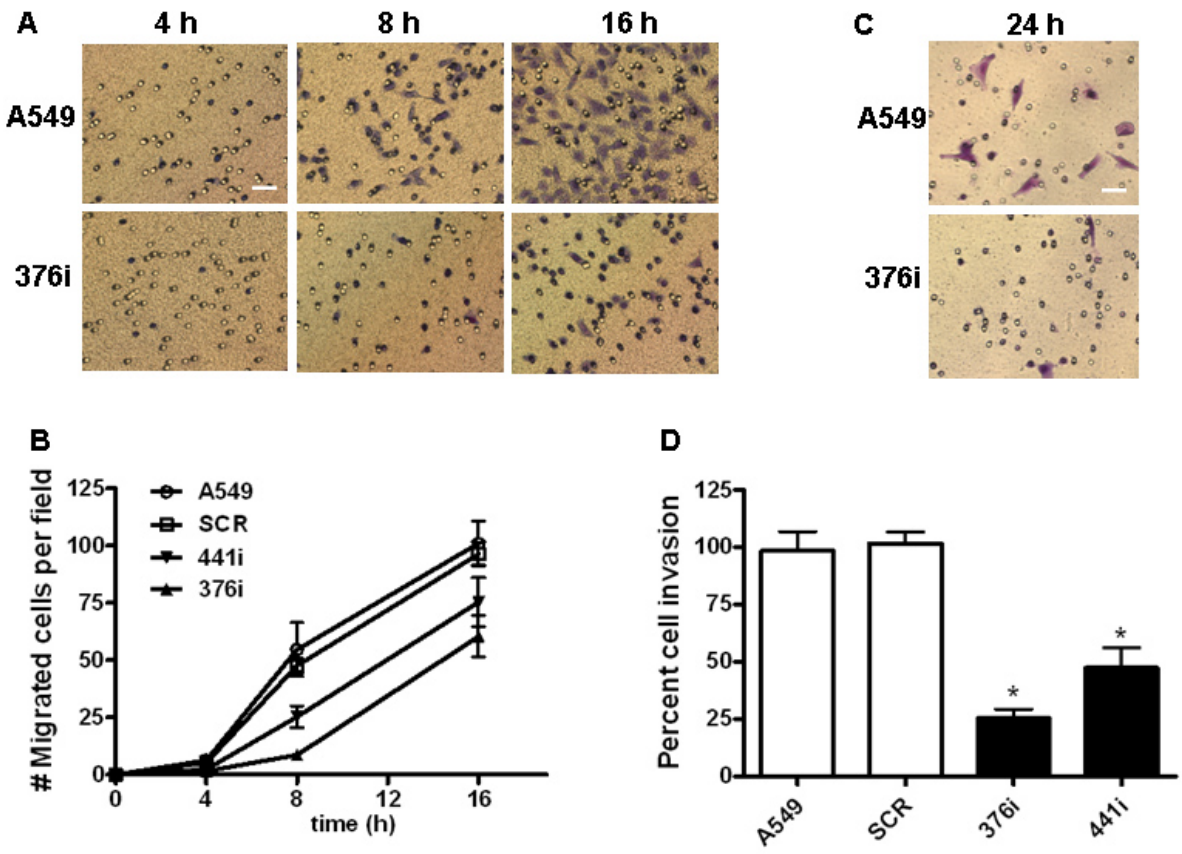

Figure 9. Cell Migration and Invasion Were Significantly Inhibited after PRL-2 Knockdown.

A) Representative images from the transwell migration assay showing Wright Giemsa stained control and PRL-2 knockdown cells that reached the lower side of the membrane 4, 8 and 16 h after plating; bar, 10 μm. B) Quantification of the cell migration time course of wild type A549 cells or A549 cells stably expressing scrambled or PRL-2 shRNA. The mean values of three independent experiments measured in triplicate are indicated; bars equal the SEM. C) Representative images of Wright Geimsa stained control and PRL-2 knockdown cells on the lower side of a membrane from the Matrigel-coated transwell invasion assay; bar, 10 μm. D) Quantification of cell invasion after PRL-2 knockdown. The mean values of three independent experiments measured in triplicate were indicated and the bars equaled the SEM. All mean values were compared to the wild type A549 cells using a two-tailed Student's t-test. *, $p<0.001$.

2.3.4 PRL-2 Depleted Cells Had No Preference for Fibronectin, Collagen, or Lamin- coated Surfaces.

PRL-1 was reported to be involved in regulating cell adhesion and invasion in A549 lung cancer cells [23]. Specifically, PRL-1 knockdown enhanced cell adhesion and spreading on fibronectin [23]. As I observed similar inhibition on cell invasion after PRL-2 suppression, I examined cell adhesion in PRL-2 knockdown cells to see if PRL-2 had a similar role in regulating cell adhesion as PRL-1.

To assess the adhesion on various extracellular matrix, I performed adhesion assay using noncoated or precoated culture dishes with various extracellular matrix, including collagen I, fibronectin, and laminin. Sixty minutes after plating the cells, I removed the non-adherent cells by gently washing with PBS. Adherent cells were stained using Hoechst staining solution and quantified with an ArrayScan II. As shown in Figure 10, more A549 cells adhered to collagen I or fibronectin-coated dishes, while less adhered on laminin-coated dishes. Although all populations clearly have a preference for noncoated, fibronectin-coated or collagen-coated dishes, there were no clear differences on cell adhesion among the cell populations when compared the PRL-2 knockdown cells to the parental A549 cells or the SCR control cells. This result was confirmed by three independent experiments in triplicates.

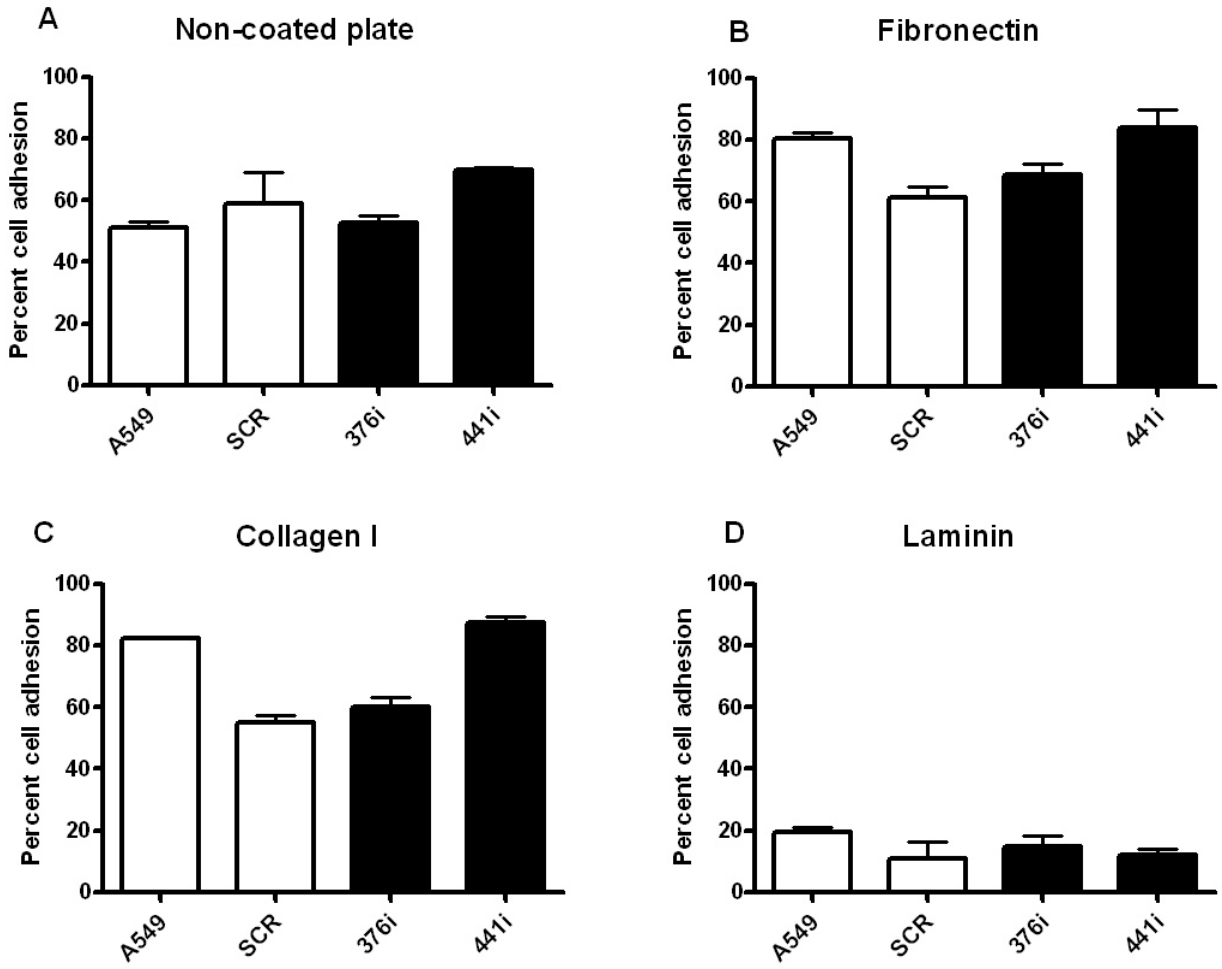

Figure 10. PRL-2 Depleted Cells Had No Preference for Fibronectin, Collagen, or Lamin- Coated Surfaces.

Cell adhesion on noncoated (A), fibronectin (B)-, collagen I (C)–, and laminin (D)-coated 96-well culture plates. After 60-min incubation, adherent cells were stained using Hoechst staining solution and analyzed with an ArrayScan II. Nine fields in three wells were counted, and each experiment was repeated thrice.

2.3.5 No Alteration on p53, Src or Akt after PRL-2 Suppression.

To study the potential mechanisms of PRL-2 mediated phenotypic changes on cell migration and invasion, I first examined the putative participants involved in PRL-1 or PRL-3 signaling pathways, such as p53 [40], Akt [39], and Src [23, 36]. As shown in the Figure 11, total Src expression was not altered after PRL-2 suppression. Moreover, there was no detectable change in c-Src stimulation as measured by phosphorylation on Tyr418, Tyr529, and the expression level of Csk, a negative Src regulator. In addition, total p53 or Akt expression levels, or their phosphorylation status were not altered after PRL-2 suppression. These data were confirmed by three independent experiments.

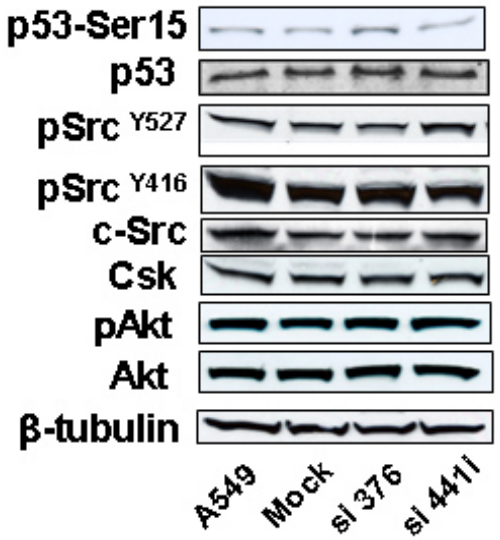

Figure 11. Effect of PRL-2 Knockdown on p53, Src, and Akt.

PRL-2 suppression did not alter total p53, Src, Csk and Akt expression and p53, Src, and Akt phosphorylation status after transient PRL-2 knockdown.

2.3.6 Effects of PRL-2 Suppression on p130Cas, Vinculin Expression, ERK phosphorylation, and Ezrin Phosphorylation

Our previous study [23] suggested that in A549 cells the close family member PRL-1 could associate with focal adhesion complex (FAC) and regulate adhesion turnover, which are involved in regulating cell migration. Thus, I examined the effect of PRL-2 knockdown on FAC and the downstream pathways. A549 cells were transiently transfected with scramble or PRL-2 siRNA. After 2 days, cells were harvested for Western blotting to permit an analysis of the changes in Ezrin status (a potential PRL substrate), p130Cas (an adaptor protein for focal adhesion complex), vinculin and paxillin (focal adhesion components that were normally recruited during FAC activation). As shown in Figure 12A, vinculin was decreased while, another FAC recruit, paxillin remain unchanged after PRL-2 suppression. Furthermore, there was a 2-3 fold increase on Ezrin phosphorylation at Tyr 146 after PRL-2 suppression, while there was no apparent effect on the Thr 567 site (Figure 12A). The results were confirmed by three independent experiments.

In addition, PRL-2 silencing significantly decreased the total expression level of p130Cas (Figure 12A), the adaptor protein for FAC. More importantly, I found that p130Cas was cleaved after PRL-2 suppression, and generated a ~80kD fragment (Figure 12B). Etoposide was used as a control to study p130Cas cleavage, because it is a known p130Cas cleavage inducer and generates a 31kD product leading to apoptosis [95]. The biological function of the 80 kD cleaved p130Cas was unknown.

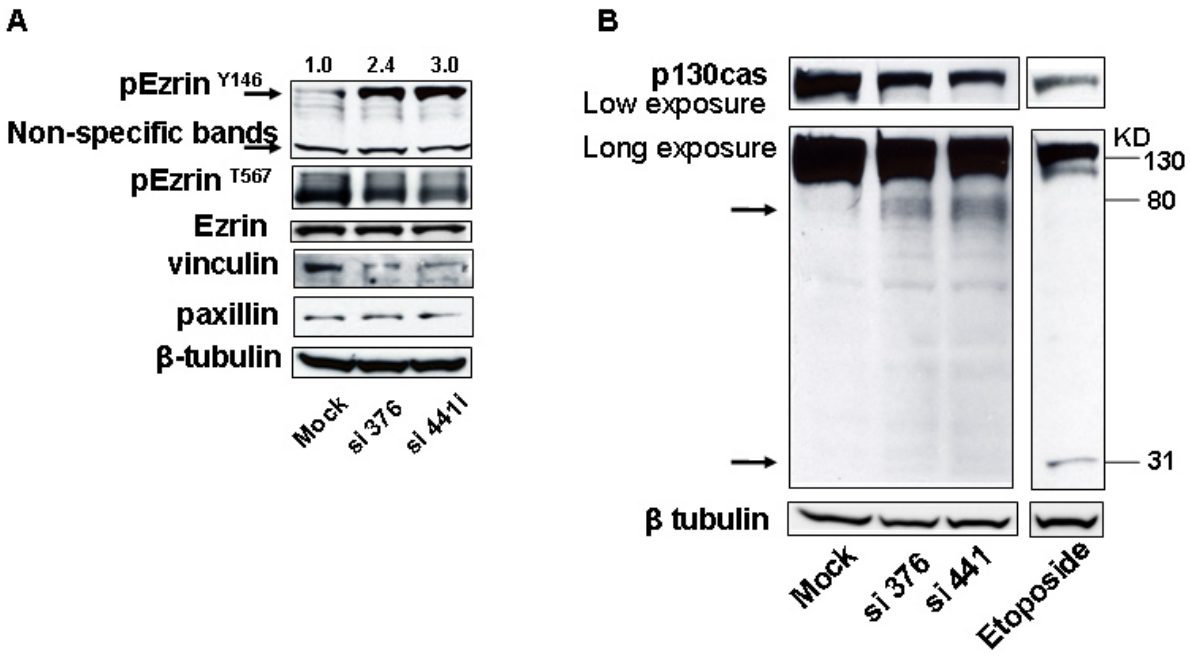

Figure 12. Effects of PRL-2 Knockdown on Ezrin, p130Cas, and Vinculin.

A) Western blotting showed hyper-phosphorylation on phosphorylated Ezrin Tyr146 and no significant change on phosphorylated Ezrin Thr567 after PRL-2 knockdown. The p130Cas and vinculin levels were decreased after transient PRL-2 knockdown. B) The cleavage of p130Cas. Etoposide was used as a control.

To examine the possible role of PRL-2 in modifying ERK activity, which is downstream of FAC and participates in cell migration and invasion, I first measured ERK1/2 phosphorylation by Western blotting. The cells were pre-treated in serum free medium for 6 h, and then serum was added as a stimulus for ERK1/2 phosphorylation. The Western blots (Figure 13A) and the quantitative data (Figure 13B) clearly showed a dramatic decrease of ERK1/2 phosphorylation in response to serum stimulation.

Collectively, the above results indicated that suppression of PRL-2 protein levels decreased p130Cas and vinculin expression, and decreased ERK1/2 phosphorylation, and eventually inhibited cell migration and invasion.

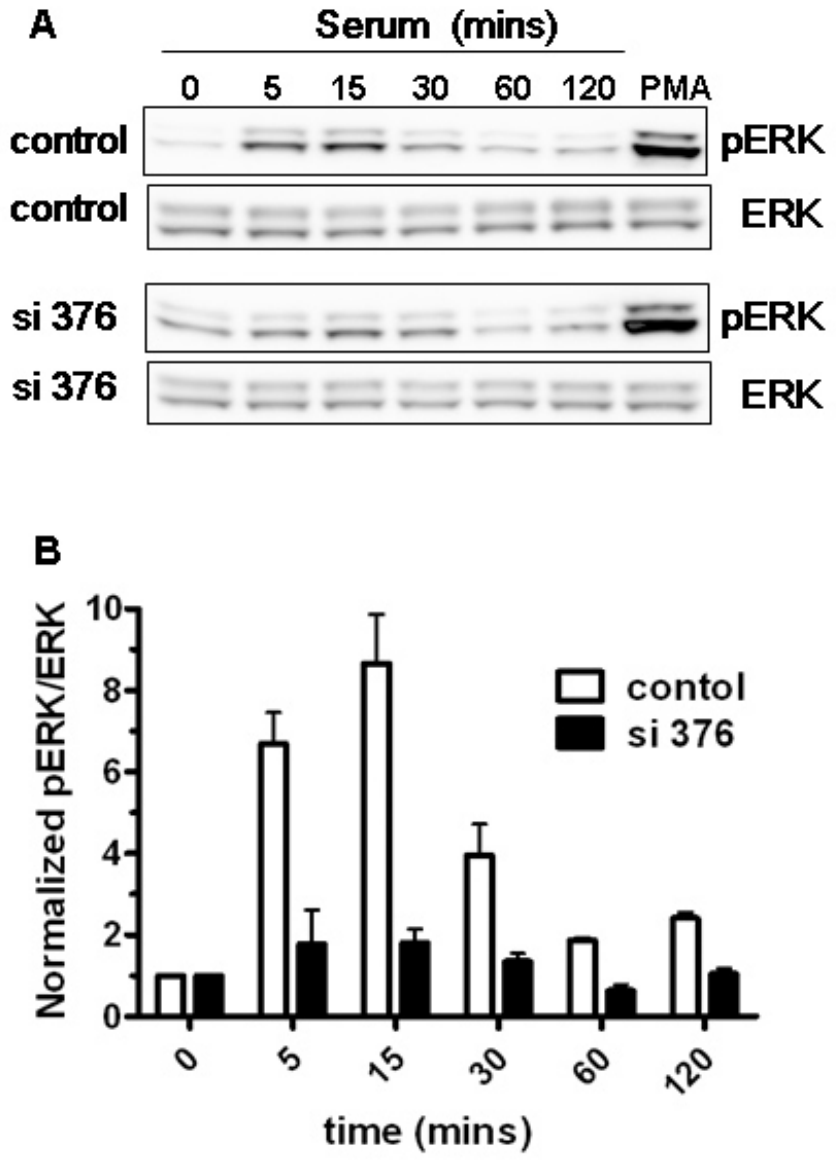

Figure 13. ERK1/2 Phosphorylation upon Serum Stimulation.

Total ERK or pERK were examined in lysates from cells with normal or silencing PRL-2. The cells were pre-treated with serum free medium for 6 h, and then serum was added for time course. Phorbol ester (PMA) was used as a positive control for ERK phosphorylation. D) Quantification of ERK phosphorylation status. N=3; bars = SEM.

2.4 DISCUSSION

Cell migration and invasion are important for embryonic development and the adult organisms in normal development. They are also crucial in cancer metastasis because metastasis is dependent on tumor cells gaining certain properties such as increased cell migration and invasion. There are several ways to assay these functions. For example, the monolayer wound healing assay is easy to complement, whereas the microliter-scale migration assay allows examining cell behavior on defined extracellular matrices. The most widely used migration assay currently is the Transwell migration assay, which I employed in this dissertation. For tumor invasion assay, the organotypic assay examines the invasion of glioma cells through a rat brain slice; while the Transwell Matrigel invasion assay, which I employed in this dissertation, is a versatile assay that is most commonly used. To limit the effect of cell proliferation, I used serum free medium when I prepared the cells, and serum was added only to the lower chamber to attract the cells moving to the lower chamber. Moreover, I measured cell migration at early time point, such as 4 h, 8 h, and 16 h, so as to minimize the interference of cell proliferation. Cell invasion was measured at 24 h when cell migration nearly saturated. This will limit the effects from cell migration in the Matrigel Transwell invasion assay when I measured cell invasion. In addition, I found the proliferation rate of PRL-2 knockdown cells was slightly slower than the parental A549 cells, but the doubling time was not significantly different (data not shown). Therefore, I believe the methodology that was employed for measuring cell migration and invasion is appropriate at the time points that I measured.

As stated previously, elevated PRL-1 or PRL-3 in a variety of cancer types correlates with the tumor progression [16, 17, 19, 20, 22]. Our laboratory also previously reported that PRL-1 silencing significantly inhibited tumor cell invasion by regulating c-Src expression level and adhesion turnover in A549 lung cancer cells [23]. In this study, I showed that suppression of PRL-2 by RNA interference significantly inhibited cell migration and invasion in A549 lung cancer cells. This result is consistent with the recent study in breast cancer, which shows that PRL-2 plays a role in breast cancer progression. Thus initial evidence has showed that PRL-2 is implicated in regulating tumor progression. These results with PRL-2 and the previous reports concerning PRL-1 or PRL-3 are consistent with my hypothesis that PRL-2, like its other family member PRL-1 and PRL-3, plays a critical role in promoting tumor cell migration and invasion.

The molecular mechanism exploited by PRLs is not firmly identified but several pathways have been proposed including p53, PI3K/Akt, and Src. PRL-3 is reported to be a p53 target involved in cell-cycle regulation [34]. PRL-3 promotes HEK293 cell invasion by down-regulation of Csk leading to Src activation [36], while PRL-1 also appears to promote cell invasion through a Src-mediated pathway [23, 38]. To study the molecular basis of PRL-2 mediated phenotype changes, I examined these potential players in the PRL signaling pathways, but did not detect any difference on the total expression level or phosphorylation status of p53, Akt or Src. As Src has some family members that closely relevant to it, it is possible that the Src or phospho-Src antibody might cross-react with the other family members. To exclude the possibility, I extended my study by examine the other Src family kinases, such as Yes, Fyn and Lyn. I found that Lyn and Yes were undetected in A549 cells, while Fyn expression level and phosphorylation status

remained unchanged after PRL-2 suppression (data not shown). The other way to confirm this is to immunoprecipitate Src from the cell lysates with Src antibody before the Western blots, which I was unable to complete due to time constraints and other experiments I was conducting. Nevertheless, my observations on p53, Akt and Src allowed me to speculate that PRL-2 may signal through a pathway different than that employed by PRL-1 or PRL-3.

This notion is reinforced when one compares my current PRL-2 data with our previously published PRL-1 data in which using the same lung cancer cell line, and the same antibody reagents, and the same methodology we found PRL-1 silencing regulates c-Src expression level and cell invasion [23]. While both PRL-1 and PRL-2 depleted cells lost their ability to invade, PRL-2 did not appear to alter Src expression or phosphorylation; and PRL-2 knockdown cells did not exhibit a preference for fibronectin nor did they appear to regulate adhesion turnover as PRL-1 silenced cells did [23]. This leads us to speculate that while PRL-2 may be functionally similar to PRL-1 and PRL-3, it could signal through different pathways.

The adaptor protein p130Cas is an important component in focal adhesion complex. The adaptor proteins do not have enzymatic or transcriptional activity, but they spatially and temporally control signaling events through their ability to undergo changes in phosphorylation or to associate with effectors in multi-molecular complexes. As p130Cas is critical adaptor in FAC, its involvement in cell motility as a component of the integrin signaling is well established [78]. Cells lacking p130Cas display impaired actin filament assembly and significantly decreased rates of migration and spreading [96]. The involvement of p130Cas in cell migration depends mainly on its tyrosine phosphorylation by Src and on the assembly of a p130Cas-Crk-DOCK180

scaffold at adhesion sites [97, 98]. Scaffold formation leads to actin polymerization and the recruitment of high affinity integrin receptors that are necessary for lamellipodia extension and cell migration. More interestingly, p130Cas dephosphorylation inhibits the formation of p130Cas dependent signaling complexes, and favors protease cleavage of p130Cas into a small fragment of 31 kD [99, 100]. The small 31 kD fragment can translocate into the nucleus, contribute to cell death by functioning as a transcriptional suppression of E2A [95]. In this work, I showed that p130Cas was cleaved and generated ~80 kD fragment after PRL-2 suppression. This might be a different cleavage pattern for p130Cas, as p130Cas has two cleavage sites [78]. The function of the 80kD fragment is unknown. In general, it appears to be too big in size for nuclear translocation, suggesting that it might have a function in cytoplasm rather than nucleus. The 80 kD was cleaved at the substrate domain. As the C-terminal domain is intact after cleavage, indicating that the cleaved p130Cas might still be able to bind Src at the C-terminal. However, as the cleavage occurs in the substrate domain, so the tyrosine phosphorylation by Src in that domain might be disrupted. In addition, the N-terminal SH3 domain was lost after cleavage. As stated previously, the N-terminal SH3 domain binds to several effectors, such as FAK and DOCK180 [78]. Thus the cleaved 80kD p130Cas might be unable to interact with FAK, DOCK180 and some other effectors. This would result in the disassembly of a p130Cas-Crk-DOCK180 scaffold at adhesion sites, which eventually inhibit cell migration. The focal adhesion pattern could be further studied by immuno-staining the critical components of FAC, such as vinculin and p130Cas.

PRLs have a clear phosphatase domain and conserved $C(X)_5R$ active site, but no substrate has been convincingly identified for the PRLs. Interestingly, Ezrin has recently been reported to be a

specific and direct target of protein PRL-3 [43]. There are three important phosphorylation sites for Ezrin: Tyr 146 at N-terminal domain, Tyr 353 in the α-helix, and Thr 567 at the C-terminal domain. The phosphorylation on Thr 567 at the C-terminal is well-studied. It is regulated by Rho GTPases. For example, RhoA regulates its effector ROCK, which could phosphorylate Thr 567 of Ezrin; while Rac can regulate PIP2 production and thereby increase Ezrin activity [101]. A novel feature of the ERM family is their ability to act both upstream and downstream of Rho GTPases, which suggests the existence of a positive feedback loop between the two families. As mentioned, Forte et al. found that the Thr 567 site of Ezrin is a direct substrate for PRL-3, which challenged the current dogma that PRL phosphatase belong to PTPs. In this work, I showed that Ezrin was hyper-phosphorylated on Tyr 146 in my PRL-2 silenced cells while no change was detected on Thr 567. To further confirm the results, I performed the pull-down assay for RhoGTPases. I found no difference on the total expression level of Rac1 or activated Rac1 after PRL-2 suppression; and similar results were obtained from the pull-down assays for RhoA and cdc42 (data not shown). This result was in support of the observation that no change was found on Thr 567 phosphorylation of Ezrin.

The kinases or phosphatases that modulate the tyrosine phosphorylation remain unclear. The two tyrosine residues (Tyr 146 and Tyr 353) could be phosphorylated upon stimulation of EGF both *in vitro* and *in vivo* [102]. LLCPK1 cells form tubules upon stimulation of HGF and this tubulogenesis requires Ezrin phosphorylation on residue Tyr 146 and Tyr 353 [103]. The tyrosine kinase $p56^{lck}$ is involved in the phosphorylation of Ezrin Tyr 146 in T lymphocytes [104]. No phosphatase is known to dephosphorylate the tyrosine of Ezrin. In this work, I showed that Ezrin was hyper-phosphorylated on Tyr 146 in my PRL-2 silenced cells, which led me to

speculate that Tyr 146 of Ezrin might be a substrate for PRL-2 phosphatase. This speculation is consistent with the current believe that PRL phosphatase belong to the PTPs. To validate Ezrin as a direct substrate for PRL-2, I could use in vitro phosphatase assay and/or pull-down assay to study the interactions between Ezrin and PRL-2. Nevertheless, I suggest that there is insufficient evidence to conclude Ezrin is a candidate for PRL-2 substrate.

It is worth noting that the PRL phosphatases have low catalytic activity as measured by traditional methods, and no literature has reported successful identification of substrates from the substrate trapping assay. At least several differences between PRLs and classical PTPs may account for their low catalytic activity *in vitro* and the difficulty in substrate trapping. PRLs have less than 30% sequence identity to DSPs, but they showed a shallow catalytic pocket, which is typical for DSPs. The catalytic pockets of PRLs are unusually wide and shallow, indicating that PRLs could accommodate a broad range of phosphorylated substrates. The substrate specificity must be determined by some other structural features from the other domains. The other notable difference is the presence of Ala instead of the highly conserved Ser/Thr residue normally next to the invariant Arg of the catalytic motif. This could lead to loss of a Ser/Thr hydroxyl group that normally facilitates the breakdown of phosphoenzyme intermediate in PTP-mediated catalysis [6]. Although it is challenging, identifying substrates for the PRL phosphotases would contribute great to the understanding the regulation of PRLs and the molecular mechanisms of PRLs signaling pathways.

The ERK phosphorylation cascade is a known downstream of integrin signaling, which functions in cellular proliferation, migration, differentiation, and survival. Its inappropriate activation is a

common occurrence in human cancers [105]. In this study, I observed the decreased ERK phosphorylation in response to serum stimulation. This was a transient effect upon serum stimulation, which occurred as quickly as 5 minutes after serum stimulation, and maximized in 15 minutes. After 2 hours, the ERK phosphorylation status went back the basal level, and the basal level of phospho-ERK was not significantly different between the control cells and the PRL-2 depleted cells. ERK clearly plays an important role in cell proliferation, so I expect PRL-2 may have an effect on cell proliferation only upon transient serum stimulation of ERK but not the basal level. This would be consistent with the observation that the proliferation rates of the controls cells and PRL-2 depleted cells were not significantly different. For Transwell cell migration assay and Matrigel invasion assay, the cells were prepared in serum free medium, and serum was present only in the lower chamber to stimulate cell migration and invasion. Thus the cells in the upper chamber response to serum stimulation, and migrated through the pore to the bottom. It is likely that the PRL-2 depleted cells in which ERK was less phosphorylated upon serum stimulation have less cell motility. Consequently, the PRL-2 depleted cells showed less invasiveness than the control cells.

3.0 INVESTIGATION OF THE ROLE OF ECTOPIC PRL-2 AND THE FUNCTIONAL DOMAINS OF PRL-2

3.1 INTRODUCTION

As shown previously, cell migration and invasion were significantly inhibited after PRL-2 suppression, indicating that PRL-2 plays a critical role in promoting tumor cell migration and invasion. If the ectopic over-expression of epitope tagged PRL-2 could promote cell migration and invasion, the consistent results could reinforce the hypothesis that PRL-2 plays an essential role in promoting tumor cell migration and invasion. Furthermore, the use of epitope tag, such as hemaglutinin (HA) epitope, would facilitate the detection of proteins such as PRL-2, which lack good commercial antibody and other agents. Therefore, I studied the roles of PRL-2 using an HA-tag at the N-terminal of PRL-2. I made the HA-tag at the N-terminal because I think the prenylation site at the C-terminal might be very important to the biological functions of PRL-2. I also made several mutations on PRL-2, including the catalytic inactive mutant C101S and the CAAX deletion mutant ΔCAAX. Expression of wild type PRL-2 significantly promoted tumor

cell migration and invasion, while expression of the mutant forms of PRL-2 did not alter cell migration or invasion. Moreover, expression of wild type but not mutant forms of PRL-2 caused ERK phosphorylation and nuclear translocation. These results support a model in which PRL-2 promotes cell migration and invasion through an ERK-dependent signaling pathway. In addition, ectopic expression of wild type PRL-2, a catalytic inactive C101S mutant, and a C-terminal CAAX deletion revealed a requirement for both the PRL-2 catalytic functionality and prenylation site.

3.2 MATERIALS AND METHODS

3.2.1 PRL-2 Constructs and Transfection

PRL-2 cDNA was subcloned into pCMV-HA vector with an HA-tag on the N-terminus. PRL-2 mutants were generated using QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacture's instructions. Mutations were confirmed by DNA sequencing. 4 μg of plasmid DNA and 10 μL LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) were combined with 500 μL Opti-MEM I reduced-serum media (Invitrogen), incubated for 20 min at room temperature, and added to each well. After 24 h, the medium was replaced with Basal Medium Eagle (Invitrogen) and 10% fetal bovine serum.

3.2.2 PRL-2 Mutations

PRL-2 mutants were generated using QuickChange Multi site-directed mutagenesis kit (Stratagene) according to the manufacture's instructions. The following mutation primers were design using Primer X http://www.bioinformatics.org/primerx/cgi-bin/protein_4.cgi :

GTGTTGCAGTGCATAGTGTTGCAGGATTGG C101Sf

CCAATCCTGCAACACTATGCACTGCAACAC C101Sr

tatataGAATTCATGAACCGTCCAGCCCCTGT CAAX-EXhof

tatataCTCGAGCTAATGCCCATTGGTATCTCTG CAAX-EXhor.

Mutations were then confirmed by DNA sequencing.

3.2.3 Antibodies and Reagents

Antibodies and reagents were obtained from the following sources: anti-p130Cas (clone 21), anti-paxillin (clone 165), and anti-Csk (clone 52) monoclonal antibodies (BD Transduction Laboratories, San Diego, CA); anti-Ezrin antibody (Sigma-Aldrich Corp, St. Louis, MO) anti-c-Src, vinculin (H-300), and anti-phospho Tyr^{146} Ezrin (Santa Cruz, CA); anti-GAPDH (14C10), rabbit polyclonal anti-ERK1/2 and phospho-Erk, Tyr^{353} Ezrin and Thr^{567} Ezrin, and Tyr^{418} Src

and Tyr^{529} Src (Cell Signaling Technology, Beverly, MA); anti-HA.11 (16B12) from Covance (Emeryville, CA).

3.2.4 Protein Extraction and Western Blotting

Cells lysates and Western blotting were done as described in Section 2.2.4.

3.2.5 Immunofluorescence

Cells were seeded on glass slides and fixed at the indicated time points with 4% paraformaldehyde at room temperature for 15 min. After three washes with PBS, cells were permeabilized for 15 min with 0.1% Triton X-100. After blocking with 2% bovine serum albumin for 45 min, cells were incubated with the anti-HA antibody or anti-pERK antibody for 2 hours; and then washed with PBS for five times. This is followed by incubating the glass slides with Alexa Fluor 588–conjugated anti-mouse IgG for HA or Alexa Fluor 594–conjugated anti-rabbit IgG for pERK, and DAPI (4',6-diamidino-2-phenylindole) for nucleus. After washing with PBS, the glass slides were mounted for image analysis. The fluorescence images of cells were captured and analyzed using an Olympus XI (Olympus, Global).

3.2.6 Cell Migration and Invasion Assay

Cell migration and invasion assays were performed as described in Section 2.2.7.

3.2.7 Statistics

Results were expressed as means ± SE of at least three independent experiments. Statistics were analyzed as described in Section 2.2.8.

3.3 RESULTS

3.3.1 Expression and Localization of HA-PRL-2 in A549 Cells.

To generate the HA-PRL-2 vector, I subcloned the PRL-2 cDNA from myc-PRL-2 vector to pCMV-HA vector using the two enzymatic digestion sites EcoRI and XhoI. The correct insertion of the PRL-2 was confirmed by digesting the two sites as shown in Figure 14A, and was also confirmed by polymerase chain reaction (PCR) as shown in Figure 14B. In addition, the correct sequence of PRL-2 insert was further confirmed by DNA sequence and DNA alignment.

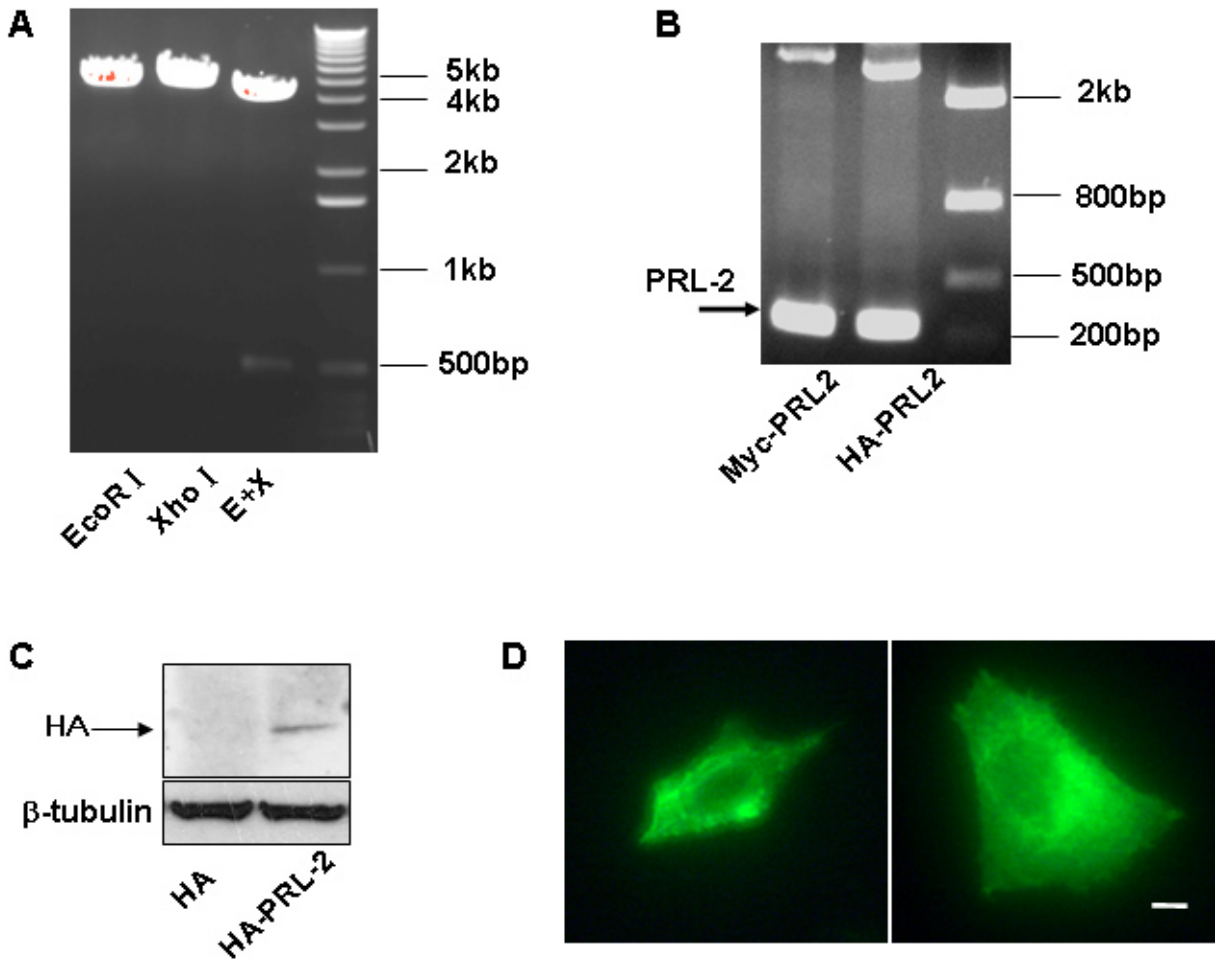

Figure 14. Expression and Localization of HA-PRL-2 in A549 Cells.

A) Enzymatic digestion of pCMV-HA-PRL-2 vector. B) PCR amplification of the pCMV-HA-PRL-2 vector. Myc-PRL-2 vector was used as a positive control. C) Western blotting showed that HA-PRL-2 protein was expressed in HA-PRL-2 transfected cells. D) Immunofluorescent staining of cell transfected with HA-PRL-2. Bar, 2 μm.

A549 cells were transfected with HA-PRL-2. After 24 h, some cells were harvested for Western blotting; while some were fixed, permealized, stained with HA antibody, and labeled with secondary goat-anti-mouse 488 (green) antibodies. The expression of HA-PRL-2 was confirmed

by Western blotting with HA antibody (Figure 14C). In addition, the localization of HA-PRL-2 was determined by immunofluorescence staining with a HA antibody. For the HA mmunofluorescence staining, the cell transfected with HA empty vector, the cells without transfection and the cells transfected with HA-PRL-2 vector but without primary antibody were used as negative controls. There was weak fluorescence signal throughout the entire cells when transfected with empty HA vector, while there was no fluorescence signal detected in the non-transfected cells or the no primary antibody stained cells (data not shown). Representative images of HA-PRL-2 over-expressing cells showed that HA-PRL-2 was present on the plasma membrane and intracellular punctate structures throughout the entire cytoplasm (Figure 14D). This observation was consistent with previously reported study using myc-PRL-2 [7].

3.3.2 PRL-2 Over-expression Promoted Tumor Cell Migration and Invasion.

I next examined the role of ectopic PRL-2 on cell migration and invasion using the Transwell migration and Matrigel invasion chambers, respectively. A549 cells were transfected with empty HA vector control or HA-PRL-2 vector. After 24 h, cells were harvested for Western blotting, Transwell migration or invasion assay. As shown in Figure 15, cells transfected with the HA-tagged PRL-2 construct migrated 35% faster than control cells transfected with the HA containing plasmid (Figure 15A). I observed a 40% increase in cellular invasion in cells transfected with the HA-PRL-2 plasmid compared to the control cells (Figure 15B).

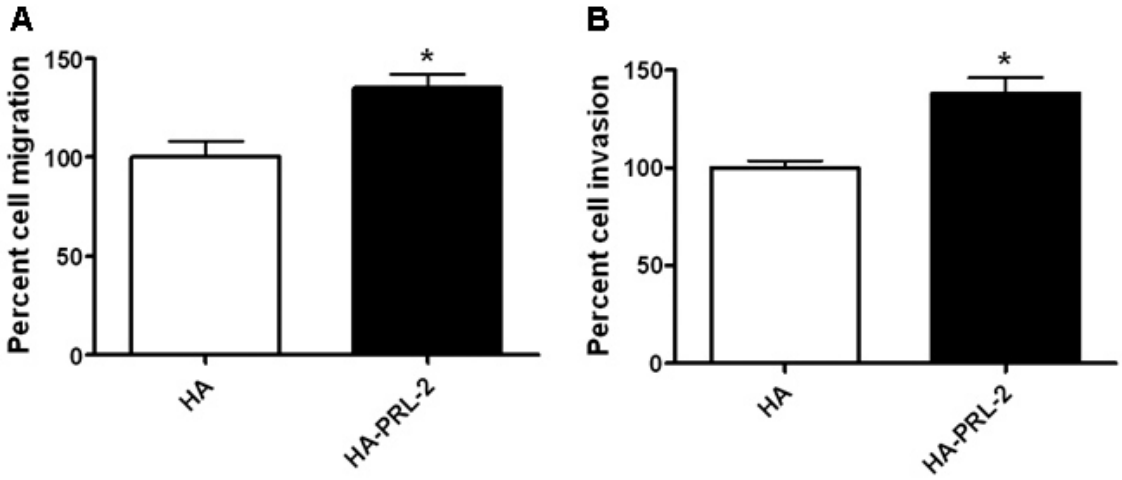

Figure 15. PRL-2 Over-expression Promoted Tumor Cell Migration and Invasion.

A) After 16 h, cells that migrate to the lower chamber were fixed, stained, and counted with a microscope. Percent cell migration was determined by normalizing the number of migrated cells in the HA-PRL-2 wells to HA vector controls. The mean values of three independent experiments measured in triplicate are indicated; bars equal SEM. The mean values were compared using a two-tailed Student's t-test. *, $p<0.01$. B) Cells were plated and after 24 h cells that invaded to the lower chamber were fixed, stained, and counted with a microscope. Percent cell invasion indicated on the ordinate was determined as invaded cells in HA-PRL-2 wells normalized to HA vector control wells. The means of three independent experiments measured in triplicate are indicated; bars are SEM and significance determined as mentioned above. *, $p<0.001$.

3.3.3 ERK Was Activated and Translocated into the Nucleus after PRL-2 Over-expression.

ERK1/2 phosphorylation has been reported to be involved in PRL-3 signaling in colon and lung cancer [41, 42]. ERK was also recently observed to be hyper-phosphorylated after over-expression of PRL-2 in breast cancer cells [28]. To determine the role of ERK in PRL-2 signaling in lung cancer cells, I first examined the total ERK and its phosphorylation status by Western blotting with ectopic HA-PRL-2 expression. A549 cells were transfected with HA control or HA-PRL-2 and harvested 24 hour later for Western blotting. I observed a 2.3 fold increase in ERK1/2 phosphorylation with no change in total ERK1/2 protein levels (Figure 16A). ERK activation was further confirmed by microscopic observation in A549 cells transfected transiently with HA-PRL-2 (Figure 16B). Endogenous pERK (red) in control cells was weak and throughout the entire cells (Figure 16B upper panels), whereas pERK in PRL-2-expressing cells was more prominent as well as being more intense in the nucleus (Figure 16B bottom panels).

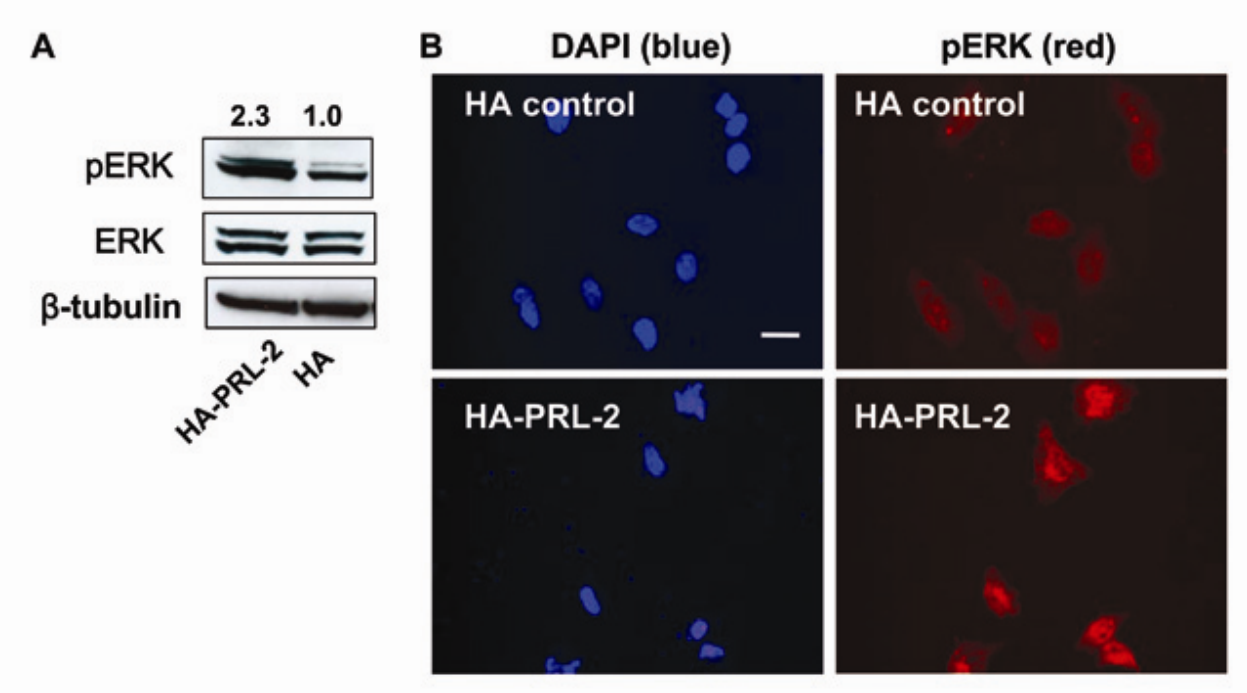

Figure 16. ERK Was Activated and Translocated into the Nucleus after PRL-2 Over-expression.

A) Western blotting showed increased ERK phosphorylation after over-expressing PRL-2. The mean values of three independent Western blots for relative pERK and ERK were shown. B) A549 cells were transfected with HA control or HA-PRL-2. After 24 h, cells were fixed, permealized, and stained with DAPI (blue) and pERK (red). In the cells with over-expressed HA-PRL-2, pERK signal was greater and intense in the nucleus; bar, 10 μm.

As stated in Section 2.3.6, I observed a significant decrease on p130Cas and vinculin levels, while increased phosphorylation on Tyr 146 of Ezrin after PRL-2 suppression. Here I examined the aforementioned molecules by Western blotting, and found no detectable difference on those molecules after transiently transfecting A549 cells with plasmids containing wild type or mutant forms of HA-PRL-2 (Figure 17).

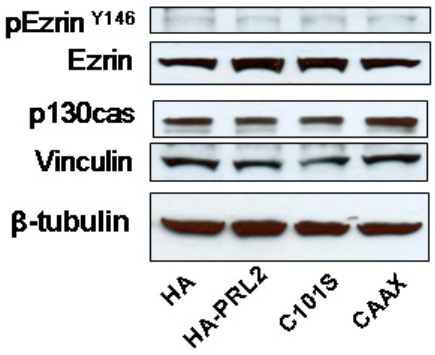

Figure 17. No Alteration on p130Cas or Vinculin levels, or Ezrin Phosphorylation after Ectopic Expression of HA-PRL-2 Vectors.

Western blotting showed no detectable alteration on phosphorylation status of Ezrin Y146 after over-expressing PRL-2 wild type vector and the mutants. The blots were representative from three independent experiments.

3.3.4 Both Catalytic Activity and C-terminal CAAX Domain Were Critical for PRL-2 Function.

To study the biochemical importance of the PRL-2 phosphatase activity and membrane localization, I constructed a phosphatase inactive mutant HA-PRL-2 by replacing the catalytic cysteine with a serine (C101S), and a C-terminal CAAX domain deleted mutant (ΔCAAX). The cells were transfected with empty HA vector control, wild type HA-PRL-2 vector, or the mutants C101S or ΔCAAX. Interestingly, only wild type PRL-2 over-expression significantly promoted cell migration (Figure 18A) and invasion (Figure 18B), but not the catalytic inactive mutant or the CAAX deleted mutant. In addition, ERK hyper-phosphorylation was only observed with wild type PRL-2 but not the mutants (Figure 18C).

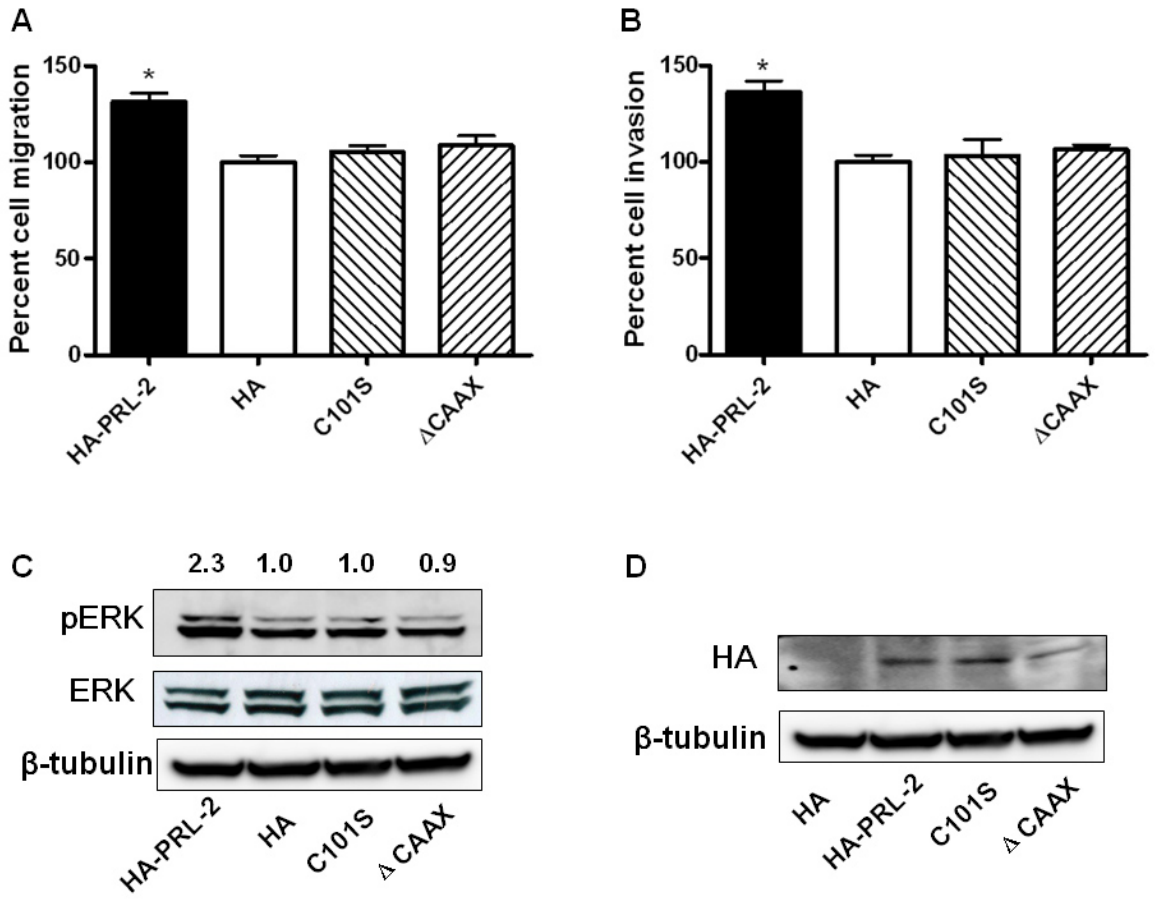

Figure 18. Both Catalytic Activity and C-terminal CAAX Domain Were Critical for PRL-2 Function.

A) A549 cells were transfected with HA control, HA-PRL-2, the catalytic dead mutant C101S or ΔCAAX deletion mutant. After 16 h, cells that migrated to the lower chamber were fixed, stained, and counted with a microscope. Percent cell migration was determined by normalizing the number of migrated cells in the HA-PRL-2 wells to HA vector controls. The mean values of three independent experiments measured in triplicate were shown; bars = SEM. *, $p<0.01$. B) Cells were plated on Matrigel matrix and after 24 h, cells that invaded to the lower chamber were fixed, stained, and counted as above. N=3; bars = SEM. *, $p<0.01$. C) Increased pERK after expression of wild type HA-PRL-2 but not the mutants. A549 cells were transfected with HA control, HA-PRL-2, the catalytic dead mutant C101S or ΔCAAX deletion mutant. Cells were harvested 24 h later for Western blotting. Average fold changes from three independent experiments normalized to HA vector control cells were shown above the Western blots. D) Western blotting confirmed the over-expression of HA-PRL-2 and its mutants.

The expression levels of ectopic HA-PRL-2 or its mutants were equivalent, which was confirmed by Western blotting using HA antibody (Figure 18D). This excluded the possibility that the wild type HA-PRL-2 promoted cell migration and invasion were due to a gene dose effect. These results suggest that both catalytic activity and C-terminal CAAX domain for prenylation are important for the biological function of PRL-2.

3.4 DISCUSSION

Although the PRL phosphatases have been implicated for tumor progression, there is a lack of good reagents to detect endogenous PRLs. For example, most PRL antibodies produced multiple non-specific bands on Western blotting gels. The specific PRL-2 antibody that I used was a test sample from Benthyl which was no longer commercially available. Moreover, I found no good PRL-2 antibodies for immuno-staining. In addition, the three PRL phosphatases share more than 80% identity in amino acid, making it difficult to develop specific antibody that only recognize one of them. Therefore, many PRL studies have been carried out by taking advantage of an epitope tag, such as myc-, HA-, FLAG- or EGF-tagged PRLs. In this work, I used HA-epitope tag at the N-terminal of PRL-2 to avoid disturbing the C-terminal CAAX box. This makes it easy to detect the ectopic PRL-2 by HA antibody for both Western blotting and immuno-staining.

As shown earlier, PRL-2 suppression inhibited tumor cell migration and invasion, indicating that the endogenous PRL-2 plays a critical in regulating tumor invasion. The work performed in this chapter further suggests the effects of ectopic PRL-2 on regulating tumor cell migration and invasion. Over-expression of wild type HA-PRL-2 significantly promotes A549 cell migration and invasion. The studies from PRL-2 knockdown and knock in experiments consistently confirmed the role of PRL-2 in promoting tumor cell invasion.

The ERK phosphorylation cascade is the most well studied MAPK pathway, which functions in cellular proliferation, migration, differentiation, and survival. Its inappropriate activation is common in human cancers [105]. In the context of PRL studies, PRL-3 over-expression was reported to positively correlate with ERK1/2 phosphorylation in colon cancer tissues [42]. Recently, Hardy et al. found that enhanced tumor growth correlated with increased ERK1/2 phorphorylation when PRL-2 was over-expressing in a mouse tumor model [28]. To study the pathways that might be altered and affected by over-expression of PRL-2 in the A549 cells, I examined the total expression level of ERK1/2 and its phosphorylation status. Consistent with the literature, I found that PRL-2 over-expression promoted tumor cell invasion, which correlated with increased ERK1/2 phosphorylation. As previously stated, PRL-2 silenced cells had significantly less ERK1/2 phosphorylation than control cells in a serum stimulated time course. These results support a model in which PRL-2 regulated tumor cell migration and invasion in an ERK-dependent pathway.

Moreover, the results of our current work further extend the evidence that the activated ERK1/2 translocated into the nucleus, where it would be in a position to regulate the transcription factors. For example, ERK1/2 could phosphorylates Thr 453 and Thr 739 sites of the transcription factor Sp1 [106]. ERK1/2 was reported to phosphorylate Ser 63 and Ser 73 of c-Jun, a critical component of the AP1 complex [107]. The ERK1/2 modulated transcription factors, such as Sp1 and c-Jun. These transcription factors could control the transcription of various target genes such as matrix metalloproteinases (MMPs), presumably controlling cell migration and invasion. Interestingly, over-expressing PRL-1 in HEK 293 cells promotes cell migration and invasion,

and correlates with increased ERK1/2 phosphorylation, increased Sp1 and AP1 luciferase activity and increased expression of MMP2 and MMP9; and these effects could be interrupted by a specific MEK1/2 inhibitor U0126, which leads to ERK1/2 inactivation [38]. As PRL-1 and PRL-2 are highly homologous, this leads me to speculate that PRL-2 promotes ERK 1/2 phosphorylation, and the activated ERK1/2 translocates into the nucleus where it regulates the transcription factors such as Sp1 and AP1, thereby controls the transcription of MMPs. The promoted transcription and expression of MMPs facilitate tumor cell invasion by degrading extracellular matrix.

Moreover, the interaction between PRL-2 and ERK1/2 must be indirect, because over-expression of the PRL-2 phosphatase would result in dephosphorylation of its target protein. The phophorylation of this target protein might be a negative regulator for ERK1/2 phosphorylation, because the removal of the phosphorylation on that target protein promoted ERK1/2 phosphorylation. There are several negative regulators of this pathway, include RAF kinase inhibitor protein (RKIP), which interferes with MEK phosphorylation [108]; RAS and RAB interactor 1 (RIN1), which competes with RAF for binding to activated RAS [109]; Impedes mitogenic signal propagation (IMP), which interacts with kinase suppressor of RAS to prevent recruitment of MEK to activated RAF [110]; and other negative regulators include AKT and serum/glucocorticoid inducible kinase (SGK), which can phosphorylate B-RAF [111, 112]. Positive regulators of RAF activity, such as protein kinase C (PKC), SRC, p21-activated kinase (PAK) and 14-3-3, activate RAF in a RAS-independent manner [87]. It is possible that PRL-2 alters the phosphorylation status of these proteins, which negatively regulates ERK1/ phosphorylation, and eventually results in the enhanced cell migration and invasion after over-

expressing PRL-2. It would be interesting to study these regulators of ERK1/2 phosphorylation to see if PRL-2 target protein(s) can be identified among them.

As stated in Chapter 2, I observed a significant decrease on p130Cas and vinculin level, while increased phosphorylation on Tyr 146 of Ezrin after PRL-2 suppression. Surprisingly, when I examined the aforementioned molecules by Western blotting, I found no detectable difference on those molecules after transiently transfecting A549 cells with the wild type or mutant forms of HA-PRL-2 vectors. I speculate the inability to detect a change in p130Cas, vinculin and Ezrin phosphorylation was due to a PRL-2 gene dose effect. In the PRL-2 knockdown cells, I depleted the endogenous PRL-2 to less than 25%. This resulted in an obvious change on p130Cas and vinculin expression level and Ezrin phosphorylation. In contrast, the A549 cells have relatively high basal level of PRL-2. Even though I tried to over-express PRL-2 in A549 cells, I only got about 20% increases in PRL-2 expression level under optimal conditions. The transfection efficacy is satisfying (over 50%). This subtle increase in PRL-2 expression level may not be sufficient to alter the expression or phosphorylation of the aforementioned molecules, such as p130Cas, vinculin and Ezrin. Nonetheless, these results suggest that PRL-2 must be a potent phosphatase in promoting tumor cell migration and invasion, because such a minor increase in PRL-2 expression level resulted in an obvious phenotype change in tumor cell migration and invasion. Further experiments could be performed by creating stable PRL-2 over-expressing cells, and select colonies that have various expression levels of PRL-2. This would be useful to study the PRL-2 gene dose effects.

A better system to study the effect of ectopic PRL-2 might be over-expressing the HA-PRL-2 vectors in the PRL-2 depleted cells. Therefore, I made a silence mutated HA-PRL-2 vector by mutating two nucleotides at the sequence to which the PRL-2 siRNA targeted. The changes in these two nucleotides do not alter the amino acids that they coded. The silence mutated vector was named AA390CC according to its coding sequence. This silence mutated vector was further confirmed by co-transfecting the AA390CC vector with the PRL-2 siRNA. Co-transfection of the wild type HA-PRL-2 and PRL-2 siRNA was used as a control. By measuring the PRL-2 expression at mRNA level, I found the AA390CC vector was over-expressed at the present of PRL-2 siRNA (data not shown). This indicated that the PRL-2 siRNA did not target to the AA390CC vector as I expected. Therefore, the silence mutated vector AA390CC will be a valuable reagent for future study. Further study should be performed to examine the phenotype changes and biochemical regulators of adhesion and invasion by over-expressing the AA390CC vector at the present of PRL-2 siRNA. It is anticipated that the expression of AA390CC could at least partially restore the phenotype changes in the PRL-2 depleted cells.

Another advantage for using the HA-tagged PRL-2 constructs is that making mutations on the PRL-2 constructs makes it possible to study the biochemical importance of PRL-2 structure. Although there is a lack of knowledge on the substrates for the PRLs, several lines of evidence suggests that the catalytic activity is important for PRL-3 functionality. For example, a catalytically inactive PRL-3 mutant has significantly reduced migration-promoting activity [35]. It is also reported that the phosphatase activity and C-terminal polybasic region are all required for PRL-1 mediated cell growth and cell migration [45]. In addition, the primary association of PRL-1, -2, and -3 with the membrane of the cell surface and the early endosome is dependent on

their prenylation and the localization of these proteins may be triggered by a regulatory event that inhibits their prenylation [7]. In this work, I made several mutations on PRL-2, including the catalytic inactive mutant (C101S) and the CAAX deletion mutant (ΔCAAX). In addition, ectopic expression of wild type PRL-2, a catalytic inactive C101S mutant, and a C-terminal CAAX deletion revealed a requirement for both the PRL-2 catalytic functionality and the prenylation site.

4.0 INVESTIGATION OF A POTENTIAL PRL INHIBITOR

4.1 INTRODUCTION

Although no substrate has been convincingly identified for the PRLs, accumulating evidence suggests that the catalytic activity is important for the PRL functionality [35, 44, 45]. Considering the importance of the catalytic activity to the biological functions of PRLs, the PRL family, making an attractive target for small molecular inhibitors, has gained attention from the cancer researchers. Potent and selective PRL inhibitors may eventually constitute novel anti-cancer agents.

Thienopyridone is the first potent PRL inhibitor that suppresses tumor cell three-dimensional growth by a novel mechanism involving p130Cas cleavage [51]. Thienopyridone potently and selectively inhibits all three PRLs but not other phosphatases *in vitro*. In addition, thienopyridone shows significant inhibition of tumor cell anchorage-independent growth in soft agar, induction of the p130Cas cleavage and anoikis [51]. Because my previously mentioned work suggests a

role of PRL-2 in tumor cell migration and invasion, I examined the effect of thienopyridone on these processes. I found that thienopyridone showed antiproliferative activity in a concentration-dependent manner with an EC 50 value of 12.4 μM. In addition, thienopyridone treatment significantly inhibited tumor cell migration and invasion, which is consistent with a role of PRL-2 in these processes.

4.2 MATERIALS AND METHODS

4.2.1 Chemical Reagents

Etoposide was obtained from (Sigma-Aldrich Corp, St. Louis, MO). Thienopyridone, also named compound KP-6-36 by Dr. Peter Wipf (Department of Chemistry, University of Pittsburgh), was provided by Dr. Peter Wipf. Hoechst 33342 was obtained from Invitrogen (Invitrogen, Carlsbad, CA). Dimethyl sulfoxide (DMSO) was obtained from Alfa Aesar (Ward Hill, MA).

4.2.2 Anti-proliferative Activity and Concentration-Response Curve

Cells (6 x 10^4) were plated on noncoated 96-well culture plates (BD Biosciences) at 37°C with 5% CO_2. The cells were treated with various concentrations of thienopyridone: 200 μM, 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.125 μM, 1.5 μM, and 0 μM. After 48 h, cells were washed with PBS three times and stained with 1 μg/ml Hoechst staining solution (Roche Applied Science, Indianapolis, IN). The plates were analyzed with ArrayScan II using a 10x objective and a Hoechst 33342 compatible filter set. Nine fields in three wells were counted, and each experiment was repeated thrice.

4.2.3 Cell Migration Assay and Cell Invasion Assay

Cell migration assay and invasion assay were performed as described in Section 2.2.7, with exception that various concentrations (12.5-50 μM) of thienopyridone were added to the medium.

4.2.4 Statistics

Results were expressed as means ± SE of at least three independent experiments. ANOVA and Student's *t* tests were performed using Graphpad Prism 5 software (Graphpad Software). Differences were considered statistically different if $P < 0.05$.

4.3 RESULTS

4.3.1 Thienopyridone Showed a Concentration-Dependent Antiproliferative Activity

To study the effect of thienopyridone (TPD) on cell proliferation of A549 lung cancer cells, I performed cell proliferation assay and analyzed using ArrayScan II. A549 cells were seeded on 96-well plates, and treated with various concentrations (1.5-200 μM) of thienopyridone. During the first 24 h, I did not observe massive cell death in cells treated with thienopyridone at concentrations of 50 μM or lower. After 48 h, I observed obvious cell death in cells treated with thienopyridone at concentrations of 6.25 μM or higher. Figure 19B showed the representative images from ArrayScan II at selected concentrations of thienopyridone treatment for 48 h. The upper panels were phase contrast images while the bottom panels were DAPI stained images. The ArrayScan II quantified the DAPI stained cells in each well. The quantitative data from ArrayScan II were then analyzed by GraphPad 5. Figure 19C showed the survival curve of A549 cell upon thienopyridone treatment. The EC 50 value from the survival curve was 12.4 μM.

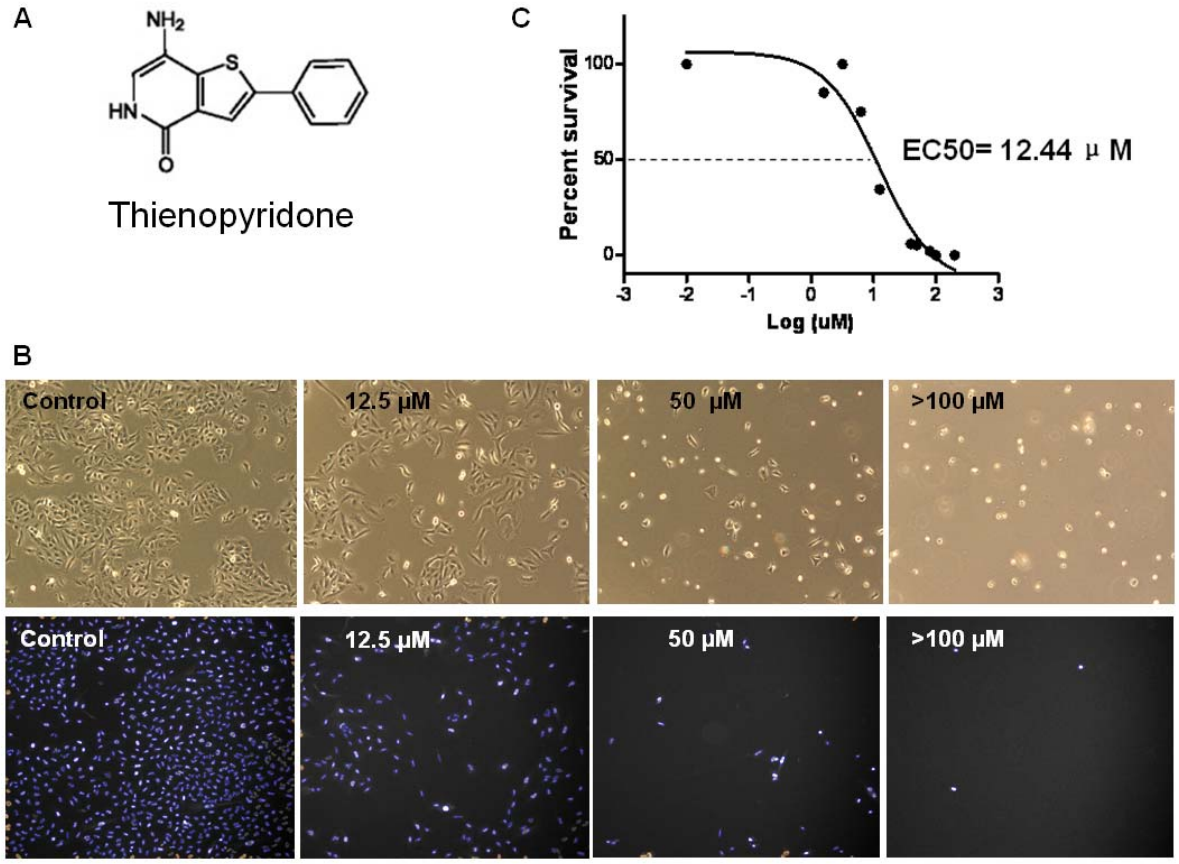

Figure 19. Thienopyridone Showed Antiproliferative Activity in a Concentration-Dependent Manner.

A) Structure of thienopyridone. B) Representative images of cells treated with various concentrations of thienopyridone from ArrayScan II. C) Quantitative survival curve after thienopyridone treatment. The data were measured mean of triplicates from three independent experiments. The mean values of three independent experiments measured in triplicate were indicated and the bars equaled the SEM.

4.3.2 Decreased Tumor Cell Migration and Invasion after Thienopyridone Treatment

To study the effect of thienopyridone on tumor cell migration and invasion, I examined cell migration and invasion after treating the A549 cells with thienopyridone. After 16 h, cells that migrated to the lower chamber were fixed, stained, and counted with a microscope. For invasion assay, cells were plated and after 24 h cells that invade to the lower chamber were fixed, stained, and counted under the microscope. I observed significantly inhibited cell migration and invasion after treating with thienopyridone, with $p<0.001$ (Figure 20A and 20B). Interestingly, both thienopyridone treatment and PRL-2 knockdown led to about 40% inhibition on cell migration and invasion (Figure 20A and 20B). Depletion of PRL-2 by either RNA interference or the pharmacological inhibitor showed similar effects on tumor cell migration and invasion.

To further study the concentration-response effect, I treated the cells with selected concentrations of thienopyridone at 0 μM, 12 μM, 25 μM, and 50 μM. As shown in Figure 20C, thienopyridone inhibited A549 cell migration at all the concentrations tested with $p<0.01$, but about 40% inhibition was the maximum it could reach. The inhibition on cell migration did not increase at higher concentrations of thienopyridone treatment. Similar results were obtained from Matrigel Transwell cell invasion assay (Figure 20D). Thienopyridone inhibited A549 cell invasion at a concentration as low as 12 μM, but the effect was not enhanced by increasing the concentration to 50 μM.

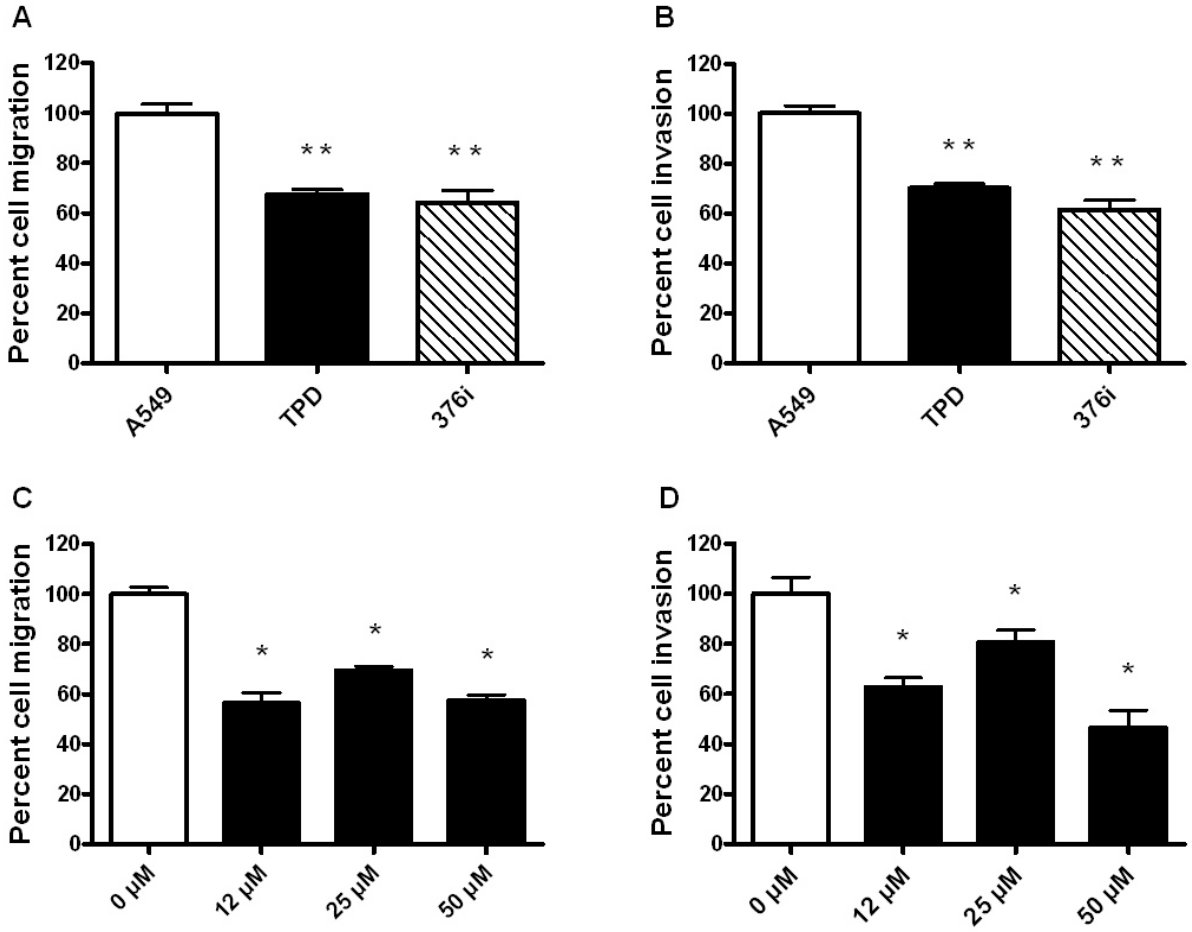

Figure 20. Decreased Tumor Cell Migration and Invasion after Thienopyridone Treatment

Percent cell migration was determined by normalizing the number of migrated cells in thienopyridone treated wells to the parental A549 controls. Percent cell invasion indicated on the ordinate was determined as invaded cells in thienopyridone treated wells normalized to the untreated wells. The mean values of three independent experiments measured in triplicate were indicated; bars = SEM. The mean values were compared using a two-tailed Student's t-test. *, $p<0.01$. A) Thienopyridone treatment and PRL-2 knockdown inhibited cell migration to the similar extent. **, $p<0.001$. B) Thienopyridone treatment and PRL-2 knockdown significantly inhibited cell invasion to the similar extent. **, $p<0.001$. C) Various concentrations of thienopyridone treatment inhibited A549 cell invasion. *, $p<0.01$. D) Various concentrations of thienopyridone treatment inhibited A549 cell invasion. *, $p<0.01$.

4.4 DISCUSSION

A number of PTPs have been implicated in oncogenesis and tumor progression, making them potential drug targets for cancer chemotherapy. As stated, pervious studies indicate that over-expression of PRL-1 and PRL-3 facilitate tumor cell invasion and metastasis in mouse models, while suppression of them inhibits tumor invasion and progression [6]. In this dissertation, I showed that PRL-2, like PRL-1 and PRL-3, is also a key contributor in promoting tumor cell migration and invasion. More importantly, the PRL-1 and PRL-3 studies show that the catalytic activity is critical for the biological functions of PRLs. This conclusion is also reinforced by my work on PRL-2. Taken together the reported literature on PRL-1 and PRL-3, and my work on PRL-2, developing small molecule inhibitors targeting to the PRL phosphatase family might be a promising strategy for anti-cancer treatment.

Thienopyridone is the first potent selective PRL inhibitor that suppresses tumor cell three-dimensional growth [51]. Thienopyridone potently and selectively inhibits all three PRLs but not other phosphatases *in vitro*. In addition, thienopyridone significant inhibits tumor cell anchorage-independent growth in soft agar [51]. The endpoint of their assay is the colony area and colony counts in the soft agar assay. The data from this assay reflects the effect of thienopyridone on tumor growth. As stated in the Introduction, the PRL family is most well-studied for promoting tumor progression, measured by cell invasion assay as well as *in vivo* mouse models. To further study the effect of thienopyridone on tumor cell migration, invasion and metastatic progression, I tested thienopyridone in A549 lung cancer cells.

Thienopyridone is a potent inhibitor for PRL phosphatases with a reported IC 50 of 0.1-0.2 μM in the affinity-based fluorescence polarization (IMAP-FP) assay, and an IC 50 of 0.46 μM in an *in vitro* phosphatase assay using DiFMUP (6,8-difluoro-4- methylumbelliferyl phosphate) as the substrate [51]. In the soft agar growth assay, thienopyridone inhibits cancer cells anchorage-dependent growth with EC 50 values of 3.29 and 3.05 μM for human colorectal cancer cells RKO and HT-29 cells, respectively [51]. In my proliferation assay, the EC 50 of thienopyridone from the survival curve was 12 μM, while 12 μM thienopyridone was also sufficient to inhibit A549 cell migration and invasion. Thienopyridone was also tested on Hela cells, and the EC 50 was about 40 μM for the proliferation assay (data not shown). Obviously, the EC 50 from my assays were higher than that was reported in the literature [51]. The difference could reflect differences in the cell types or the methodologies employed. The IC 50 from *in vitro* phosphatase assay was less than 0.5 μM, while the EC 50 from the cell based assay was higher. This could be explained as the cells did not takeup thienopyridone very well, or thienopyridone had binding proteins in the cells which resulted in less inhibitory effect. From the cell based assays, The EC 50 for human colorectal cancer cells in soft agar assay was about 3 μM [51], while the EC 50 from my survival curve was 12 μM. It is possible that thienopyridone is more effective in inhibiting tumor cell anchorage-dependent growth, and is relatively less potent in inhibiting cell migration and invasion. Or it is also likely that A549 lung cancer cells that I used are more resistant to thienopyridone than the colorectal cancer cells RKO and HT-29 that the previous investigation used. It seems that Hela cells might be even more resistant to thienopyridone than A549 cells. They showed a higher EC 50 value of 40 μM in the proliferation assay.

Another possibility would be compound stability. I found that with the compound lost the activity with storage and the EC 50 increased. This could be due to oxidation, reduction or other modifications of thienopyridone. For example, 7-amino-2-phenylthieno[3,2-c]pyridin-4-ol (thienopyridone) would be oxidized under air conditions to form 7-imino-2-phenylthieno[3,2-c]pyridin-4(7H)-one. Thus, I speculate it would be worthwhile studying what modification makes thienopyridone chemically instable, and then develop thienopyridone derivatives for better PRL inhibitor and better candidates for cancer chemotherapy.

Further studies would be performed to examine the aforementioned biochemical regulators in the PRL-2 signaling pathway, such as p130Cas, Ezrin, and ERK, after thienopyridone treatment. As thienopyridone treatment and PRL-2 depletion led to similar inhibitory effects on tumor cell migration and invasion, it is anticipated that the molecules that were altered in PRL-2 depleted cells would be altered after thienopyridone treatment. For example, I anticipate observing less vinculin and p130Cas expression, less ERK phosphorylation upon serum stimulation, and more Ezrin phosphorylation on Tyr 146 after thienopyridone treatment. More interestingly, it was reported that thienopyridone treatment induced p130Cas cleavage and generated a 31 kD cleaved p130Cas, which led to caspase-mediated cell apoptosis [51]. In my experiments using A549 cells, I did not observed apoptotic cells after thienopyridone treatment. In light of the different cleavage pattern I found in the PRL-2 depleted cells, I speculate that there might be a different p130Cas cleavage pattern which generate the 80 kD cleavage product in A549 cells.

As stated, thienopyridone inhibited all three PRL phosphatases with similar IC 50 values [51], indicating that the effects of thienopyridone were due to the inhibition on all PRL phosphatases. To study the specific effect of thienopyridone on one of the PRL phosphatase, it would be very interesting to combine thienopyridone with PRL siRNAs. For example, treating the PRL-2 depleted cells with thienopyridone would facilitate studying the inhibitory effect of thienopyridone on PRL-1, as in A549 cells PRL-3 is undetected. Similarly, treating the PRL-1 depleted cells with thienopyridone would facilitate studying the inhibitory effect of thienopyridone on PRL-2. It is anticipated that the inhibitory effect of thienopyridone in PRL-1 depleted cells would mimic the observation from the PRL-2 depleted cells.

As stated, there are no substrates identified from the attempted substrate trapping assay. At least several differences between PRLs and classical PTPs may account for the difficulty in substrate trapping. PRLs have less than 30% sequence identity to DSPs, but they showed a shallow catalytic pocket, which is typical for DSPs. The other notable difference is the presence of Ala instead of the highly conserved Ser/Thr residue normally next to the invariant Arg of the catalytic motif. This could lead to loss of a Ser/Thr hydroxyl group that normally facilitates the breakdown of phosphoenzyme intermediate in PTP-mediated catalysis [6]. Although it is challenging, identifying substrates for the PRL phosphotases would not only contribute great to the understanding of the molecular mechanisms of PRLs signaling pathways, but also provide better substrates for *in vitro* phosphastase assay to evaluate PRL inhibitors.

5.0 GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1 GENERAL DISCUSSION

Despite attention that has been directed toward understanding the potential roles of PRL-1 and PRL-3 in tumor development and metastasis, there has been a noticeable absence of information about PRL-2. By studying the role and mechanisms of PRL-2 in tumor cell migration and invasion, I attempted to gain insights into the biological functions and signaling pathways of PRL-2, and to better understand its regulatory mechanism of tumor invasion and metastasis. Here my studies of PRL-2 in A549 lung cancer cells suggest for the first time that PRL-2 plays a critical role in promoting tumor cell migration and invasion. I found that suppression of endogenous PRL-2 by RNA interference inhibited cell migration and invasion in A549 lung cancer cells, while ectopic expression of PRL-2 significantly promoted tumor cell migration and invasion. These results with lung cancer cells also coincide with recent observations about PRL-2 in breast cancer [28], so the regulatory role of PRL-2 may be general for multiple tumor types.

Placed in the context of previous work, these results suggest that PRL-2 shares a similar functionality with the other two PRL family members. For example, both PRL-1 and PRL-3 have been found to be over-expressed in a variety of different cancer cell lines and tumor tissues, and the elevated PRLs are associated with enhanced tumor progression [3, 32, 35]. Ectopic PRL-1 or PRL-3 expression in different cancer cell types correlate with the induction of metastatic phenotypes, such as enhanced motility and invasiveness [35, 36]. Moreover, over-expression of PRL-1 or PRL-3 promotes experimental metastasis in mice [35, 44]. Likewise, ablation of endogenous PRL-1 or PRL-3 by small interfering RNA has the opposite effect [16, 23, 38].

The biochemical signaling pathways exploited by PRLs are not firmly established but several pathways have been proposed including p53, PI3K/Akt, and Src. Nonetheless, when I suppressed PRL-2 in A549 cells, I observed decreased p130Cas and vinculin expression, and decreased ERK phosphorylation, but did not detect any difference on the total level or phosphorylation status of p53, Akt or Src. Furthermore, when one compares the current PRL-2 data with our previously published PRL-1 data, in which using the same lung cancer cell line we showed that PRL-1 silencing regulates c-Src level and cell invasion [23]. While both PRL-1 and PRL-2 depleted cells lost their ability to invade, PRL-2 did not seems to alter Src expression or phosphorylation; and PRL-2 knockdown cells did not exhibit a preference for fibronectin nor did they appear to regulate adhesion turnover as PRL-1 silenced cells did. This leads me to speculate that while PRL-2 may be functionally similar to PRL-1 and PRL-3, it could signal through somewhat different pathways.

Focal adhesions are dynamic structures through which the cytoskeleton of a cell connects to the extracellular matrix via interactions with integrin receptors. Ligand binding by integrins results in recruitment of adaptor, kinases and other components to the FAC. Besides Src family kinases and focal adhesion kinase, the adaptor protein p130Cas is critical downstream component in this integrin signaling, and its involvement in cell motility as a component of the integrin signaling machinery is well established [78]. Vinculin and paxillin are also important components that usually recruited to FAC [113]. In the context of PRL-3 studies, p130Cas phosphorylation is increased as is the interaction between p130Cas and vinculin in PRL-3 expressing HEK 293 cells [36], whereas vinculin and paxillin levels are reduced and the focal adhesion formation is defective in the PRL-3 expressing HeLa and CHO cells [39]. The discrepancy might be due to different cell type or conditions, and also possibly reflect different aspects of PRL-3 in regulating focal adhesion turnover, as both enhanced focal adhesion formation and disassembly are important in regulating cell migration.

In this study, I observed decreased p130Cas and vinculin expression while paxillin levels were unchanged after PRL-2 suppression in A549 cells. This is consistent with our previous observation in PRL-1 silencing A549 cells [23], in which p130Cas were also decreased while paxillin unchanged (vinculin levels unknown). The fact that paxillin level remained unchanged is most likely because the association between p130Cas and vinculin is more important in A549 cells as the paxillin expression is hardly detected in this cell line. Further studies could be performed to study the focal adhesion pattern by immuno-staining the focal adhesion components such as vinvulin. In addition, I observed p130Cas cleavage after PRL-2 suppression, which generated the 80 kD cleavage product. This cleavage occurred at the

substrate domain, and led to the loss of the N-terminal SH3 domain, which binds to several effectors, such as FAK and DOCK180 [78]. Thus the cleaved 80kD p130Cas might be unable to interact with FAK, DOCK180 and some other effectors. This would result in the disassembly of a p130Cas-Crk-DOCK180 scaffold at adhesion sites, which eventually inhibit cell migration. In sum, these results indicate that PRL-2 might regulate tumor cell migration and invasion through a Src or p53 independent p130Cas signaling pathway.

The Ras-Raf-ERK signal transduction cascade is one of four mitogen-activated protein kinase (MAPK) signaling pathways. Its inappropriate activation is a common occurrence in human cancers [105]. ERK phosphorylation is a well-known target of FAK-Src signaling [114], downstream of FAC. The local activation of ERK can be facilitated by association with paxillin [115]. FAK could also recruit ERK to adhesions, where the local activation of ERK regulates adhesion disassembly and migration [116]. ERK could also be translocated into the nucleus and control cell migration by regulating the transcription factors [81]. For PRL studies, it was reported [42] that PRL-3 over-expression correlated with ERK1/2 phosphorylation in colon cancer tissues. Recently, Hardy et al. found that enhanced tumor growth correlated with increased ERK1/2 phorphorylation when PRL-2 was over-expressed in a mouse tumor model [28]. Consistent with this observation, I found that PRL-2 silenced cells had significantly less ERK1/2 phosphorylation than control cells in a serum stimulated time course. In addition, PRL-2 over-expression promoted tumor cell invasion, which correlated with increased ERK1/2 phosphorylation. Importantly, I found the activated ERK1/2 translocated into the nucleus, where it could regulate the transcription of various target genes, presumably controlling cell migration

and invasion. Clearly, identifying the PRL-2 regulated genes in the future will be important for uncovering the roles of PRL-2 in tumor progression.

Although no substrate has been convincingly identified for the PRLs, evidence exists to suggest that the catalytic activity is important for PRL-3 functionality. For example, a catalytically inactive PRL-3 mutant has significantly reduced migration-promoting activity [35]. EGF-PRL-3 expressing CHO cells rapidly induced metastatic tumor formation in lung while the catalytic inactive mutant expressing ones lose this metastatic activity [44]. As PRL-2 has conserved catalytic domain with PRL-3, I hypothesized that the catalytic activity is also required for PRL-2 function. To study the biochemistry importance of the catalytic domain and the C-terminal CAAX domain, I generated an HA-tagged wild type PRL-2 vector as well as the catalytic inactive mutant C101S or C-terminal CAAX deletion ΔCAAX. It was demonstrated in this dissertation that the PRL-2 functionality requires both phosphatase activity and prenylation site of PRL-2.

Although PRLs have a clear phosphatase domain, their substrates are not well defined. Interestingly, only Ezrin, a linker between plasma membrane and cytoskeleton, has been reported to be a direct target of protein PRL-3 [43]. *In vitro* dephosphorylation assays suggest that Ezrin-Thr 567 is a substrate of PRL-3, which challenges the current believe that PRLs belongs to PTP family. This work has not been independently confirmed by another study. Interestingly, I found that Ezrin was hyper-phosphorylated on Tyr 146 in my PRL-2 silenced cells while there were no changes on Thr 567. My observation is in support of the current dogma that PRLs are PTPs, which leads me to speculate that Tyr 146 of Ezrin might be a direct substrate for PRL-2.

Nevertheless, there is currently insufficient evidence to propose Ezrin as a direct substrate for PRL-2.

Collectively, I summarized my work in a testable model shown in Figure 21. PRL-2 phosphatase regulates the components in the FAC, such as vinculin and p130Cas expression level. ERK phosphorylation is a well-known target of FAK-Src signaling [114], downstream of FAC. ERK can be recruited to adhesions, and the local activation of ERK regulates adhesion disassembly and migration [98]. The activated ERK could also translocate into the nucleus, where it modulates the transcription factors and their target genes, presumably control cell migration and invasion. Interesting, Ezrin-mediated early metastatic survival was reported to be partially dependent on the activation of ERK [117]. This is also in support of the model that ERK might be downstream of Ezrin. There might be other unknown regulators that are involved in the PRL-2 mediated cell migration and invasion; therefore, I use some question marks in the model.

In this work, I used RNA interference to suppress PRL-2 expression at mRNA level, and PRL-2 suppression significantly inhibited tumor cell migration and invasion. Furthermore, I found that the catalytic activity and prenylation site were required for the biological functions of PRL-2. Therefore, I used a potent PRL inhibitor to suppress tumor cell migration and invasion by inhibiting the PRL phosphatase activity. In addition, prenylation inhibitors could be used to inhibit PRL-2 prenylation and thereby suppress PRL-2 mediated cell migration and invasion. The prenylation inhibitors are not preferable in my work because it would non-specifically inhibit the functions of all the prenylation-dependent proteins.

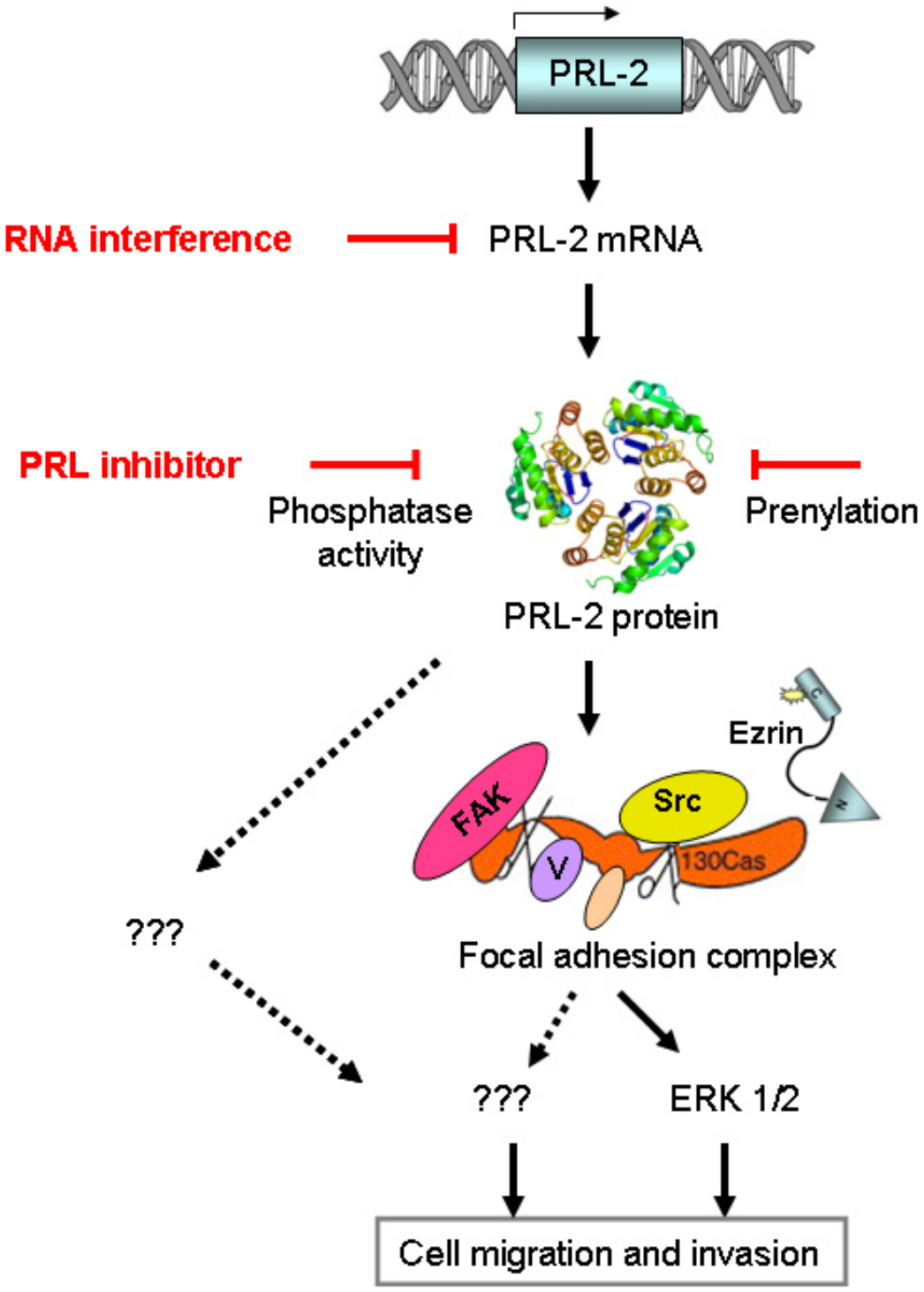

Figure 21. A Schematic Model of the PRL-2 Pathways.

In sum, my results provide support for the involvement of PRL-2 in promoting tumor cell invasion via ERK signaling pathway. To date, most studies have focused on the role of PRL-1 and PRL-3 in tumor progression. Here I demonstrated for the first time that PRL-2 regulated cell migration and invasion in non-small cell lung cancer. Notably, I showed that the PRL-2 stimulated cell invasion was associated with ERK1/2 phosphorylation, and activated ERK in the nucleus might participate in PRL-2 mediated tumor cell invasion. In this speculative scenario, the functional role and molecular mechanism of PRL-2 were studied in A549 lung cancer cells. Future studies need to employ mouse models to test this hypothesis *in vivo*.

5.2 FUTURE DIRECTIONS

PRL-2 has been shown to play important regulatory roles in tumor invasion, but much less is known about PRL-2 controlled pathways as well as the molecular mechanisms of tumor progression. Therefore, further studies should be performed to fully understand its functions, regulation mechanisms and signaling pathways associated with PRL-2 in tumor cells, in order to better conceptualized the high levels of PRL-2 in tumor cell lines and tissues. The interaction of PRL-2 and p130Cas, as well as the biological functions of the 80kD cleavage product of p130Cas are well worth investigating to better understand the correlation between the two and the involved signaling network. Moreover, how ERK phosphorylation is regulated in the PRL-2 signaling pathways is unknown. Therefore, I would suggest future studies should employ co-immunoprecipitation or yeast two hybrid methods to identify the molecules that directly interact with PRL-2. This might permit one to study how PRL-2 interacts with and regulates ERK phosphorylation and determine if it is a direct effect. In addition, this work could be reinforced by re-introducing silence mutated PRL-2 into the PRL-2 knockdown cells.

A question remains about whether or not Ezrin is a direct substrate for PRL-2. I believe it would be extremely helpful to use *in vitro* phosphatase assay to validate Ezrin as a substrate for PRL-2, or to perform substrate trappings studies to identify substrates for PRL-2. This could provide more insights into the PRL-2 mediated tumor cell invasion, and could also facilitate the evaluation of PRL inhibitor by providing better substrate for *in vitro* phosphatase assay.

Furthermore, as my work suggests that the catalytic activity is critical for the biological functions of PRL-2, it would be quite useful to identify and develop potent and selective PRL inhibitors. These could be valuable chemical probes and lead structures for future cancer chemotherapy candidates.

As PRL-2 shares a highly conserved sequence and structure to PRL-1 and PRL-3, one could further study the effect of these PRL phosphatases in tumor progression by investigating the possible compensation from the other two family members using different tumor cell lines. To fully understand the role of PRL-2 in human tumors, it would also be quite useful to study the effect and pathways of PRL-2 in different tumor cell lines as well as mouse models. In addition, characterizing the regulation of PRL-2 expression and function under different circumstances would be equally important for constructing the general picture of PRL-2 during embryogenesis, organogenesis, tumorigenesis and metastasis. This might be helpful in predicting the side effects of the novel PRL inhibitors.

APPENDIX A

ANTIBODIES FOR WESTERN BLOTTING

Antibodies	Dilutions	Vendors
Anti-Akt	1:1000	Cell Signaling
Phospho-Akt	1:1000	Cell Signaling
Anti c-Src	1:1000	Santa Cruz
Phospho-Src	1:1000	Cell Signaling
Anti-ERK1/2	1:1000	Cell Signaling
Phospho-ERK 1/2	1:1000	Cell Signaling
Anti-Ezrin	1:2000	Covance
Phospho-Ezrin-Tyr^{146}	1:200	Santa Cruz
Phospho-Ezrin- Thr^{567}	1:500	Cell Signaling
Anti-GAPDH	1:1000	Cell Signaling
Anti-HA.11	1:1000	Covance
Anti-p130Cas	1:500	BD Transduction Lab.
Anti-p53	1:1000	Cell Signaling
Phosphor-p53-Ser^{15}	1:1000	Cell Signaling
Anti-paxillin	1:500	BD Transduction Lab.
Anti-PRL-2 polyclonal	1:500	Bethyl
Anti-Pan-PRL	1:500	R&D Systems
Anti-β-tubulin	1:10000	Cedarlane Laboratories
Anti-vinculin	1:200	Santa Cruz

APPENDIX B

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