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TETRASPANIN KAI1/CD82 INHIBITS CELL MIGRATION-RELATED CELLULAR EVENTS VIA REORGANIZING ACTIN NETWORK

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By
Wei Liu
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DEDICATION

This dissertation is dedicated to

My wife, Ruohong Zhou

My son, Chang Liu

My daughter, Emma Liu

My parents, Xiaoyang Liu and Bihui Zhong

My brothers Kesheng Liu and Jian Liu

My Mother-in-law, Qiuyun Huang

My Father-in-law, Zhiguang Zhou

For their love and support

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ABSTRACT

To determine how tetraspanin KAI1/CD82, a tumor metastasis suppressor, inhibits cell migration, we assessed which motility-related cellular events are altered by KAI1/CD82 and how KAI1/CD82 regulates these events. We found that KAI1/CD82-overexpressing cancer cells exhibit various morphologies but typically display elongated cellular extensions and a lack of lamellipodia. Live imaging demonstrated that the formation of lamellipodia and retraction of extensions were deficient upon KAI1/CD82 overexpression.

The deficiency in developing motility-related cellular events was accompanied by defects in actin cortical network and stress fiber formations. Notably, actin polymerization was attenuated by KAI1/CD82. Although Rac1 activity was diminished upon KAI1/CD82 expression, Rac1 could not rescue lamellipodia formation because Rac1 activity is not required for this process in Du145 prostate cancer cells. Surprisingly, RhoA activity was upregulated upon KAI1/CD82 overexpression despite the loss of stress fiber and lack of cellular retraction, suggesting that enhanced RhoA activity is a compensatory effect resulting form impaired actin polymerization. Cofilin, an effector of both Rac and Rho, cannot translocate to the cell periphery in KAI1/CD82-overexpressing cells to facilitate lamellipodia formation, though the total and active cofilin proteins remain unchanged.

In summary, we demonstrate that KAI1/CD82 inhibits protrusion and retraction events crucial for cell movement by disrupting actin cortical network

and stress fiber formations. At the molecular level, KAI1/CD82 impairs actin polymerization by unbalancing Rac1 and RhoA activities. KAI1/CD82-induced disruption of actin organization likely results from the suppression of common signaling steps of multiple pathways but is alleviated by cell-cell adhesion.

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LIST OF ABBREVIATIONS

A	Ala, Alanine
ABP	Actin binding protein
AIP1	Actin-interacting protein 1
ARP	Actin-Related Proteins
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
C3	C3 transferase
CAP	Cyclase-associated protein
CCD	Charge-coupled device
CD82	Also known as R2; 4F9; C33; IA4; ST6; GR15; KAI1; SAR2
Cdc42	Cell division cycle 42
C-Met	Hepatocyte growth factor receptor
Crk	V-Crk avian sarcoma virus (Chicken Retroviruses); CT10 oncogene homolog
CXCR-4	Chemokine CXC motif receptor 4
DARC	Duffy antigen receptor for chemokines
DIC	Differential Interference Contrast
DMEM	Dulbecco's Modified Eagle's Medium
DN	Dominant negative
ECM	Eextracellular matrix
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FN	Fibronectin
GAPs	GTPase-activating proteins
GDIs	Guanine nucleotide dissociation inhibitors
GEFs	Guanine nucleotide exchange factors
GM2	GalNAcβ4[NeuAcα3]Galβ4Glcβ1Cer
GST	Glutathione S-transferase
GTPase	Guanosine triphosphatase
HGF	Hepatocyte Growth Factor
IPTG	Isopropyl β-D-thiogalactopyranoside
KAI1	Kangai 1
L	Leu, Leucine
LIMK	LIM domain kinase
LM	Laminin
mAb	Monoclonal antibody
MDFR	Morphological deviation from roundness
mDia	Mouse Diaphanous-related formin
MIEG	MSCV-based bicistronic (EGFP) retroviral vector
MLC	Myosin light chain
MTOC	Microtubule organizing center
N	Asn Asparagine

NP40	Nonidet-P40
P130/Cas	p130Crk-associated substrate, Bcar1 (breast cancer resistance);
pAb	Polycolnal antibody
PAK	p21-activated kinase
PAX	Paired box gene
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PIP2	Phosphatidylinositol-4,5-bisphosphate
PI3k	Phosphatidylinositol3-kinase
Rac	Ras-related C3 botulinum toxin substrate 1
Rho	RAS homolog
RIPA	Radioimmunoprecipitation
ROCK	Rho-associated coiled-coil-containing protein kinase
SDF-1	Stromal cell-derived factor 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Src V-SRC	Avian sarcoma (Schmidt-ruppin A-2), viral oncogene
TESK1	Testicular protein kinase 1
TM4	Tetraspanin
WASP	Wiskott-Aldrich Syndrome protein

CHAPTER 1. INTRODUCTION

1.1 Importance of cell migration in normal development and diseases

Cell migration is a life-long event. It accompanies us throughout our life, from shortly after conception to death. During embryonic development, cell migration is pivotal for morphogenesis (Ridley et al., 2003). For example, massive cell migration directly contributes to three-layer-embryo formation during gastrulation and subsequent tissue and organ development (Ridley et al., 2003). Cell migration is also actively involved in normal renewal of skin and intestine, in which the newly differentiated epithelial cells migrate up from the basal layer and the crypts, respectively. However, the detailed mechanisms of cell migration and its importance in a variety of biological events are still far from delineated (Ridley et al., 2003).

Cell migration is not only key for normal development and biological processes, but also heavily involved in a vast number of pathological processes, for example, vascular disease, osteoporosis, chronic inflammatory diseases (rheumatoid arthritis and multiple sclerosis), and cancer. During wound healing and immune defense processes, circulating leukocytes, which are attracted by the chemotactants released from the inflammatory lesion, migrate into the tissue surrounding the lesion to attack the pathogens, destroy infected cells, and clean up debris.

For all above pathological processes, to unveil the fundamental mechanisms underlying cell migration will shed light on the development of more effective therapeutic methods and for a variety of illnesses.

In recent years, scientists have made many important progresses about how cancers initiate and develop. Surgery, chemotherapy and radiation can effectively treat the primary tumor lesion. However, relatively little is known about how cancers are able to metastasize to distant tissues and organs (Steeg, 2006). Metastasis is a multiple step process including invasion, survival and arrest of cancer cells in the bloodstream, and finally the formation of metastatic colonization to the target organs (Steeg, 2006) .

The metastasic cancer lesion can lead to catastrophic consequences, such as the functional failure of target organs due to cancer growth and infiltration. In fact, the local invasion and distal metastasis of the cancer are the leading cause of death in many common cancer cases, such as lung metastasis and destruction in thyroid cancer (Mazzaferri, 2006), and the bone, lungs, liver and brain metastasis and destruction in breast cancer (Price, 1994), et al. It is very important to understand the mechanisms for our body to fight against tumor metastasis. However, the genetic foundation of suppressing cancer metastasis remained unclear. It has been reported that at least three genes are involved in this process. They are Nm23 (Fournier et al., 2002; Hartsough et al., 2002; Palacios et al., 2002; D'Angelo et al., 2004; Engel et al., 2004), medroxyprogesterone (MPA) (Ouatas et al., 2003; Palmieri et al., 2005), and KAI1 (Bandyopadhyay et al., 2006; Zijlstra and Quigley, 2006; Gellersen et al., 2007; Iiizumi et al., 2007). Here, Iwill focus on how KAI1/CD82 inhibits cancer cell metastasis.

1.2 KAI1/CD82 is a cell motility-inhibitory tetraspanin

1.2.1 The discovery of KAI1/CD82

KAI1/CD82 (also called R2, C33, IA4, or 4F9) was initially identified from T cell activation studies (Gaugitsch et al., 1991). The KAI1/CD82 gene encodes a 267 amino acid protein that contains four putative transmembrane domains (Gaugitsch et al., 1991). Later studies showed that immuno-crosslinking of KAI1/CD82 triggered the intracellular calcium mobilization in lymphocytes and the T cell adhesion, the cell surface level of KAI1/CD82 was up-regulated upon cell activation and cytokine stimulation, and KAI1/CD82 played an accessory role in T cell activation (Fukudome et al., 1992; Gil et al., 1992; Nojima et al., 1993). Interestingly, KAI1/CD82 was also identified as a target of the monoclonal antibody that inhibited syncytium formation induced by human T-cell leukemia virus (Fukudome et al., 1992).

The role of KAI1/CD82 in cancer progression was discovered by a genetic screen attempting to identify metastasis suppressing genes (Ichikawa et al., 1991). Using microcell-mediated chromosome transfer, human gene(s) responsible for suppressing metastasis of the highly metastatic rat AT6.1 prostate cancer cells was mapped to the short arm of human chromosome 11 (Ichikawa et al., 1992). Dong, Isaacs, and Barrett made important progress in 1995 as they cloned the metastasis suppressor gene located at human chromosome 11 p11.2-13 and designated this suppressor as KAI1(Dong et al., 1995). KAI1 is identical to CD82 (Dong et al., 1995). KAI1/CD82 expression

leads to a marked suppression of lung metastases of AT6.1 prostate cancer cells, without affecting the growth rate of the primary tumor (Dong et al., 1995).

1.2.2 KAI1/CD82 as a tumor metastasis suppressor

Although KAI1/CD82 was initially identified as a metastasis suppressor of prostate cancer, a plethora of evidence supports KAI1/CD82 as a wide-spectrum invasion- and metastasis-suppressor during the progression of a variety of solid tumors (Dong et al., 1995; Hemler, 2001; Stipp et al., 2003). In the past decade, KAI1/CD82 expression in tumors has been thoroughly analyzed. In normal tissues, KAI1/CD82 is ubiquitously expressed. Its mRNA levels are high in spleen, placenta, lung, liver, kidney, and prostate, moderately abundant in the pancreas, skeletal muscle, and thymus; and detectable at low level in brain, heart, ovary, stomach, and uterus (Nagira et al., 1994; Dong et al., 1995). KAI1/CD82 is evolutionarily conserved (Nagira et al., 1994; Dong et al., 1995) . For example, KAI1/CD82 protein displays 76% identity and 82% similarity between human and mouse (Nagira et al., 1994). Thus, although KAI1/CD82 is important for cancer progression, the ubiquitous tissue expression pattern and evolutionary conservation of KAI1/CD82 implicate that KAI1/CD82 also plays a significant role in development. In malignant solid tumors, the presence of KAI1/CD82 expression predicts a better prognosis for cancer patients (Dong et al., 1996; Dong et al., 1997b; Higashiyama et al., 1998; Ow et al., 2000), whereas the down-regulation or loss of KAI1/CD82 expression is constantly found in the clinically advanced cancers (Dong et al., 1997b; Kawana et al.,

1997; Yu et al., 1997; Uchida et al., 1999; Yang et al., 2000). Consistent with these observations, an inverse correlation between KAI1/CD82 expression and the invasive and metastatic potentials of cancer has been frequently observed in a wide range of malignancies such as prostate (Ueda et al., 1996; Bouras and Frauman, 1999; Tricoli et al., 2004), gastric (Hinoda et al., 1998), colon (Takaoka et al., 1998a; Lombardi et al., 1999), cervix (Liu et al., 2001; Schindl et al., 2002; Liu et al., 2003), breast (Yang et al., 1997; Yang et al., 2000; Yang et al., 2001), skin (Geradts et al., 1999), bladder (Yu et al., 1997; Jackson et al., 2000a; Ow et al., 2000; Jackson et al., 2002), lung (Adachi et al., 1996; Adachi et al., 1998), pancreas (Guo et al., 1996; Friess et al., 1998; Friess et al., 2001), liver (Guo et al., 1998; Sun et al., 1998), and thyroid cancers (Chen et al., 2004). KAI1/CD82 overexpression inhibits cell migration and cancer invasion in vitro and suppresses cancer metastasis in animal models (Dong et al., 1995; Yang et al., 2001). In addition to the suppression of cancer progression, it has been occasionally reported that KAI1/CD82 induces senescence (Bandyopadhyay et al., 2006) and apoptosis (Ono et al., 1999; Schoenfeld et al., 2003).

1.2.3 KAI1/CD82 as a tetraspanin

KAI1/CD82 is a type III transmembrane protein that belongs to the tetraspanin superfamily (Figure 1.1). Tetraspanins are characterized by 4 membrane-spanning domains, 4-6 well-conserved extracellular cysteine residues that presumably form 2-3 disulfide bonds, a Cys-Cys-Gly motif in the large

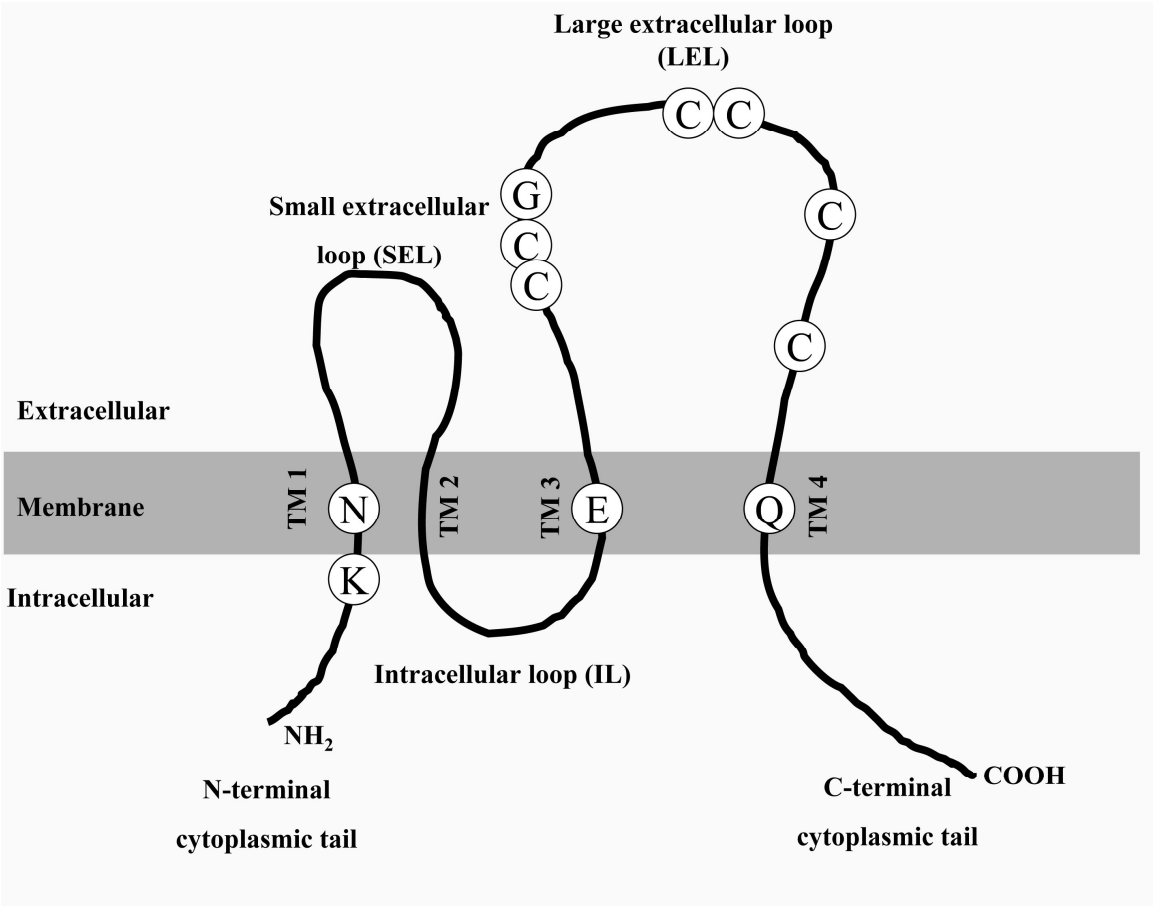


Figure 1.1 KAI1/CD82 is a tetraspanin

KAI1/CD82 is a type III transmembrane protein and belongs to the tetraspanin superfamily. KAI1/CD82 is characterized by 4 membrane-spanning domains, 6 well-conserved extracellular cysteine residues that presumably form 3 disulfide bonds, a Cys-Cys-Gly motif in the large extracellular loop, and polar residues within the transmembrane domains.

extracellular loop, and polar residues within the transmembrane domains (Wright MD & Tomlinson MG., 1994; Hakomori, 2002; Stipp et al., 2003). Tetraspanins participate in a wide variety of biological events such as immune response, viral entry, synapse formation, neurite outgrowth, and sperm-egg fusion (Maecker et al., 1997). Although the mechanism remains unclear, tetraspanins regulate migration, fusion, adhesion, and proliferation at the cellular level (Maecker et al., 1997). Among these, regulation of cell motility stands out as a prominent feature for many tetraspanins. For example, tetraspanins KAI1/CD82 and CD63 inhibit cell motility, while tetraspanins CD151 and CO-029 promote it. The structural elements in KAI1/CD82 required for its function are still not well understood. Characterization of these elements will shed light on the mechanism that governs KAI1/CD82 function. In the following, Iwill discuss the biochemical and structural features of human KAI1/CD82 protein. (Higashiyama et al., 1995; Berditchevski, 2001; Boucheix and Rubinstein, 2001; Levy and Shoham, 2005).

1.2.3.1 Large extracellular loop (LEL)

Among tetraspanins, the LELs usually share less homology than the transmembrane and intracellular domains. The LELs can be further divided into variable and constant regions based on the sequence homology (Hemler, 2001). The variable region is situated in the middle and usually mediates heterogeneous protein-protein interactions. The constant regions are situated at N- and C-termini of LEL and predicted to be responsible for the dimerization (Stipp et al., 2003; Levy and Shoham, 2005). The variable region of the KAI1/CD82 LEL (residues

110-227) contains six highly conserved cysteines (Gaugitsch et al., 1991; Fukudome et al., 1992; Gil et al., 1992; Nojima et al., 1993). These cysteine residues form disulfide bonds which are critical for the correct folding of the LEL (Berditchevski, 2001; Hemler, 2001; Stipp et al., 2003). Also in the LEL variable region, KAI1/CD82 contains 3 sites for N-glycosylation, Asn129, Asn157 and Asn198 (Ono et al., 1999). Although KAI1/CD82 glycosylation has been experimentally demonstrated (Ono et al., 2000), the contribution of each site to the glycosylation remains to be determined. The C-terminal constant region of the KAI1/CD82 LEL may contain the structural elements needed for its function since an alternatively spliced form of KAI1/CD82, which lacks the C-terminal part of LEL and a part of the 4th transmembrane region, no longer exerts the inhibitory activity on cell migration (Lee et al., 2003b).

1.2.3.2 Small extracellular loop (SEL)

The function of KAI1/CD82 SEL has not yet been studied. The studies from other tetraspanins such as CD81 suggest that the SEL is involved in the optimal folding of the LEL (Masciopinto et al., 2001).

1.2.3.3 Transmembrane (TM) domains

TM domains are highly conserved among tetraspanins (Levy and Shoham, 2005). The specific hydrophobic interactions of TM domains between tetraspanins are important for the maintenance of Tetraspanin Web (Stipp et al., 2003). Like other tetrapsanins, KAI1/CD82 TM1, 3, and 4 domains contain polar

residues Asn, Gln, and Glu, respectively. These polar residues are predicted to situate in the internal TM-TM interface rather than the external TM-lipid interface and serve as both hydrogen bond donors and recipients mediating the tetraspanin dimerization or polymerization (Zhou et al., 2000; Bienstock and Barrett, 2001; Gratkowski et al., 2001; Senes et al., 2001). Moreover, the TM domains are needed for KAI1/CD82 maturation. A truncated KAI1/CD82 lacking TM1 (TM2-4) can't leave the endoplasmic reticulum (Cannon and Cresswell, 2001). Co-expression of TM1 facilitates the transport of TM2-4 to the cell surface (Cannon and Cresswell, 2001), suggesting the TM1 may either contain a surface-targeting signal or be required for the proper TM domain assembly of KAI1/CD82.

1.2.3.4 Intracellular loop (IL)

The IL only contains 4 or 5 amino acid residues including one acidic residue (Stipp et al., 2003; Levy and Shoham, 2005). Like tetraspanins CD9 and CD151, KAI1/CD82 is constitutively palmitoylated in the cysteine residues located in or near the interface of TM and cytoplasmic regions (Zhou et al., 2004). The Cys83 residue at the IL and Cys74 near the IL can be palmitoylated (Zhou et al., 2004).

1.2.3.5 Cytoplasmic domains

The N- and C-terminal cytoplasmic domains of KAI1/CD82 only contain 11 and 15 residues, respectively. There are 3 cysteine residues (Cys5, Cys251, and Cys253) in the tails. All are adjacent to TM domains and found to be

palmitoylated (Zhou et al., 2004). The glycine residue (Gly2) in the N-terminal tail is a putative myristoylation site. Acylation likely assists the anchorage of short tails to membrane and the interactions between TM domains in the Tetraspanin Web. In addition, the Lys10 and Tyr11 residues in N-terminal tail are well conserved among tetraspanins (Stipp et al., 2003), but the biochemical relevance of these residues awaits to be addressed. The C-terminal cytoplasmic domain contains a tyrosine-based, functional internalization motif (Bonifacino and Dell'Angelica, 1999; Stipp et al., 2003).

1.2.4 How KAI1/CD82 suppresses cancer invasion and metastasis

1.2.4.1 KAI1/CD82 regulates the functions of its associated proteins

KAI1/CD82 likely suppresses cancer metastasis by primarily inhibiting cancer cell migration and invasion. There are two mechanisms possibly involved: one is that KAI1/CD82 per se directly initiates signals to diminish cell motility. This mechanism is less likely because the simplistic structure of KAI1/CD82 and the lack of enzyme motif that within its cytoplasmic domain. However, there is example, ligands for tetraspanins may exist (Nakamura et al., 2000; Crotta et al., 2001; Waterhouse et al., 2002; Nakajima et al., 2005) even though tetraspanins are not documented as typical receptors (Stipp et al., 2003). Also, the cross-linking of KAI1/CD82 proteins with a monoclonal antibody produces profound morphological changes and signaling (Nojima et al., 1993). For example, the crosslinking of KAI1/CD82 with its mAb in T-cells trigger the tyrosine

phosphorylation of Vav1 (Delaguillaumie et al., 2002), SLP76 (Delaguillaumie et al., 2002), and Cas-L (Delaguillaumie et al., 2002; Iwata et al., 2002). However, these effects could be alternatively interpreted as a result of clustering of the KAI1/CD82-associated tetraspanin web.

Alternatively, KAI1/CD82 inhibits cell motility through regulating the signaling initiated or mediated by KAI1/CD82 associated protein such as integrin and EGFR in tetraspanin web. Growing evidence supports this notion. The tetraspanin web components that KAI1/CD82 associates with include:

1. Tetraspanins such as CD9 and CD81 (Claas et al., 2001; Hemler, 2003);
2. Integrins such as β_1 and β_2 integrins (Mannion et al., 1996; Ono et al., 1999; Shibagaki et al., 1999; Sugiura and Berditchevski, 1999; Iwata et al., 2002; Lee et al., 2003),
3. Immunoreceptors such as MHC-I and -II molecules (Szollosi et al., 1996; Lagaudriere-Gesbert et al., 1997; Shibagaki et al., 1999); CD4 (Imai et al., 1992; Imai et al., 1995; Shibagaki et al., 1999), CD8 (Imai et al., 1992), EWI2/PGRL (Zhang et al., 2003), FPRP/CD9P-1 (Charrin et al., 2001), CD19 (Bradbury et al., 1992), and CD46 (Lozahic et al., 2000) ;
4. Growth factors and growth factor receptors such as heparin-binding epidermal growth factor (Nakamura et al., 2000), EGF receptor (Odintsova et al., 2000), and hepatic growth factor receptor c-Met (Sridhar and Miranti, 2006; Todeschini et al., 2007);
5. Intracellular signaling proteins such as PKC (Zhang et al., 2001), and

6. Other proteins such as KITENIN (Lee et al., 2004; Rowe and Jackson, 2006) and gamma-glutamyl transpeptidase (Nichols et al., 1998); In the following, Iwill discuss the functional significance of these associations.

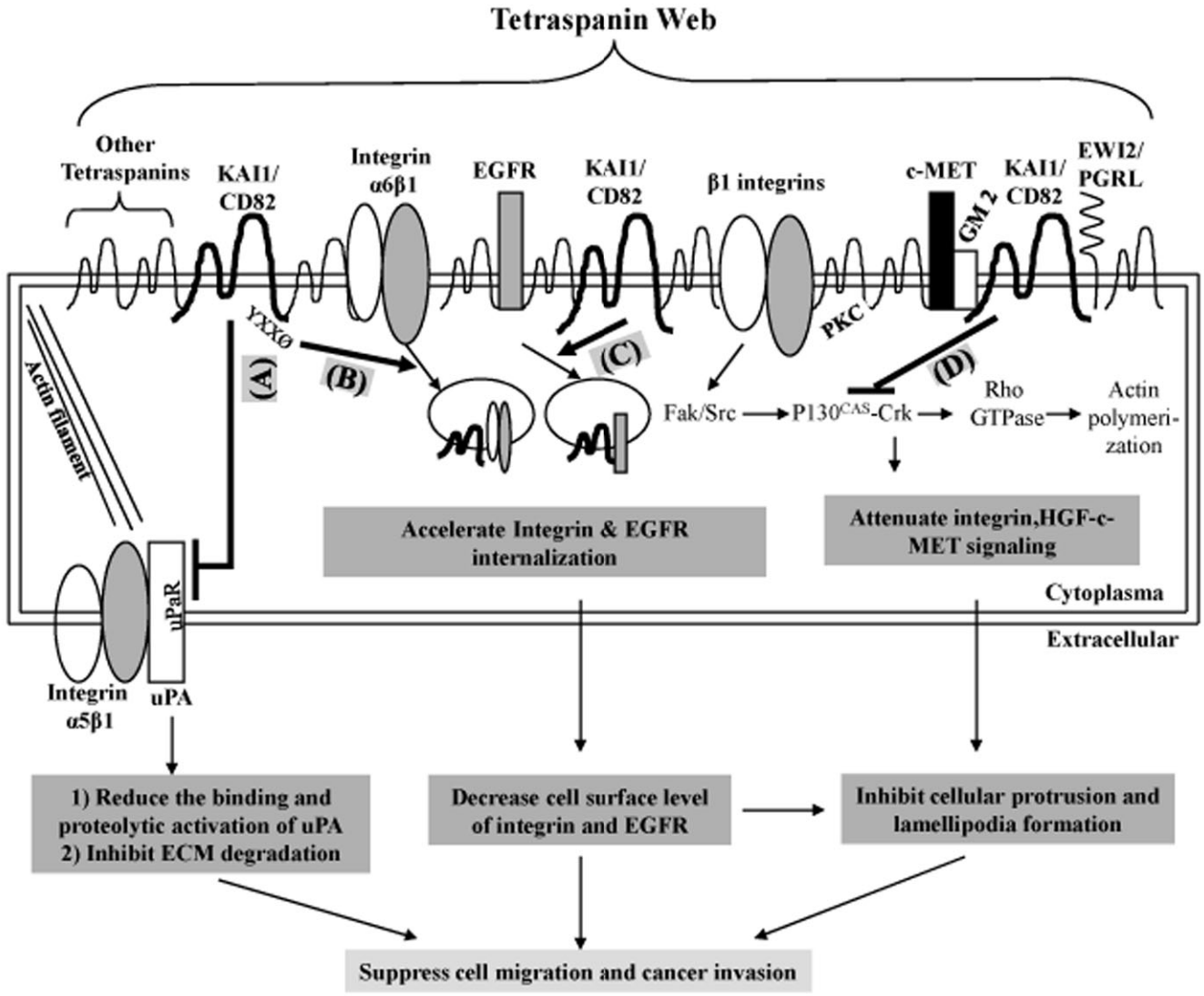
1.2.4.1.1 Integrin

KAI1/CD82 associates with various integrins including $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, and $\alpha L\beta 2$ (Mannion et al., 1996; Shibagaki et al., 1999; Sugiura and Berditchevski, 1999; Ono et al., 2000; Iwata et al., 2002; Lee et al., 2003) (Figure 1.2). Since integrins are indispensable for cell adhesion and migration, this association likely plays a role in KAI1/CD82 metastasis-suppressive function. Indeed, KAI1/CD82 regulates integrin-mediated signaling and -dependent adhesion.

1. KAI1/CD82 directly alters the downstream signaling events of integrin (Zhang et al., 2003). KAI1/CD82 has been reported to regulate the activities or levels of Src family kinases though Src kinases are not necessarily activated by integrins (Lagaudriere et al., 1998; Jee et al., 2003; Zhang et al., 2003)'. KAI1/CD82 down-regulates the formation $p130^{CAS}$-Crk complex, a molecular switch for cell motility, leading to the inhibition of cell migration (Zhang et al., 2003). Rho small GTPases are activated by the clustering of KAI1/CD82 in T cells (Delaguillaumie et al., 2002). The inhibition of Rho GTPases blocks the cellular protrusions triggered by KAI1/CD82 clustering and impairs KAI1/CD82-stimulated phosphorylation of Rho GTPases guanosine exchange factor Vav1 and

Figure 1.2 The mechanisms contributing KAI1/CD82-mediated suppression of cancer cell migration and invasion

A. KAI1/CD82 indirectly inhibits the uPA binding to uPAR and the pericellular plasminogen activation through inducing the redistribution of uPAR into focal adhesions and the association of uPAR with integrin $\alpha 5\beta 1$. Presumably, pericellular ECM degradation will be consequently reduced. **B** and **C.** KAI1/CD82 accelerates the internalization of both integrin $\alpha 6\beta 1$ and EGFR. The internalized proteins might be delivered to lysosomes for degradation. Hence, the levels of integrin and EGFR at thecell surface are reduced, and cell adhesion and cell migration are impaired. **D**. KAI1/CD82 and other tetraspanins interact with each other and form tetraspanin web. KAI1/CD82 attenuates integrin signaling such as FAK-Src-$p130^{CAS}$-Crk and Rho small GTPases signaling. The KAI1/CD82-containing tetraspanin web also recruits activated PKC to $\beta 1$ integrins and regulates the phosphorylation of laminin-binding integrins. As a consequence, cellular protrusion, possibly cellular retraction, and actin cytoskeleton reorganization are inhibited, and cell migration and cancer invasion are suppressed. **E**. The KAI1/CD82-EWI2/PGRL association enhances the motility-inhibitory activities of both molecules, while the KAI1/CD82-KITENIN association attenuates the motility-promoting activity of KITENIN. *Modified with permission. Liu WM, and Zhang XA, 2006, KAI1/CD82, a tumor metastasis suppressor: Cancer Letters, v. 240, no. 2, p. 183-194.*


Tetraspanin Web
Other Tetraspanins
KAI1/ CD82
Integrin α6β1
EGFR
KAI1/ CD82
β1 integrins
c-MET
GM2
KAI1/ CD82
EWI2/ PGRL
YXXØ
(A)
(B)
(C)
(D)
PKC
Actin filament
Fak/Src
P130CAS-Crk
Rho GTPase
Actin polymeri-zation
Accelerate Integrin & EGFR internalization
Attenuate integrin,HGF-c-MET signaling
Cytoplasma
Extracellular
uPaR
Integrin α5β1
uPA
1) Reduce the binding and proteolytic activation of uPA
2) Inhibit ECM degradation
Decrease cell surface level of integrin and EGFR
Inhibit cellular protrusion and lamellipodia formation
Suppress cell migration and cancer invasion

adapter protein SLP76. However, the activity of Rho GTPases are not required for the inducible interaction between actin cytoskeleton and KAI1/CD82 (Delaguillaumie et al., 2002), suggesting a downstream role of Rho or other mechanisms.

2. KAI1/CD82 directly modulates integrin-dependent cell-extracellular matrix adhesion. For instance, integrin α6-dependent adhesion to laminin are diminished upon KAI1/CD82 overexpression and enhanced by KAI1/CD82 knockdown (He et al., 2004). It was also reported that KAI1/CD82 expression inhibited adhesion to fibronectin of DLD-1 and CT-26 colon cancer cells (Lombardi et al., 1999; Lee et al., 2003b), while KAI1/CD82 silencing promoted the adhesion to fibronectin of BM314 colon cancer cells (Lombardi et al., 1999).

1.2.4.1.2 Epidermal growth factor receptor (EGFR)

EGFR belongs to the ErbB family of receptors, and the ErbB pathway is frequently hyper-activated in cancer via the overproduction of ligands and receptors or the constitutive activation of receptors (Yarden and Sliwkowski, 2001). KAI1/CD82 associates with EGFR, ErbB2, and ErbB3 (Odintsova et al., 2000; Odintsova et al., 2003). Through this association, KAI1/CD82 accelerates the desensitization of EGF signaling and endocytosis of EGFR (Odintsova et al., 2000) (Figure 1.2). Meanwhile, KAI1/CD82 specifically inhibits ligand-induced EGFR dimerization and alters the distribution of EGFR in plasma membrane (Odintsova et al., 2003).

1.2.4.1.3 EWI2/PGRL

EWI2/PGRL belongs to a novel Ig subfamily and associates with tetraspanins such as CD9, CD81, and KAI1/CD82 (Clark et al., 2001; Stipp et al., 2001) (Figure 1.2). KAI1/CD82 can form a complex with EWI2/PGRL in a CD9- and CD81-independent manner (Zhang et al., 2003b). EWI2/PGRL overexpression inhibits cell migration (Clark et al., 2001; Zhang et al., 2003b) and enhances the motility-inhibitory activity of KAI1/CD82 (Clark et al., 2001; Zhang et al., 2003b; Lee et al., 2004).

1.2.4.1.4 KITENIN

KITENIN was recently found to directly bind the C-terminal cytoplasmic domain of KAI1/CD82 (Lee et al., 2004). KITENIN is also a transmembrane 4 protein but does not belong to the tetraspanin superfamily. In contrast to KAI1/CD82, KITENIN promotes cell migration, cancer invasion, and cancer metastasis (Lee et al., 2004).

1.2.4.1.5 Protein kinase C (PKC)

Among tetraspanin-associated intracellular Signaling proteins, KAI1/CD82 interacts with the activated PKC (Zhang et al., 2001a) (Figure 1.2) but not phosphatidylinositol-4 kinase (PI-4K) (Zhang et al., 2001a). Upon phorbol ester stimulation, the activated PKC is recruited to interact with integrin-tetraspanin complex (Zhang et al., 2001a) and regulate integrin-dependent cell migration (Zhang et al., 2001a).

1.2.4.1.6 c-MET and GM2

c-MET is the cell surface receptor of HGF. HGF-cMET axis promotes integrin-dependent cell migration. KAI1/CD82 can attenuate this process and thus inhibit cell migration (Sridhar and Miranti, 2006). GM2 is a glycosphingo-lipid (GSLs), which interacts with KAI1/CD82. GM2 is required for KAI1/CD82's inhibition on cell migration through regulation of HGF-cMET axis (Todeschini et al., 2007).

1.2.4.1.7 Duffy Antigen Receptor for Chemokines (DARC)

DARC can inhibit the colonization of metastasized cancer cells, probably through the interaction with KAI1/CD82(Bandyopadhyay et al., 2006; Rinker-Schaeffer and Hickson, 2006). This interaction can trigger the senescene of cancer cell and completely abolish the lung metastasis of rat prostatic carcinoma cell line AT6.1, the human breast carcinoma cell line MDA-MB-435, and the mouse melanoma cell lines B16BL6 and B16F10 (Bandyopadhyay et al., 2006; Zijlstra and Quigley, 2006; Gellersen et al., 2007; Iiizumi et al., 2007). However, in DARC knockout mice, this inhibition of metastasis has been significantly impaired.

1.2.5 KAI1/CD82 redistributes the plasma membrane components

Cell surface molecules undergo dynamic redistributions during cell migration. KAI1/CD82 overexpression leads to the redistribution of urokinase-type plasminogen activator receptor (uPAR) into focal adhesions to form a stable complex with integrin $\alpha5\beta1$, though no physically interaction was found between KAI1/CD82 and uPAR (Bass et al., 2005). The redistribution of uPAR markedly reduces its ability to bind the ligand uPA, and consequently to cleave and activate plasminogen (Bass et al., 2005) (Figure 1.2). Similarly, the redistribution of EGFR and gangliosides within plasma membrane were also found upon KAI1/CD82 overexpression (Odintsova et al., 2000; Odintsova et al., 2003).

Besides re-compartmentalization of plasma membrane components, KAI1/CD82 likely redistributes Tetraspanin Web components such as EGFR into distinct cellular compartments through endocytosis. In addition, the diminished integrin $\alpha6$-dependent cell adhesion upon KAI1/CD82 expression results from the decreased levels of $\alpha6$ integrins at cell surface (He et al., 2004). As to the effect on EGFR, KAI1/CD82 accelerates the internalization of $\alpha6$ integrins (Figure 1.2). Since the total cellular $\alpha6$ integrins are not altered by KAI1/CD82, $\alpha6$ integrins are likely redistributed into endocytic compartments. The mechanism KAI1/CD82 use to redistribute the tetraspanin web components is obscure. The answer will likely rely on the identification of intrinsic trafficking motifs in KAI1/CD82.

1.2.6 Post-translational modifications of KAI1/CD82: the essential makeup for its motility-suppressive activity

1.2.6.1 Palmitoylation

Palmitoylation is a posttranslational acylation process in which saturated fatty acids, predominantly palmitate, are covalently linked to the cysteine residues in proteins. Palmioylation contributes to membrane targeting of proteins, protein trafficking, localization of proteins into organized membrane microdomains such as lipid rafts, and regulation of functional activities of proteins (Dunphy and Linder, 1998; Resh, 1999; Bijlmakers and Marsh, 2003). Tetraspanin palmitoylation contributes to the organization of Tetraspanin Webs (Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002). KAI1/CD82 is palmitoylated in all 5 cysteines proximal to the plasma membrane (Cys5, Cys74 , Cys83 , Cys251 , and Cys253) (Zhou et al., 2004). Removal of palmitoylation reverses KAI1/CD82-dependent inhibition of cell migration and invasion, which is at least partly due to less KAI1/CD82 reaching the cell surface and joining the Tetraspanin Web (Zhou et al., 2004).

1.2.6.2 Glycosylation

KAI1/CD82 is N-glycosylated, and the degrees of glycosylation vary among cells or tissues (Fukudome et al., 1992; Imai et al., 1992; White et al., 1998). The glycosylation appears to be important for the cell surface targeting of KAI1/CD82

(Fukudome et al., 1992; Imai et al., 1992), motility regulation (Ono et al., 2000), and integrin association (Ono et al., 1999; Ono et al., 2000).

These studies suggest that reaching the cell surface or the presence in plasma membrane is the prerequisite for KAI1/CD82 to exert its motility-inhibitory activity.

1.2.7 KAI1/CD82 induces apoptosis: a distinctive or concurrent mechanism?

Along with several other tetraspanins, KAI1/CD82 induces apoptosis in a variety of cell lines upon overexpression (Ono et al., 2000; Schoenfeld et al., 2003). Meanwhile, KAI1/CD82 sensitizes cells to the pro-apoptotic stimuli (Schoenfeld et al., 2003). KAI1/CD82 overexpression promotes the generation of intracellular reactive oxygen intermediates, which possibly result from the Cdc42-dependent release of intracellular anti-oxidant glutathione (Schoenfeld et al., 2003). It remains to be determined whether KAI1/CD82-induced apoptosis contributes to the metastasis suppression. Despite no effects on cancer cell proliferation in vitro and/or tumor growth in vivo for prostate, colon, and skin cancers (Dong et al., 1995; Takaoka et al., 1998a; Lombardi et al., 1999; Kim et al., 2005), KAI1/CD82 was found to decrease the size of primary tumors of gastric and breast cancers in animal models (Dong et al., 1995; Lee et al., 2003a; Lee et al., 2003b). It is therefore possible that, at least in some cancers, the induction of apoptosis may serve as a concurrent mechanism for KAI1/CD82.

1.2.8 How KAI1/CD82 expressions are diminished or lost in invasive and metastatic cancers

How KAI/CD82 gene expression is regulated becomes an increasingly important question. The answer will not only shed light on the mechanisms of cancer invasion and metastasis but also likely lead to clinical benefits. The regulatory mechanisms of KAI1/CD82 gene expression have started to emerge from recent studies and are summarized as follows and in Table 1.1.

1.2.8.1 The alternative splicing of KAI1/CD82

KAI1/CD82 contains at least one alternative spliced form, in which the exon 7 is deleted (Lee et al., 2003b). As a result, this spliced form lacks the distal part of the second extracellular loop and part of the fourth transmembrane region (also see section 1.3.1). Compared to wild type KAI1/CD82, this spliced KAI1/CD82 cannot efficiently suppress the metastasis of gastric carcinoma (Lee et al., 2003).

1.2.8.2 The transcriptional regulation of KAI1/CD82 expression

Down-regulated KAI1/CD82 expression does not result from loss of heterozygosity (Dong et al., 1997a; Kawana et al., 1997; Tagawa et al., 1999), the hyper-methylation of the CpG island within KAI1/CD82 promoter region (Jackson et al., 2000b), or the mutations within the KAI1/CD82 coding region (Dong et al., 1995; Miyazaki et al., 2000). Rather, it is likely due to increased transcription repressor activity and/or decreased activator activity.

Table 1.1 Molecules involved in KAI1/CD82 transcription regulation

Molecules involved in activation	Molecules involved in repression
Transcription factors:	*Transcription factors:*
NFκB* (Shinohara et al., 2001; Li et al., 2001; Baek et al., 2002; Telese et al., 2005)	NFκB* (Baek et al., 2002; Telese et al., 2005; Kim et al., 2005)
Tip60; (Telese et al., 2005)	β-Catenin (Kim et al., 2005);
Fe65; (Telese et al., 2005)	Reptin (Kim et al., 2005)
SET (Telese et al., 2005)	NcoR (Baek et al., 2002);
P53; (Dong et al., 1997a)	TAB2 (Baek et al., 2002);
AP1; (Dong et al., 1997a)	HDAC3 (Baek et al., 2002)
AP2; (Dong et al., 1997a)	
C-Jun & JunB (Mashimo et al., 1998; Mashimo et al., 2000; Gao et al., 2003; Marreiros et al., 2005)	
Other molecules:	
Amyloid β precursor protein (APP) (Baek et al., 2002)	
PKC (Akita et al., 2000)	
IL-1(Baek et al., 2002)	
Nerve growth factor (Sigala et al., 1999)	

* NFκB could serve as either activator or repressor depending on whether co-activator or co-repressor is recruited (see text for detail).

1.2.8.2.1 NFκB

NFκB P50 subunit was found to bind to a region between -6631 to -6996 bp upstream of the KAI1/CD82 gene transcription start site. This region contains a sequence homologous to the NFκB binding sequence (Baek et al., 2002; Telese et al., 2005). The overexpression or activation of NFκB elevates the mRNA and protein levels of KAI1/CD82 in murine pro-B cells (Li et al., 2001) and various human adenocarcinoma cell lines (Li et al., 2001; Shinohara et al., 2001), while specific inhibition of NFκB by IκBα down-regulates KAI1/CD82 transcription (Li et al., 2001). In contrast to p50, NFκB p65 subunit does not bind to the KAI1/CD82 promoter. These observations strongly suggest that NFκB p50 subunit dimerize and regulate the transcription of KAI1/CD82. Whether NFκB activates or represses KAI1/CD82 transcription actually depends on the nature of the transcription co-factors recruited to NFκB p50. Co-activator Tip60/Fe65 complex or co-repressor N-CoR/TAB2/HDAC3 complex could exchange with each other to bind NFκB p50 and turn on and off KAI1/CD82 transcription, respectively (Baek et al., 2002; Telese et al., 2005).

1.2.8.2.2 β-Catenin and Reptin

A recent discovery has linked the transcription regulation of KAI1/CD82 to β-catenin (Kim et al., 2005). β-catenin, in combination with reptin chromatin remodeling complex, binds directly to the p50 docked on the NFκB response element in the KAI1/CD82 promoter region and replaces the Tip60 co-activator complex (Telese et al., 2005). Consequently, the co-repressor HDACs are

recruited to β-catenin-reptin complex to repress KAI1/CD82 transcription. Therefore, the exchange of β-catenin co-repressor and Tip60 co-activator also regulates the KAI1/CD82 transcription.

1.2.8.2.3 p53, AP-1, AP-2, JunB, and c-Jun

Although no typical TATA- and CAAT-binding motifs are found in KAI1/CD82 promoter, KAI1/CD82 promoter does contain binding sites for the transcription factors such as p53, AP1, AP2, and Sp1 (Dong et al., 1997). Two minimal promoter elements have bee reported: one is located from -197 to -1 bp and another from +1 to +351 bp (Gao et al., 2003). Both *in vitro* and *in vivo* analyses indicated that p53 is able to bind the KAI1/CD82 promoter and turn on its expression (Mashimo et al., 2000). Furthermore, p53 is involved in etoposide-induced KAI1/CD82 gene expression in A549 non-small cell lung cancer cells (Mashimo et al., 2000). Although KAI1/CD82 transcription is up-regulated by p53 (Marreiros et al., 2003; Marreiros et al., 2005), KAI1/CD82 can still be expressed in cells containing p53 mutant deficient in DNA binding (Duriez et al., 2000; Shinohara et al., 2001). In addition, c-Jun and Jun-B was also found to participate in KAI1/CD82 transcription regulation (Mashimo et al., 2000; Marreiros et al., 2003; Marreiros et al., 2005).

1.2.8.2.4 Other transcription factors

Besides NFκB, other transcription factors in IL-1 and TNF signaling pathways may also regulate KAI1/CD82 transcription since IL-1 and TNF induce KAI1/CD82 gene expressions (Baek et al., 2002; Bao et al., 2007). Also, a novel transcription co-activator, SET, can be recruited by p50-Tip60-Fe65-APP complex to KAI1/CD82 promoter region (Telese et al., 2005; Bao et al., 2007). Moreover, PKC activation promotes KAI1/CD82 gene transcription and expression (Akita et al., 2000), suggesting that the transcription factors responsive to PKC activation are involved. Finally, transcription factors responsive to nerve growth factor may also elevate the transcription of KAI1/CD82 (Sigala et al., 1999).

Taken together, the replacement of transcription activators from the KAI1/CD82 promoter by the dominant co-repressor complex may serve as one mechanism to repress KAI1/CD82 expression. How NFκB, N-CoR, β- catenin, p53, AP-1, and AP-2 coordinately regulate the transcription of KAI1/CD82 in a temporal-spatial manner is an interesting and important issue that remains to be addressed.

1.3 The actin cytoskeleton rearrangement, cell migration, and small Rho GTPases

1.3.1 Cytoskeleton components, actin polymerization and cytoskeletal rearrangement

The cytoskeleton can be described as cellular "scaffolding". It provides a traffic track for all organelle, within the cytoplasm. It presents in all eukaryotic and prokaryotic cells (Albert, 1994). The cytoskeleton provides a dynamic structure to maintain cell shape, as well as protects the cell. Furthermore, it participates in cell motion. In addition to all the roles above, it plays pivotal roles in cellular division. Thus, the cytoskeleton functions as a bone-like structure within the cytoplasm (Albert, 1994). The cytoskeleton consists of three major classes of proteins. They are microfilaments, intermediate filaments and microtubules.

1. Microfilaments. The major protein residings in the microfilaments is actin. Actin monomer can be polymerized to form filaments, which are the major microfilaments. An actin filament is approximately 7 nm in diameter. It is composed of two actin chains oriented in a circular shape. They are heavily accumulated just beneath the plasma membrane, so as to keep cellular shape, and form cytoplasmic protuberances such as pseudopodia and microvilli, and mediate inter-cellular signaling transduction in some cell-to-cell or cell-to-matrix junctions. Moreover, they participate in cytokinesis and cell contraction in coordination with myosin. The

interaction of actin and its motor, myosin, can reduce cytoplasmic streaming in most cells.

2. Intermediate filaments**.** The major proteins are vimentins, keratin, neurofilaments, and lamin. Unlike microfilaments, which only consist of actin, intermediate filaments consist of a variety of structural proteins. Different intermediate filaments and their major functions are listed as following:

   a. Vimentins – ubiquitously expressed as structural support of many cells.

   b. Keratin – found mostly in skin cells, hair and nails.

   c. Neurofilaments – present in neural cells.

   d. Lamin - structural support to the nuclear envelope.

3. Microtubules**.** The main cytoskeletal protein is microtubules. Microtubules are hollow cylinders of approximately 25 nm in diameter. They are made of 13 protofilaments consisting of polymers of alpha and beta tubulin. They constantly undergo rapid turnover as they bind GTP for polymerization or GDP for depolymerization. They are organized by the centrosome. The key functions of microtubules are listed as follows:

   a. Intracellular transport (provides track for dyneins and kinesins as that transport organelles such as mitochondria or vesicles).

   b. Axoneme of cilia and flagella.

   c. Mitotic spindle.

   d. Cell wall in plants.

As stated in the above, the major force that drives cell to migration is microfilaments, more specifically, actin filaments. Actin filaments are the building block of lamellipodia and filopodia, which play central roles in plasma membrane protrusion and afterwards cell migration (Albert, 1994). Understand how actin is polymerized is very important for understanding of cell migration and its regulation (Albert, 1994; Pollard and Borisy, 2003; Raftopoulou and Hall, 2004). The first step of locomotion, ie; the protrusion of a leading edge is driven by the actin polymerization that pushes the plasma membrane outward. Types of protrusive structures are various in different cell types. Protrusion can be divided into three different catalogories, filopodia (also known as microspikes), lamellipodia, and pseudopodia. All of them are filled with a dense core of filamentous actin and contain no membrane-enclosed organelles. These three structures differ basically in the way at actin is organized, in two or three dimensions respectively. The actin associating proteins involved in the formation of these structures are also varied, and their interactions with actin are regulated in a spatial and temporal manner. These structures named above are introduced as follows:

1. Filopodia**.** Filopodia are growth-cone like the protrusions found commonly in some types of fibroblasts. They contain long, bundled actin filaments, similar to those in microvilli but longer, thinner, and more dynamic (Albert, 1994; Pollard and Borisy, 2003; Raftopoulou and Hall, 2004).
2. Lamellipodia**.** Lamellipodia are more likely to be present in epithelial cells, although fibroblasts and some neurons may also use similar structures to

migrate. Unlike filopodia, lamellipodia are complicated by consisting of two-dimensional, sheet-like structures. They contain a dense, branched "dendritic" mesh network of actin filaments, lying on a plane parallel to the solid substratum. The geometry of lamellipodia makes it convenient to observe under the phase contrast light microscope. Thus, people know more about the dynamic organization and protrusion mechanism of lamellipodia than that of filopodia and pseudopodia (Albert, 1994; Pollard and Borisy, 2003; Raftopoulou and Hall, 2004).

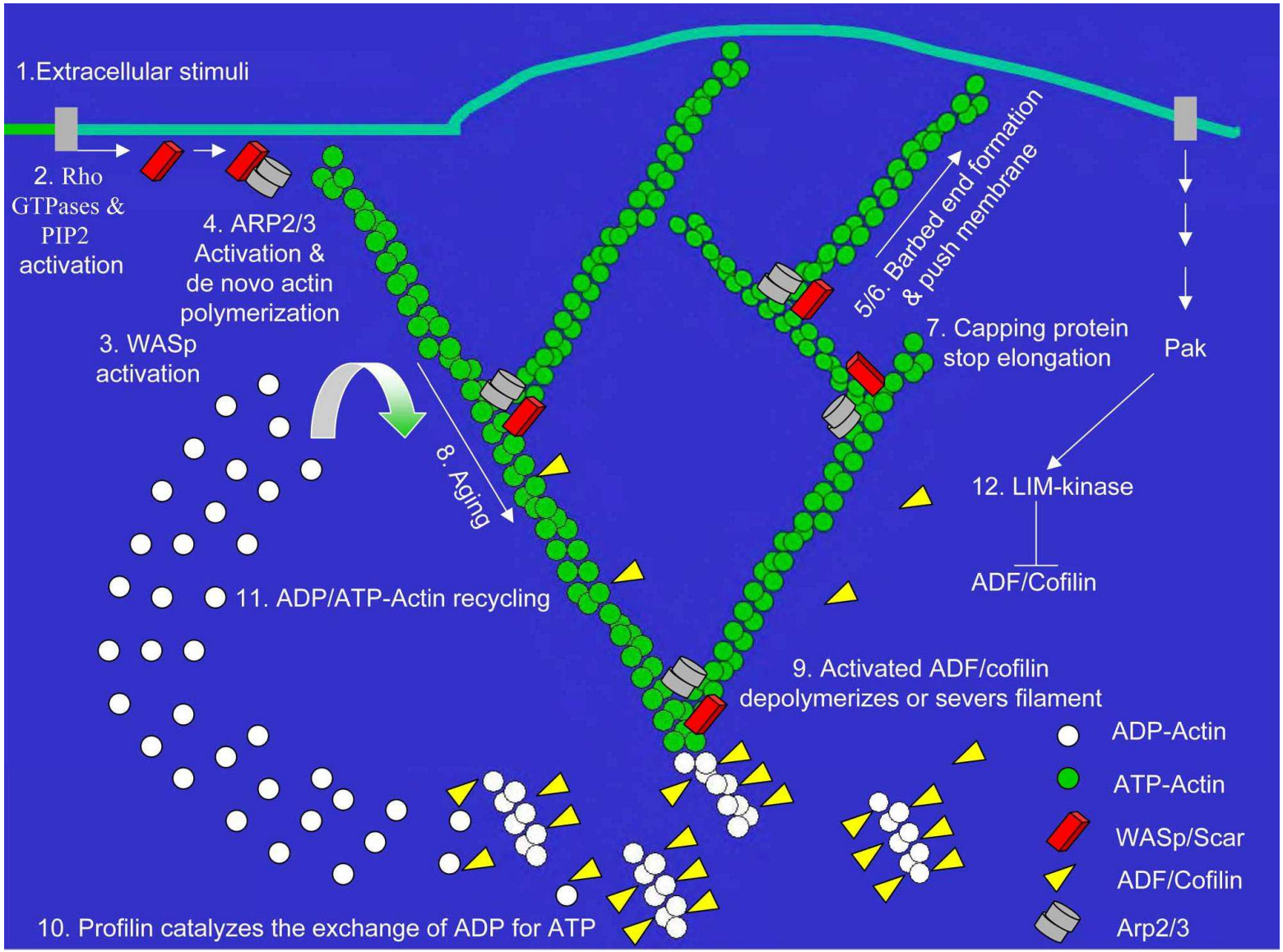
3. Pseudopodia. Pseudopodia are stubby three-dimensional projections filled with an actin-filament gel. They can be found in amoebae and neutrophils (Albert, 1994; Pollard and Borisy, 2003; Raftopoulou and Hall, 2004).

Since lamellipodia is the most simplistic machinery that is required for cell motility, I here use them as an example to illustrate how actin undergoes polymerization during cell migration.

Actin filaments are double helical polymers of globular subunits that are assembled from head-to-tail to endow the filament a molecular polarity (Pollard and Borisy, 2003). According to the arrowhead pattern heralded by decoration with myosin (also see Figure 1.3), one end is called the barbed end and another is called the pointed end. This polarity is the core mechanism driving actin assembly in cells. The barbed end usually points outward towards the plasma membrane (Green in Figure 1.3). It is strongly oriented to favor the elongation of the actin filaments (Small et al., 1978). For example, when the permeabilized cells were fed with fluorescent dye-tagged actin, actin was found to be added to

Figure 1.3 Key factors participating in actin polymerization

1. Extracellular stimuli activate receptors. **2**. The correspondent signal transduction pathways activate small Rho GTPases and PIP2. **3**. Activate WASp/Scar proteins. **4**. WASp/Scar proteins recruit Arp2/3 complex and an actin monomer to the side of a preexisting filament to initiate a new branch. **5**. Rapid growth at the dendritic barbed end of the new branch. **6**. pushes the membrane outward and forms membrane protrusion and lamellipodia. **7**. Capping proteins shut down growth within seconds. **8**. Filaments aged by hydrolysis of ATP-actin subunit followed by dissociation of the γ phosphate (green circle turn white). **9**. Dephosphorylated / activated ADF/cofilin induces phosphate dissociation and promotes disassembly of ADP-actin from pointed filament ends (during steady-state protrusion when G-actins are depleted). Dephosphorylated / activated ADF/Cofilin can also sever ADP-actin filaments (during stimulated protrusion and lamellipodia formation when G-actins become abundant). **10**. Profilin catalyzes the exchange of ADP for ATP (turning the white circles subunits green again), **11**. The recycling pool of ATP-actin bound to profilin can return to where membrane protrusion and the actin dendritic mesh network locate, ready to generate more barbed ends. **12**. Small Rho GTPases also activate PAK and LIM kinase. Once LIM kinase has been acti-vated, it phosphorylates and inactivates ADF/cofilin. Phosphorylation or inacti-vation of ADF/cofilin slow down the turnover of the filaments and actin tread-milling. *Modified with permission. Pollard TD, and Borisy GG, 2003, Cellular Motility Driven by Assembly and Disassembly of Actin Filaments: Cell, v. 112, no. 4, p. 453-465.*


1.Extracellular stimuli
2. Rho GTPases & PIP2 activation
3. WASp activation
4. ARP2/3 Activation & de novo actin polymerization
5/6. Barbed end formation & push membrane
7. Capping protein stop elongation
8. Aging
9. Activated ADF/cofilin depolymerizes or severs filament
10. Profilin catalyzes the exchange of ADP for ATP
11. ADP/ATP-Actin recycling
12. LIM-kinase
ADF/Cofilin
Pak
ADP-Actin
ATP-Actin
WASp/Scar
ADF/Cofilin
Arp2/3

the barbed ends at the leading edge of the lamellum (Symons and Mitchison, 1991; Chan et al., 2000; Pollard and Borisy, 2003). In steady state, ATP-actin associates at the barbed end as ADP-actin dissociates from the pointed end, resulting in very slow recycling and treadmilling of actin from the barbed end to the pointed end, which can be captured directly by fluorescence time-lapse microscopy (Fujiwara et al., 2002). However, in highly motile state, actin treadmilling at the leading edge turns faster. During the actin treadmilling, ATP hydrolysis in the filament also plays an essential role to maintain this process (Pollard and Borisy, 2003).

A series of questions regarding the actin cytoskeleton rearrangement during cell migration remains unanswered: how does the growth of actin filaments turn faster when cells switch from steady state to highly motile state? How is the *de novo* actin polymerization initiated and terminated? How do actin filaments form dendritic mesh network to push forward the membrane at the leading edge? How are ATP- and ADP- bound actin monomers in the barbed end and pointed end of the actin filament network recycled? How do environmental and internal signals closely govern these reactions?

Answers to these questions are assessed by a theory called the dendritic nucleation/array treadmilling (Pollard and Borisy, 2003) (Figure 1.3). In short, cells contain a pool of unpolymerized actin monomers bound to groups of protein including profilin (Vinson et al., 1998; Kaiser et al., 1999) and thymosin-β4 (Safer and Nachmias, 1994; De La Cruz et al., 2000). *De novo* actin polymerization will be initiated when extracellular stimuli turn on signaling pathways and sequentially

activate nucleation-promoting factors, primarily the WASp/Scar family of proteins (Takenawa and Miki, 2001; Weaver et al., 2003).

WASp was named after the first patient with human bleeding disorder and immunodeficiency, called Wiskott - Aldrich syndrome. A mutation of the WASp gene has been identified as the cause of this syndrome (Rengan et al., 2000).

Soon after the activation of nucleation-promoting factors, Arp2/3 complex becomes then activated to initiate *de novo* polymerization of a actin filament that forms a branch on the side of a previously existing filament (Machesky et al., 1994; Mullins et al., 1998) (thorough discussion of Arp2/3 can be found in section 1.3). Fed by actin-profilin from the abundant subunit pool, newly synthesized branches grow rapidly and push the plasma membrane outward (Sagot et al., 2002). The elongation of the newly synthesized actin filament is transient, as the capping proteins terminate growth (Cooper and Schafer, 2000). Actin subunits in this new dendritic network hydrolyze their bound ATP rapidly but dissociate the γ-phosphate slowly. Dissociation of γ-phosphate triggers disassembly reactions, accompanying with debranching and binding of ADF/cofilin (Pollard and Borisy, 2003).

Cofilin then promotes the severing and dissociation of ADP-subunits from the pointed ends (Pollard and Borisy, 2003) (Detailes discussion of Cofilin can be found in section 1.3). Profilin functions as the nucleotide exchange factor for actin. It catalyzes the transition of ADP-actin into ATP-actin and the recycling of subunits to the ATP-actin-profilin pool (Vinson et al., 1998; Kaiser et al., 1999), thus ready for the next round of assembly. Beside dissembling and recycling

actin from the pointed end during steady state movement, ADF/cofilins may also contribute to initiate protrusions by severing filaments to expose barbed ends for elongation (Zebda et al., 2000; Ghosh et al., 2004). In this case, an individual actin filament does not treadmill in the way of simultaneous elongating at one end and shortening at another. A new filament is synthesized at a branch point and grows towards its barbed end while its pointed end is capped at the branch; the filament becomes capped at its barbed end, debranches and shortens after being severed by cofilin (Pollard and Borisy, 2003). Thus, the actin filament bundle are recycling and reproducing themselves at the leading edge as a whole. However, actin treadmills dismantle themselves and these dismantling processes keep some distance from the very leading edge in the front (Figure 1.3).

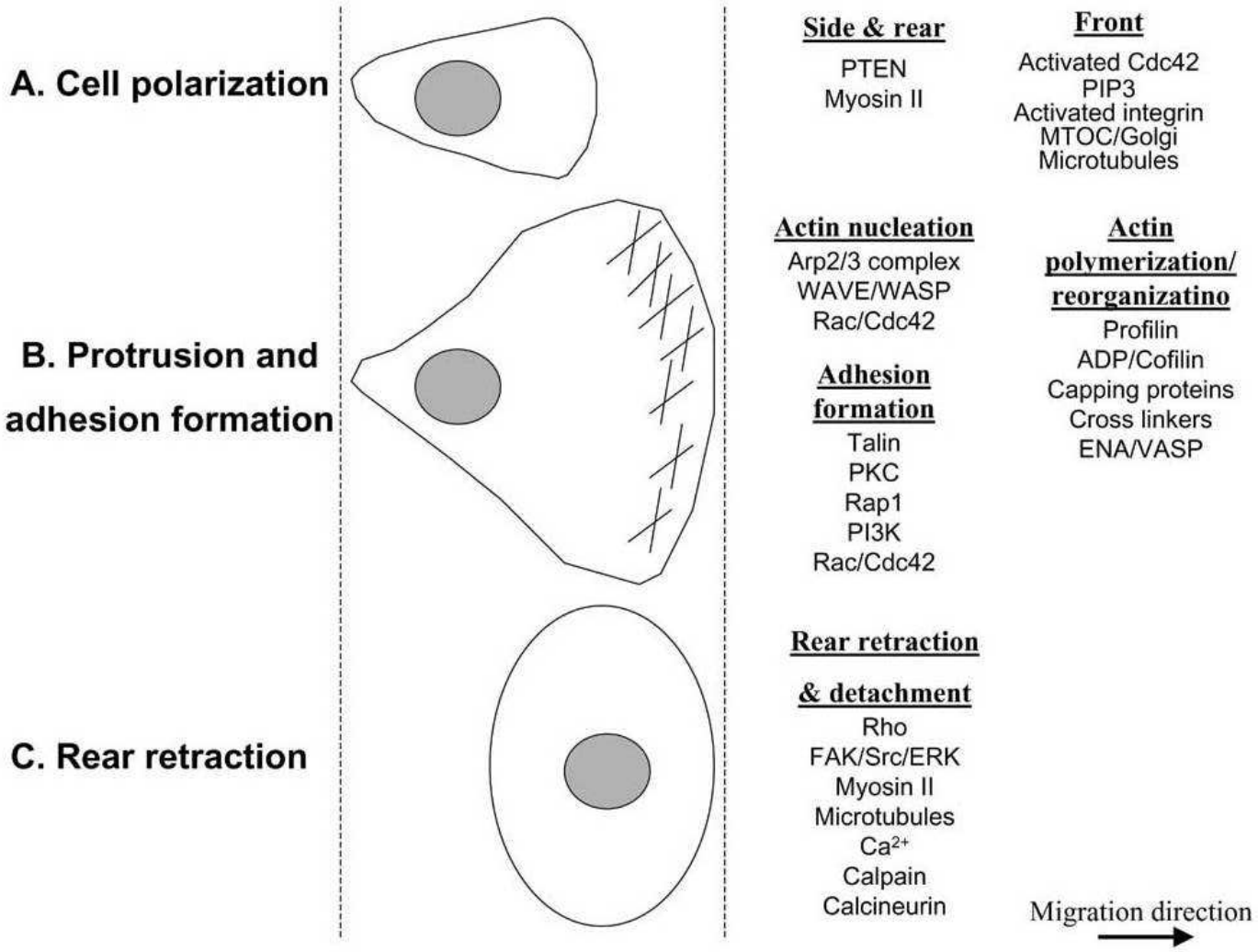
1.3.2 Steps in cell migration and signals that govern those steps

Directed cell movement is depended upon the rearrangement of cytoskeletal filament throughout the cytoplasm (Elson, 1988; Raftopoulou and Hall, 2004).The cytoskeleton consists of three distinct types of protein filaments: Actin filaments (Pollard and Borisy, 2003), microtubules (Watanabe et al., 2005) and intermediate filaments (Goldman et al., 1996).

Cell migration requires a highly well organized and cyclical program starting from transient local signaling events to initiate polarization and ending with rear retraction (Figure 1.4) (Horwitz and Parsons, 1999; Parent and Devreotes, 1999; Horwitz and Webb, 2003; Ridley et al., 2003; Van Haastert and Devreotes, 2004;

Figure 1.4 Cell migration steps

Cell migration can be divided into three sequential steps: **A**. Polarity is intrinsic to a migrating cell. Cdc42, as well as Par proteins and aPKC, participate in the initiation of polarity. Those proteins synergize to promote directed vesicle trafficking, such as translocation of Golgi apparatus towards the front of the nucleus and toward the leading edge, re-organization of microtubules , and the formation of the MTOC. In the presence of chemotactic agents, PIP3 start to accumulate at the leading edge as the result of the localized PI3K activation. PTEN, a PIP3 phosphatase localized to the cell margins and rear, restrict protrusions to the front along with mysosin II. **B**. Protrusion heralds the start of cell migration. Sequential activation of Rac and Cdc42, their down-stream effectors WASP/WAVE proteins, and then Arp2/3 promote the formation of actin branches on pre-existing actin filaments. In the combination effect of profilin (control the availability of activated actin monomers), ADF/cofilin (de-branching, depolymerizing and severing proteins), and capping protein, promote actin polymerization and thus actin treadmilling. Protrusions are stabilized by the new adhesions formation. Integrin activation and clustering is pivotal for nascent adhesions. Talin binding and PKC-, Rap1-, and PI3K-mediated pathways activate integrins. Rac participates in Integrin clustering. **C**. Rear retraction and detachment are mediated by Src/FAK/ERK, Rho, myosin II, calcium, calcineurin, calpain, microtubules. *Modified with permission. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, and Horwitz AR, 2003, Cell Migration: Integrating Signals from Front to Back: Science, v. 302, no. 5651, p. 1704-1709.*


A. Cell polarization
B. Protrusion and adhesion formation
C. Rear retraction
Side & rear
PTEN
Myosin II
Front
Activated Cdc42
PIP3
Activated integrin
MTOC/Golgi
Microtubules
Actin nucleation
Arp2/3 complex
WAVE/WASP
Rac/Cdc42
Actin polymerization/ reorganizatino
Profilin
ADP/Cofilin
Capping proteins
Cross linkers
ENA/VASP
Adhesion formation
Talin
PKC
Rap1
PI3K
Rac/Cdc42
Rear retraction & detachment
Rho
FAK/Src/ERK
Myosin II
Microtubules
Ca2+
Calpain
Calcineurin
Migration direction

Vicente-Manzanares et al., 2005). Cell migration consists of several key steps as follows:

1. Polarization. When a non-migrating cell encounters chemotactic molecules, its cell body will transform into two distinct portions: the “front” portion, ie., the forward moving portion, and the”back” portion, ie., the retracting rear portion localizes. Small Rho GTPases family member Cdc42 (Stowers et al., 1995; Wedlich-Soldner et al., 2003; Watanabe et al., 2004; Etienne-Manneville et al., 2005), PAR proteins (PAR3 and PAR6) (Nagai-Tamai et al., 2002; Suzuki et al., 2002; Macara, 2004; Suzuki et al., 2004; Gerard et al., 2007; Totong et al., 2007), and atypical protein kinase C (aPKC) (Suzuki et al., 2002; Suzuki et al., 2004) are participating in polarization. After polarization has occurred, multiple overlapping signaling pathways from small RhoGTPases, PI3K, integrin, microtubules and vesicular transport will maintain the stability of the polarization.

2. Protrusion. Protrusion heralds the actual beginning of the migration cycle. The cardinal event during the formation of protrusion is the actin polymerization (Pollard and Borisy, 2003). The actin cytoskeleton undergoes polarization, forms barbed ends and pointed ends under the induction of ARP2/3 complex, and thus drives the actin treadmilling and membrane protrusion (Bailly et al., 1999). Protrusions can be present in the forms of either filopodia (spike –like) or lamellipodia (large and broad). They provide the direction of cell migration (Pollard and Borisy, 2003).

Several proteins are proposed to regulate the fast-turnover actin cytoskeletal rearrangement. They include the following: ADF/cofilin (Kiuchi et al., 2007), capping proteins (Eddy et al., 1997; Miyoshi et al., 2006), profilin (Didry et al., 1998; Gutsche-Perelroizen et al., 1999; Blanchoin et al., 2000a; Battersby et al., 2004), dendritic network stabilizers within lamellipodia such as α-actinin (Laukaitis et al., 2001), cortactin (Weed et al., 2000), and filamin A (Hou et al., 1990; Flanagan et al., 2001). Cofilin can sever actin filaments, promote the dissociation of actin, generate more barbed ends for dendritic actin mesh network formation, increase actin turnover rate, and promote lamellipodia formation in conjunction with ARP2/3 complex (Svitkina and Borisy, 1999).

3. Traction. At this stage, the newly formed adhesions at the leading edge of a migrating cell are stabilized by the recycling of the integrin from the rear end to the front end (Pollard and Borisy, 2003; Caswell and Norman, 2006).
4. Retraction**.** Rear retraction of the cell is the hallmark of finishing the migration cycle and cell translocation. During retraction, myosin II plays a pivotal role (Uchida et al., 2003; Wylie and Chantler, 2003).

1.3.3 Small RhoGTPases and their role in actin cytoskeleton rearrangement and cell migration

Small Rho GTPases are ubiquitously expressed in various species. So far, 20 members have been found in mammals. Members 7, 5 and 15 were

uncovered in *Drosophila melanogaste*r, *Caenorhabditis elegan*s and *Dictyostelium discoideum* respectively (Schultz et al., 1998).

During cell migration, small Rho GTPases play a central role as the major molecular switch (Burridge and Wennerberg, 2004). They are typically activated by upstream signaling mechanisms such as integrin (Hood and Cheresh, 2002) and EGF (Ridley and Hall, 1992; Ridley et al., 1992; Nobes et al., 1995; Malliri et al., 1998). Rho GTPases switch the on and off downstream signal transduction pathways by cycling between a GDP-bound, inactivated form and a GTP-bound, activated form (Raftopoulou and Hall, 2004). This transition is tightly regulated by guanine exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine dissociation inhibitors (GDIs) (Raftopoulou and Hall, 2004). In their GTP-bound activated state, Rho GTPases bind and activate downstream targets (effectors) such as kinases and scaffold proteins. Then they trigger a series of intracellular responses to regulate actin dynamics, cell migration and invasion (Figure 1.4).

These small Rho GTPases coordinate with each other and play very distinctive but well cooperative roles to propel cell migration. For example, RhoA regulates the assembly of contractile and actin filament-myosin crosslinking, while Rac and Cdc42 promotes actin polymerization and dendritic mesh network to form peripheral lamellipodia and filopodia, respectively (Hood and Cheresh, 2002; Ridley et al., 2003; Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004). Moreover, all three GTPases also induce inside-out signaling to promote the assembly of integrin-based, matrix adhesion complexes (Ridley and Hall,

1992; Ridley et al., 1992; Nobes et al., 1995). Based on such a broad spectrum of functions, it is not surprising that these three regulatory proteins play such pivotal roles in controlling cell migration. Beside actin cytoskeleton rearrangement, Rho GTPases are also implicated in a variety of other biochemical processes, ranging from cell polarity, microtubule assembly to gene transcription. Most notably, Cdc42 is required for the establishment of cell polarity, while all three can, in distinct ways, affect the microtubule cytoskeleton and gene transcription (Etienne and Hall, 2002; Hood and Cheresh, 2002; Ridley et al., 2003; Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004). The functions of each Rho GTPases are listed as follows for further discussion (See also Figure 1.5).

1.3.3.1 Upstream of Rho GTPases

The p130Cas/CrkII/DOCK180 pathway, as illustrated in Figure 1.5, the SH2/SH3 domain-containing adaptor protein Crk (Hasegawa et al., 1996; Buday et al., 2002) interacts with another adaptor molecule p130Cas. The presence of Crk/p130Cas complex activates Rac. Crk/p130Cas complex serves as a “molecular switch” to promote cell migration (Figure 1.5) (Klemke et al., 1998). DOCK180 is a downstream component of integrin signaling (Hood and Cheresh, 2002). DOCK180 was originally portrayed as a binding partner of CrK. Expression of DOCK180 can enhance further p130Cas/Crk/Rac-induced cell migration (Cheresh et al., 1999). The mechanism of how p130Cas/Crk/DOCK180 complex activates Rac might be as follows: DOCK180/ELMO recruits a DH-

Figure 1.5 Small RhoGTPases, their upstream regulators and downstream effectors, and their roles in actin cytoskeleton rearrangement and cell migration

Small RhoGTPases, like Rac, can be activated by conjugation of integrin and its ECM ligand. After integrin activation, GEF DOCK180 form complex with CrKII and p130CAS and thus activates Rac. PI3-K also activates Rac. Once Rho GTPases are activated, they will execute distinct but well coordinated tasks to promote cytoskeletal rearrangement and thus cell migration. Rac and cdc42 promote lamellipodia / new adhesion formation and filopodia / polarization formation respectively. Two important effectors have been identified downstream of Rac and cdc42. 1) Ser/Thr kinase p65PAK activates Arp2/3 and plays an important role in regulating actin dynamics to form lamellipodia. 2). WASp/SCAR/ WAVE family of scaffold proteins. Once they are activated, each of these proteins is able to activate the Arp2/3 complex, which in turns promotes actin polymerization either *de novo* or at the barbed end or sides of preexisting filaments. In this way, the dendritic morphology of lamellipodia (by Rac1) or filopodia (by Cdc42) is generated. Under the similar mechanism, cdc42 also promotes cell polarization. Rho promotes focal adhesion formation, cell body contraction and rear end retraction. 1) In leukocytes and macrophages, Rho stimulates actin-myosin filament crosslinking and in turn contractility through Ser/Thr kinase p160ROCK. Rock inhibits MLC phosphatase, which in turn allows MLC to remain phosphorylated. The overall effect is to activate MLC and induce actin-myosin crosslinking and thus rear contraction and tension. 2) p160ROCK, like p65PAK, can phosphorylate and activate LIMK, which in turn phosphorylates and inactivates cofilin. Cofilin temporarily lose its function to sever actin filaments, resulting in stabilization of actin filaments within actin:myosin filament bundles, as well as favoring focal adhesion formation and maintenance. Solid arrows symbolize direct activating signals. *Modified with permission. Raftopoulou M, and Hall A, 2004, Cell migration: Rho GTPases lead the way: Developmental Biology, v. 265, no. 1, p. 23-32.*

Actin cytoskeletal rearrangement & cell migration
Lamellipodia / New adhesion
Filapodia / Polarization
Rear contraction / Tension / Focal adhesion at cell body
Actin Polymerization
Actin Polymerization
Actin Polymerization
F-actin stabilization
Actin:myosin crosslinking
Arp2/3
Cofilin
Arp2/3
Cofilin
MLC phosphatase
MLC phosphorylation
Scar/WAVE
p65PAK
LIMK
WASp/ WAVE
mDia
P160 ROCK
LIMK
Citron kinase
Rac
Cdc42
RhoA
p130CAS
PI3-K
DOCK180 /CrkII
EGFR
ECM
ECM
ECM
α
β
α
β
α
β
Extracellular
Membrane
Intracellular

containing GEF to the complex, which in turn activates Rac by adding GTP (Raftopoulou and Hall, 2004). In lymphocytes, Vav might function as a GEF since it is associated with DOCK2. Other GEFs might still exist, because the blocking of Vav activity by dominant negative Vav is not sufficient to block activation of Rac by DOCK2 (Nishihara et al., 2002).

1.3.3.2 Rac signaling: lamellipodia and new adhesion in the front

1.3.3.2.1 Rac1 promote actin polymerization, lamellipodia formation and cell migration

As a major downstream target of integrin and growth factor signaling pathways, Rac promotes actin polymerization in the leading edge to form membrane protrusion, lamellipodia (Nobes and Hall, 1995; Etienne and Hall, 2002; Hood and Cheresh, 2002; Ridley et al., 2003; Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004), and cell adhesion (Souza-Schorey et al., 1998; Burridge and Wennerberg, 2004).

How does Rac promote membrane protrusion and lamellipodia formation? Expression of the constitutively activated Rac induces membrane ruffling. Furthermore, suppression of Rac activity by expressing a dominant-negative form of Rac, N17Rac1, impairs the ruffle formation induced by growth factors (Ridley et al., 1992). It is worthwhile to mention here that membrane ruffles closely resemble lamellipodia, and those terms are interchangeably used (Burridge and Wennerberg, 2004). Frequently, lamellipodia can develop into

membrane ruffles, especially when these membrane protrusions can't attach to the matrix and thus flip backward on the dorsal surface (Burridge and Wennerberg, 2004). On the other hands, membrane ruffles can be induced *de novo* from the dorsal surfaces of cells. However, membrane protrusion may be related more to macropinocytosis than cell migration (Burridge and Wennerberg, 2004), as the activated Rac promotes the accumulation of large vesicles that contain the engulfed materials from the extracellular environment (Ridley et al., 1992). Interestingly, macropinocytosis is also found when the activated Ras is introduced into cells (Bar-Sagi and Feramisco, 1986), and this Ras-induced membrane ruffling can be inhibited by the dominant-negative Rac (Ridley et al., 1992; Burridge and Wennerberg, 2004).

Unlike the controversial role of RhoA in actin polymerization, Rac is a robust actin polymerization activator. Although Rac1 can activate PI 5-kinase and this suggests that Rac1 promotes actin polymerization by activating the uncapping process of actin filaments, through lipids such as PIP_2 (Tolias et al., 1995). Arp2/3 complex, the potent actin nucleater and polymerizer, turns out to be a more prominent in inducing barbed ends and dendritic actin mesh network at the leading edge of lamellipodia. What is the linkage between Rac and Arp2/3? Does Rac directly or indirectly regulate actin polymerization? Why does the regulation of Arp2/3 by Rac1 play such an exceptionally important role in the formation of lamellipodia and cell migration?

First, WAVE/Scar protein bridges between Rac and Arp2/3 (Figure 1.5). WAVE/Scar protein belongs to the WASP family (Machesky and Insall, 1998;

Miki et al., 1998). Unlike Cdc42, which binds WASP and N-WASP and induces a conformational change that sequentially activates downstream effectors such as Arp2/3 to nucleate actin polymerization, Rac does not interact directly with the WAVE/Scar proteins. Instead, Rac can activate Arp2/3 through WAVE/Scar by two different and indirect ways, by IRSp53 (Miki et al., 2000), or alternatively by Rac binding proteins Nap125, PIR121, and probably HSPC300 and Abi2 (Eden et al., 2002). However, IRSp53 has later been found to preferentially bind Cdc42 over Rac, indicating that IRSp53 may be the downstream of Cdc42 activation (Govind et al., 2001; Krugmann et al., 2001). As for Nap125 and PIR121, along with HSPC300 and Abi2, they bind WAVE and restrict its activity during the steady state. Once activated, Rac will induce the dissociation of Nap125, PIR121, and Abi2 from WAVE and HSPC300, the release of WAVE to stimulate the actin polymerization activity of Arp2/3 (Eden et al., 2002). Interestingly, another adaptor protein, Nck, also binds Nap125, which similarly frees WAVE. Both Rac and Nck are the downstream effectors of a variety of receptors and provide an alternative or synergistic role in mediating extracellular signals to actin polymerization and cytoskeletal rearrangement (Burridge and Wennerberg, 2004).

Another downstream effector of Rac that is related to cytoskeletal rearrangements and membrane ruffling is PAK. Both Rac and Cdc42 activate PAK. It then can promote lamellipodia formation (Sells et al., 1997) and disrupt stress fibers and focal adhesions (Manser et al., 1997). Although the mutagenesis study of PAK shows that the direct binding and activation of PAK to

Rac or Cdc42 might not be required for its functions in promoting lamellipodia formation, such a physical binding and activation are indispensable for attenuating the formations of stress fiber and focal adhesion (Manser et al., 1997). Besides this, PAK also affects polarization (Sells et al., 1999). PAK also binds to several other substrates or binding partners other than Rac, such as the actin binding protein filamin, LIM kinase, myosin, the paxillin/Pix/PKL complex, and the adaptor protein Nck (Figure 1.5) (Burridge and Wennerberg, 2004).

Filamin is the major actin binding protein and a substrate for PAK (Vadlamudi et al., 2002). Filamin is enriched in the cell cortex where F actin is concentrated, especially in membrane ruffles. Filamin is essential for the growth factor- or PAK-induced membrane ruffling. The binding of filamin to PAK activates PAK. Filamin promotes lamellipodia and ruffle formation by crosslinking F actin and stabilizing the actin networks underneath the membrane of protrusions. Moreover, filamin also serves as a scaffold for PAK and other signaling proteins (Stossel et al., 2001). Finally, filamin also binds either GTP- or GDP- loaded forms of Rho, Rac, and Cdc42 (Ohta et al., 1999). Since filamin is enriched in the actin cortex and is important for lamellipodia formation, the binding to small Rho GTPases may regulate the differential localization of Rac and Rho to the leading edge or rear contractile region (Burridge and Wennerberg, 2004).

As another downstream target of Pak, LIM kinase can be phosphorylated and activated by both PAK (Edwards and Gill, 1999; Edwards et al., 1999) and Rho-kinase (Maekawa et al., 1999). In turn, LIM kinase phosphorylates and inactivates cofilin, which promotes depolymerization of F-actin (Blanchoin et al.,

2000b; Burridge and Wennerberg, 2004). The net effect of PAK-LIM kinase activation is to stabilize actin filaments and filament bundles. This is somewhat paradoxical, as the stabilization of F-actin may slow down the turnover rate of actin filaments, which will to some extent impede the formation of lamellipodia or ruffles. During cell migration, lamellipodia formation demands active cofilin and rapid actin recycling between polymer and monomer to sustain the actin treadmilling (Figure 1.3 and 1.6) (Blanchoin et al., 2000a; Zebda et al., 2000). How to explain such a discrepancy? One possible explanation would be that cofilin activation and inactivation are under elegant spatial or temporal control. During the cell migration, active cofilin was found to be more concentrated at the leading edge to promote actin polymerization, in cooperation with Arp2/3 complex. In contrast to the active cofilin, inactive cofilin, i.e, the one phosphorylated by PAK-LIM kinase may concentrate further back from the leading edge of lamellipodia (Burridge and Wennerberg, 2004). This subtle difference may allow active cofilin to promote polymerization, synergizing with the Arp2/3 complex at the leading edge of lamellipodia, while simultaneously the inactive cofilin distributes further back in the body of the lamellipodium or ruffle, leading to stabilization of the newly formed actin filaments in the dendritic actin mesh network (Burridge and Wennerberg, 2004). Furthermore, the stage of the cell (with or without stimulation) may also need to be included into the consideration. During the steady state, the need of cofilin activation at the leading edge is relatively low and cells may only form new actin branches by the effort of Arp2/3. The actin bundles are relatively stabilized under this state. However,

once the cell turns to the active migratory stage, the demand for active cofilin rises dramatically as the newly formed barbed end, actin mesh network and actin treadmilling will do the following: 1. Consume lots of actin monomers, which are generated from depolymerization of F actin by active cofilin. 2. Form new barbed ends severed by active cofilin to develop into dendritic mesh network and consequently lamellipodia (DesMarais et al., 2005).

The association of PAK with the Pkl/paxillin/Pix complex potentially has multiple effects on downstream signals. Pkl is an Arf GAP, therefore implicating a convergent point between Rac and Arf GTPases signaling (Burridge and Wennerberg, 2004). Pix is a Rac and Cdc42 GEF (Turner et al., 2001). The interaction of a downstream Rac effector with an upstream Rac regulator strongly suggests at the existance of signaling feedback loops. The pairing of downstream effectors with the same GEF also implies that a specific GEF may preferentially activate one out of the possible downstream pathways (Burridge and Wennerberg, 2004).

Another possibility for PAK to promote actin polymerization is that the adaptor protein Nck can recruit PAK receptor tyrosine kinases and may participate in the activation of both WASP and WAVE/Scar and actin polymerization (Bokoch, 2003). As a consequence, Rac1 promotes cell migration (Hood and Cheresh, 2002; Ridley et al., 2003; Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004).

1.3.3.2.2 Rac1 promotes adhesion formation

Other than stimulating lamellipodia formation and cell migration, Rac1 also promotes cell adhesion (Burridge and Wennerberg, 2004). In lymphocytes, activated Rac promotes adhesion, probably through enhancing cell spreading, actin cytoskeleton reorganization, and inside-out signaling to the clustered integrin that engage with ECM (Souza-Schorey et al., 1998). Cofilin inactivation by Rac activation is essential for integrin mediated adhesion (Toshima et al., 2001; Marcoux and Vuori, 2005). Other actin severing proteins such as gelsolin, are also the downstream effectors of Rac-phospholipase C-calcium signaling pathway to sever actin filaments (Snyder et al., 2003).

In addition to promoting adhesion, Rac1 also controlls focal adhesion formation within the new adhesions through the local inside-out activation of integrins (Kiosses et al., 2001). Active Rac may inhibit cofilin activation through PAK-LIM kinase pathway, to stabilize the stress fiber where focal adhesions are developing (Burridge and Wennerberg, 2004). Recently, this has been confirmed by the study using Rac1 knock out animal (Guo et al., 2006). However, Rac also perturbs stress fiber and focal adhesion formation via activating PAK and antagonizing Rho activity (Sander et al., 1999), resulting into inhibition of myosin light chain kinase (MLCK) activity and less MLC phosphorylation (Sander et al., 1999). How to explain this discrepancy? The answer may reside in the temporal and spatial regulation of distribution of Rac and Rho, respectively (Xu et al., 2003). Within a cell, Rac activity dominates tje behavior at the front, while Rho activity is more prominent at the rear. The antagonism between Rac and Rho

signaling pathways determines the migration direction of the cell in response to chemotactic signals. The activities of Rho and myosin at the rear inhibit protrusive activity in this region and confine the protrusion to the front of the migratory cell (Worthylake and Burridge, 2003; Xu et al., 2003)

Moreover, a recent study has indicated that Rac1 might also regulate the genomic stability and senescence (Debidda et al., 2006).

1.3.3.3 RhoA signaling: stress fibers, tension and contractile at the rear

RhoA strengthens the formations of focal adhesions and stress fibers (Nobes and Hall, 1995; Etienne and Hall, 2002; Hood and Cheresh, 2002; Ridley et al., 2003; Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004). It also regulates rear contraction (Nobes and Hall, 1995; Machesky and Hall, 1997; Etienne and Hall, 2002; Hood and Cheresh, 2002; Ridley et al., 2003; Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004).

Rho promotes actin nucleation, polymerization, and stress fibers formation in focal adhesions (Ridley and Hall, 1992), probably through activating of myosin light chain (MLC) by phosphorylation (Chrzanowska-Wodnicka and Burridge, 1996). Situating in between Rho and MLC phosphatase is Rho-kinase (ROCK, ROK), a downstream Rho effector. ROCK increases MLC phosphorylation by inhibiting the MLC phosphatase (Kimura et al., 1996). Rho-kinase can also directly phosphorylate MLC and thereby activate myosin (Amano et al., 1996) (Figure 1.5). The important relationships among MLC, contractile tension, and stress fiber / focal adhesion formation are as follows: in a single cell in which

myosin activity was inhibited, applying extrinsically mechanical force to this single cell could mimic an increasing intrinsic tension, promoting the assembly of stress fibers and focal adhesions (Riveline et al., 2001).

Taken together, Rho drives the formation of stress fibers and focal adhesions, promotes myosin contractility, and thereby raises contractile tension (Nobes and Hall, 1995; Machesky and Hall, 1997; Etienne and Hall, 2002; Hood and Cheresh, 2002; Ridley et al., 2003; Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004) (Figure 1.5).

The next interesting question would be this: is Rho-induced actin polymerization relevant to stress fiber development? Surprisingly, the level of actin polymerization in response to Rho activation was relatively modiocre (Machesky and Hall, 1997). On the other hand, constitutively active form of Rho-kinase induced robust stellate-like stress fibers. Unlike the stress fibers induced by active Rho, those ROCK-induced stellate-like stress fibers are much thicker (Watanabe et al., 1999).

Interestingly, activated mammalian homolog of diaphanous (mDia), another Rho effector, induces the formations of more stress fibers (Watanabe et al., 1999) (Figure 1.5). As a member of the formin family proteins, mDia induces actin polymerization (Li and Higgs, 2003). However, how this elevated actin polymerization is relevant to stress fiber organization has yet to be determined. As mentioned above in the external force on cells study, mDia turned out to be an important impact factor. In the presence of Rho specific inhibitor C3 exotransferase, external force cannot stimulate focal adhesion formation.

However, overexpression of active mDia, but not ROCK, could rescue this phenotype (Riveline et al., 2001).

Other targets for Rho relevant to actin cytoskeleton rearrangement (Bishop and Hall, 2000) include: 1) Citron kinase, a Rho-kinase like protein, which promotes MLC phosphorylation and myosin activation (Madaule et al., 1998; Fujiwara et al., 2002). 2) LIM kinase, which phosphorylates and inactivates cofilin/ADF, thereby leading to the inhibition of actin depolymerization and increasing stability of actin filament arrays including stress fibers and the cortical actin ring (Maekawa et al., 1999; Raftopoulou and Hall, 2004), and 3) Adducin, which is member of the ERM family. It is a substrate for Rho-kinase and is activated by phosphorylation. Once it is phosphorylated, adducin increases its affinity to F-actin (Kimura et al., 1998).

Surprisingly, when cells lose stress fibers and focal adhesions, such as cell rounding up during early mitosis or experimental manipulation, Rho activity is higher instead of lower (Ren et al., 1999; Maddox and Burridge, 2003). Based on Rho's role in promoting focal adhesion and stress fiber formation, the rounded cells should have decreased Rho activity. Why? The answer may be that the presence of high Rho activity would contribute to the disassembly of stress fibers and the construction of more rigid cell cortex (Burridge and Wennerberg, 2004). The rigid cortex of a rounded cell increases the resistance to mechanical forces and protects the cell from damage. This may be important for leukocytes to survive and function in the circulation. How can high Rho activity achive this? The potential explanations are as follows: 1). High Rho activity promotes

phosphorylation and inactivation of cofilin through the ROCK / LIM kinase pathway (Figure 1.5); 2) High Rho activity enhances MLC phosphorylation and activation; thereby promote actin-myosin crosslinking, rear contractility, and probably cell rounding up (Figure 1.5). On the other hand, decreased Rho activity can be observed when cells become adhered to ECM (Ren et al., 1999). The declining in Rho activity may facilitate the transition from a rigid cortex to a more dynamic actin cortex that allows cell spreading and cell migration (Arthur and Burridge, 2001).

Besides focal adhesion, Rho also regulates cell adhesion. Rho induces integrin clustering to provide firm attachment to the ends of stress fibers in focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996). The inhibition of Rho in fibroblasts not only abolishes stress fibers, but also decreases adhesion, causing the retraction of lamellae and rounding of the cell body (Chrzanowska-Wodnicka and Burridge, 1996). Merely inhibiting focal adhesions cannot explain for all these effects, because many cells without the structures adhere and spread well (Burridge and Wennerberg, 2004). It is possible that the disruption of Rho function leads to a collapse of the cortical actin organization (Burridge and Wennerberg, 2004).

In contrast to fibroblasts, Rho inhibits the integrin clustering within leukocytes, which lack both stress fibers and focal adhesions. Inhibition of Rho promotes integrin-mediated adhesion (Worthylake et al., 2001). One explanation is that, during the resting / suspended state, integrins are distributed evenly on the cell surface. Integrin clustering has been inhibited by being tethered to the relatively

rigid cortical actin network. Since the stability of the cortical actin is controlled by Rho-ROCK-LIM kinase-cofilin pathway, active Rho may contribute to the maintaining of a rigid cortical actin network by inhibiting the activation of actin filament-severing protein, cofilin (Figure 1.5). Once Rho activity is suppressed, cofilin becomes activated. Activated cofilin will sever and depolymerize the cortical actin network, and consequently integrins are clustered by binding the multivalent ECM ligands or by inside-out signals (Worthylake and Burridge, 2003).

1.3.3.4 Cdc42 signaling: A master regulator of cell polarity and filopodia

1.3.3.3.1 Cdc42 and cell polarity

Cdc42 defines cell polarity in a variety of eukaryotic organisms ranging from yeast to humans (Ridley et al., 2003). Despite the fact that those macrophage would still be able to move in a Rac-dependent manner, inhibition of Cdc42 in macrophage cells impairs their ability to undergo chemotaxis (Ridley, 2001). The mechanisms for Cdc42 to control cell polarity are the following:

1. Cdc42 regulates the positive feedback loop between Rac and PI (3,4,5) P3 which is important for initiating neutrophil asymmetry and polarity when the external chemotactic gradient is present (Weiner et al., 2002).
2. Cdc42 determines where lamellipodia form, thereby regulating the direction of polarization (Labrousse et al., 1999; Ridley et al., 2003). Cdc42 is activated close to the leading edge of migrating cells (Smirnova et al.,

1998). Both inhibition and global activation of Cdc42 are able to perturb the directionality of migration (Etienne and Hall, 2002).

3. Cdc42 regulates the orientation of the microtubule-organizing center (MTOC) and Golgi apparatus by positioning them in front of the nucleus and toward the leading edge in slowly migrating cells through PAR6/PAR3/aPKC/APC pathway (Etienne and Hall, 2002; Rodriguez et al., 2003). The polarized migration of many cells is heralded by reorganization of the microtubule cytoskeleton and centrosome to form MTOC. The orientation of MTOC usually indicates the direction of migration. Cdc42-induced MTOC orientation is important for cell migration. It directs microtubule growth into the lamella as well as trafficking of Golgi-derived vesicles to the leading edge along those growing microtubule. Those vesicles provide the source of membrane and associated proteins required for forward protrusion (Etienne and Hall, 2002; Ma and Chisholm, 2002; Rodriguez et al., 2003). How does Cdc42 regulate MTOC? In astrocytes, PAR6, an effector of Cdc42, forms a complex with PAR3 and an atypical protein kinase C (aPKC) and mediates Cdc42 regulated of MTOC position (Etienne and Hall, 2003). PAR6/PAR3/aPKC complex orients MTOC by local capture of microtubules at the leading edge through a protein called APC, which binds tubulin at the ends of microtubules (Etienne and Hall, 2002). CLIP170 and IQGAP (Rodriguez et al., 2003) and/or the microtubule-based dynein/dynactin motor protein complex

(Etienne and Hall, 2003) might also participate in this PAR6/PAR3/aPKC complex-APC mediated MTOC formation.

4. Cdc42 crosstalks with Rac to promote and control cell polarity through the interaction of APC, Asef and other proteins. Interestingly, APC associates with Asef, a Rac-specific GEF. This interaction indicates a potential crosstalk between Rac and Cdc42 signalings at the leading edge (Kawasaki et al., 2000). APC is found to be able to move along microtubules, probably via binding to the microtubule-associated protein EB1 and the kinesin/KAP3 motor complex (Nakamura et al.2001; Gundersen, 2002; Jimbo et al., 2002), supporting the idea that the APC-Asef interaction is important for polarity formation. The track by which APC moves towards the plus ends of microtubules in migrating cells could be used to track Asef down to where Rac-dependent actin polymerization is taking place (Bienz, 2002; Jimbo et al., 2002).

1.3.3.3.2 Cdc42 and filopodia

Cdc42 induces filopodia formation (Nobes and Hall, 1995; Kozma et al., 1995), through its binding to its downstream effector WASP and N-WASP (Kozma et al., 1995; Nobes and Hall, 1995; Raftopoulou and Hall, 2004) (Figure 1.5).

Another downstream effector of Cdc42 is PAK1. Pak1 activation induced by Cdc42 shares a common downstream events with the one to Rac (see above Rac section), i.e., activate LIMK and then inactivate cofilin, thereby stabilizing the

actin filament (Figure 1.5) (Arber et al., 1998; Edwards et al., 1999; Raftopoulou and Hall, 2004). Pak1 also interacts with downstream targets of heterotrimeric GTP-binding protein–coupled receptors activated by variety of chemoattractants. These interactions underline a positive feedback loop between Cdc42 and PAK1, and contribute to the high Cdc42 activity at the leading edge (Li et al., 2003). Similarly, integrins also maintain the local Cdc42 activity through this positive feedback loops (Etienne and Hall, 2001; Etienne and Hall, 2002; Raftopoulou and Hall, 2004).

1.3.3.5 Crosstalk among Rho GTPases

1.3.3.5.1. Cdc42 and Rho can be activated following the Rac activation

In serum-starvated fibroblasts, the activation of Rac during membrane ruffling usually leads to the formation of stress fibers, which is dependent on another member in the same Rho GTPase family, Rho (Ridley and Hall, 1992). This strongly indicates that Rac activation might in turn activate Rho. Moreover, Cdc42 is also found to be activated by a similar manner after the Rac activation (Kozma et al., 1995; Nobes and Hall, 1995).

1.3.3.5.2. RhoA and Rac counteract with each other

In contrast to the early observation, activation of Rac could suppress the activation of RhoA and vice versa in many cell types (Sander et al., 1999) (Figure 1.5). How does Rac suppress Rho? 1) Rac could increase the intracellular

reactive oxygen species (ROS) levels. Rising ROS inhibits low molecular weight protein tyrosine phosphatase (LMW-PTP), thereby in turns stimulates the phosphorylation and activation of p190RhoGAP, which inactivates RhoA (Nimnual et al., 2003). 2). PAK, a downstream effector of Rac1, induces the loss of stress fibers and focal adhesions through either inhibiting MLCK (Sander et al., 1999) , or directly phosphorylating myosin II heavy chain (Kiosses et al., 1999; van Leeuwen et al., 1999).

1.3.3.6 Pathogens, diseases and pharmaceutical compounds that target Rho GTPases

1.3.3.6.1 Pathogens modulate Rho GTPases

Interestingly, various bacterial pathogens have developed strategies to escape from the host cell defense system by regulating Rho GTPases proteins. Those strategies include preventing phagocytosis and thus escaping from being uptaken and destroyed, and promoting phagocytosis to occupy the immunologically inaccessible locations within the host cells (Burridge and Wennerberg, 2004). The prototypical agent is the C3 exotransferase from Clostridium botulinum. It ADP-ribosylates RhoA and inhibits nucleotide exchange catalyzed by GEFs, and in turn immediately inactivates RhoA proteins (Barbieri et al., 2002).

Other toxins that influence Rho GTPases activity may inactivate or activate Rho GTPases: Clostridial toxins A and B glucosylates Rho, Rac, and Cdc42

proteins and thus inactivates them (Barbieri et al., 2002). Cytotoxic-necrotizing factor (CNF) produced from E. coli activates Rho by deamidation of Gln63 (Barbieri et al., 2002). This blocks the GAP-activated GTPase activity, resulting in the constitutively active RhoA. Salmonella can also activate Rac and Cdc42 by injecting SopE and SopE2 proteins into host intestine epithelial cells (Stebbins and Galan, 2001). These toxins are such potent GEFs for Rac and Cdc42 that they stimulate robust membrane ruffling at the attached sites of bacteria, thereby facilitating the phagocytosis of the bacterium (Stebbins and Galan, 2001). After entry into host cells, Salmonella immediately starts to secret another protein, SptP, to suppress the Rac and Cdc42 activity. Inhibition of active cytoskeletal rearrangement by SptP will provide the bacteria more advantage to survive. Enteropathic Yersinia also express several proteins that can regulate Rho GTPase activity (Finlay and Cossart, 1997; Bliska, 2000; Burridge and Wennerberg, 2004).

1.3.3.6.2 Cancer metastasis, Rho GTPases, their inhibitors and modulators

As discussed in detail above, one of the consequences of the activation of Rho GTPases is the elevated cell migration. For cancer, that means increased chance of metastasis. Compounds that inhibit Rho GTPases activity are also tested for their obvious clinical cancer metastasis prevention and treatment. One of those compounds is Rac1 specific compound, NSC23766 (Gao et al., 2004)., NSC23766 was identified based on a structure-based virtual screening of compounds that fit into a surface groove of Rac1 known to be important for GEF

specification. NSC23766 effectively disrupts the binding of Rac1 with its specific GEF, Trio or Tiam1 in a dose-dependent manner, thereby specifically inhibiting the Rac1 activation without interfering with other Rho GTPases such as Cdc42 or RhoA. In the presence of NSC23766 the Rac1-dependent lamellipodia formation will be blocked. It also inhibited Rac1-induced proliferation, anchorage-independent growth, and invasion (Gao et al., 2004).

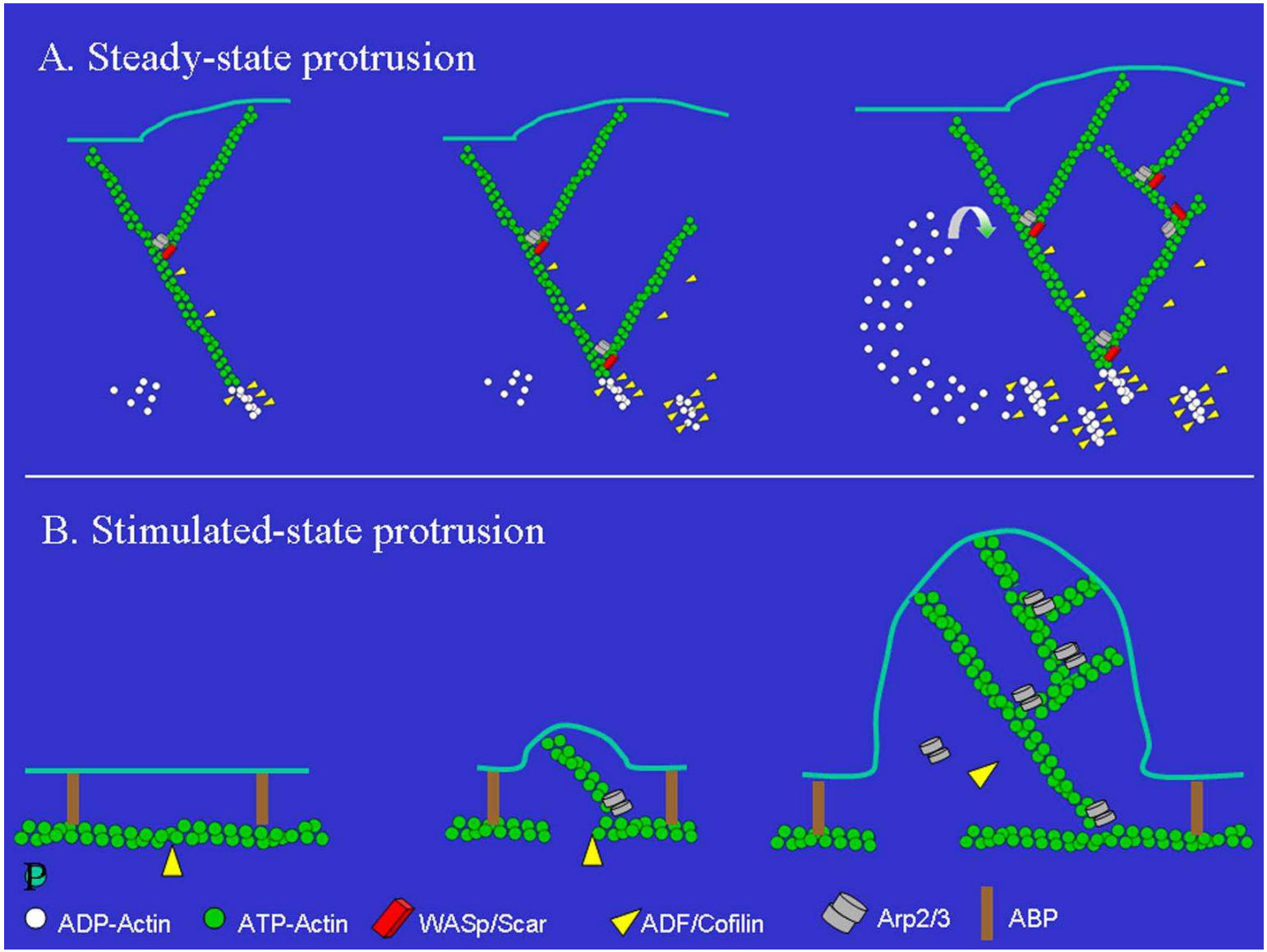
1.3.3.7 Major downstream effectors of Small RhoGTPases – Cofilin, an actin severing and depolymerizing protein

During the cytoskeletal rearrangement, protrusion and cell migration, the most important downstream effectors of small Rho GTPases are cofilin and Arp2/3 (Figure 1.6).

Cofilin is a 19kDa protein that is ubiquitously expressed (Carlier et al., 1997; Maciver, 1998; Maciver et al., 1998; Bamburg et al., 1999; Wang et al., 2007). Cofilin can be inactivated by the phosphorylation on Ser3 position (Agnew et al., 1995; Moriyama et al., 1996). Although cofilin binds to both G- and F-actin, it preferentially binds to ADP-bound actin subunits and promotes actin monomer disassembly from the pointed end of an actin filament. (Carlier et al., 1997; Maciver, 1998; Maciver et al., 1998; Bamburg et al., 1999; Wang et al., 2007). Thus, Cofilin plays an important role in actin cytoskeletal rearrangement, especially during membrane protrusion (Bamburg et al., 1999; Condeelis et al., 2001; Ono, 2003; Pollard and Borisy, 2003; Wang et al., 2007). Active cofilin can also sever actin filaments and in turn generate free actin barbed ends to provide

Figure 1.6 Roles of Cofilin during different state protrusion

A. In steady state protrusion formation, such as in keratocytes, a low G-actin level limits polymerization. Cofilin functions exclusively as an actin-recycling factor by depolymerizing filaments to generate free G-actin. In this state, cofilin localizes at the base of the lamellipodium to sustain steady-state actin polymerization at the leading edge. Meanwhile, Arp2/3 complex near the cell membrane is responsible for dendritic nucleation. (Loisel et al., 1999; Svitkina and Borisy, 1999). **B**.In contrast, during the stimulated protrusion stage, such as in crawling cells like fibroblasts and chemotactic carcinoma cells, cofilin synergistically cooperates with Arp2/3 complex. Cofilin seveing is omportant r in initiating reassembly of actin filaments and governing the migratory direction during this stage (Ghosh et al., 2004) .Cofilin is found enriched within barbed ends at the very leading edge, but not at the base of lamellipodia (Chan et al., 2000). Cofilin itself is sufficient to generate barbed ends, actin polymerization and protrusion (Ghosh et al., 2004). Actin polymerization depends on the pre-existing G-actin pool and is not necessarily tightly coupled to depolymerization. Cofilin also determines the site of activation of the Arp2/3 complex to form protrusion. *Modified with permission. DesMarais V, Ghosh M, Eddy R, and Condeelis J, 2005, Cofilin takes the lead: Journal of Cell Science, v. 118, no. 1, p. 19-26.*


A. Steady-state protrusion
B. Stimulated-state protrusion
P
ADP-Actin
ATP-Actin
WASp/Scar
ADF/Cofilin
Arp2/3
ABP

a platform for the later development of the dendritic network beneath the plasma membrane (Maciver et al., 1991; Du and Frieden, 1998; Chan et al., 2000; Ichetovkin et al., 2002). How does cofilin depolymerize or sever actin filaments? The answer is that cofilin bind to F-actin and causes a bend in the actin filament. This twist destabilizes the actin-actin interactions and eventually fragments the filament (McGough et al., 1997). The next questions are as following: how are cofilin's functions executed in vivo to contribute to cell motility and its related morphology? Is cofilin level positively correlated with the cell motility? In early studies, the activation of cofilin was thought to be required for cell motility (Carlier et al., 1997; Condeelis et al., 2001). In supporting this notion, an elevated cofilin level or declined phosphorylated cofilin level can be detected within the cells or tissues from different species. Elevated total cofilin have been found in variety of tumor cell lines, such as highly invasive C6 rat glioblastoma cell line (Gunnersen et al., 2000), A549 human lung cancer cells (Keshamouni et al., 2006), and human pancreatic cancer cells (Sinha et al., 1999). The decreased level of phosphorylated / inactivated cofilin may also be an indicator of upregulated cofilin activity. Phosphrylated cofilin level is downregulated in T-cell lymphoma (Jurkat), carcinomas from the cervix (HeLa), colon (KM12), liver (HepG2) and kidney (COS1) (Nebl et al., 1996; Wang et al., 2007). In clinical tumor samples, the increased cofilin expression is detected in oral squamous-cell carcinoma (Turhani et al., 2006), renal cell carcinoma (Unwin et al., 2003), and ovarian cancer using both proteomic and genomic approaches (Martoglio et al., 2000).

However, is cofilin always upregulated in highly motile cells, such as cancer cells? The answer is no. In high motility cells such as potent metastatic hepatocellular carcinoma (HCC) cells line MHCC97-H (Ding et al., 2004) and ovarian surface epithelium (OSE) cells derived from a woman with a family history of ovarian and/or breast cancer and bearing BRCA1 mutation (Smith-Beckerman et al., 2005), cofilin had been found to be downregulated instead of upregulated. If endogenous cofilin levels fluctuate among different cell types instead of positively proportional to cell motility, then what would happen if lectopically overexpress cofilin into those cells? Will they have higher motility or less? Similar to the controversial effect of endogenous cofilin on cell motility, the overexpression of wild-type cofilin can either promote invasion in Melanoma cell (Dang et al., 2006), or inhibit the invasiveness of human lung cancer H1299 cells (Lee et al., 2005).

Therefore, the expression level and phosphorylation level of cofilin are not sufficient to determine the motility and invasion status of cells (Wang et al., 2007). What are the roles of cofilin in controling the cell motility and cytoskeletal rearrangement in vivo? Without understanding the cofilin pathway in each individual situation, it is very hard to predict the consequence by simply altering the level of one component of the whole pathway, such as cofilin in those above cases. The well-balanced of the activity of cofilin as well as other molecules in the cofilin pathway are required for chemotaxis and motility in tumor cells (Wang et al., 2007).

What are the cofilin pathways? What factors / molecules control the activation, inactivation and translocation of cofilin? The cofilin pathway includes several kinases and phosphatases that regulate cofilin phosphorylation, dephosphorylation, and / or translocation in response to extracellular stimuli (Figures 1.3; 1.6). Those stimuli include epidermal growth factor (EGF) (Mouneimne et al., 2004), transforming growth factor-α (TGF_{α}) , stromal cell-derived factor 1 (SDF1) and heregulin, which are positively correlated with cancer metastasis (Wang et al., 2007).

Cofilin is regulated in a temporal and spatial manner by the following processes:

1. Phosphorylation / inactivation. Cofilin can be phosphorylated at the serine 3 residues by LIM kinase (LIMK1 and LIMK2) (Arber et al., 1998; Yang et al., 1998; Dan et al., 2001) and testicular protein kinase 1 (TESK1, TESK2) (Rosok et al., 1999; Toshima et al., 2001) . This phosphorylation inhibits the binding of cofilin to actin. LIMKs can be either activated by p21-activated kinase 1 (PAK1) and Rho-dependent protein kinase (ROCK1) (Figure 1.5) (Dan et al., 2001; Bamburg and Wiggan, 2002) through phosphorylation, or inhibited by SSH1 through dephosphorylation (Soosairajah et al., 2005).
2. Dephosphorylation / activation. Dephosphorylation of cofilin at Ser3 is achieved by phosphatase types 1, 2A (Ambach et al., 2000), phosphatase types 2B (Meberg et al., 1998), slingshot (SSH) (Niwa et al., 2002) and

chronophin phosphatases (Gohla et al., 2005). Dephosphorylation of cofilin results in the activation of cofilin and its binding to actin.

3. Blockage of the actin-binding capability of cofilin by protein-protein interaction.

   a. Phosphatidylinositol-4,5-bisphosphate (PIP2) binds to cofilin which blocks the binding of cofilin to actin (Yonezawa et al., 1990; Yonezawa et al., 1991). The binding of PIP2 to cofilin is regulated by phospholipase C (PLCγ). PLCγ hydrolyses PIP2 and thus releases cofilin to be available to bind to actin (Mouneimne et al., 2004; Mouneimne et al., 2006). In non-carcinoma cell type such as platelets and fibroblasts, PLCγ promotes the plasma membrane protrusion by regulating both F actin severing protein: cofilin and gelsolin (DesMarais et al., 2005). Like cofilin, gelsolin also severs actin filaments, but in Ca^{2+}- and PIP2- dependent manners (Sun et al., 1999). In contrast to cofilin, the actin severing and protrusion formation rates induced by gelsolin are a relatively slow (the half life is of actin severing is 15 minutes after growth factor stimulation) (Allen, 2003). In platelets and fibroblasts, gelsolin seems to be important for the generation of barbed ends (Azuma et al., 1998; Falet et al., 2002). Other actin or cofilin binding proteins such as tropomyosin, cyclase-associated protein, gelsolin, and AIP1 may also regulate cofilin's actin severing function as well (DesMarais et al., 2005; Wang et al., 2007).

b. Tropomysosin. The binding of tropomysosin to actin prevents actin filaments from being either depolymerized (Bernstein and Bamburg, 1982) or severed (DesMarais et al., 2002) by cofilin. Within the leading edge of lamellipodia of carcinoma cell, tropomyosin is absent wherever cofilin and Arp2/3 dominate and actively promote actin polymerization (DesMarais et al., 2002).

c. Cyclase-associated protein (CAP). CAP induces the release of cofilin from the cofilin–G-actin heterodimer (Moriyama and Yahara, 2002), and in turn stimulates actin depolymerization from the pointed end (Moriyama and Yahara, 2002; Balcer et al., 2003). CAP might also accelerate actin recycling and thus actin polymerization (Moriyama and Yahara, 2002).This may also contribute to the localization of cofilin in crawling cells and therefore migration direction (Bertling et al., 2004).

d. Actin-interacting protein 1 (AIP1). AIP1 binds to cofilin (Aizawa et al., 1999; Okada et al., 1999; Rodal et al., 1999; Ono, 2003) and this binding might stimulate the depolymerization activity of cofilin (Ono, 2003).

4. Intracellular pH. Elevating PH by Na-H exchanger protein potentiates cofilin's actin-severing efficiency (Bernstein et al., 2000; Bamburg and Wiggan, 2002; Patel and Barber, 2005; Srivastava et al., 2007).

It is worthwhile to discuss subcellular localization of cofilin specifically. The reason is that cofilin mediates two relatively controversial processes: barbed-end formation and subsequent actin polymerization (Condeelis, 2001) and actin

depolymerization (Carlier et al., 1997; Lappalainen and Drubin, 1997). How are these two opposing processes balanced during protrusion and cell motility? In fact, the role that cofilin plays varies from cell type and functional states of cells (DesMarais et al., 2005).

1. Cell type. In migrating chick fibroblasts, cofilin promotes depolymerization of actin filaments to generate more actin monomers for treadmilling (Cramer, 1999); In neurons, cofilin severs actin filaments to open more free barbed ends for dendritic network formation which is an important driving force for growth cone motility (Endo et al., 2003).
2. Cell migration stages (Figure 1.6). In steady state protrusion formation, such as in keratocytes, the low G-actin level limits polymerization. Cofilin functions exclusively as an actin-recycling factor by depolymerizing filaments to generate free G-actin. Meanwhile, Arp2/3 complex near the cell membrane is responsible for the dendritic nucleation (Loisel et al., 1999; Svitkina and Borisy, 1999). In contrast, during the stimulated protrusion stage of crawling cells like fibroblasts and chemotactic carcinoma cells (Figure 1.6), cofilin synergistically cooperate with Arp2/3 complex. The severing activity of cofilin initiates reassembling of actin filaments and governs the migratory direction during this stage (Ghosh et al., 2004). Cofilin is found to be enriched at barbed ends at the very leading edge, but not at the base of lamellipodia (Chan et al., 2000). Cofilin itself is sufficient to generate barbed ends to promote actin polymerization and protrusion (Ghosh et al., 2004). The two models are

not mutually exclusive (Figure 1.6). The transition from stimulated to steady protrusion formation state, or vice versa, could happen if cell kept moving until G-actin supply become limited to support further polymerization. At this point, cofilin-mediated actin depolymerization and elevation of actin turn-over rate become critical for further polymerization.

Then, what is the difference between these two models? Cofilin defines the sites of dendritic nucleation, and in turn cell protrusion, only in the stimulated protrusion model (DesMarais et al., 2005; Wang et al., 2007) .

In addition to all these functions described above, the cofilin pathway contributes to morphogenesis, such as cell polarity in Drosophila (Blair et al., 2006), and blastocyst positioning and body wall formation (Ono et al., 2003).

The final question: why is cofilin enriched at the leading edge of lamellipodia, but not at the base of the lamellipodia and other compartments of the cell during cell migration? Is cofilin activated in those regions or activated at other locations and then undergo translocation into the leading edge? If this is the case, then what mechanisms control cofilin translocation after activation?

Driven by variety of stimuli, cofilin becomes dephosphorylated or activated, then translocated to the plasma membrane (Suzuki et al., 1995; Nagaishi et al., 1999; Nagata-Ohashi et al., 2004; Verdijk et al., 2004) where it interacts with the actin cytoskeleton and promotes actin cortical meshwork formation, actin recycling, and consequently lamellipodia formation (Raftopoulou and Hall, 2004). Translocation of cofilin to the plasma membrane constitutes a key step for cofilin activation (Suzuki et al., 1995; Nagaishi et al., 1999; Nagata-Ohashi et al., 2004;

Verdijk et al., 2004). For example, in T leukemia cells and NIH3T3 cells, phosphorylated cofilin undergoes the translocation to lamellipodia upon activation or dephosphorylation (Verdijk et al., 2004). Still, how cofilin activation couples with the translocation to the leading edge of lamellipodia remains largely unknown. In fact, the EGF stimulation of invasive mammary tumor cells doesn't always result in either cofilin activation / dephosphorylation or inactivation / phosphorylation (Song et al., 2006c), so models of which cofilin activation couples with stimuli stimulation (Nishita et al., 2005b) may not always reflect the invasion potential in all types of tumors. Thus, one needs to be cautious in evaluating models.

The next question is this: will the level of dephosphorylated cofilin really portray the cofilin activity within the cells? As Ialready mentioned above, there are at least four mechanisms to regulate cofilin activity. Simply measuring the ratio of dephosphorylated cofilin to the total cofilin may not be an ideal way to assess the activity of cofilin in cells. Interestingly, cofilin dephosphorylation is uncoupled from the EGF stimulated membrane protrusion (Mouneimne et al., 2004). In fact, the levels of phosphorylated cofilin in mammary tumor cells increase in response to EGF-stimulation (Mouneimne et al., 2004). However, EGF can activate PLCγ, which in turn hydrolyzes PIP2, releases cofilin from the cofilin-PIP2 complex, and thus activates cofilin (Mouneimne et al., 2004). Shortly after the release of cofilin, LIMK1 phosphorylates it and results in a net elevation of phosphorylated cofilin level. Although the total inactivated cofilin may increase after stimulation, the sharpening asymmetrical distribution of the cofilin activity

inside the tumor cell may account for the more active motility (Mouneimne et al., 2004; Blair et al., 2006; Song et al., 2006b; Wang et al., 2007).

1.4 Novelty of this study

Overall, cell migration plays roles in a wide variety of pivotal biological processes ranging from embryonic development to cancer cells metastasis. Elucidating the mechanisms that govern cell migration will not only have a tremendous impact on the deciphering of those mystery biological processes, but will also lead to the discovery of novel and more effective treatment of many common diseases. KAI1/CD82 is a key suppresser of cell migration. So far, how KAI1/CD82 regulates cell migration is not clear, especially how KAI1/CD82 regulates cancer cell migration. Previously, there were several studies on how KAI1/CD82 regulates hematopoietic cell morphology and cytoskeletal rearrangement (Liu and Zhang, 2006). However, those studies focused on suspension cells, which behave quite differently from cells with epithelial origin in many aspects, including cell adhesion, cytoskeletal rearrangement and cell migration. Instead of inhibiting migration of epithelial and fibroblast cells, KAI/CD82 promotes actin cytoskeleton rearrangement and cell migration in hematopoietic cells, probably through enhancing co-stimulation of T-cell surface receptors TCR (Liu and Zhang, 2006). In this dissertation, I use prostate cancer cell lines Du145 and PC3 cells as models to study the role of KAI1/CD82 in cytoskeletal rearrangement. I conclude that KAI1/CD82 inhibits cancer cell migration through impairing actin cytoskeletal rearrangement.

CHAPTER 2. KAI1/CD82 INHIBIT CANCER CELL MIGRATION THROUGH ALTERING MOTILITY RELATED CELL MORPHOLOGY

2.1 Introduction

Regulation of cell motility is a common feature of many tetraspanins (Maecker et al., 1997; Berditchevski, 2001; Boucheix and Rubinstein, 2001 Hemler, 2001). Although it remains largely unclear how tetraspanins modulate cell motility, the possible mechanisms start to emerge from recent progress (Higashiyama et al., 1995; Shi et al., 2000; Odintsova et al., 2003). Lines of evidence suggest that tetraspanins could regulate the functional status of cell adhesion molecules and growth factor receptors (or membrane-bound growth factor). They physically and/or functionally associate with each other and then alter cellular behaviors through these partners (Higashiyama et al., 1995; Maecker et al., 1997; Shi et al., 2000; Berditchevski, 2001; Boucheix and Rubinstein, 2001; Hemler, 2001; Odintsova et al., 2003). For example, KAI1/CD82 attenuates epidermal growth factor (EGF) signaling and integrin function by accelerating the endocytosis of its associated EGF receptor and integrin, respectively (Odintsova et al., 2000; Berditchevski, 2001; He et al., 2004). In parallel, experimental data support the notion that tetraspanins per se solicit outside-in signals to modulate cellular functions (Maecker et al., 1997; Berditchevski, 2001; Hemler, 2001). Again, taking KAI1/CD82 as an example, immuno-crosslinking of cell surface KAI1/CD82 demonstrates that KAI1/CD82 functions as a costimulatory molecule during the T cell activation (Nojima et al., 1993; Lebel-Binay et al., 1995; Lagaudriere et al., 1998; Shibagaki et al., 1998; Delaguillaumie et al., 2002; Iwata et al., 2002), indicating that KAI1/CD82 plays a direct role in signal initiation and/or transduction.

Regardless which of the two possible mechanisms plays a more predominant role, tetraspanins and/or their associated molecules must act on the cytoskeleton to alter cell motility and motility-related cellular events. For example, clustering the cell surface KAI1/CD82 proteins using immobilized KAI1/CD82 mAb induces profound dendritic cellular protrusions in T cells, accompanied by the rearrangement of the actin cytoskeleton and the connection of KAI1/CD82 to the actin cytoskeleton, in a protein kinase A activity-dependent but Src kinase activity-independent manner (Nojima et al., 1993; Lagaudriere et al., 1998). Further studies indicated that Rho small GTPases are required for KAI1/CD82-induced dendritic processes in T cells (Delaguillaumie et al., 2002).

Cell migration requires the polarized formation and extension of cellular protrusions, the transmembrane connection of cytoskeleton to ECM for the generation of traction force to propel cell body forward, and the retraction of the rear cellular portion (Raftopoulou and Hall, 2004).

In this chapter, I will focus on how KAI1/CD82 inhibits cell migration at the cell morphological level, such as polarization, protrusion, lamellipodia formation, and random cell migration on ECM.

2.2 Materials

The monoclonal antibodies (mAbs) used in this study were human CD81 mAb M38 (Fukudome et al., 1992), human KAI1/CD82 mAb M104 (Fukudome et al., 1992), 4F9 (Schlossman et al., 1994; Iwata et al., 2002), and TS82b (Tepnel, Stamford, CT). A mouse monoclonal IgG2 was used as a negative control

antibody (Sigma). The secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or -rabbit IgG antibody (Sigma) and rhodamine-conjugated goat-anti-mouse IgG antibody (Biosource International, Camarillo, CA).

Cell culture medium DMEM was purchased from Invitrogen (San Diego, CA) Extracellular matrix (ECM) proteins used in this study were human plasma fibronectin (FN) (Invitrogen, San Diego, CA), mouse laminin (LN)-1 (Invitrogen, San Diego, CA), and rat LN-5 (Desmos Inc., San Diego, CA).

Growth factors or chemokine used in this study include epidermal growth factor (EGF) (Upstate Biotechnology, Lake Placid, NY), platelet-derived growth factor (PDGF) (Upstate Biotechnology), hepatocyte growth factor (HGF) (Sigma), and stromal cell derived factor 1 (SDF-1) (R&D, Minneapolis, MN).

2.3 Methods

2.3.1 Establishment of KAI1/CD82 transfectants

Prostate cancer cell lines Du145 and PC3 were obtained from ATCC (Manassas, VA) and cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. The full-length KAI1/CD82 cDNA was constructed in a eukaryotic expression vector pCDNA3.1 (Invitrogen). Du145 or PC3 cells were transfected with the plasmid DNA using either Superfectin (Qiagen, Valencia, CA) or Lipofectamine 2000 (Invitrogen), respectively, and selected with 1 mg/ml geneticin (Invitrogen). The geneticin-resistant clones were

pooled, and the KAI1/CD82-positive cells were collected by flow cytometric cell sorting (Zhang et al., 2003a).

2.3.2 Western blot

Western blot was performed as previously described (Zhang et al., 2003a). For of total cellular proteins, an equivalent number of cells were lysed using RIPA buffer, the protein concentrations of lysates were normalized, and then the lysates were separated by SDS-PAGE. After being transferred electrically, nitrocellulose membranes (Schleicher & Schuell, Keene, NH) were sequentially blotted with primary antibody and horseradish peroxidase conjugated anti-mouse or -rabbit IgG (Sigma) and then detected with chemiluminesence reagent (PerkinElmer Life Sciences). In some cases, membranes were stripped and reblotted with mAbs or pAbs according to the manufacturer's instruction.

2.3.3 Flow cytometry

Cell surfact expression of CD82 was analyzed as discribed before (Zhang et al., 2001b). Du145-Mock and Du145-CD82 transfectant cells were incubated with either negative control mAb, integrin mAbs, or specific TM4SF mAbs M104. In turns, cells were stained with FITC-conjugated goat-anti-mouse IgG. The stained cells were analyzed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA). Fluorescence with negative control mAb was subtracted to give specific mean fluorescence intensity (MFI) units.

2.3.4 Cell spreading experiment

Du145-mock and Du145-CD82 transfectants were rinsed with PBS, then detached by trypsin/EDTA treatment. After centrifugation, the supernatant was removed and cells were resuspended in DMEM containing 10% FCS (Invitrogen, San Diego, CA). Cells were then plated on ECM protein (FN or LM 1)-coated glass bottom dishes (MatTek Corp., Ashland, MA) in DMEM medium for 3-6 h. Cells attached and spread for 20 min before being fixed with 3.7% formaldehyde in PBS with 5% sucrose and 2 mM $MgCl_2$ for 15 min at RT. Images were taken with a Hamamatsu cooled CCD camera run by Metamorph (Universal Imaging, PA) or a Bio-Rad 1024 microscope (Bio-Rad, Hercules, CA). Areas of cells (20–45 cells per coverslip) were calculated using the Scion Image v1.62 program (Scion Corp.).

2.3.5 Time-lapse video microscopy

The time-lapse video-microscopic experiments were performed basically as previously described (Hinz et al., 2004; Prass et al., 2006). The Du145- or PC3-transfectant cells were plated on ECM protein (FN or LM 1)-coated glass bottom dishes (MatTek Corp., Ashland, MA) in DMEM medium for 3-6 h before the time-lapse imaging experiment. In the imaging experiment, each transfectant cell was observed for 1 to 6 h on an Axiovert 135 TV microscope (Carl Zeiss, Thornwood, NY) using DIC optics with either an oil-immersion 40X Plan Fluar or an oil-immersion 63X Plan Apo objective. The microscope was equipped with a heated

stage and the temperature was kept at 37°C. CO_2 was kept by covering the medium with mineral oil. Images were taken with a Hamamatsu cooled CCD camera run by Metamorph (Universal Imaging, PA) in 1, 2, 5, or 10 min interval during the observation periods. The cellular motile behaviors and the translocation of cells were assessed in movies made from the saved images. Cellular motile behaviors were analyzed under different conditions such as serum-free DMEM, DMEM containing EGF, DMEM containing KAI1/CD82 mAb, etc. The images were analyzed by Nikon EZ-C1 FreeViewer software.

2.3.6 Cell migration assay

Migration experiments were performed in Transwell membrane filter inserts in 24-well tissue culture plates (BD Labware, Bedford, MA) as described previously (Zhou et al., 2004). The Transwell filters were 6.5 mm in diameter, and the pore size for polycarbonate membranes was 8 μm. Filters were spotted with FN diluted in 10 mM $NaHCO_3$ or LM 1 diluted in PBS on the lower surface of the Transwell inserts at 4°C overnight and then blocked with 0.1% heat-inactivated BSA at 37°C for 30 minutes. Cells were detached at 90% confluence with 2 mM EDTA/PBS, washed once in PBS, and resuspended in serum-free DMEM containing 0.1% BSA. A 300-μL cell suspension was added to inserts at a density of 3×10^4 cells/insert. DMEM containing 1% FCS was added to the lower wells. Migration was allowed to proceed at 37°C for 3 hours. Cells that did not migrate through the filters were removed using cotton swabs, and cells that migrated through the inserts were fixed and stained with Diff-Quick (Baxter Healthcare

Corp., McGraw Park, IL). The number of migrated cells per insert was counted under a light microscope at magnification x40. Data from several independent experiments were pooled and analyzed using a two-tailed, Student's t test.

2.3.7 Image analysis and MDFR measurement

For cell morphology evaluation, phase contrast or fluorescent digital images of cells were analyzed by using the Image J software from NIH image (NIH image, http://rsb.info.nih.gov/nih-image/). The margin of each individual cell was traced by using the software's polygon selection tool and the cell perimeter and cell area were automatically calculated by the software. Then, as described previously (Szabo et al., 1995), the morphological deviation from roundness (MDFR), i.e., the deviation of each cell shape from perfect roundness, was calculated by dividing the theoretical maximum area for a given perimeter (perimeter^2/4π) by the observed pixel area. The value for a perfectly round cell equals 1.0, and larger values represent increasing levels of deviation from roundness.

2.4 Results

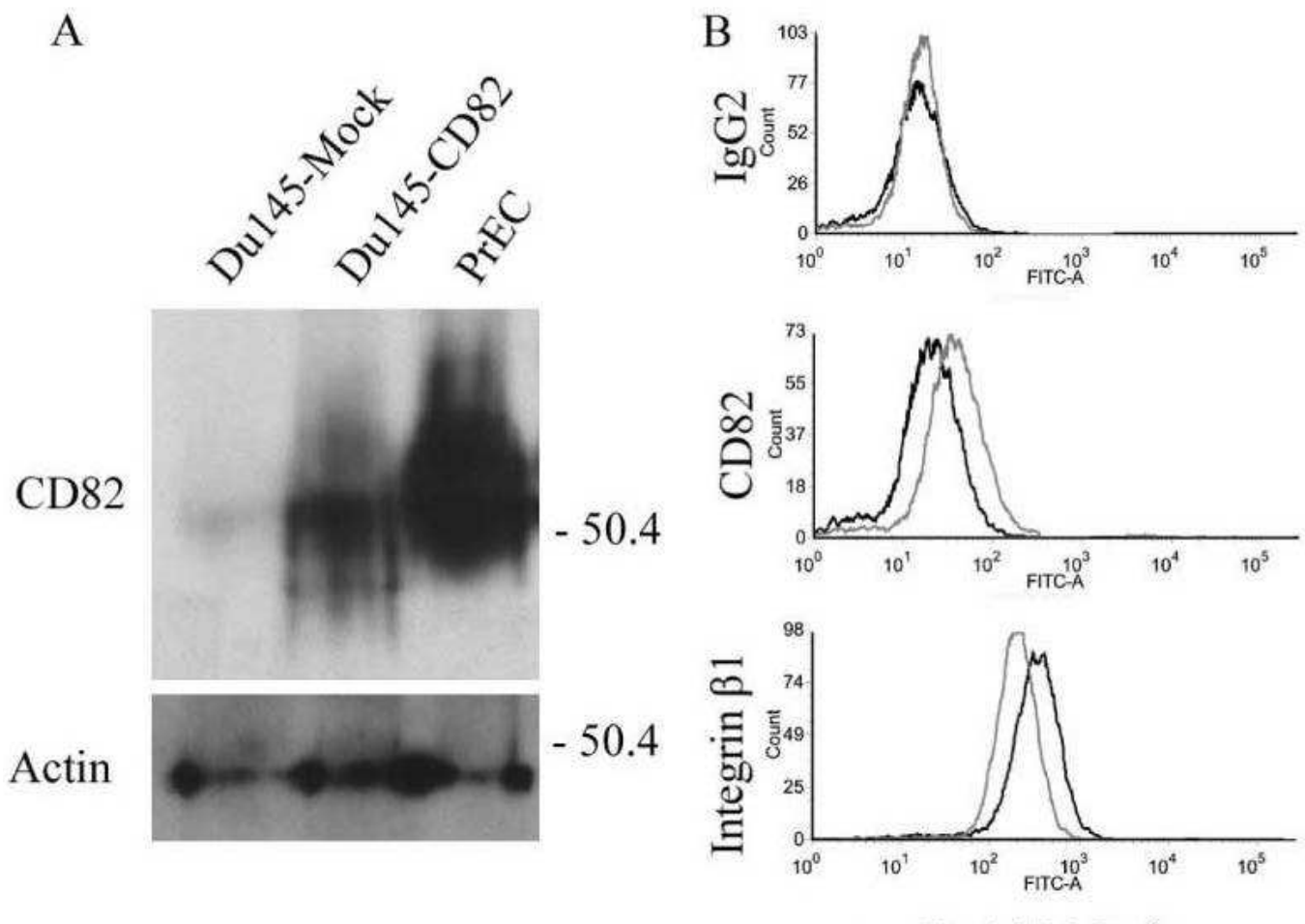
2.4.1 Detection and comparison of CD82 expression in Du145-mock and Du145-CD82 stable transfectants

After generating Du145-mock and Du145-CD82 transfectants as described above in Methods 2.3.1, KAI1/CD82 expression levels were determined by both flow cytometry and western blot (Figure 2.1). CD82 expression is not obviously detected in Du145-mock cell, whereas strong signals of CD82 has been detected (Figure 2.1A). The CD82 expression level within Du145-CD82 is not higher than the endogenous level of those cells sharing similar origin as prostate epithelial, which naturally express CD82, such as normal prostate epithelial cell line PrEC (Figure 2.1A). The expression of CD82 on the surface of Du145-CD82 transfectants was further confirmed by FACS (Figure 2.1B). Compared to the absence of CD82 expression on Du145-mock transfectants, there was a clear peak shift indicating successful overexpression and correct conformation of CD82 on the surface of Du145-CD82 transfectants, as they were able to be recognized by CD82 specific antibody.

Thus, Isuccessfully re-introduced CD82 back to Du145 cells, which lose their endogenous expression of CD82 due to epigenetic events which is still largely unknown at current stage (Liu and Zhang, 2006). As a positive control, cell surface β1 integrin level was also detected simultaneously. It is not a surprise to see that there were slightly decreasing expression levels of cell surface β1

Figure 2.1 Detection and comparison of CD82 expression in Du145-mock and Du145-CD82 stable transfectants

Upon establishing stable transfectants of Du145-mock and Du145-CD82, CD82 expression is determined by the following: **A**. Western blot. CD82 expression is barely detectable in Du145-mock cell. However, Du145-CD82 transfectants shows decent protein level of CD82. In order to compare how high the expression level of CD82 in Du145-CD82 cells, cell lysate from those cells sharing similar origin as prostate epithelial, which naturally express CD82, such as normal prostate epithelial cell line PrEC, was also collected. The ectopic overexpression level of CD82 within D145-CD82 was contrasted and compared with the endogenous expression level of that in PrEC cells in this blot. The bottom panel shows the actin loading control. **B**. FACS analysis of cell surface CD82 expression. Both Du145-mock and Du145-CD82 transfectants were detached, then blocked with 1% BSA. Cells were then stained with negative control mouse IgG2 mAb, specific TM4SF mAbs M104, or integrin mAbs. In turns, cells were stained with FITC-conjugated goat-anti-mouse IgG as secondary antibody. The stained cells were analyzed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA).


A
Du145-Mock
Du145-CD82
PrEC
CD82
- 50.4
Actin
- 50.4
B
IgG2
CD82
Integrin β1
Count
FITC-A

integrin, since CD82 may accelerate its internalization and thus reduce its cell surface level (Liu and Zhang, 2006).

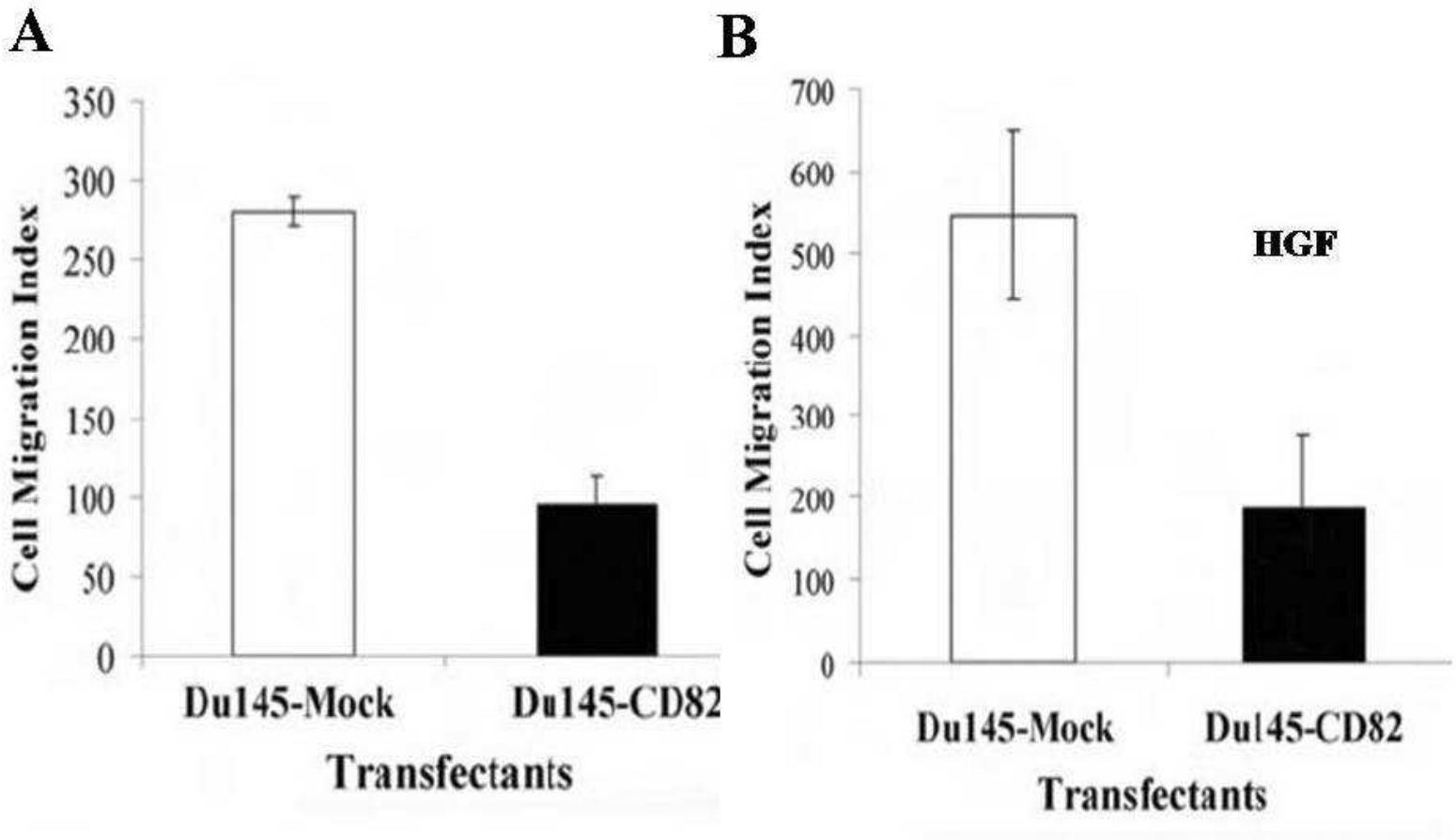
2.4.2 KAI1/CD82 inhibits Du145 cell motility

Upon confirming the expression of KAI1/CD82, Du145 transfectants were in turn tested for their directional motility, either by transwell haptotactic migration on FN, or chemohaptotactic migration on FN under the stimulation of HGF, respectively (Figure 2.2). As shown in Figure 2.2A, the ability of Du145-CD82 cells to migrate toward ECM was greatly reduced compared with that of the Du145-Mock cells. The attenuated haptotactic cell migration in Du145-KAI1/CD82 cells could also be replicated on LN5-coated substrata in our previous published data (Zhang et al., 2003a). Those results indicated that Du145-CD82 has robust inhibitory effects on cell migration mediated by both FN- and LM-binding integrins such as $\alpha_5\beta_1$ and $\alpha_3\beta_1$, respectively.

Besides inhibiting the haptotactic cell migration, CD82 expression also suppressed chemohaptotactic migration, i.e. cells migrated toward both the chemoattractant gradient and immobilized gradient (Figure 2.2B). In the presence of HGF, a strong cell migration promoter, the KAI1/CD82 transfectant still migrated significantly less on FN. Moreover, the difference between Mock and CD82 cells migrating towards FCS and HGF are approximately the same, aound 3-4:1. This indicates that KAI1/CD82 impairs cell migration capability regardless the activation of c-Met. c-Met activation has been documented to be important for KAI1/CD82's inhibition of cell migration in several cell types

Figure 2.2 KAI1/CD82 inhibits Du145 cell motility

A, KAI1/CD82 inhibits haptotactic cell migration on fibronectin. Haptotactic migration of the Du145 transfectants was measured using the transwell inserts coated with FN (20 μg/ml). The migration medium in top and bottom wells was DMEM containing 0.1% heat-inactivated BSA and antibiotics. Cells that migrated onto the lower surface were fixed, stained, and photographed. In each individual experiment, cells that migrated through the filters were counted from at least three randomly selected fields. Results were obtained from at least three individual experiments and represented as the cell migration index, which is the number of cells per a high power field. The bar graph representgraph represents means ± S.D. $p < 0.001$ on FN between the Mock and KAI1/CD82 transfectants. **B**, KAI1/CD82 inhibits chemohaptotactic cell migration. Chemohaptotactic migration assays were carried out toward chemoattractants on FN (20 μg/ml)-coated filters in the medium of the bottom well. The chemoattractants in the medium of the bottom well is HGF (100 ng/ml). The results were obtained from three experiments (mean ± S.D.). $p < 0.001$ toward HGF between the Mock and KAI1/CD82 transfectants.


A
Cell Migration Index
350
300
250
200
150
100
50
0
Du145-Mock
Du145-CD82
Transfectants
B
Cell Migration Index
700
600
500
400
300
200
100
0
HGF
Du145-Mock
Du145-CD82
Transfectants

including YTS1 bladder cancer cells (Todeschini et al., 2007), and metastatic prostate cell line PC3 (Sridhar and Miranti, 2006). Although it has been reported that c-Met activation can fully overturn the motility inhibition induced by CD82 (Todeschini et al., 2007), activation of c-Met by HGF in Du145-CD82 cells can't rescue the impaired cell migration, which is previously inhibited by overexpression of CD82 (Figure 2.2B).

2.4.3 KAI1/CD82 contributes to deficiencies in cellular extension retraction and lamellipodia formation

Cell movement on or through different ECMs is substantially diminished when KAI1/CD82 is forced to express in invasive or metastatic tumor cells, in which KAI1/CD82 expression is usually lost (Dong et al., 1995; Hemler, 2001; Stipp et al., 2003). When spread on ECM such as FN and LM, Du145-KAI1/CD82 transfectant cells exhibited profound differences in cellular morphology from the Mock cells. KAI1/CD82 overexpression typically results in polygonal, dumbbell, liquid-drop, spindle, and arboreal cell shapes (Figures 2.3 and 2.4). In dumbbell-, spindle- and liquid drop-like cells, the elongated cellular extensions are probably caused by the deficiency in retraction of elongated cellular extensions during cell spreading and migration. Although the elongated cellular extensions can also be found in Mock cells, the occurrence frequency was much fewer than the KAI1/CD82-expressing cells. These cellular extensions could be observed when cells spread or migrate on substratum coated with FN or LN 5 (Figure. 2.4B) in the presence or absence of serum or growth factor. For example, extraordinarily

Figure 2.3 Morphological phenotypes of KAI1/CD82-overexpressing cells

A. KAI1/CD82-expressing cells displayed altered morphology. Upper panel, Du145-Mock and -KAI1/CD82 transfectant cells were plated on either FN (10 μg/ml)- or LM 5 (10 μg/ml)-coated glass coverslips at 37oC, 5% CO_2 overnight in serum-free DMEM medium. Lower panel, PC3-Mock and -CD82 transfectant cells were plated on tissue culture flasks at 37°C, 5% CO_2 overnight in DMEM medium containing 10% FCS. **B**. Quantitative analysis of cell morphology. Based on the images of Du145-Mock and -CD82 cells on FN, the MDFR was analyzed as described Experimental Procedures. Asterisk stands for statis-tically significant difference between Mock and CD82 transfectants ($p < 0.05$). **C**. Lack of lamellipodia in KAI1/CD82-overexpressing cells. Upper panel, Du145-Mock and -CD82 cells were spread on FN (10 μg/ml)-coated plates in DMEM containing 1% FCS at 37°C for overnight. The DIC images of cells were captured on an Axiovert inverted microscope using DIC optics with a 40 x F Fluar oil immersion objective. The arrows indicate lamellipodia. Lower panel, PC3-Mock and -KAI1/CD82 transfectant cells were spread on FN- or LN 1-coated plates and stained with CD81 mAb M38 using immunofluorescence as described (Zhou et al., 2004) to visualize cell peripheries. **D**. CD82-overexpressing cells frequently exhibit elongated cellular extensions. Du145-Mock and -CD82 cells were spread on FN (10 μg/ml)-coated plates in DMEM containing 10% FCS and HGF (100 ng/ml) at 37°C for 3-6 h. The DIC images of cells were captured using DIC optics with a 40 x F Fluar oil immersion objective. Scale bar, 50 μm.

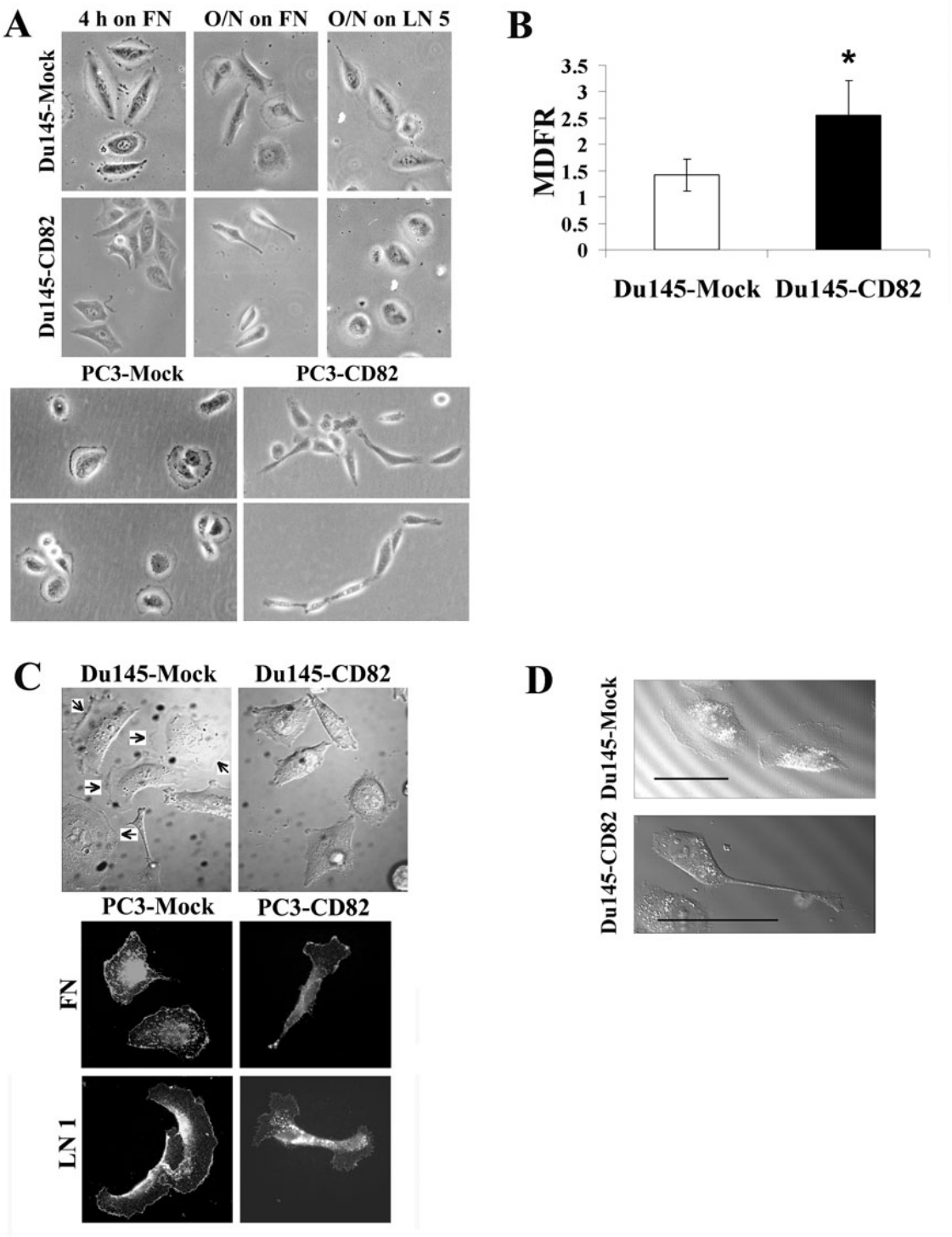

A
4 h on FN
O/N on FN
O/N on LN 5
Du145-Mock
Du145-CD82
PC3-Mock
PC3-CD82
B
MDFR
3.5
3
2.5
2
1.5
1
0.5
0
*
Du145-Mock
Du145-CD82
C
Du145-Mock
Du145-CD82
PC3-Mock
PC3-CD82
FN
LN 1
D
Du145-Mock
Du145-CD82

Figure 2.4 KAI1/CD82 attenuates the formation of lamellipodia and retraction of cellular extensions

A. KAI1/CD82 inhibits lamellipodia and protrusion formations. Du145-Mock and –KAI1/CD82 transfectant cells were plated on FN-coated glass coverslips. DIC images were acquired using time-laps vidoemicroscopy as described in experimental procedures. Arrows indicate lamellipodia. **B**. KAI1/CD82 inhibits the retraction of rear tail. Du145-Mock and -KAI1/CD82 cells were placed on FN (10 μg/ml)-coated coverslip for 3-6 h and treated with 100 ng/ml HGF for 4-6 h. The cell morphology was photographed by using time-lapse videomicroscopy for 3 h. Arrow heads indicate the retraction processes in Du145-Mock cells and arrows the rear tail in Du145-KAI1/CD82 cells. The time-lapse intervals are labeled inside images. Scale bar, 20 μm.

A
Mock
CD82
0 min
8 min
16 min
24 min
32 min
40 min
48 min
56 min
64 min
72 min
B
Mock
CD82
0 min
3 min
6 min
9 min
12 min
15 min
18 min
21 min
24 min
27 min
Mock
CD82
30 min
45min
60 min
75 min
90 min
105 min
120 min
180 min

long cellular extensions were observed in KAI1/CD82-expressing cells treated with HGF (Figure 2.3D). Time-lapse video microscopy demonstrated that the elongated cellular extensions were generated due to the lack of contractile retraction (Figure. 2.3C, Figure 2.4B).

For KAI1/CD82-expressing cells with multilateral morphology, cell edges usually become flat or slightly concave, reflecting the lack of lamellipodia formation. Indeed, as reported earlier (Berditchevski, 2001; Zhou et al., 2004), lamellipodia formation was diminished upon KAI1/CD82 overexpression (Figures 2.3C and 2.4A). In Du145-KAI1/CD82 cells, lamellipodia formation was completely lost (Figures 2.3C and 2.4A), while in PC3-KAI1/CD82 cells, localized lamellipodia were typically limited to one or two cellular ends (Figure 2.3C). Time-lapse video microscopy demonstrated that the Mock cells could rapidly generate broad lamellipodia while the KAI1/CD82-expressing cells failed to produce lamellipodia (Figure 2.4A). Because of attenuated protrusive activities, some KAI1/CD82-expressing cells displayed more rigid edges.

Moreover, when spread on FN-coated plates or grow in cell culture dishes (Figure 1A), Du145-KAI1/CD82 cells showed significantly more MDFR, a parameter to describe the extent of irregularity departing from roundness in cell shape, than the Mock cells (Figure 2.3B). However, when spread on LN 5, Du145-KAI1/CD82 cells exhibited a more roundup morphology (Figure 2.3), reminiscent to the cell flattening process on poly-L-lysine coated plates, suggesting the attenuated outside-in signaling from laminin-binding integrins α3β1 and/or α6β4. The morphology of KAI1/CD82 transfectant cells also reflects

the loss of polarization during spreading, consistent with the loss of directionality of these cells during cell migration (Zhang et al., 2003a). The morphological evidences for the loss of polarization include bipolar cellular extensions or lamellipodia (Figure 2.3C bottom panel), and cell flattening on LN 5 (Figure 2.3A top panel). The KAI1/CD82 expressionexpression level of KAI1/CD82 proteins in Du145-CD82 cells is slitely lower thandid not exceed the endogenous expression level of the endogenous protein in an immortalized human normal prostate epithelial cell line PERrEC (Figure 2.1).

2.5 Discussion

Alterations in cellular morphology upon KAI1/CD82 expression reflect deficiencies in protrusion and retraction - could this explain the motility-inhibitory mechanism at the cellular level?

KAI1/CD82 expression alters the cellular morphology in various aspects. These alterations can be attributed to both diminished protrusive activities such as the lack of formation of lamellipodia and cellular protrusion and attenuated retraction of cellular extension and trailing edge. Since migrating cells typically display these motility-related morphological events, loss and disruption of these events are therefore predicted to be important for the cell movement-inhibitory activity of KAI1/CD82.

In Du145 prostate cancer cells, KAI1/CD82 expression abolishes the lamellipodia formation on fibronectin-coated, laminin-coated, or regular cell culture flask surface. Only on laminin 5-coated substratum did KAI1/CD82-

expressing cells undergo cell flattening, a symmetric cell spreading process. In PC3 prostate cancer cells, KAI1/CD82 grossly inhibits the lamellipodia formation on fibronectin but partially inhibits it on laminin. Because PC3 cells could form fan-like, well-developed lamellipodia on laminin, KAI1/CD82 more likely inhibits the development or prevailence of fan-like lamellipodia. Of note, the definition of lamellipodia referrenced herein covers both subdomains of recently designated lamellipodia and lamella (Ponti et al., 2004). Since both Du145 and PC3 cells form lamellipodia during cell migration, the lack or disruption of lamellipodia formation or development is very likely to be crucial for the motility-inhibitory activity of KAI1/CD82, though some PC3-KAI1/CD82 cells can still form incomplete lamellipodia on laminin.

The presence of elongated cellular extensions is another genuine effect of KAI1/CD82 overexpression in Du145 and PC3 cells. The elongated cellular extensions could be the consequence of excessive protrusive activity, but they could also reflect the retraction deficiency. Our study clearly demonstrated that various cellular extensions in KAI1/CD82-expressing Du145 and PC3 cells are caused by deficiency in the retraction process. Earlier studies showed that the immobilized-KAI1/CD82 mAbs induce profound dendritic extensions in hematopoetic cells (Nojima et al., 1993; Lagaudriere et al., 1998), morphologically reminiscent of some types of cellular extensions seen in KAI1/CD82-expressing Du145 and PC3 cells. Despite the differences between overexpression and Ab engagement and between adherent and suspension

cells, these dendritic extensions more likely represent the phenotype of less retraction if the motility-inhibitory activity of KAI1/CD82 is considered.

KAI1/CD82 not only blocked the cellular protrusion and retraction processes that were induced by the integrin-ECM engagement, but also blocked these events stimulated by growth factors or serum (Figure 1), For example, KAI1/CD82-caused deficiency in rear tail retraction is manifested in the presence of HGF. These observations indicate that KAI1/CD82 inhibits both integrin and growth factor signaling. Another novel observation in this study is that the effect of KAI1/CD82 on these motility-required cellular processes appears to be independent of cell-cell contacts. In other words, KAI1/CD82 can induce these morphological changes without directly engaging a cellular receptor from adjacent cells because those morphological effects are pronounced when cells do not form cell-cell contacts. On the contrary, the cell-cell contacts alleviate KAI1/CD82 effects on the actin cytoskeleton: the foundation of morphological changes and this will be shown in Chapter 3.

CHAPTER 3. KAI1/CD82 DISRUPTS ACTIN CYTOSKELETON REARRANGEMENT THROUGH REGULATION OF THE SMALL-RHO GTPASES SIGNALING PATHWAY

3.1 Introduction

Cell migration consists of several critical steps: polarization formation, extension of cellular protrusions, the transmembrane connection of cytoskeleton to ECM for the generation of traction force to propel the cell body forward, and the retraction of the rear cellular portion (Horwitz and Parsons, 1999; Parent and Devreotes, 1999; Horwitz and Webb, 2003; Ridley et al., 2003; Raftopoulou and Hall, 2004; Van Haastert and Devreotes, 2004; Vicente-Manzanares et al., 2005). Thus, by nature, cell migration is a process of global reorganization of thecytoskeleton. For example, actin polymerization drives the formation and extension of the protrusions such as lamellipodia at the leading edge (Condeelis, 1993; Small et al., 1993; Mitchison and Cramer, 1996; Mogilner and Oster, 1996;), while the asymmetric distribution and crossing linking of myosin and actin produce the force for cellular contractility and lead to the retraction of the trailing edge (Kolega, 2003; Kolega, 2006; Sato et al., 2007). Rho small GTPases are clearly pivotal in all of these cytoskeletal rearrangement processes (Raftopoulou and Hall, 2004). For example, Rac is primarily responsible for generating a protrusive force through localized actin polymerization, while Rho is responsible for the contraction of the cell body and the retraction of the rear end (Jaffe and Hall, 2005). As a downstream effector of Rho GTPases (Raftopoulou and Hall, 2004), cofilin severs actin filaments to generate barbed ends and thus facilitates the actin treadmilling (Bamburg and Wiggan, 2002; Raftopoulou and Hall, 2004). Arp2/3, an effector complex, nucleates new actin filaments from the sides of preexisting filaments (Weaver et al., 2003; Raftopoulou and Hall, 2004). The

severing activity of cofilin and branching activity of Arp2/3 function coordinately to promote the formation of branched actin network or cortical actin meshwork at the leading edge and generate propulsive force for migrating cells (Blanchoin et al., 2000a).

Tetraspanin KAI1/CD82 is an inhibitor of cell movement (Dong et al., 1995; Guo et al., 1996; Higashiyama et al., 1998; Takaoka et al., 1998a; Takaoka et al., 1998b; Miyake et al., 1999; Ono et al., 1999; Uchida et al., 1999; Yang et al., 2001; Delaguillaumie et al., 2002; Zhang et al., 2003a; Zhang et al., 2003b). Previous studies have shown that KAI1/CD82 suppresses cancer metastasis by inhibiting cell migration. Mechanisms may include the following: 1) Inhibiting EGF and integrin signaling by accelerating their endocytosis (Berditchevski, 2001; Odintsova et al., 2003; He et al., 2004; Liu and Zhang, 2006). In turns KAI1/CD82 attenuates outside-in signals and thus modulates cellular functions (Maecker et al., 1997; Berditchevski, 2001; Hemler, 2001). 2) Regulating cytoskeletal rearrangement. In T cells, CD82 clustering promotes cellular protrusions and actin cytoskeleton rearrangement. This activity may depend on protein kinase A (Nojima et al., 1993; Lagaudriere et al., 1998). Rho small GTPases are also important for KAI1/CD82-induced dendritic protrusion in T cells (Delaguillaumie et al., 2002).

Recent studies revealed that, in solid tumor cells, KAI1/CD82 attenuates the signaling derived from integrin (Ridley et al., 2003), EGFR (Yarden and Sliwkowski, 2001), and c-Met (Dugina et al., 1995; Sridhar and Miranti, 2006; Todeschini et al., 2007) and that FAK-Src-p130^{CAS}-Crk pathway is a major

downstream signaling pathway affected by KAI1/CD82 (Zhang et al., 2003a; Sridhar and Miranti, 2006). At the cellular level, besides the aforementioned induction of cellular protrusions via immobilized KAI1/CD82 mAbs, earlier studies showed KAI1/CD82 inhibits lamellipodia formation (Odintsova et al., 2000). The cellular and molecular events critical for the function of KAI1/CD82 and the signaling altered by KAI1/CD82, however, have not been elucidated in depth and details. The goal of this study is to determine the subcellular event and cytoskeletal events crucial for the motility-inhibitory activity of KAI1/CD82. In this study, Ifound that KAI1/CD82 inhibits the formation of lamellipodia and the retraction of cellular extension, which result from deficient development of actin cortical meshwork and stress fibers. Not surprisingly, actin polymerization becomes attenuated upon KAI1/CD82 overexpression, due to the deregulation of Rho small GTPase activities and aberrant functions of Rho GTPase effectors.

3.2 Materials

The monoclonal antibodies (mAbs) used in this study were human integrin α3 mAb X8 (Sauer et al., 2003), human integrin α5 mAb PUJ-2 (Pujades C, 1996), human integrin β1 mAb A1A5 and TS2/16 (Hemler et al., 1984), human CD81 mAb M38 (Fukudome et al., 1992), human KAI1/CD82 mAb M104 (Fukudome et al., 1992), 4F9 (Schlossman et al., 1994; Iwata et al., 2002), and TS82b (Tepnel, Stamford, CT), Rac1 mAb (BD Pharmingen, San Jose, CA), and β-tubulin mAb (Sigma, St. Louis, MI). A mouse monoclonal IgG2 was used as a negative control antibody (Sigma). The polyclonal Ab (pAb) for cofilin was purchased from

Cytoskeleton (Denver, CO). The pAb for phosphorylated cofilin were either the gift from Dr. Bombarg of University of Colorado or purchased from Cell Signalling (Danvers, MA). The pAb for p34 of Arp2/3 complex was purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal Abs for RhoA, Cdc42, and Rac1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or -rabbit IgG antibody (Sigma) and rhodamine-conjugated goat-anti-mouse IgG antibody (Biosource International, Camarillo, CA).

Extracellular matrix (ECM) proteins used in this study were human plasma fibronectin (FN) (Invitrogen, San Diego, CA), mouse laminin (LN)-1 (Invitrogen, San Diego, CA), and rat LN-5 (Desmos Inc., San Diego, CA).

Growth factors or chemokine used in this study include epidermal growth factor (EGF) (Upstate Biotechnology, Lake Placid, NY), platelet-derived growth factor (PDGF) (Upstate Biotechnology), hepatocyte growth factor (HGF) (Sigma), and stromal cell derived factor 1 (SDF-1) (R&D, Minneapolis, MN).

Other reagents include cell permeable C3 transferase (Cytoskeleton), Texas Red- or Alexa 488-conjugated α-phallodin (Molecular Probe, Eugene, OR), Rac1 inhibitor 23766 and its negative control 23767 (NIH, Bethesda, MD), and actin polymerization enhancer jasplakinolide (J7473) (Molecular Probe).

3.3 Methods

3.3.1 Transfectants

Retrovirus containing the Rac1 dominant negative mutants Rac1N17, and constitutively active mutants Rac1L61 were kindly provided by Dr. Yi Zheng of University of Cincinnati. Those constructs were ligated in frame with the nucleotides encoding a three-hemagglutinin (HA_3) tag at the 5′ end of the retroviral vector MIEG3 that expresses enhanced green fluorescent protein bicistronically. Recombinant retroviruses were produced using the Phoenix cell packaging system as described before (Guo and Zheng, 2004). Du145-mock and Du145-CD82 were infected with the respective retroviruses and harvested 48 to 72 h postinfection. The enhanced green fluorescent protein-positive cells were isolated by fluorescence-activated cell sorting (FACS). pEGFP, pEGFP-actin , pEGFP-N-WASp (kindly provided by Dr. A. Weaver of Vanderbilt University), pEGFP-Cofilin (kindly provided by Dr. J Vandekerckhove of University Ghent)and pEGFP-PLCδ PH domain (kindly provided by Dr. John Cox of University of Tennessee) were transiently transfected into Du145-KAI1/CD82 and -Mock cells using Lipofectamine 2000 by following the manufacturer's protocol.

3.3.2 Time-lapse video microscopy

The time-lapse video-microscopic experiments were performed as previously described in Chapter 2 method 2.3.5. For intracellular GFP-actin polymerization study, GFP-actin was transfected 48 h prior to experiment, then plated on FN-

coated glass bottom plates (MatTek Corp., Ashland, MA). Images were taken with a C1Si confocal system mounted on an Eclipse TE2000-E microscope (Nikon, Melville, NY), using a Plan Fluor oil-immersion 40X objective (N.A. 1.3), or an oil-immersion Plan Apo 60X objective (N.A. 1.45). The system is equipped with an environmental-control chamber (InVivo Scientific, St Louis, MO). Temperature was kept at 37^{o}C and 5% CO_2. DIC and fluorescent images were recorded every 1-2 minute. Polymerization of GFP-actin was assessed in movies made from the saved images

3.3.3 Fluorescent and confocal microscopy

The immunofluorescence staining was carried out as described (Zhang et al., 2001b). In brief, glass coverslips were coated with either FN (50 μg/ml) in 10 mM $NaHCO_3$ or LM 1 (50 μg/ml) in phosphate buffer saline (PBS) at 4^{o}C overnight, and then blocked with 0.1% heat-inactivated bovine serum albumin (BSA) at 37^{o}C 45 min. Du145 transfectants were harvested in PBS containing 2 mM EDTA, washed once, and plated on the ECM-coated coverslips in serum-free or serum DMEM for 4 to 6 h at 37^{o}C. Then the cells were fixed with 3% paraformaldehyde (Sigma) and permeated with 0.1% Brij99 (Sigma). Nonspecific binding sites were blocked with 20% goat serum in PBS for 1 h at room temperature. The cells were incubated sequentially with primary mAbs (~1 μg/ml) and with Rhodamine-conjugated secondary antibody. For F-actin staining, cells were incubated with Texas Red-conjugated phalloidin. Each incubation lasted 30 min at RT in 20% goat serum/PBS and followed by 4 washes with PBS.

Finally the coverslips were mounted on glass slides in FluroSave reagent (Calbiochem-Novabiochem, San Diego, CA), analyzed using an Axiophot fluorescent microscope (Carl Zeiss) or a Bio-Rad 1024 confocal microscope (Bio-Rad, Hercules, CA) and photographed with Optronics digital camera (Southern Micro Instrument, Marietta, GA) at 40X or 63X magnification.

3.3.4 Rac1, Cdc42 and RhoA effector pull-down assay

The cellular Rho GTPase activities were measured by an effector domain pull-down assay as described (Gao et al., 2004). Du145 transfectant cells were washed with PBS buffer once and lysed in RIPA buffer (1% NP40, 0.2% SDS, 150 mM NaCl, 25 mM HEPES, 2 mM phenylmethylsulfonyl-fluoride, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 2 mM sodium vanadate, and 2 mM sodium fluoride) on ice. Cell lysates were clarified by centrifugation at 14, 000 g at 4^{o}C for 5 min, and equal amounts of lysate were incubated with GST fusion beads at 4^{o}C for 45 min. The beads were washed three times with ice-cold RIPA buffer. To assay the activity of Cdc42, cell lysate was affinity-precipitated with the beads adsorbed with the WASP Cdc42 binding domain-GST fusion that only binds to the active, GTP-bound form of Cdc42. With the same principle, Rac1 or RhoA activity was pulled down with PAK Rac1 binding domain-GST fusion or Rhotekin RhoA binding domain-GST fusion beads, respectively. The total cell lysates and the affinity-precipitated products were run on a SDS-PAGE gel, transferred to nitrocellulose membrane, and then immunoblotted for Cdc42, Rac1, or RhoA, respectively. The blots were detected with chemiluminesence reagent (Perkin-

Elmer, Boston, MA). The intensities of the bands from four separate experiments were measured by NIH Scion Image program (Kroczynska et al., 2006).

3.3.5 Cell motility assay

Different Rac1 mutants within either Du145-mock or Du145-CD82 were tested by the transwell migration described as above in Chapter 2 method 2.3.6.

3.3.6 Actin polymerization analysis

As described above, intracellular GFP-actin polymerization was analyzed using live fluorescent imaging under a time-lapse confocal microscope. In addition, intracellular F-actin level was measured by using flow cytometry (Miyake et al., 2001). Briefly, Du145 transfectant cells at the confluent culture stage were detached with trypsin and washed once with sterile PBS. The cells were permeabilized with 0.01% Triton X-100 in PBS at 4ºC for 1 min and then washed 3 times with PBS. The permeabilized cells were fixed with 3.7% formaldehyde at RT for 5 min and subsequently washed 3 times with PBS. For F-actin staining, the cells were incubated with 66 nM Alexa 488-conjugated phalloidin in PBS containing 1 % BSA at 37ºC for 1 h. The stained cells were washed 3 times with PBS and analyzed in BD LSR II flow cytometer. The flow cytometry data were analyzed by using FlowJo 7.1 or FCS express V3 software.

3.3.7 Rac1 inhibitor assay

To evaluate whether Rac1 specific inhibitor, NSC23766, can inhibit lamellipodia formation in Du145 cell, Du145 cells grown in 10% calf serum were treated with 100 μM NSC23766 or DMSO overnight according to previous description (Gao et al., 2004), and then the cell were detached. Cell spreading experiment were then done as described in Chapter 2 method 2.3.4.

3.3.8 Cytokine stimulation assay

Du145-Mock or -KAI1/CD82 transfectant cells were spread on FN-coated plates in complete DMEM and treated with integrin β1 activating mAb A1A5 (8 μg/ml), EGF (100 ng/ml), HGF (100 ng/ml), or SDF-1 (100 ng/ml) at 37^{o}C for 4-6 h. Cells were then fixed, permeabilized, and incubated with Texas Red-conjugated α-phalloidin. Digital images were captured under a fluorescent microscope. Scale bar, 20 μM.

3.3.9 RhoA inhibition assay

Du145 cells were plated on coverslips overnight in DMEM full medium containing 10% FBS, then were untreated or treated with 2.0 μg/ml of C3 Transferase (Cytoskeleton) for 4-6h at 37°C. Cell were then fixed, stained with Rhodamine-labeled Phalloidin, and visualized by fluorescence microscopy. Images were taken at a magnification of 60X.

3.3.10 Jasplakinolide inducing actin polymerization

It has been reported that Jasplakinolide (Molecular probe, J7473) can induce actin polymerization (Bubb et al., 1994c). Effect of Jasplakinolide on actin polymerization of Du145-Mock and Du145-CD82 were then determined. Cells were treated with 50nM Jasplakinolide in full DMEM containing 10% FCS for 24 hours, Cell were then fixed, stained with Rhodamine-labeled Phalloidin, and visualized by fluorescence microscopy. Images were taken at a magnification of 60X.

3.3.11 Image analysis

For quantitation of F-actin intensity, the cell perimeters were outlined as described above in chapter 2 method 2.3.7. fluorescent digital images of cells were analyzed by using the Image J software from NIH image (NIH image, http://rsb.info.nih.gov/nih-image/). The margin of each individual cell was traced by using the software's polygon selection tool and the cell perimeter and cell area were automatically calculated by the software.Then, based on the fluorescent digital images of α-phalloidin staining, the total cellular F-actin intensity, F-actin intensity inside the inner edge of cortical actin ring, and F-actin intensity of membrane protrusion or outside the inner edge of cortical actin ring were measured using the “analyze” function in Image J software. Theoretically, total cellular F-actin intensity = F-actin intensity inside the inner edge of cortical actin ring + F-actin intensity of membrane protrusion. Relative F-actin intensity of

membrane protrusion = F-actin intensity of membrane protrusion / total cellular F-actin intensity (Figure 3.1).

3.4 Results

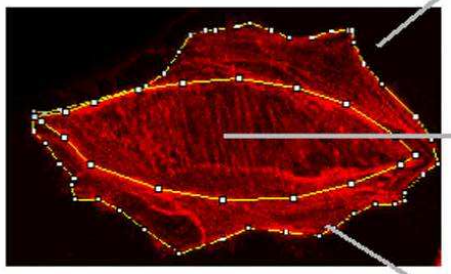
3.4.1 KAI1/CD82 inhibits the formations of actin cortical network and stress fibers

Dynamic actin reorganization is the hallmark of a motile cell (Steinmetz et al., 1997). Usually, the loss of stress fibers and formation of extensive cortical meshwork accompany the enhanced cell migration (Steinmetz et al., 1997). It was reported that KAI1/CD82 could induce F-actin rearrangement either by the immobilized KAI1/CD82 mAb (Delaguillaumie et al., 2002) or in response to EGF stimulation (Lagaudriere et al., 1998). In Du145-Mock cells, actin was extensively polymerized into continuous fibers when cells were spread on both FN- and LM1-coated substrata (Figure 3.2A). The actin fibers distributed in the cell peripheral areas were assembled into cortical actin ring or meshwork, while the ones in cell central areas are the stress fibers (Figure 3.2A). In contrast, the F-actin in Du145-KAI1/CD82 cell was either stained as patches or formed discontinuous fibers (Figure 3.2A), suggesting a defect in actin polymerization. In some cells, F-actin was concentrated in cell periphery to form dense actin bundles (Figure 3.2A), which were found much more frequently in KAI1/CD82-expressing cells than in the Mock cells. Similar abnormality of actin organization was also found in PC3-KAI1/CD82 cells (data not shown).

Figure 3.1 Illustration of the calculation of F-actin intensity quantification and membrane protrusions

For quantitationquantization of F-actin intensity, the cell perimeters were outlined by image J software as described above. Then, based on the fluorescent digital images of α-phalloidin staining, the total cellular F-actin intensity, F-actin intensity inside the inner edge of cortical actin ring, and F-actin intensity of membrane protrusion or outside the inner edge of cortical actin ring were measured using the “analyze” function in Image J software. Theoretically, total cellular F-actin intensity = F-actin intensity inside the inner edge of cortical actin ring + F-actin intensity of membrane protrusion, so F-actin intensity inside the inner edge of cortical actin ring = total cellular F-actin intensity - F-actin intensity of membrane protrusion . Finally, Relative F-actin intensity of membrane protrusion = F-actin intensity of membrane protrusion / total cellular F-actin intensity.

How to quantitate relative F-actin intensity of membrane protrusion?



Total cellular F-actin intensity

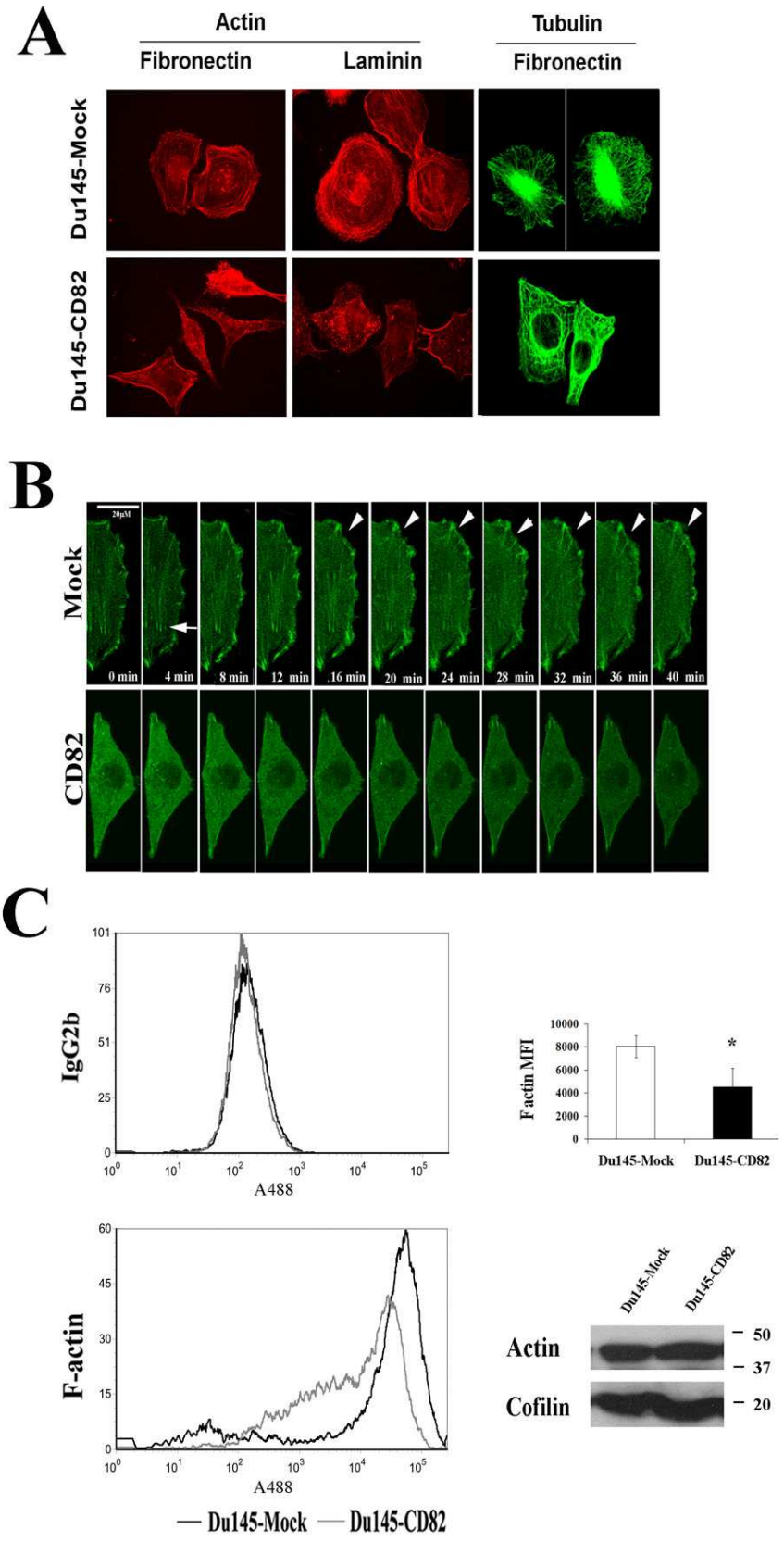
F-actin intensity inside the inner edge of cortical actin ring

F-actin intensity of membrane protrusion = Total cellular F-actin intensity − F-actin intensity inside the inner edge of cortical actin ring

Relative F-actin intensity of membrane protrusion = F-actin intensity of membrane protrusion / Total cellular F-actin intensity

Figure 3.2 KAI1/CD82 regulates the activities of Rho GTPases

A. After being spread on FN (50 μg/ml)- or LM1 (50 μg/ml)-coated coverslips at 37^{o}C, 5% CO_2 for 6 h, Du145 transfectant cells were fixed, permeabilized, and then stained with TRITC-conjugated α-phalloidin. The fluorescent images were captured under an Axiophot fluorescent microscope equipped with an Optronics digital camera at magnification 63X. **B**. Kymography of actin polymerization in Du145-Mock and -KAI1/CD82 cells. The cells were transiently transfected with pEGFP-actin construct. They were spread on FN-coated coverslip in complete DMEM and photographed using time-lapse confocal videomicroscopy. Arrowheads and arrows indicate actin polymerization during the development of mini-protrusion and stress fiber, respectively. **C**. Less F-actin in Du145-KAI1/CD82 cells. Top panel, Du145-Mock and -KAI1/CD82 cells were fixed, permeabilized, incubated with Alexa 488-conjugated phalloidin or IgG2b, and then analyzed using flow cytometry. Bottom panel, Quantization of the mean fluorescence intensity (MFI) of F actin shown in the top panel. Data are expressed as the mean MFI of three independent experiments ($p < 0.05$ between Mock and KAI1/CD82 cells). Right bottom panel, the cell lysates from the experiment were analyzed in Western blot for total cellular actin proteins. Cofilin blot was used as the protein loading control.


A
Actin
Tubulin
Fibronectin
Laminin
Fibronectin
Du145-Mock
Du145-CD82
B
Mock
CD82
0 min
4 min
8 min
12 min
16 min
20 min
24 min
28 min
32 min
36 min
40 min
C
IgG2b
A488
F-actin
A488
Du145-Mock
Du145-CD82
F actin MFI
Du145-Mock
Du145-CD82
Actin
Cofilin
50
37
20

Since microtubules also play an important role in cell movement and morphogenesis (Wittmann and Waterman-Storer, 2001), Ianalyzed the effect of KAI1/CD82 expression on microtubule organization. As showed in Figure 3.2A, no apparent difference was found in microtubule number, length, and distribution between Du145-Mock and -KAI1/CD82 cells, indicating that KAI1/CD82 specifically affects the actin cytoskeleton. In contrast to the pronounced defect in actin cytoskeletal rearrangement, microtubule cytoskeletal structure remained less affected in terms of length, continuity, and number in Du145-CD82 cells, though microtubule organization centers in these cells become less profound than those in Mock cells (Figure 3.2A).

3.4.2 Actin polymerization is impaired upon KAI1/CD82 overexpression

To determine the biochemical nature of the aberrant actin organization in KAI1/CD82-expressing cells, Ianalyzed actin polymerization. Consistent with the F-actin staining at steady state under immunoflurescence microscopy, Ifound that actin polymerization was impaired in Du145-KAI1/CD82 cells under live imaging using GFP-actin as a tracer (Figure 3.2B). Actin polymerization in stress fibers (Figure 3.2B, arrow), cortical ring, and peripheral bundles (Figure 3.2B, arrow head) in the membrane protrusions occurred in Mock cells, but they are significantly attenuated in KAI1/CD82 cells (Figure 3.1B). In KAI1/CD82 cells, actin polymerization could still be found at the end of cellular extensions, and were sometimes accompanied by the formation of large endocytic or exocytic vesicles.

To confirm this observation, Idirectly measured and compared the intracellular quantity of F-actin using fluorescent phalloidin in permeabilized Du145 transfectant cells. As shown in Figure 3.2C, the F-actin level, reflected by the mean fluorescent intensity of intracellular phalloidin staining, was significantly decreased in KAI1/CD82-expressing cells, compared with the one in Mock cells. The levels of total actin protein or G-actin were equal between Du145-Mock and -KAI1/CD82 cells (Figure 3.2C bottom panel).

Thus, the results from the qualitative live imaging and quantitative flow cytometry experiments agree with each other and indicate impaired actin polymerization within KAI1/CD82 cells.

3.4.3 The roles of cell adhesion- and growth factor-signaling in KAI1/CD82-induced morphological and cytoskeletal changes

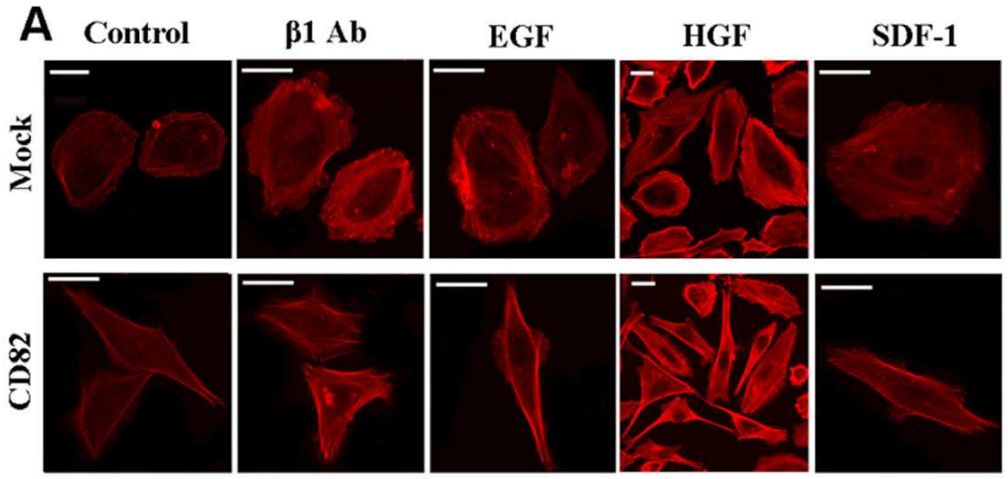
It has been reported that signaling initiated by integrin-ECM adhesion and growth factors is diminished by KAI1/CD82 and these diminutions correlate KAI1/CD82-induced inhibition of cell movement (Dong et al., 1995; Hemler, 2001; Stipp et al., 2003). Inext investigated if increased input of the signaling from cell adhesion, growth factor, and chemokine could reverse KAI1/CD82-induced morphological and cytoskeletal changes. For example, growth factors such as EGF and HGF were reported to promote actin cortical meshwork and stress fiber formation or development (Gohla et al., 1999; Kodama et al., 2000; Liu et al., 2002; Marcoux and Vuori, 2005). By evaluating the quantity and distribution of F-actin in Du145-KAI1/CD82 cells, Ifound that KAI1/CD82-induced

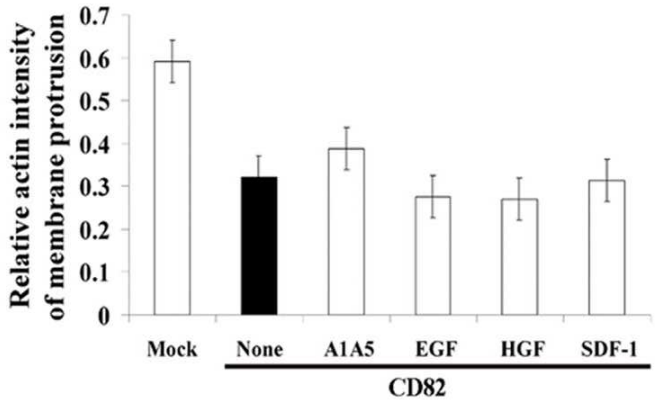
changes such as the loss of actin cortical meshwork or less F-actin in protrusions could not be rescued by the activation of following signaling either alone (Figure 3.3A) or in combination (data not shown): β1 integrins, EGFR, c-Met, and CXCR4. Thus, KAI1/CD82 affects actin reorganization likely through inhibiting either multiple signaling pathways or the signaling step after the convergence point of multiple pathways. To further explore other possibly involved signaling, Iinvestigated the effects of cholesterol remover MβCD and PKC activator PMA on the morphological and cytoskeletal changes induced by KAI1/CD82. Ifound that both reagents could not override the changes induced by KAI1/CD82 (our unpublished data).

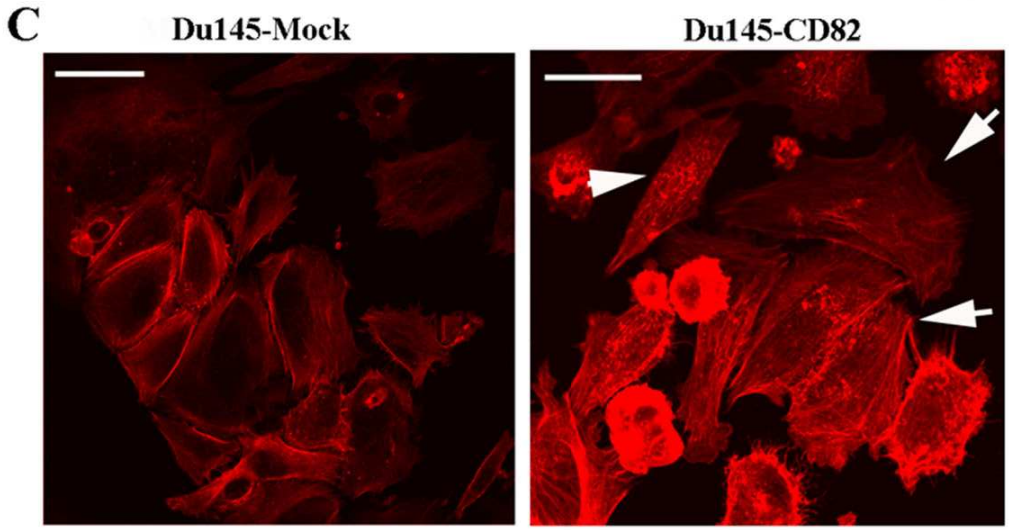
Surprisingly, Irealized that KAI1/CD82-induced morphological and cytoskeletal changes became alleviated when cell-cell contacts were formed (Figure 3.3B). Namely, stress fibers and cortical actin meshwork were partially or largely restored, respectively, in the KAI1/CD82-expressing cells when they were placed in a cell-cell contact microenvironment. This observation suggests that the signaling resulting from cell-cell adhesion acts against KAI1/CD82 signaling by rescuing actin polymerization probably through either the downstream of KAI1/CD82's signaling target or a pathway parallel to KAI1/CD82 signaling. HGF was reported to override the motility-inhibitory effect of KAI1/CD82 in YTS1 bladder cancer cells (Todeschini et al., 2007). However, Idid not observe a similar effect on cell migration for HGF in Du145-KAI1/CD82 cells (Figure 2.2). The method to calculate relative F-actin intensity of the membrane protrusion is illustrated in Figure 3.1.

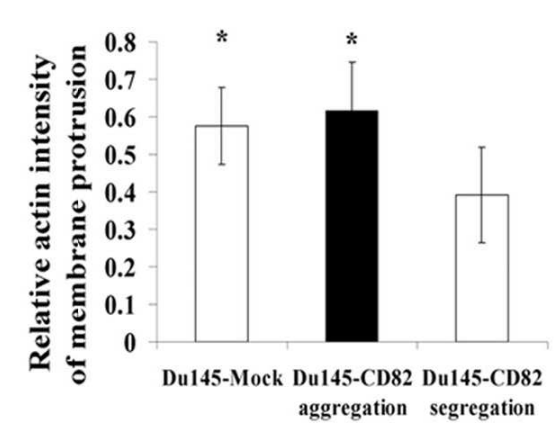
Figure 3.3 Effects of cell-ECM adhesion, cell-cell adhesion, chemokine, and growth factor on KAI1/CD82-induced cytoskeletal changes

A. Du145-Mock or -KAI1/CD82 transfectant cells were spread on FN-coated plates in complete DMEM and treated with integrin β1 activating mAb A1A5 (8 μg/ml), EGF (100 ng/ml), HGF (100 ng/ml), or SDF-1 (100 ng/ml) at 37^{o}C for 4-6 h. Cells were then fixed, permeabilized, and incubated with Texas Red-conjugated α-phalloidin. Digital images were captured under a fluorescent microscope. Scale bar, 20 μM. **B**. Quantitative analysis. The cortical F-actin intensity was quantitated as described in Experimental Procedures. Bars denote the average intensity of 50~95 cells from three individual experiments. The differences between Mock and all CD82 groups are statistically significant (p values < 0.05), while the differences between untreated CD82 and each treated CD82 group are not statistically significant (p values > 0.05). **C**. Effect of cell-cell contact on actin polymerization in Du145-Mock and -KAI1/CD82 cells. Scale bars, 100 μM in Du145-Mock cells and 20 μM in Du145-CD82 cells. Arrows indicate the well-developed actin cortical meshwork seen in the KAI1/CD82-expressing cells with cell-cell contacts. Arrowheads indicate that no well-developed cortical network was found in KAI1/CD82-expressing cells without cell-cell contacts. **D**. Quantitative analysis. The cortical F-actin intensity was quantitated as described in Experimental Procedures. Bars denote the average intensity of 32-40 cells from three individual experiments. The differences between the Mock and the KAI1/CD82 cells without cell-cell contacts and between the KAI1/CD82 cells with and without cell-cell contacts are statistically significant (p values < 0.05), while the differences between the groups of Mock and KAI1/CD82 cells containing cell-cell contacts are not statistically significant (p values > 0.05).


A
Control
β1 Ab
EGF
HGF
SDF-1
Mock
CD82


B
Relative actin intensity of membrane protrusion
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Mock
None
A1A5
EGF
HGF
SDF-1
CD82


C
Du145-Mock
Du145-CD82


D
Relative actin intensity of membrane protrusion
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
Du145-Mock
Du145-CD82 aggregation
Du145-CD82 segregation

3.4.4 KAI1/CD82 regulates Rac1 and RhoA activities

Since a previous study found that Rho GTPases are involved in KAI1/CD82-mediated signaling in T cells (Delaguillaumie et al., 2002) and Rho GTPases are the key regulators of actin cytoskeleton organization (Nobes and Hall, 1995; Nimnual et al., 2003), Ianalyzed the Cdc42, Rac, and Rho activities in Du145-Mock and -KAI1/CD82 cells. By the effector domain pull-down experiments, Ifound that Rac1 activity was significantly downregulated by KAI1/CD82 while RhoA activity was markedly upregulated by KAI1/CD82. Cdc42 activity remained unchanged upon the overexpression of KAI1/CD82 (Figure 3.4).

Since Rac1 and RhoA play crucial roles in actin polymerization and cell migration as described above, Inext determined whether Rac1 activity is essential for KAI1/CD82-induced morphological and cytoskeletal changes, Iexpressed the constitutively activated form of Rac1, i.e., Rac1 L61 mutant, in Du145-KAI1/CD82 cells. Rac1 L61 mutant could not induce the cortical meshwork formation (Figure 3.5A), lamellipodia formation (Figure 3.7D), and cell migration (Figure 3.5C) in Du145-KAI1/CD82 cells. Surprisingly, in Du145-Mock cells, the dominant negative form of Rac1, i.e., Rac1 N17 mutant could not inhibit the formations of cortical meshwork (Figure 3.5A), lamellipodia formation (Figure 3.5A), and cell migration (Figure 3.5C) either. In addition, inhibition of Rac1 activity by a Rac1 specific inhibitor 23766 (Gao et al., 2004) can't suppress the lamillapodia formation of Du145 cells (Figure 3.5B). Thus, Rac1 appears not to be required for lamellipodia formation in Du145 cells. Moreover, the inhibition of

Figure 3.4 KAI1/CD82 regulates the activities of Rho GTPases

Upper panel, KAI1/CD82 inhibits Rac1 activity. Du145-Mock or -KAI1/CD82 transfectant cells were lysed in a lysis buffer containing 1% NP-40 and 0.2% SDS detergents. Cell lysates were subjected to the affinity precipitation using GST-PAK1, which only binds to the activated or GTP-bound Rac. The co-precipitated, GTP-bound Rac GTPase was detected by Rac mAb. The intact cell lysates were blotted with Rac mAb to demonstrate equivalent levels of total Rac proteins between Mock and KAI1/CD82 transfectant cells. Blots show results from a single representative experiment; the graph represents the relative density of the Rac band summarized from four individual experiments (mean ± SD), based on the densitometric analyses. P value between Mock and KAI1/CD82 is < 0.05. *Middle panel,* KAI1/CD82 enhances RhoA activity. Du145 transfectants were pretreated as described above. GTP-bound RhoA was pulled down by GST-Rhotekin and detected by RhoA mAb. The picture shows the results from a single representative experiment; the graph represents the relative density of the RhoA bands summarized from four individual experiments (mean ± SD), based on the densitometric analyses. P value between Mock and KAI1/CD82 is < 0.05. *Lower panel*, KAI1/CD82 does not significantly alter Cdc42 activity. GTP-bound Cdc42 was pulled down by GST-PAK1 and detected by Cdc42 mAb. The picture shows the results from a single representative experiment; the graph represents the relative density of the Cdc42 bands summarized from four individual experiments, based on the densitometric analyses. P value between Mock and KAI1/CD82 is < 0.05. In all experiments, tubulin protein levels in cell lysates were detected via Western blot and served as protein loading controls.

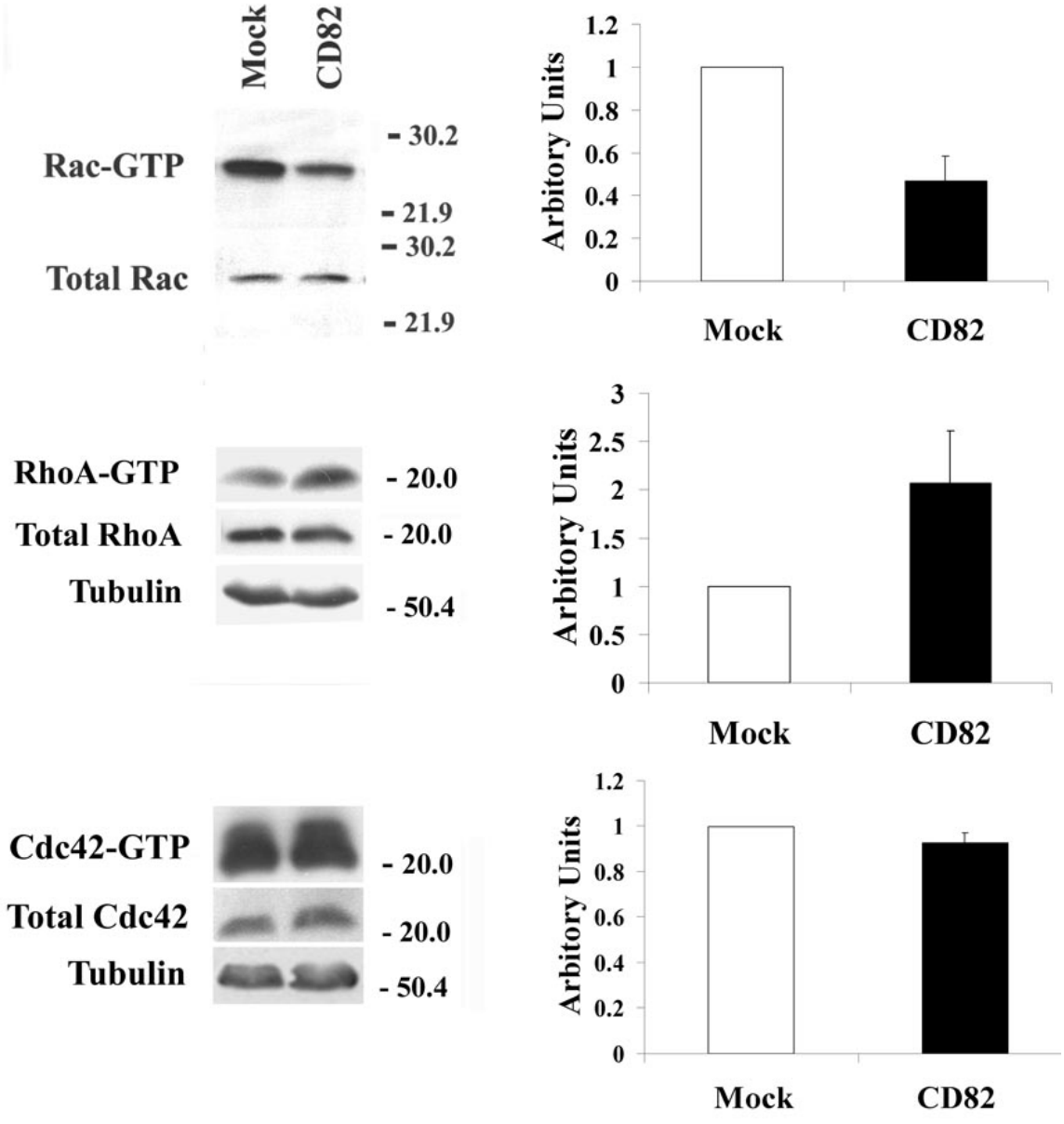
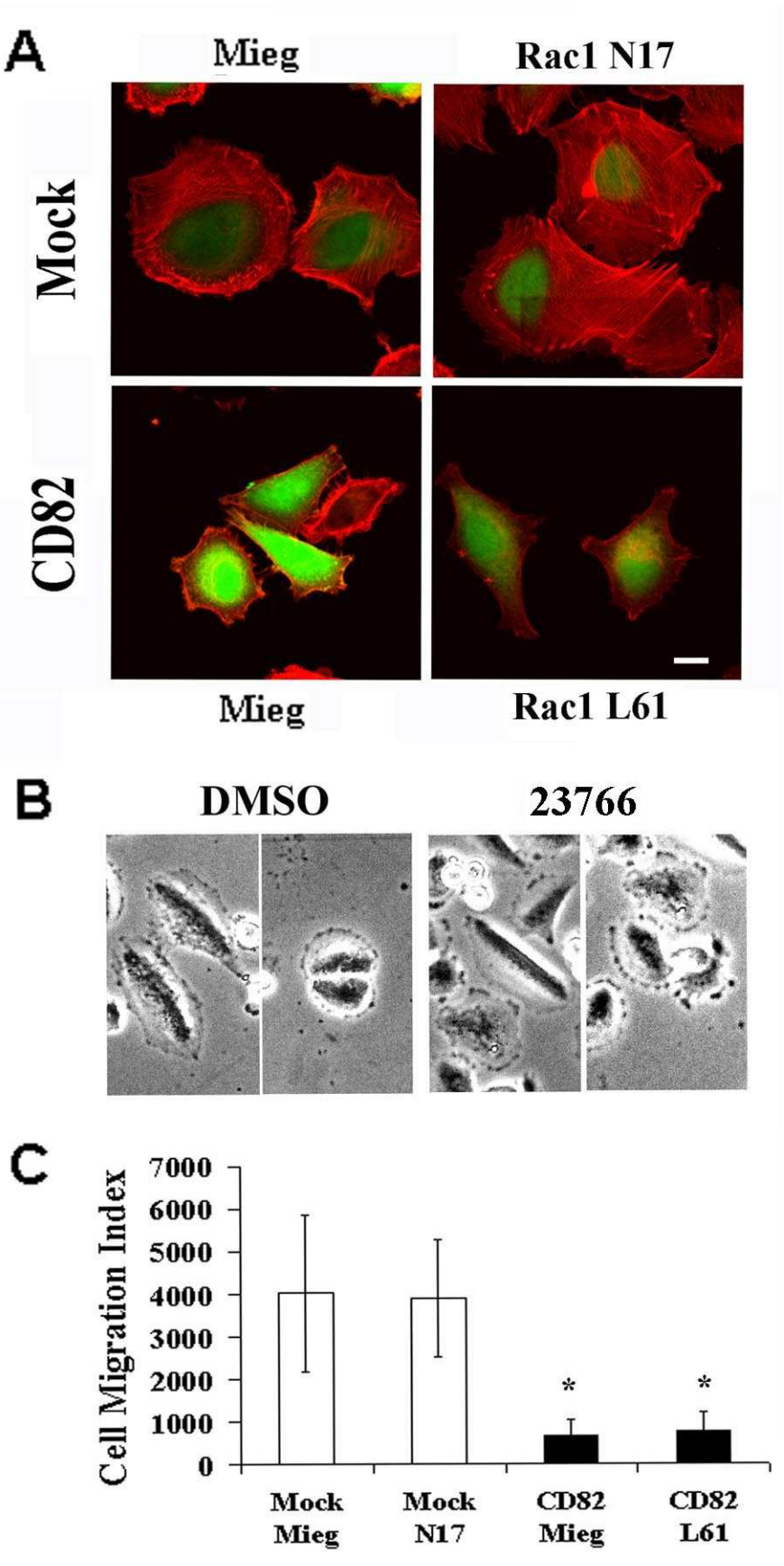

Mock
CD82
Rac-GTP
Total Rac
- 30.2
- 21.9
- 30.2
- 21.9
Arbitory Units
1.2
1
0.8
0.6
0.4
0.2
0
Mock
CD82
RhoA-GTP
Total RhoA
Tubulin
- 20.0
- 20.0
- 50.4
Arbitory Units
3
2.5
2
1.5
1
0.5
0
Mock
CD82
Cdc42-GTP
Total Cdc42
Tubulin
- 20.0
- 20.0
- 50.4
Arbitory Units
1.2
1
0.8
0.6
0.4
0.2
0
Mock
CD82

Figure 3.5 Rac1 cannot rescue KAI1/CD82-altered cellular morphology and actin cytoskeletal organization

A. Du145-Mock cells expressing GFP or Rac1 N17-GFP and Du145-KAI1/CD82 cells expressing GFP or Rac1 L61-GFP were spread on FN-coated coverslips at 37^{o}C, 5% CO_2 for 3-6 min. Cell morphology images were captured using a digital camera at magnification of 60X, and F-actin were stained and photographed as described in Experimental Procedures, Bar = 20 um. **B**. Effect of Rac1 inhibition on lamellipodia formation in Du145 cells. Rac1 specific inhibitor 23766, no-activity control 23766, and DMSO control were add to DMEM media to treat Du145 cell overnight. Cell morphology images were captured using a digital camera at magnification of 60X. **C**. Rac 1 activities in the cells expressing Rac1 N17 or L61 and treated with 23766. (D) Cell migration of Du145 transfectants were measured on FN-coated substratum.


A
Mieg
Rac1 N17
Mock
CD82
Mieg
Rac1 L61
B
DMSO
23766
C
Cell Migration Index
7000
6000
5000
4000
3000
2000
1000
0
*
*
Mock Mieg
Mock N17
CD82 Mieg
CD82 L61

RhoA activity in Du145-KAI1/CD82 cells by C3 toxin cannot rescue KAI1/CD82-induced morphological and cytoskeletal alterations (Figure 3.6).

3.4.5 KAI1/CD82 blocks cofilin translocation

Cofilin is a downstream effector of both Rac and Rho and promotes actin reorganization (Raftopoulou and Hall, 2004). Despite that the levels of total, inactive (phosphorylated), and active (= total - phosphorylated) cofilin proteins remain unchanged in KAI1/CD82-overexpressing cells (Figure 3.7A), cofilin was concentrated in membrane protrusions such as lamellipodia (Figure 3.7B and D, arrow) as well as in nuclei in Du145-Mock cells (Figure 3.7B), resulting in relatively less cofilin within the cytoplasm (Figure 3.7B and D, arrow head). In contrast to Du145-Mock cells, cofilin was distributed across the cytoplasm but not enriched at the cell periphery in Du145-KAI1/CD82 cells (Figure 3.7B). The phosphorylated or inactivated cofilin was present in the perinuclear cytoplasm, and there was no difference in subcellular distribution of phosphorylated or inactivated cofilin between Mock and KAI1/CD82 cells (Figure 3.7C). Thus, although cofilin was still translocated to peripheral cytoplasm in Du145-KAI1/CD82 cells, KAI1/CD82 expression inhibits the translocation of cofilin to or the enrichment of cofilin at the cell periphery, implying a putative mechanism by which Du145-KAI1/CD82 cells fails to form lamellipodia. Moreover, this blockage of cofilin translocation was not overturned by the increased activity of Rac1 (Figure 3.7D), which activates cofilin, promotes cofilin translocation to membrane ruffles, and initiates the lamellipodia formation (Suzuki et al.,1995; Nagaishi et

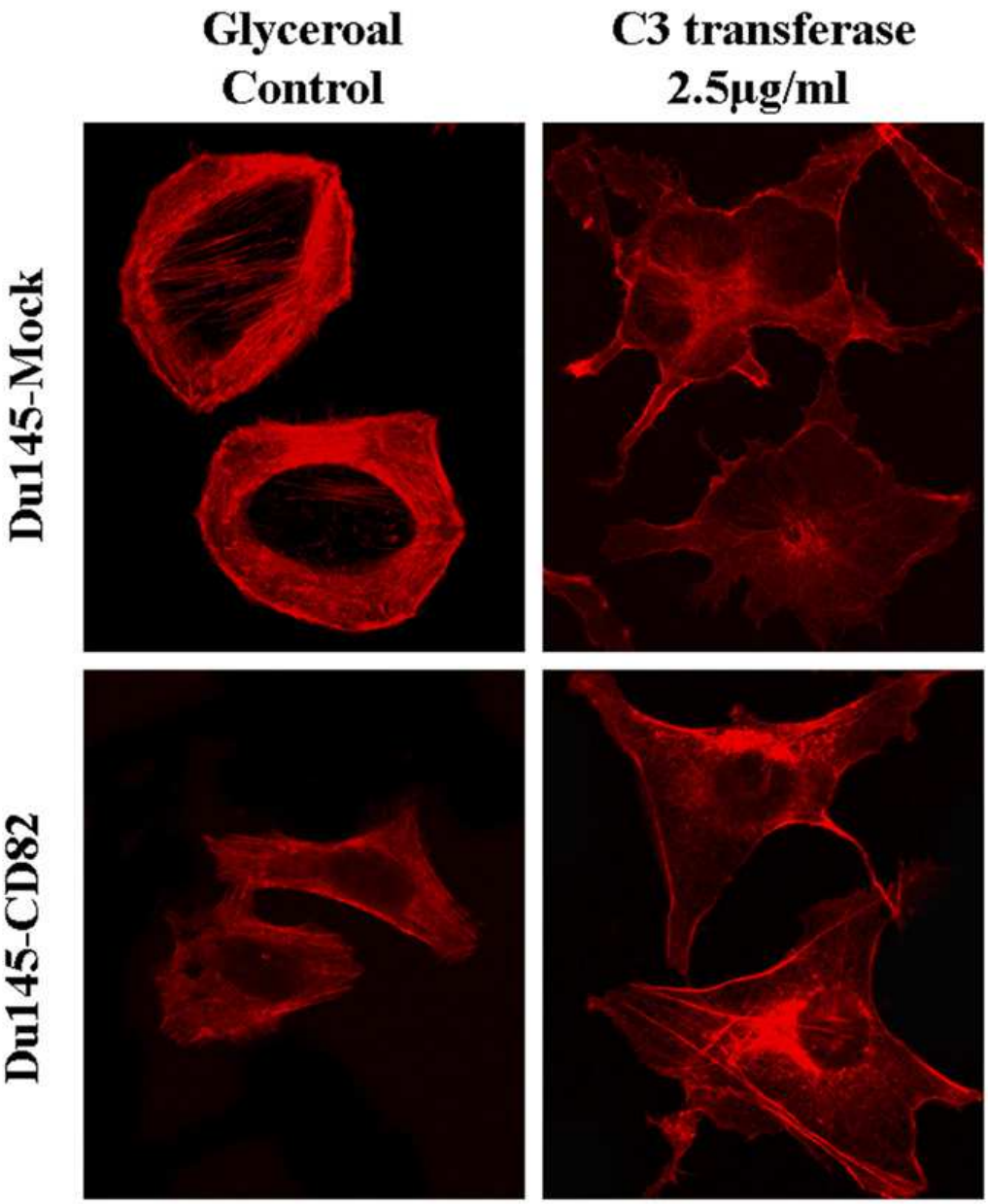
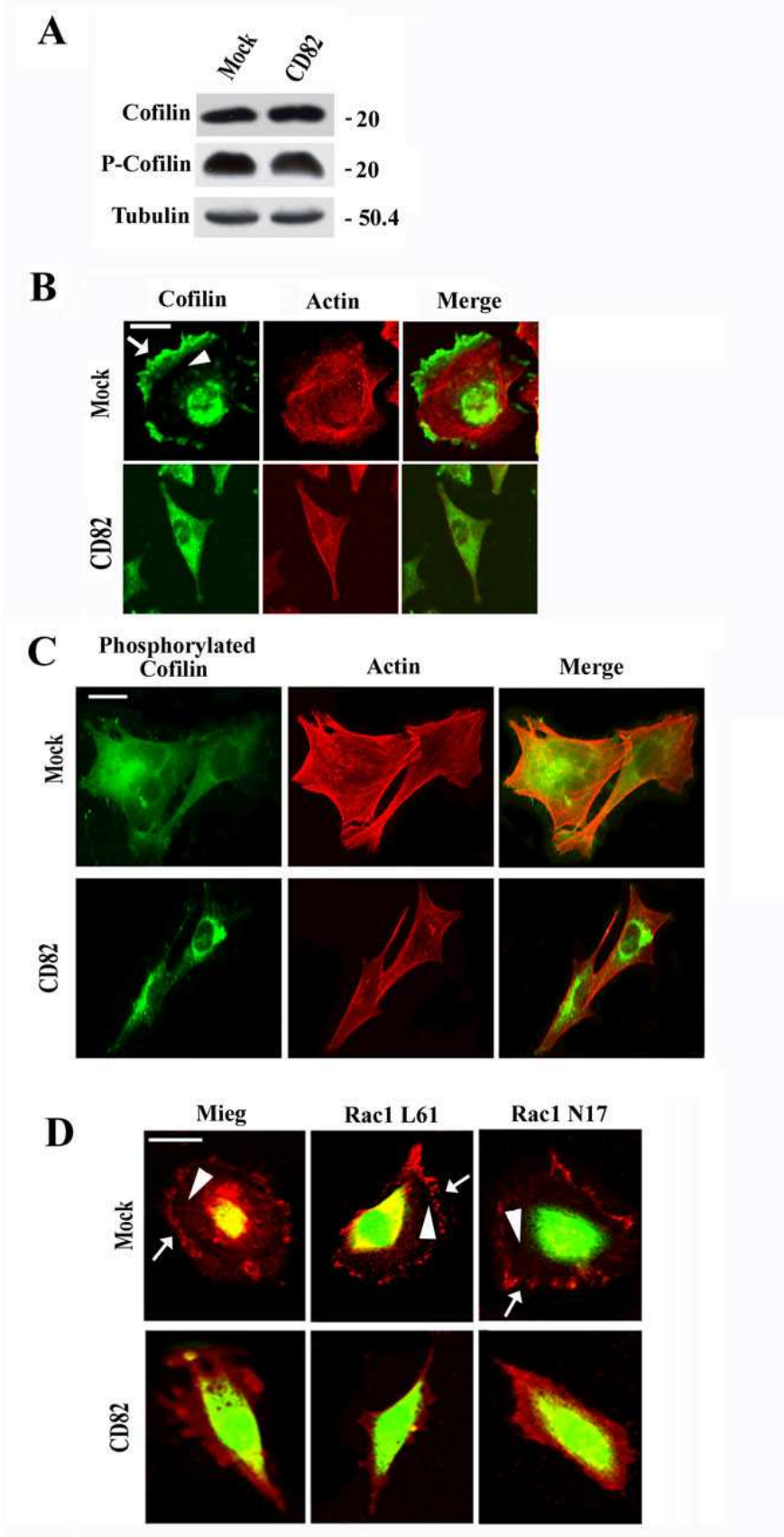


Figure 3.6 RhoA specific inhibtor – C3 transferase's effect on actin reorganization

Du145-Mock and Du145-CD82 cells were spread on 10 µg/ml FN-coated Mak-Tek chamber and treated with either glycerol control or C3 (2.5 ug/ml) for 4-6 hours according to manufacturer's manual. Cells were then fixed with 3% paraformaldehyde, permeabilized by 0.1% Brij 98. F-actin werewas stained with Texas Red-conjugated phalloidin and analyzed using confocal microscopy. Scale bar, 50 µm

Figure 3.7 KAI1/CD82 blocked the translocation of cofilin

A. The levels of total and phosphorylated cofilin proteins in Du145-Mock and -KAI1/CD82 cells were assessed by Western blot using pAbs against cofilin and phosphorylated cofilin, respectively, as described in Experimental Procedures. Tubulin blot is used a control for protein loading. **B**. KAI1/CD82 prevents cofilin from being targeted to the cell periphery. Du145 transfectant cells were spread on FN-coated coverslips in complete DMEM for 3-6 h. The cells were fixed, permeabilized, and incubated with cofilin pAb and TRITC-conjugated α-phalloidin, followed by the FITC-conjugated 2^{nd} Ab staining. Digital images were captured under a confocal microscope and each image represents a single XY section. Arrow indicates the translocation of cofilin into lamellipodia, while arrowhead indicates the relatively transparent zone beneath the actin cortical meshwork and within the cytoplasm. Scale bar, 20 μm. **C**. Comparison of the subcellular distribution of phosphorylated cofilin between Du145-Mock and -KAI1/CD82 cells. The experiment was performed as described in **B** except that the pAb against phosphorylated cofilin was used as the primary Ab. Scale bar, 20 μm. **D**. Du145-Mock cells expressing GFP or Rac1 N17-GFP and Du145-KAI1/CD82 cells expressing GFP or Rac1 L61-GFP were spread on FN-coated coverslips at 37^{o}C, 5% CO_2 for 3 ~ 6 h. Cofilin was visualized using immunofluorescence as described above. The digital images were captured under a confocal microscope. Scale bar, 20 μm.


A
Mock
CD82
Cofilin
- 20
P-Cofilin
- 20
Tubulin
- 50.4
B
Cofilin
Actin
Merge
Mock
CD82
C
Phosphorylated Cofilin
Actin
Merge
Mock
CD82
D
Mieg
Rac1 L61
Rac1 N17
Mock
CD82

al., 1999; Nagata-Ohashi et al., 2004; Verdijk et al., 2004). Neither can the inhibition of Rac1 by dominant negative N17 suppress the translocation of cofilin in Du145-mock cell (Figure 3.7D).

To further elucidate how important cofilin is in the KAI1/CD82 signaling pathway, Ioverexpressed GFP-cofilin into both Du145-Mock and Du145-CD82 transfectants (Figure 3.8). Upon cofilin overexpresion, Du145-Mock cells were still able to form lamellipodia (Figure 3.8 upper panel), whereas Du145-CD82 cells still failed to form membrane protrusion effectively (Figure 3.8 lower panel). Although cofilin still actively participated in membrane protrusion in both Du145-Mock and Du145-CD82 cells, Icouldn't determine whether cofilin is the primary driven force for membrane protrusion formation or not in this spectacular experiment. This is not a surprise to us as overexpression of cofilin may impair the actin cytoskeleton rearrangement and cell migration (Lee et al., 2005) or enhance the same process (Dang et al., 2006).

As Imentioned above in Chapter 1, PIP2 binds to cofilin and restricts its activation. It is thus worthwhile to determine if there are any difference regarding to the sub-cellular localization of PIP2 within Du145-Mock and Du145-CD82 cells, as it might give us some useful hints on why there are less cofilin present in the leading edge of Du145-CD82 cells. As shown in Figure 3.9, PIP2 subcellular distribution, more accurately, the plama membrane PIP2 distribution has not been altered by KAI1/CD82.

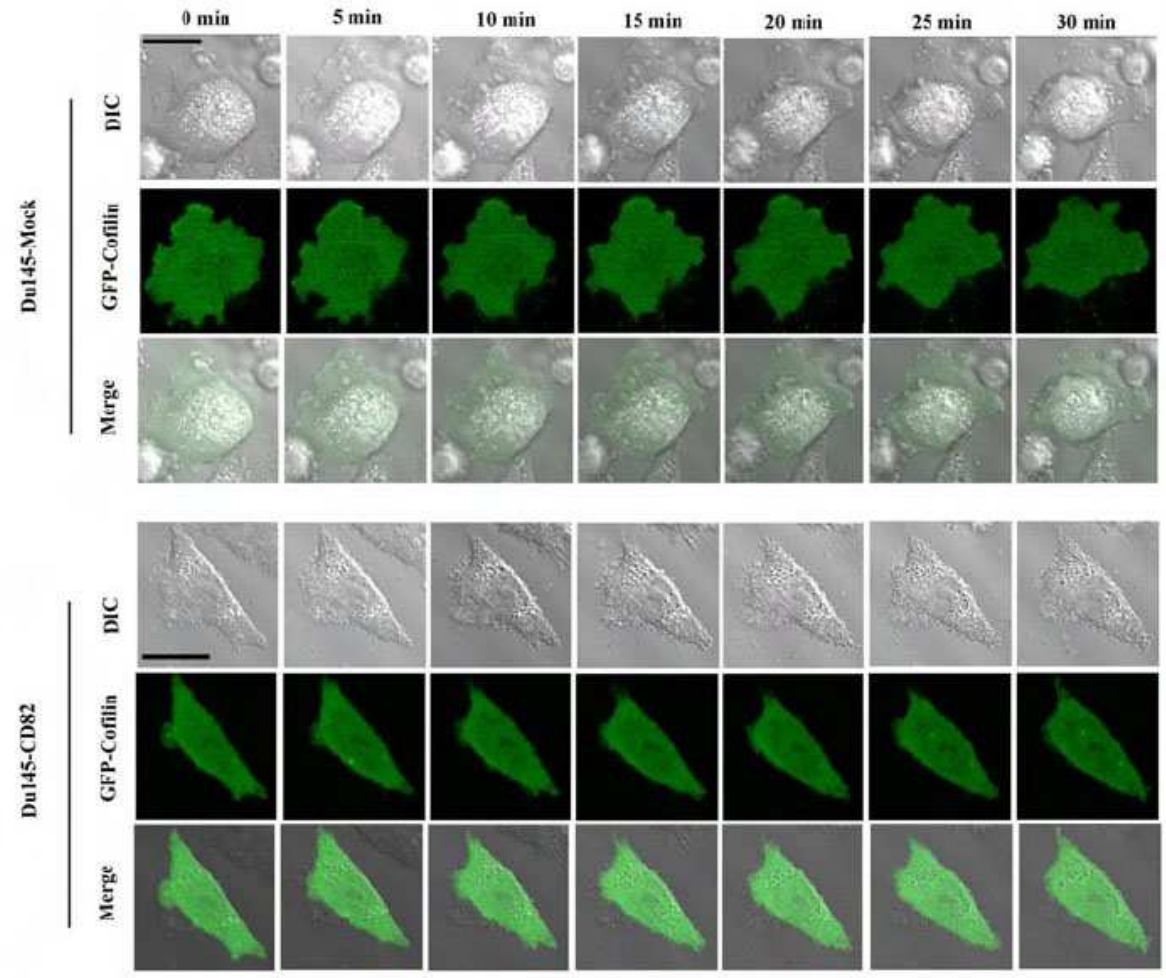
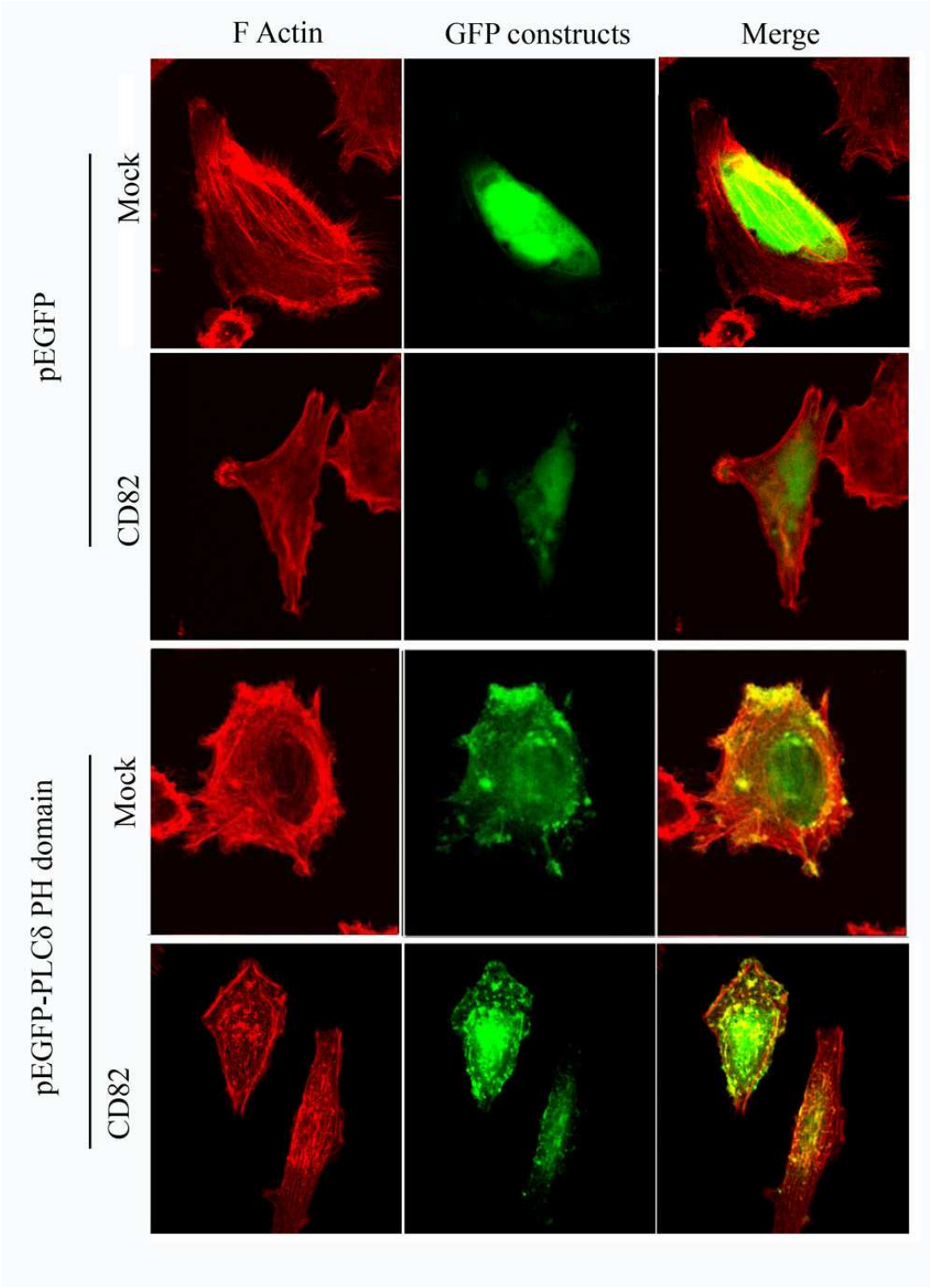


Figure 3.8 GFP-cofilin overexpression cannot overturn the inhibited lamellipodia formation by CD82 in Du145 cells

The pEGFP-Cofilin construct was transiently transfected into Du145-Mock and Du145-CD82 transfectants. 48 h after transfection, the cells were spread on 10 μg/ml FN-coated plates in complete DMEM at 37^{o}C for 4-6 h. Cells were then photographed using time-lapse confocal videomicroscopy for 30 minutes. Representative pictures from 0, 5,10,15,20,25,30 minutes are shown. Green color stands for the expression and localization of GFP-cofilin. Images were taken at a magnification of 60X. Scale bar, 20 μm

Figure 3.9 No alteration in PIP2 level upon KAI1/CD82 expression

The pEGFP and pEGFP-PLCδ PH domain constructs were transiently transfected into Du145-Mock and Du145-CD82 transfectants. At the 48 h after transfection, the cells were spread on 10 μg/ml FN-coated Mak-Tek chamber and analyzed using confocal microscopy. A Z-stack of digital images was captured as described above. Representative images of different transfectants were shown. Green color stands for the expression and localization of either pEGFP or pEGFP-PLCδ PH domain.


F Actin
GFP constructs
Merge
pEGFP
Mock
CD82
pEGFP-PLCδ PH domain
Mock
CD82

3.4.6 Effect of KAI1/CD82 expression on Rac effector Arp2/3

Arp2/3 complex is a downstream effector of Cdc42 and Rac1 and plays pivotal roles in nucleation and polymerization of actin (Pollard and Borisy, 2003), especially for the branched cortical actin network (Mullins et al., 1998) at the leading edge of lamellipodia (Svitkina and Borisy, 1999). By analyzing p34, a component of Arp2/3 complex, Ifound that Arp2/3 complex remains unchanged in both subcellular localization and protein level between Du145-Mock and -KAI1/CD82 cells (Figure 3.10).

3.4.7 KAI1/CD82 inhibiting actin polymerization can't be overturned by N-WASp or jasplakinolide

N-WASp and jasplakinolide are potent actin polymerization inducers. N-WASp promotes actin polymerization via Arp2/3 (Kozma et al., 1995; Nobes and Hall, 1995; Raftopoulou and Hall, 2004). Jasplakinolide is a cyclic peptide isolated from the marine sponge, Jaspis johnstoni. It has been shown to bind to, de novo nucleate, and stabilize actin filaments (Bubb et al., 1994b; Bubb et al., 2000). Neither overexpression of GFP-N-WASp nor treatment of jasplakinolide (50nM), can rescue the defective actin polymerization induced by KAI1/CD82 (Figures 3.11 and 3.12)

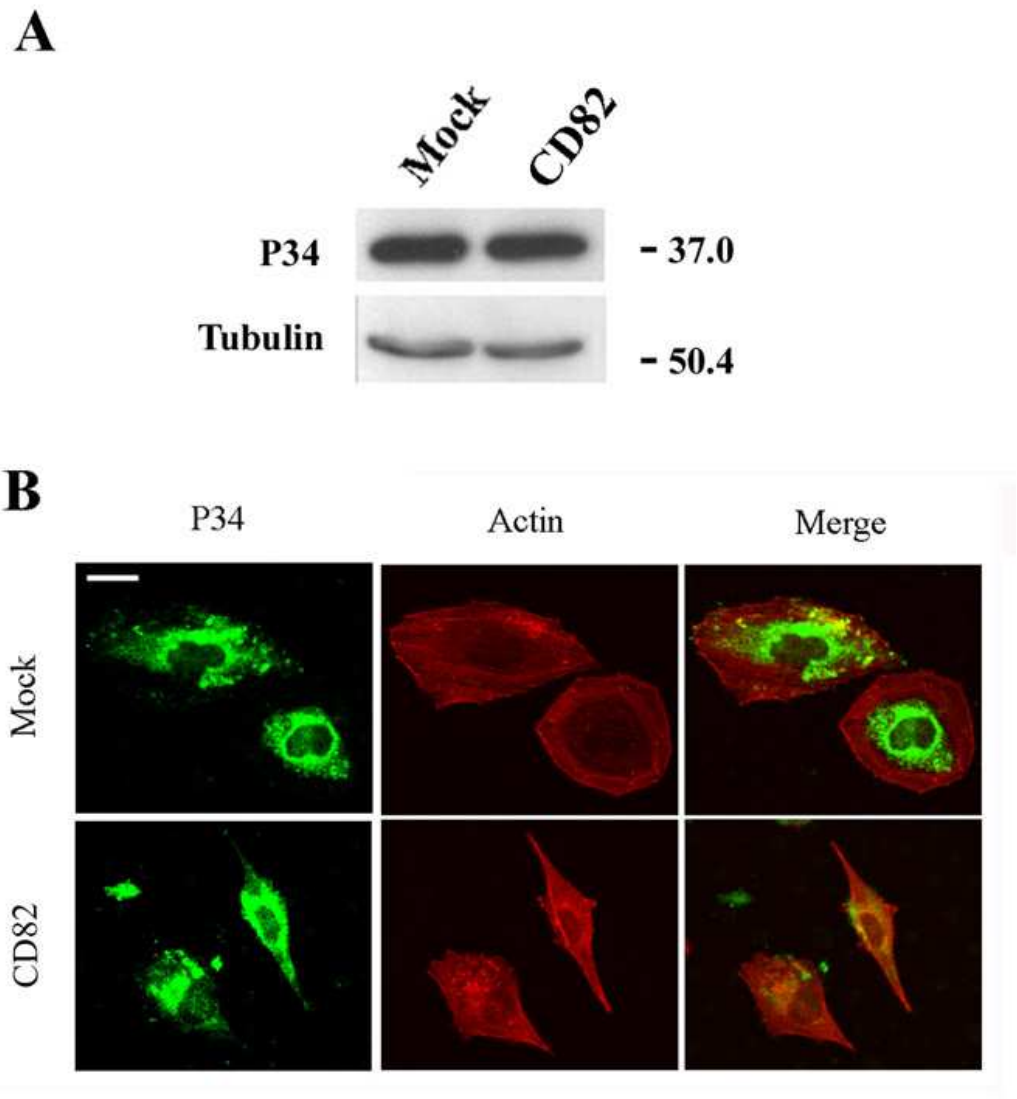
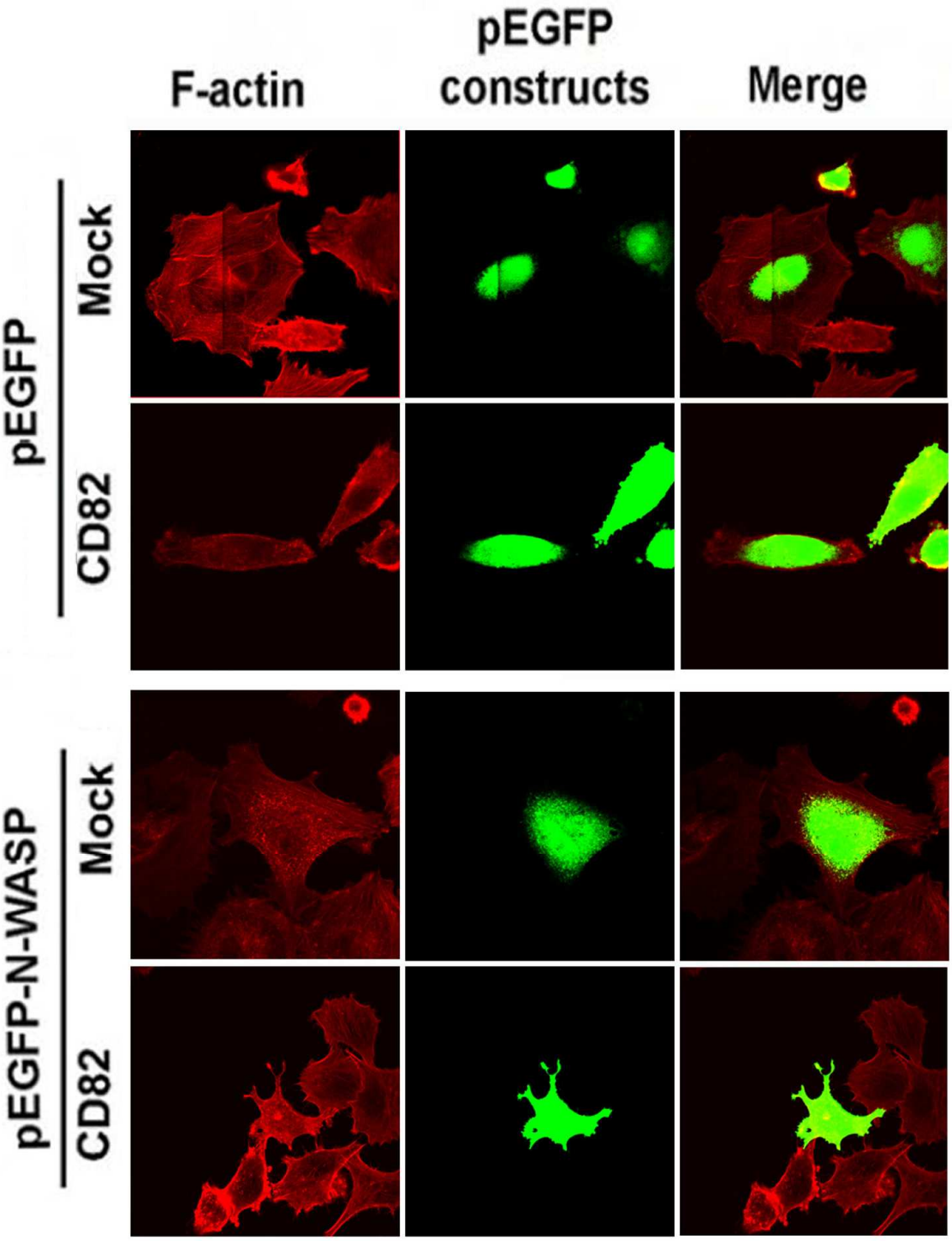


Figure 3.10 KAI1/CD82 doesn't alter the protein level and subcellular localization of the p34 protein in Arp2/3 complex

A. The level of p34 proteins in the Arp2/3 complex in Du145-Mock and -KAI1/CD82 cells was assessed by Western blot using pAbs against p34 protein as described in Experimental Procedures. Tubulin blot is used a control for protein loading. **B**. Du145 transfectant cells were spread on FN-coated coverslips in complete DMEM for 3-6 h. The cells were fixed, permeabilized, and incubated with p34 pAb and TRITC-conjugated α-phalloidin, followed by the FITC-conjugated 2nd Ab staining. Digital images were captured under a confocal microscope, and each image represents a single XY section. Scale bar, 20 μm.

Figure 3.11 Effect of N-Wasp on actin polymerization in Du145 transfectants

The pEGFP and pEGFP-N-WASp constructs were transiently transfected into Du145-Mock and Du145-CD82 transfectants. At the 48 h after transfection, the cells were spread on 10 μg/ml FN-coated Mak-Tek chamber. Cells were then fixed. F actin was stained with Rhodamine-labeled Phalloidin, and analyzed using confocal microscopy. Representative images of different transfectants were shown. Green color stands for the expression and localization of either pEGFP or pEGFP-N-WASp. Images were taken at a magnification of 60X. Scale bar, 50 μm


F-actin
pEGFP constructs
Merge
pEGFP
Mock
CD82
pEGFP-N-WASP
Mock
CD82

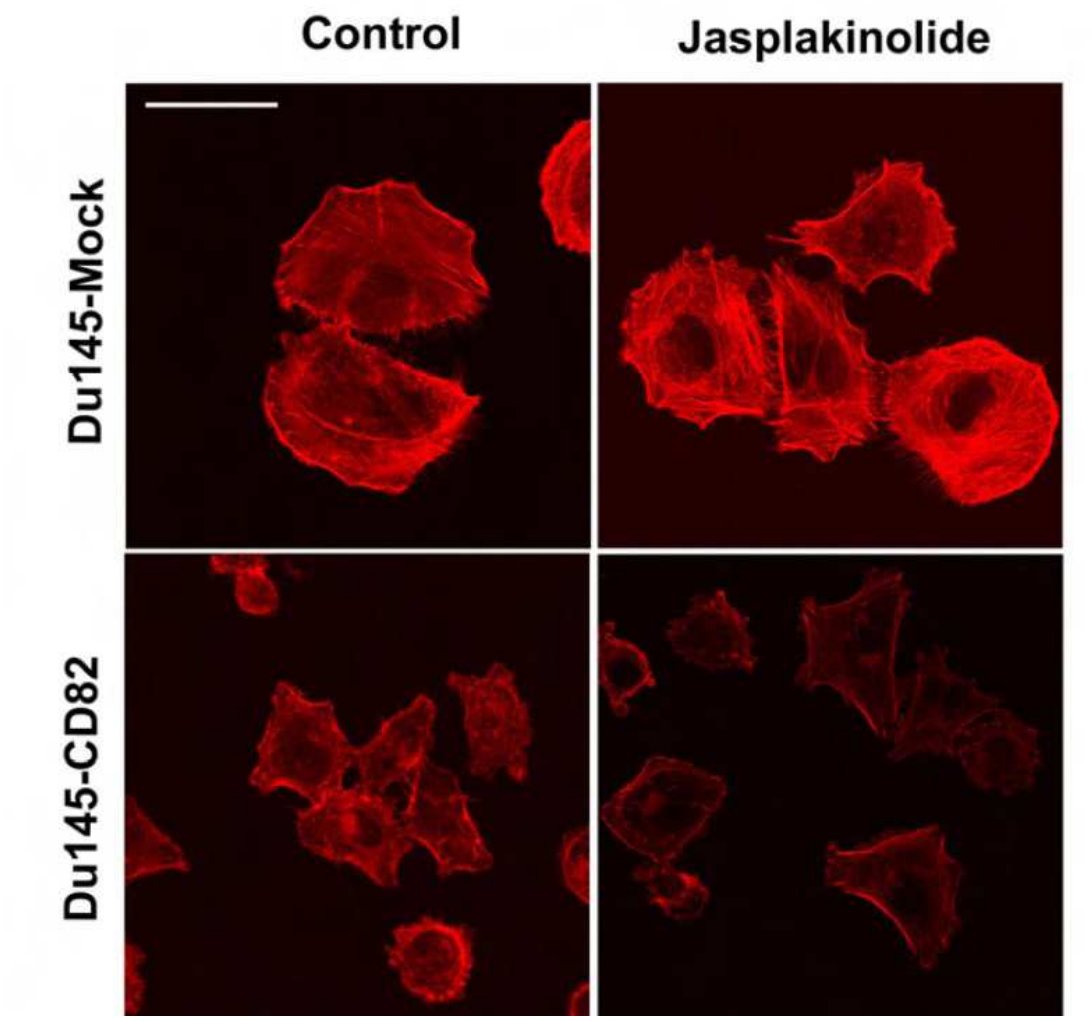


Figure 3.12 Jasplakinolide cannot overturn the actin polymerization defect in Du145-CD82 cells

It has been reported that Jasplakinolide (Molecular probe, J7473) can induce actin polymerization (Bubb et al., 1994a). Jasplakinolide's effect on actin polymerization that was suppressed by CD82 was determined. Du145-Mock and Du145-CD82 transfectants cells were spread on 10 μg/ml FN-coated Mak-Tek chamber and treated with 50nM Jasplakinolide in full DMEM containing 10% FCS for 24 hours, Cell were then fixed, stained with Rhodamine-labeled Phalloidin, and visualized by fluorescence microscopy. Images were taken at a magnification of 60X. Scale bar, 50 μm

3.5 Discussion

3.5.1 Alterations in cellular morphology upon KAI1/CD82 expression reflect the deficiencies in protrusion and retraction - the motility-inhibitory mechanism at the cellular level

KAI1/CD82 expression alters the cellular morphology in various aspects. These alterations can be attributed to both diminished protrusive activities such as the lack of formation of lamellipodia and cellular protrusion and attenuated retraction of cellular extension and trailing edge. Since migrating cells typically display these motility-related morphological events, loss and disruption of these events are therefore predicted to be important for the cell movement-inhibitory activity of KAI1/CD82.

In Du145 prostate cancer cells, KAI1/CD82 expression abolishes the lamellipodia formation on fibronectin-coated, laminin-coated, or regular cell culture flasks. Only on laminin 5-coated substratum KAI1/CD82-expressing cells underwent cell flattening, a symmetric cell spreading process. In PC3 prostate cancer cells, KAI1/CD82 grossly inhibits the lamellipodia formation on fibronectin but partially inhibits it on laminin. Because PC3 cells could form fan-like, well-developed lamellipodia on laminin, KAI1/CD82 more likely inhibits the development or prevailing of fan-like lamellipodia. Of note, the definition of lamellipodia referred herein covers both subdomains of recently designated lamellipodia and lamella (Ponti et al., 2004). Since both Du145 and PC3 cells form lamellipodia during cell migration, the lack or disruption of lamellipodia

formation or development is very likely to be crucial for the motility-inhibitory activity of KAI1/CD82 though some PC3-KAI1/CD82 cells can still form incomplete lamellipodia on laminin.

The presence of elongated cellular extensions is another genuine effect of KAI1/CD82 overexpression in Du145 and PC3 cells. The elongated cellular extensions could be the consequence of excessive protrusive activity, but they could also reflect the retraction deficiency. Our study clearly demonstrated that various cellular extensions in KAI1/CD82-expressing Du145 and PC3 cells are caused by deficiency in retraction processes. Earlier studies showed that the immobilized-KAI1/CD82 mAbs induce profound dendritic extensions in hematopoetic cells (Nojima et al., 1993; Lagaudriere et al., 1998), morphologically reminiscent of some types of cellular extensions seen in KAI1/CD82-expressing Du145 and PC3 cells. Despite the differences between overexpression and Ab engagement and between adherent and suspension cells, these dendritic extensions more likely represent the phenotype of less retraction if the motility-inhibitory activity of KAI1/CD82 is taken into consideration.

MTOC is important for promoting cell polarization at the front edge of a migrating cell (Etienne and Hall, 2002; Ridley et al., 2003; Rodriguez et al., 2003). The diminished MTOC in Du145-CD82 may play a role in indicating a potential mechanism; however, the major regulator of the MTOC, Cdc42 activity, has not been changed by KAI1/CDC42 (Figure 3.4).

KAI1/CD82 not only blocked the cellular protrusion and retraction processes that were induced by the integrin-ECM engagement but also blocked these events stimulated by growth factors or serum (Figure 3.2), For example, KAI1/CD82-caused deficiency in real tail retraction is manifested in the presence of HGF. These observations indicate that KAI1/CD82 inhibits both integrin and growth factor signaling. Another novel observation in this study is that the effect of KAI1/CD82 on these motility-required cellular processes appears to be independent of cell-cell contacts. In other words, KAI1/CD82 can induce these morphological changes without directly engaging a cellular receptor from adjacent cells because those morphological effects are pronounced when cells do not form cell-cell contacts. On the contrary, the cell-cell contacts alleviate KAI1/CD82 effects on the actin cytoskeleton, the foundation of morphological changes.

3.5.2 KAI1/CD82-induced deficiencies in protrusion and retraction processes are caused by the aberrant actin polymerization

The profound morphological changes induced by KAI1/CD82 apparently result from the aberrant organization and/or reorganization of cellular cytoskeleton networks. Actin is the cytoskeletal system that plays a central role in cell movement-related subcellular events including protrusion, traction, and retraction (Horwitz and Parsons, 1999; Parent and Devreotes, 1999; Horwitz and Webb, 2003; Ridley et al., 2003; Van Haastert and Devreotes, 2004; Vicente-Manzanares et al., 2005). As predicted, the actin cytoskeleton becomes

globally aberrant upon KAI1/CD82 expression. The lack of or defect in cortical meshwork and stress fibers is evident in KAI1/CD82-expressing cells, suggesting the aberrancy in actin polymerization. Indeed, KAI1/CD82 overexpression in Du145 cells results in the attenuation of actin polymerization, which is likely caused by the disregulated activities and/or localization of Rac, Rho, and their effectors.

The development of the cortical actin network generates protrusive force, morphologically evidenced by lamellipodia formation (Ridley et al., 2003). In addition, actin reorganization drives the extension of lamellipodia at the leading edge and creates the traction force (Ridley et al., 2003). Cell movement needs both protrusion and traction forces (Ridley et al., 2003). Therefore, the formation of the actin cortical meshwork and simultaneous extension of leading lamellipodia are the major subcellular morphological features of many migrating cells. In KAI1/CD82-overexpressing Du145 cells, the losses of these morphological characteristics are apparently caused by the aberrant actin polymerization, particularly the polymerization of the branched actin network.

In parallel, the retraction process is also essential for the movement of many cells (Ridley et al., 2003). The defects in retraction upon KAI1/CD82 expression are displayed either as elongated trailing tails when Du145-KAI1/CD82 cells were treated with HGF, or as long cellular extensions in cells with bipolar-or dumbbell-shape, or simply as persistent vertices in cells with polygonal shape. Mechanistically, the deficiency in retraction could result from either functional and/or structural abnormality of actin-myosin retraction machinery. For example,

loss of ROCK activity will cause functional incompetence of this machinery while disrupted actin fibers due to the suppressed actin polymerization will make the retraction process lose the structural base in Du145-KAI1/CD82 cells.

3.5.3 Aberrant actin polymerization in KAI1/CD82-expressing cells results from the deregulations of Rac, Rho, and their effectors - the molecular mechanism

Rho small GTPases are the master regulators of actin reorganization. Rac activation stimulates membrane ruffling through polymerization of cortical actin around the cell periphery, while Rho activation stimulates cell contractility through assembly of mainly radial-oriented actin stress fibers (Hall, 1998; Raftopoulou and Hall, 2004). In many cell types, Rac activation negatively regulates Rho activity through generating reactive oxygen species and subsequently activating p190RhoGAP (Nimnual et al., 2003). The delicate balance between the antagonistic activities of Rac and Rho is crucial for proper cell movement and also specifies cell morphology (Sander et al., 1999; Nimnual et al., 2003). The aberrant cytoskeleton reorganization upon KAI1/CD82 expression very likely results from the imbalance of Rho GTPase activities, namely, increased RhoA and decreased Rac1 activities. Iextrapolate that this imbalance ultimately leads to the defect in actin polymerization, aberrant cellular morphology, and diminished cell motility.

Consistent with the current understanding of the roles of Rac in cell morphology and movement, the suppressed lamellipodia and cell movement

caused by KAI1/CD82 expression correlate well with the diminished Rac1 activity in Du145 cells. However, Rac1 activity is, unexpectedly, not required for the lamellipodia formation in Du145 cells and the dominant-active mutant of Rac1 did not enhance the movement of Du145 cells either. Hence, departing from the doctrine, our finding in Du145 cells underlines a Rac1-independent mechanism for lamellipodia formation. In fact, Rac-independent lamellipodia formation has been sporadically reported elsewhere (Spaargaren and Bos, 1999; West et al., 2000), though the underlying mechanism remains obscure. In $Rac1^{-/-}$ mouse embryonic fibroblasts, lamellipodia formation is abolished (Guo et al., 2006; Vidali et al., 2006), but the cells are still able to migrate in a lamellipodia-independent manner (Vidali et al., 2006). Du145 cells were isolated from the brain metastatic lesion of a prostate cancer patient and may not behave like fibroblasts. The mechanism responsible for lamellipodia formation in Du145 cells could vary from the one for fibroblasts. Notably, cell migration appears to be driven by, at least partially, lamellipodia in both PC3 and Du145 cells though lamellipodia are not essential for cell migration in fibroblasts (Vidali et al., 2006).

As expected, a lower Rac1 activity is accompanied with a higher RhoA activity in Du145 cells expressing KAI1/CD82 because Rac and Rho are mutually antagonistic in a variety of cells (Sander et al., 1999; Caron, 2003; Nimnual et al., 2003). Based on the Bar-Sagi pathway (Nimnual et al., 2003), Ipredict that the decreased Rac1 activity results in the enhanced RhoA activity through a less p190RhoGAP activity at the plasma membrane. If so, KAI1/CD82-expressing Du145 cells may not experience the defect in retraction because an enhanced

RhoA activity typically promotes cellular retraction (Nobes and Hall, 1995; Alblas et al., 2001; Raftopoulou and Hall, 2004).

Meanwhile, an enhanced RhoA activity will promote stress fiber formation, which is apparently attenuated upon KAI1/CD82 expression. Alternatively, the enhanced RhoA activity in KAI1/CD82-expressing Du145 cells plays a compensatory role in reacting to the suppressions of downstream events such as ROCK or cofilin. If so, cells are unable to retract even with the enhanced RhoA activity. More likely, both possibilities coexist in KAI1/CD82-expressing Du145 cells and the elevated RhoA per se is probably a combinatory effect.

The third possibility is that KAI1/CD82 activates RhoA at the leading edge of migrating cells. Since an increased RhoA activity at the leading edge of migrating cells inhibits membrane protrusion, RhoA is suppressed in lamellipodia probably through the local enrichment of Rac1(Raftopoulou and Hall, 2004). Because KAI1/CD82 inhibits membrane protrusive events such as lamellipodia, the upregulation of RhoA by KAI1/CD82 in the leading edge leads to an increased local activity of RhoA that inhibits lamellipodia formaiton. Surprisingly, our observation is not unique. When cells lose stress fibers and focal adhesions, such as cell rounding up by entering mitosis or experimental manipulation, Rho activity are higher instead of lower (Ren et al., 1999; Maddox and Burridge, 2003). According to Rho's role in promoting focal adhesion and stress fiber formation, the logical consequence of round up cell should be a decreasing in Rho activity. Why? The answer may be that the presence of high Rho activity would disassemble stress fibers (Burridge and Wennerberg, 2004)

KAI1/CD82 expression did not alter Cdc42 activity in Du145 cells, in contrast to an earlier report that Cdc42 activity was upregulated during the transient overexpression of KAI1/CD82 (Schoenfeld et al., 2003). The enhanced Cdc42 activity was reported to serve as a mechanism for a mass scale of apoptosis induced by KAI1/CD82 (Schoenfeld et al., 2003). In KAI1/CD82 stable transfectants, apoptosis is apparently not a major phenotype though KAI1/CD82-expressing Du145 or PC3 cells are more prone to apoptosis (our unpublished data). As mentioned above, Ido realize that KAI1/CD82-expressing Du145 cells exhibit more symmetric morphology and are less polarized on laminin 5. Since Cdc42 regulates polarity, one may expect KAI1/CD82 to down-regulate Cdc42 activity. However, Cdc42 is not the sole controller for cell polarity because Cdc42 null fibroblasts still retain polarity. Thus, the connection between Cdc42 and KAI1/CD82-induced morphological effects may not be immediate.

As a key effector of both Rac and Rho, cofilin plays an important role in membrane ruffling (Aizawa et al., 1996) or lamellipodia formation (Chan et al., 2000; Zebda et al., 2000; DesMarais et al., 2004; Ghosh et al., 2004; Raftopoulou and Hall, 2004; Cai et al., 2007) and in cell migration and invasion (Bamburg et al., 1999; Raftopoulou and Hall, 2004; Wang et al., 2006; Cai et al., 2007; Iwasa and Mullins, 2007).

Driven by the activated Rac, LIM kinase (LIMK) phosphorylates or inactivates cofilin. The net effect of PAK-LIM kinase activation is to stabilize actin filaments and filament bundles. This is somewhat paradoxical, as the stabilization of F-actin may slow down the turnover rate of actin filaments, which will at some

extent retard the formation of lamellipodia or ruffles. During cell migration, lamellipodia formation demand active cofilin and rapid actin recycling between polymer and monomer to sustain the actin treadmilling (Blanchoin et al., 2000a; Zebda et al., 2000). How to explain such a discrepancy? One possible explanation would be that cofilin activation and inactivation are under elegant spatial or temporal control. During the active cell migration stage, active cofilin was found more distally in a lamellipodium / ruffle to promote polymerization in cooperation with the Arp2/3 complex at the leading edge of a protrusion.

In contrast to the active cofilin, inactive cofilin phosphorylated by PAK-LIM kinase phosphorylation may be concentrated further back from the leading edge of lamellipodia. This subtle difference may allow cofilin to promote polymerization, synergizing with the Arp2/3 complex at the leading edge of lamellipodia, while simultaneously the inactive confilin distributes further back in the body of the lamellipodium or ruffle to stabilize the newly formed actin filaments within the dendritic actin mesh network (Burridge and Wennerberg, 2004). Consequently, activated cofilin is translocated to the plasma membrane (Suzuki et al., 1995; Nagaishi et al., 1999; Nagata-Ohashi et al., 2004; Verdijk et al., 2004) where it interacts with the actin cytoskeleton and promotes actin cortical meshwork formation, actin recycling, and lamellipodia formation (Raftopoulou and Hall, 2004).

Translocation of cofilin to the plasma membrane is considered as an indicator of the cofilin activation (Suzuki et al., 1995; Nagaishi et al., 1999; Nagata-Ohashi et al., 2004; Verdijk et al., 2004). For example, in T leukemia cells and NIH3T3

cells, phosphorylated cofilin undergoes the translocation to lamellipodia upon activation or dephosphorylation (Verdijk et al., 2004). In resting cells, cofilin is distributed evenly throughout cytosol. Once the cells are activated, cofilin becomes accumulated at the plasma membranes (Suzuki et al., 1995; Nagaishi et al., 1999; Nagata-Ohashi et al., 2004; Verdijk et al., 2004).

Interestingly, the subcellular localizations of total and inactivated cofilin in Du145-Mock and -KAI1/CD82 cells display similar distribution patterns as the ones in migrating and non-migrating fibroblasts cell (Dawe et al., 2003), respectively. The novel observation made from this study is that, upon KAI1/CD82 expression, cofilin is no longer enriched at the cell periphery though it can be translocated to the peripheral cytoplasm. This observation strongly suggests that KAI1/CD82 expression block the translocation and therefore activation of cofilin, underlining a putative mechanism by which KAI1/CD82 impairs lamellipodia formation. If so, one would expect more phosphorylated or inactive cofilin in Du145-KAI1/CD82 cells. In fact, the level of phosphorylated cofilin proteins remains unchanged upon KAI1/CD82 expression. This could be explained by the fact that, in Du145-KAI1/CD82 cells, more cofilin dephosphorylation due to the enhanced RhoA activity offsets less cofilin dephosphorylation due to the decreased Rac activity. Alternatively, cofilin underwent dephosphorylation during the translocation to the peripheral cytoplasm in Du145-KAI1/CD82 cells, as it did during the translocation to the cell periphery in Mock cells.

Interestingly, cofilin is still able to translocate to lamellipodia in the Du145 cells expressing dominant-negative Rac1, implying a Rac1-independent translocation mechanism. Surprisingly, the overexpression of GFP-cofilin (Figure 3.8), N-WASP (Figure 3.11) or or treatment of actin polymerization inducer jasplakinolide (Figure 3.12) in Du145-CD82 cells can't rescue KAI1/CD82-caused defects in actin cytoskeleton rearrangements such as cortical ring, stress fiber, and/or lamellipodia.

Another key player in cortical actin polymerization is the Arp2/3 complex, which is localized at the leading edge of the lamellipodia in Xenopus laevis keratocytes and fibroblasts (Svitkina and Borisy, 1999). However, Ididn't observe the expected localization of P34, a component of Arp2/3 complex, in lamellipodia in Du145 prostate cancer cells (Figure 3.10B), probably due to the partial overlapping in subcellular distribution between P34 and Arp2/3, a seven-protein-complex.

3.5.4 KAI1/CD82 intercepts multiple signaling pathways: cell-cell adhesion stands out

KAI1/CD82 can initiate outside-in signaling. For example, KAI1/CD82 engagement by the immobilized KAI1/CD82 mAb in T cells leads to the tyrosine phosphorylations of adaptor protein SLP76 and Rac guanosine exchange factor Vav1(Delaguillaumie et al., 2002) and actin reorganization (Lagaudriere et al., 1998; Delaguillaumie et al., 2002; Delaguillaumie et al., 2004). This signaling may intercept the pro-migration signaling derived from integrin and growth factor

receptor. In parallel, since KAI1/CD82 physically interacts with β1 integrin and EGFR and down-regulates their function, KAI1/CD82 can directly inhibit the pro-migration signaling at the very upstream. If the complexity of TEM constituents is taken into the consideration, there are multiple signaling pathways susceptible for KAI1/CD82 inhibition. Indeed, Ifound in this study that KAI1/CD82-induced morphological and cytoskeletal changes cannot simply be overridden by one or two signaling mechanisms. For example, the signaling originated from β1 integrin (Ridley et al., 2003) ,EGFR (Yarden and Sliwkowski, 2001), C-Met (Matsumoto et al., 1994; Dugina et al., 1995; Guasch et al., 1998; Sridhar and Miranti, 2006; Todeschini et al., 2007), and CXCR4 (Lapidot, 2001; Kayali et al., 2003; Bartolome et al., 2004; Marchesi et al., 2004; Tan et al., 2006) promote cell migration and actin reorganization through Rho small GTPases. Activation of these signaling pathways, however, cannot override KAI1/CD82-exerted signaling and reverse the morphological and cytoskeletal effects. Iunderline this finding as a major advent of the molecular mechanism of KAI1/CD82.

Interestingly and also surprisingly, cell-cell adhesion significantly alleviates the morphological and cytoskeletal effects of KAI1/CD82. Although KAI1/CD82 has been reported to upregulate cell-cell adhesion or aggregation (Lagaudriere-Gesbert et al., 1997b; Shibagaki et al., 1998; Shibagaki et al., 1999; Jee et al., 2003), associate with E-cadherin in colon cancer cell (Lee et al., 2003b), and bind to the counter-receptor DARC, the interplay between KAI1/CD82 and cell-cell adhesion remains to be understood. Likely, cell-cell adhesion corrects, to some degree, the imbalanced Rac and Rho activities in Du145-KAI1/CD82 cells

as cell-cell contacts typically promote Rac but suppress Rho activities. In contrast to the notion that KAI1/CD82 inhibits cell migration by engaging DARC through cell-cell adhesion, it appears that KAI1/CD82 only exerts the cell movement-related morphological and cytoskeletal impacts on cells when cell-cell adhesion falls apart.

In conclusion, Idemonstrated in this study that KAI1/CD82 likely intercepts multiple signaling events and/or common steps of various signaling pathways, resulting in the imbalance of Rac and Rho activities. Consequently, actin organization and reorganization become aberrant. The cytoskeletal changes in KAI1/CD82-expressing cancer cells cause the inhibitions of both cellular protrusion and retraction processes, which ultimately leads to the suppression of cell movement.

It has been reported that CD82 can induce apoptosis through reducing Redox species, and thus raising the ROS (Ono et al., 2000; Schoenfeld et al., 2003). Here, Ifound that CD82 can also inhibit migration through impairing actin cytoskeleton reorganization. Interestingly, disruption of cytoskeleton can trigger and accompany apoptosis (Rao et al., 1999). So far, whether CD82 inhibit cancer cell metastasis and trigger apoptosis through common pathway or different pathway remain unclear. In our study, Idiscovered that CD82 can downregulated Rac1, which also contribute to the reduction of the Redox. Finally, this study on actin cytoskeleton rearrangement will definitely shed lights on how CD82 suppress cancer metastasis.

CHAPTER 4. DISCUSSION

4.1 Summary: how KAI1/CD82 regulates cytoskeleton rearrangement and cell migration at molecular and cellular level

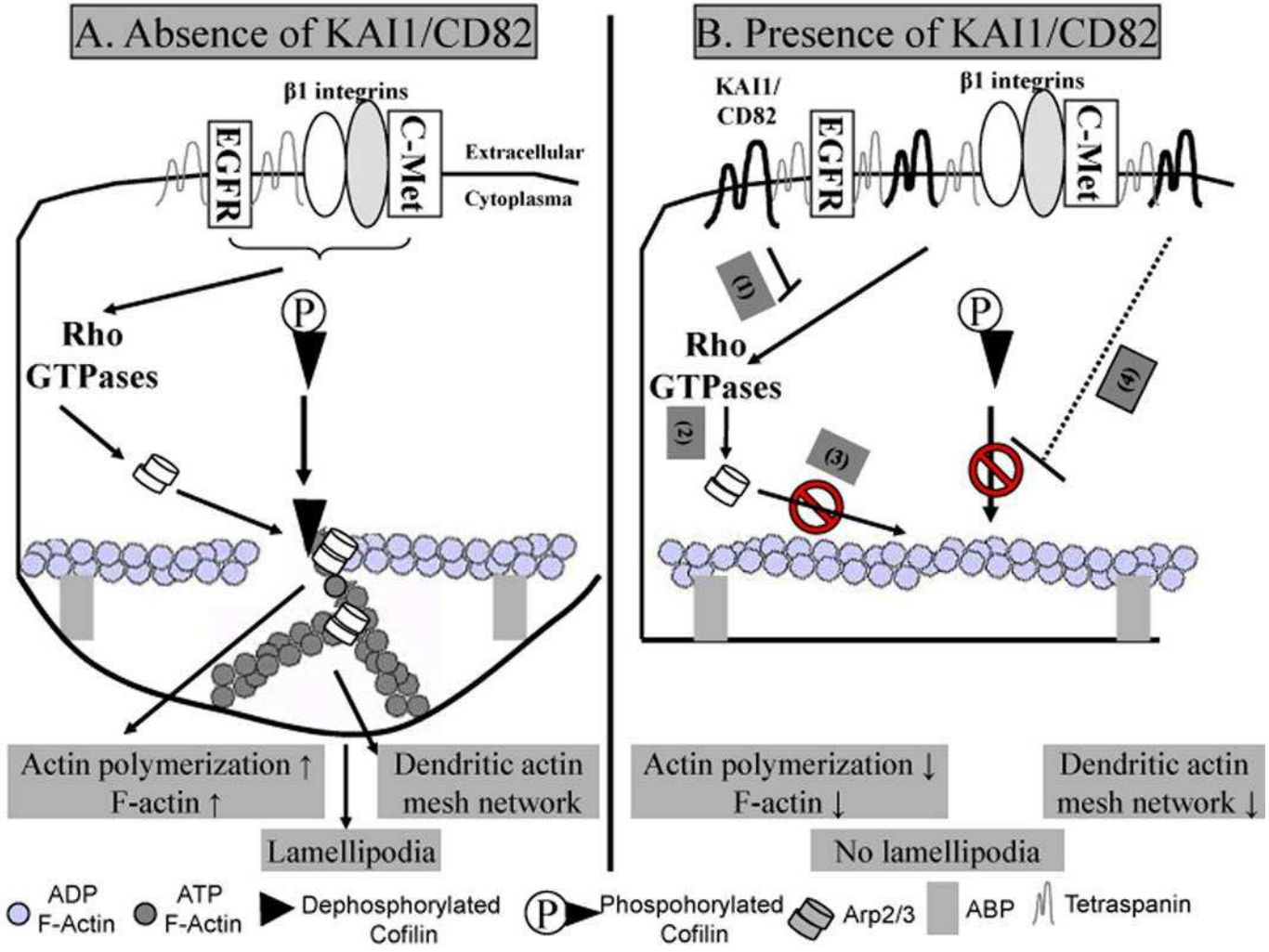
4.1.1 Molecular level: KAI1/CD82 inhibits cell migration by inhibiting actin polymerization and lamellipodia formation

KAI1/CD82 impairs the actin cytoskeleton rearrangement through four possible mechanisms (Figure 4.1):

In the absence of KAI1/CD82, such as cancer cells that lose their KAI1/CD82 expression due to epigenetic events, stimulation of motogenic signaling pathways such as integrin, cMet, and EGFR will activate small RhoGTPases through DOCK/Crk-p130CAS or PI-3K-Akt axis (Hasegawa et al., 1996; Klemke et al., 1998; Buday et al., 2002; Hood and Cheresh, 2002) (Figure 1.5). In turn, as one of the downstream effector of RhoGTPases, cofilin is phosphorylated and stabilizes newly synthesized actin filaments. In addition, activation of those cell surface receptors by stimuli might also couple (Nishita et al., 2005a) or uncouple (Song et al., 2006a) with the the dephosphorylation activation of cofilin. Cofilin then undergoes translocation to the plasma membrane at the leading edge of migrating cells to sever actin filaments (Suzuki et al., 1995; Nagaishi et al., 1999; Nagata-Ohashi et al., 2004; Verdijk et al., 2004). The consequence of cofilin activation is to generate more barbed ends fo actin filaments (Bamburg et al., 1999; Condeelis et al., 2001; Ono, 2003; Pollard and Borisy, 2003; Wang et al., 2007). On the other hand, small GTPase activation will also activate Arp2/3 (Raftopoulou and Hall, 2004). The synergic effects of Arp2/3 and cofilin

Figure 4.1 Summarization of how KAI1/CD82 inhibits cell migration at molecular level

Detail of how KAI1/CD82 impairs actin cytoskeleton rearrangement by inhibiting actin polymerization and lamellipodia formation. **A**. In the abesenceabsence of KAI1/CD82, as in cancer cells which lose their KAI1/CD82 expression due to epigenetic events, multiple pro-migration signalingssignals including integrin, cMet, and EGFR will activate small RhoGTPases. In addition, those stimuli might also promote the dephosphorylation and thus activation of the cofilin. Cofilin then undergoes translocation beneath the plasma membrane to sever actin filaments to generate more barbed ends. Small GTPases then activate Arp2/3. Subsequentially Arp2/3 and active cofilin work synergistically to promote actin polymerization and in turn form those pro-migration morphology such as dendritic actin mesh network and lamellipiodia. **B**. The presence of KAI1/CD82 will inhibit multiple signaling pathways: (1).Accelerate the internalization of cell surface pro-adhesiveness and pro-migration proteins, such as integrin and EGFR; (2). Inhibit Rac1 activity and disrupt the balance among individual small RhoGTPases, (3) inhibit the activation of Arp2/3 and thus actin polymerization and generates less F actin fiber, less dendritic actin mesh network and less or no lamellipodia; (4). Inhibit cofilin translocation to membrane protrusion and lamellipodia.

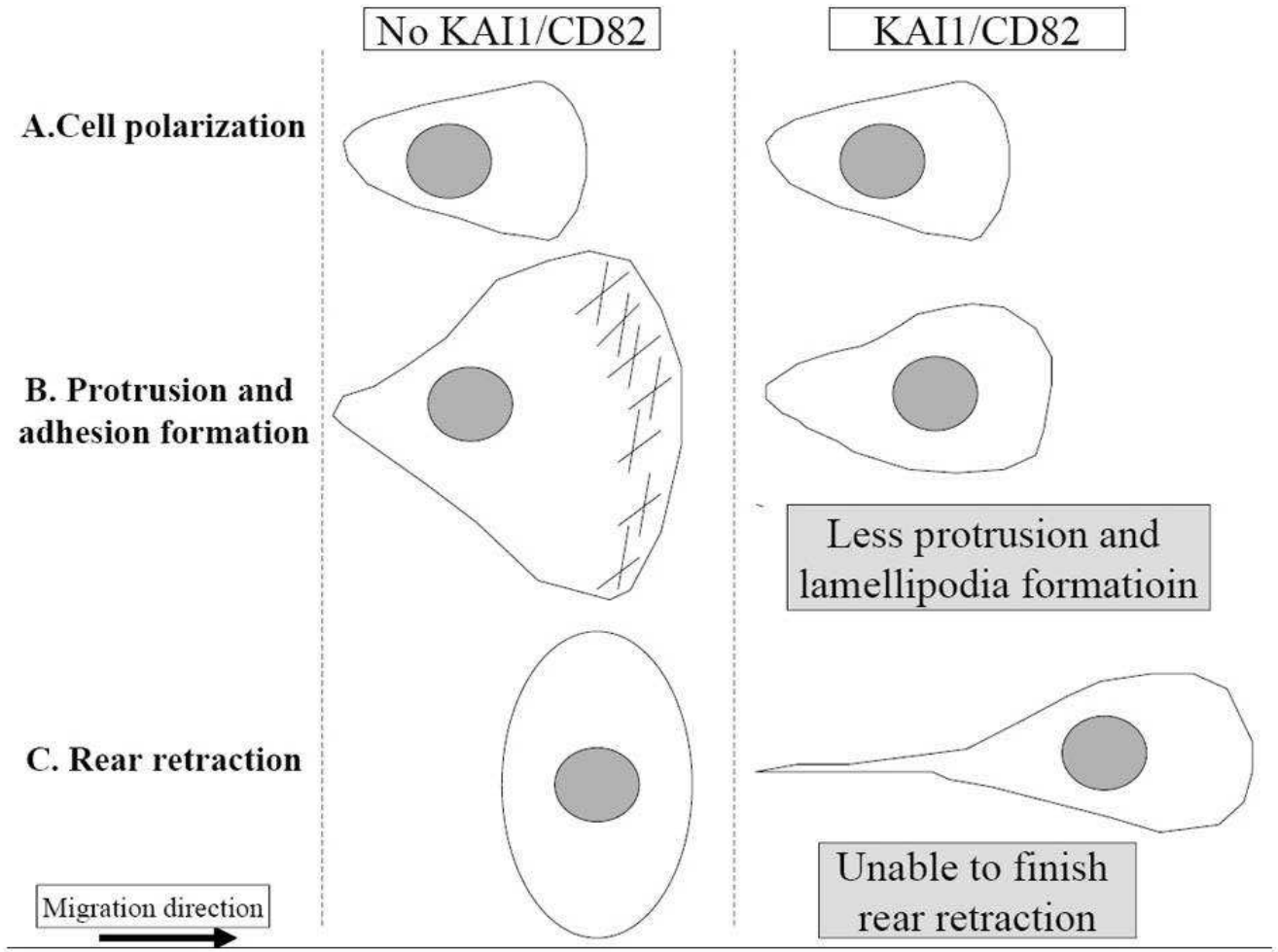

A. Absence of KAI1/CD82
β1 integrins
EGFR
C-Met
Extracellular
Cytoplasma
Rho
GTPases
P
Actin polymerization ↑
F-actin ↑
Dendritic actin
mesh network
Lamellipodia
B. Presence of KAI1/CD82
KAI1/
CD82
β1 integrins
EGFR
C-Met
(1)
Rho
GTPases
P
(2)
(3)
(4)
Actin polymerization ↓
F-actin ↓
Dendritic actin
mesh network ↓
No lamellipodia
ADP
F-Actin
ATP
F-Actin
Dephosphorylated
Cofilin
P
Phospohorylated
Cofilin
Arp2/3
ABP
Tetraspanin

activations are to induce actin polymerization, increase F-actin level, and consequently promote pro-migration events such as the formation of dendritic actin mesh network and lamellipoidia (Bamburg et al., 1999; Condeelis et al., 2001; Ono, 2003; Pollard and Borisy, 2003; Wang et al., 2007) (Figure 4.1A).

In the presence of KAI1/CD82, multiple signaling pathways crucial for actin cytoskeletal rearrangement and cell migration are interrupted (Figure 4.2) (Liu and Zhang, 2006): First, KAI1/CD82 can accelerate the internalization of cell surface pro-adhesion and pro-migration proteins, such as integrin and EGFR (Liu and Zhang, 2006), and subsequently attenuate their responses to external and internal stimuli. The outcomes of this attenuation are that cytoskeleton rearrangement is slowed down and cells become less motile; KAI1/CD82 might also directly or indirectly inhibit Rac1 activation and disrupt the delicate balance between individual small RhoGTPases, such as the Rac1 / RhoA ratio (Figure 3.4). Rac1 and RhoA are important for proper initiation and maintenance of cell migration (Etienne and Hall, 2002; Hood and Cheresh, 2002; Ridley et al., 2003; Burridge and Wennerberg, 2004; Raftopoulou and Hall, 2004;). Rac1 promotes actin polymerization and lamellipodia formation, whereas RhoA isRhoA is responsible for stress fiber formation to provide the cell a transient firm attachment on the substratum during membrane protrusion and rear contratilecontractility to finish the final step of cell migration. Rac1 and RhoA activities are under tightly temperaltemporal and spatial control so that Rac1 and RhoA cooperate with each other during cell migration (Etienne and Hall, 2002; Hood and Cheresh, 2002; Ridley et al., 2003; Burridge and Wennerberg; 2004;

Figure 4.2 Summarization of how KAI1/CD82 inhibits cell migration at cellular level

Cell migration contains three distinct steps. They are polarization, protrusion formation and rear retraction. In this dissertation, Ilearned that KAI1/CD82 may intercept all of those three processes, and thus suppress cell migration. During polarization, KAI1/CD82 might disturb the MTOC organization. Although Cdc42 activity remain equal in the present or absent of KAI1/CD82 in Du145 cells, other regulatory mechanisms importantsimportant for cell polarization may be affected by KAI1/CD82 (Also see Figure 1.4). . During protrusion formation, KAI1/CD82 profoundly inhibits the membrane protrusion and lamellipodia formation, probably through disturbing actin cytoskeletal rearrangement as discussed in Figure 5.1. Iconclude that unbalancing Rho GTPases, especially Rac1 / RhoA and blockage of cofilin translocation to leading edge induced by KAI1/CD82, account for those defects. During rear retraction, KAI1/CD82 induces an exceptionally prolonged cell morphology that can be exaggerated by pro-migratory stimuli such as HGF. These defects strongly imply dysfunction of rear retraction during cell migration. RhoA upregulation and Rac1/RhoA unbalancing may be important for this defect.


No KAI1/CD82
KAI1/CD82
A.Cell polarization
B. Protrusion and adhesion formation
Less protrusion and lamellipodia formatioin
C. Rear retraction
Unable to finish rear retraction
Migration direction

Raftopoulou and Hall, 2004). In our study, I found that this balance is disrupted as Rac1 is downregulated and RhoA is upregulated upon overexpression of KAI1/CD82 (Figure 3.4). This imbalance may account for the declined actin polymerization, dendritic actin cortical mesh network formation, lamellipoidia formation and cell motility (Figure 4.1B).

As downstream effectors of small Rho GTPases, Arp2/3 and cofilin might also be affected in function when KAI1/CD82 overexpression causes the imbalance of Rac1 and RhoA. As described above, the downregulated Arp2/3 and coflilin activities will lead to decreased actin polymerization, less F actin fiber, less dendritic actin mesh network formation and less or no lamellipodia (Figures 2.3, 2.4, 3.2- 3.12); Although MTOC became obscure upon KAI1/CD82 overexpression, RhoGTPase member Cdc42, which is responsible for cell polarization (Ridley et al., 2003), was unchanged. Cofilin translocation to the leading edge of membrane protrusion is important for membrane protrusion or lamellipodia formation (Bamburg et al., 1999; Condeelis et al., 2001; Ono, 2003; Pollard and Borisy, 2003; Wang et al., 2007). In this study, Iobserved an interesting phenominanphenomenon: KAI1/CD82 can block cofilin translocation to the cell periphery (Figure 3.7). Overexpression of cofilin, dominant negative Rac1, or constitutively active Rac1 cannot rescue this inhibitory effect (Figures 3.7, 3.8).

The inhibition of actin cytoskeletal rearrangement by KAI1/CD82 is strong. Overexpression of an Arp2/3 activator, N-WASp, or stimulation with an actin

polymerization inducer, Jasplakinolide, cannot overturn the inhibition by KAI1/CD82 (Figures 3.11, 3.12).

In short, these evidences strongly indicate that KAI1/CD82 inhibits multiple pro-migratory signaling pathways rather than just one or two specific pathways (Liu and Zhang, 2006).

4.1.2 How KAI1/CD82 inhibits cell migration at cellular level

Cell migration can be divided into three distinct steps: polarization, protrusion formation and rear retraction. In this dissertation study, Ilearned that KAI1/CD82 might have impacts on all three steps to suppress cell migration (Figure 4.2). 1). Polarization. In Figure 3.2A, MTOC in Du145-CD82 became vague as compared to a strong present in the Du145-Mock cells. Although Cdc42 activity is not altered by KAI1/CD82 in Du145 cells, other regulatory mechanisms importantsimportant for cell polarization may be affected by KAI1/CD82 (Figure 1.4). 2). Protrusion formation. KAI1/CD82 can profoundly inhibit the membrane protrusion and lamellipodia formation (Figures 3.1, 3.2, 3.3, 3.5, 3.7, 3.8, 3.11, and 3.12). Ipropose that deregulation of Rho GTPases, especially Rac1and RhoA and blockage of cofilin translocation to leading edge account for those defects. 3) Rear retraction. As shown in Figure 3.1 and Figure 3.3A, cells overexpressing KAI1/CD82 exhibit an elongated cell morphology that can be substaintiated by pro-migratory stimuli such as HGF. This defect strongly suggests a malfunction of rear retraction during cell migration. RhoA upregulation may be important for this defect since loss of focal adhesion and stress fiber

usually accompany by the upregulated RhoA activity (Ren et al., 1999; Maddox and Burridge, 2003). In addition, transiently decreased Rho activity may facilitate the transition of actin network from a rigid cortex to a more dynamic actin cortex that allows cell spreading and cell migration (Ren et al., 1999).

4.2 Summary: a historic perspective of KAI1/CD82

Based on the current understanding, a summary of the mechanisms by which KAI1/CD82 inhibits cell migration and cancer invasion is illustrated in Figure 1.2. In the effort to delineate these mechanisms, there are a list of intriguing questions remain to address, and some of them have been long-standing since the first paper about KAI1/CD82 was published 14 year ago. For example, KAI1/CD82 appears to be functionally versatile as shown in Figure 1. If so, is there really a master or major mechanism for KAI1/CD82 to inhibit cell migration and caner invasiveness? Or does KAI1/CD82 inhibit migration and invasion by regulating a specific signaling pathway? If such mechanism(s) indeed exist, can the migration- and invasion-inhibitory mechanism of KAI1/CD82 be applied to its suppression of metastasis? Some early studies found that KAI1/CD82 expression in leukemia and lymphoma cells was actually upregulated (Lebel-Binay et al., 1995; Shibagaki et al., 1998; Shibagaki et al., 1999; Delaguillaumie et al., 2002; Delaguillaumie et al., 2004), which is distinctive from what Ilearned from solid tumors. Does KAI1/CD82 behave differently in solid tumor and hematopoetic malignancy? With the emergence of other metastasis suppressors, are there any mechanistic links between KAI1/CD82 and other cancer metastasis

suppressors such as BRMS1, KISS1, CD44, and NM-23 (Welch et al., 2000)? In contrast to KAI1/CD82, some tetraspanins like CO-029 and CD151 promote cell migration and cancer metastasis. What are the structural bases responsible for the functional differences between KAI1/CD82 and these motility-promoting tetraspanins? Since most of KAI1/CD82-associated proteins are residents in the Tetraspanin-enriched microdomain, what is the role of Tetraspanin-enriched microdomain in KAI1/CD82-mediated suppression of cell migration, cancer invasion and metastasis? Although Ihave focused on the cell-extracellular matrix adhesion mediated by KAI1/CD82-associated integrins, several studies indicated that KAI1/CD82 regulates calcium-independent cell-cell adhesion. How does KAI1/CD82 regulate cell-cell adhesion? Is the altered cell-cell adhesion a part of mechanism for KAI1/CD82's metastasis-suppressive activity? As Idiscussed earlier, KAI1/CD82 likely takes part in development and immune response. Do the physiological functions of KAI1/CD82 share the same mechanism with its suppression of cancer metastasis? Ibelieve the answers to these questions will elaborate the understanding of the nature of KAI1/CD82 as well as the general mechanism of cancer progression.

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VITA

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