Understanding the role of Rudhira in cell migration

A Thesis submitted for the degree of

Master of Science

by

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Declaration

I hereby declare that this thesis entitled '**Understanding the role of Rudhira in cell migration**' is an authentic record of research work carried out by me under the guidance of Professor Maneesha S. Inamdar at Laboratory of Stem Cell Biology and Vascular Biology, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore.

In keeping with the norm of reporting scientific observations, due acknowledgement has been made whenever work described here has been based on the findings of other investigators. Any omission owing to oversight or misjudgement is highly regretted.

Zeenat Diwan

Bangalore

Date:

Certificate

This is to certify that the work described here in this thesis entitled '**Understanding the role of Rudhira in cell migration**' is the result of investigations carried out by **Ms. Zeenat Diwan** in Laboratory of Stem Cell Biology and Vascular Biology, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under my guidance and that the results presented here have not previously formed the basis for the award of any other diploma, degree or fellowship.

Prof. Maneesha S. Inamdar

Bangalore

Date:

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Abbreviations

ICM	Inner cell mass
EB	Embryoid bodies
EC	Endothelial Cell
RNA	Ribonucleic Acid
RNAi	RNA interference
siRNA	small interfering RNA
mRNA	Messenger RNA
RISC	RNA induced silencing complex
ES	Embryonic Stem cell
shRNA	Short Hairpin RNA
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
TALEN	Transcription Activator-Like Effector Nucleases
FAK	Focal Adhesion Kinase
BCAS3	Breast Cancer Amplified Sequence 3
MTA-1	Metastasis Associated Protein-1
Cdc42	Cell Division Control protein 42 homolog
NCBI	National Centre for Biotechnology Information
CDD	Conserved Domain Database
Pfam	Database of Protein Families
KO	Knockout
WT	Wildtype
dpc	Days Post Coitum
PyMT	Polyoma Middle T
CMV	Cytomegalovirus
CPCSEA	Committee for the purpose of Control and Supervision of Experiments on animals
PBS	Phosphate Buffered Saline

ECGS	Endothelial Cell Growth Supplement
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcription-PCR
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
ECL	Enhanced Chemiluminescence
DiI-Ac-LDL	DiI-labelled Acetylated Low Density Lipopolysaccharide
PECAM	Platelet Endothelial Cell Adhesion Molecule
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
CCD	Charge-Coupled Device
eNOS	Endothelial Nitric Oxide Synthase
VE-cadherin	Vascular Endothelial Cadherin
KD	Knockdown
HEK293	Human Embryonic Kidney cells 293
IRES	Internal Ribosome Entry Site
EGFP	Enhanced Green Fluorescent Protein
FACS	Fluorescence-Activated Cell Sorting
NIH	National Institute of Health
FL	Full Length
ECM	Extracellular Matrix
GTPase	Guanosine Triphosphate hydrolase
MTOC	Microtubule-Organizing Center
FA	Focal Adhesion
ROCK	Rho-associated protein kinase
LIMK	LIM domain kinase
ADF	Actin Depolymerizing Factor

WASp	Wiskott–Aldrich Syndrome protein
WAVE	WASP-family Verprolin homologous protein
FBS	Fetal Bovine Serum
DAPI	4',6-diamidino-2-phenylindole
IgG	Immunoglobulin G
SEM	Standard Error of Mean
EB1	End-Binding protein 1

Synopsis of the thesis entitled

Understanding the role of Rudhira in cell migration

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This thesis describes the functional analysis of the cytoskeletal protein Rudhira in cell migration. Rudhira is predominantly expressed in the embryonic vasculature and adult neo-angiogenic processes such as wound healing and pathological processes such as tumor progression. Rudhira is a cytoskeletal protein that plays a key role in directional cell migration, by facilitating actin cytoskeleton reorganization and focal adhesion turnover. Several *in vivo* and *in vitro* model systems such as the fruit fly, mouse, zebrafish and endothelial cell lines are used for characterization of the fine-tuned and complex regulatory networks that regulate blood vessel formation. Two such model systems that are used in our laboratory to study the functions of Rudhira are *rudhira* knockout mice and *rudhira* knockdown endothelial cell lines. I have generated a *rudhira* knockout endothelial cell line, which could serve as a resource and also overcome some of the limitations of the previous models. The inherent difference in the predicted domain architecture of the two halves of Rudhira suggests differences in their molecular functions primarily with respect to cell migration. Thus, using techniques like *in vitro* wound healing assay and immunostaining, I have attributed some of the cell migration steps regulated by Rudhira, including, microtubule stabilization and stress fiber reduction, to the two halves of the protein. Taken together, this study offers fresh insights into the mechanism of regulation of cell migration by Rudhira and may be applicable to better understanding the role of cell migration in blood vessel formation.

Development of the cardiovascular system requires the formation of blood vessels, which occurs by two main processes: vasculogenesis and angiogenesis. Blood vessel formation occurs during embryogenesis as well as in adults, both in physiological conditions like wound healing and menstrual cycle and pathological conditions such as ischemia, retinopathies and tumors. Dysregulation of such a critical process is often associated with vascular diseases or metastatic tumors. The cellular and molecular mechanisms of physiological and pathological angiogenesis are quite similar. Hence, molecules which are expressed in both contexts are of great significance since they would not only provide a better understanding of vascular development, but may prove to be potential therapeutic targets.

Earlier studies from our laboratory have shown that Rudhira is a novel cytoskeletal protein expressed during developmental and adult angiogenesis and in tumors. Rudhira plays a crucial role in directional cell migration. Mouse knockout of *rudhira* is embryonic lethal with severe cardiovascular defects, arising primarily due to a defect in directional endothelial cell migration resulting in aberrant vascular patterning. Rudhira interacts with both the cytoskeletal components, namely microtubules and intermediate filaments. In response to a migratory stimulus Rudhira rapidly relocalizes towards the leading edge, activates Cdc42 and controls both actin reorganization and cell polarity. However, the exact mechanism by which Rudhira mediates cell migration needs further investigation.

Concordant with my first aim, endothelial cell lines were generated from *rudhira* knockout and wildtype embryos using PyMT virus mediated immortalization of endothelial cells. These cell lines could serve as a resource for studying the functions of Rudhira and also overcome some of the shortcomings of the previous models used in the lab. Moreover, since the cells have already undergone all the aberrant processes that occurred from the beginning of development of the embryo till the lethality sets in (as a result of *rudhira* being absent), they would show true pathological phenotype.

Identification of functional domains facilitates understanding of the mechanism of action of the protein. Given that Rudhira regulates cytoskeleton architecture and cell migration, assigning these functions to specific regions of the protein would help in deciphering the details of its molecular functions. Bioinformatics analysis shows that Rudhira contains predicted WD40 domains in its N-terminal half, and a predicted BCAS3 domain and majority of the

phosphorylation sites in the C-terminal half. This inherent difference between the predicted domain architecture of the two halves of the protein was suggestive of differences in their molecular functions, primarily cell migration.

Thus, the second aim of my project was to determine whether the two halves of Rudhira are involved in regulating different aspects of cell migration. *In vitro* wound healing assay showed that both RudA and RudB can independently promote cell migration in non-migratory cells. Interestingly, RudA was able to increase the rate of cell migration considerably more compared to RudB or RudFL, implying an inherent difference between the two halves with respect to their contribution to cell migration. Contrary to this, no difference was observed between the two halves with respect to three of the processes involved in cell migration. Both RudA and RudB could increase microtubule stabilization and decrease stress fibers, and neither could rescue the defect in focal adhesion turnover. However, the effect of the two halves on other aspects of cell migration, such as microtubule alignment, filopodia formation, etc., needs to be determined to conclude whether there is indeed any difference between their molecular functions. Moreover, ascertaining their interaction with the already known interactors of Rudhira will help us in better understanding their role in cell migration.

In summary, we observed a functional difference between the two halves of Rudhira at the cellular level, but there does not seem to be a difference between them at the molecular level. Further investigation into their effects on other features of cell migration would help us in better understanding the function of Rudhira in cell migration.

Understanding the role of Rudhira in cell migration

1. Introduction

The cardiovascular system, comprising heart, blood and blood vessels, is one of the first functional organ systems to develop in the vertebrate embryo. The vasculature has many essential roles during mammalian development – from serving as a "nutrient and waste pipeline" to being a major communication system between distant organs and tissues (Risau and Flamme, *Annu Rev Cell Dev Biol*, 1995). Blood vessel formation is thus a tightly regulated process, and occurs at two distinct locations - extra-embryonic yolk sac and embryo proper (Coultas *et al*., *Nature*, 2005).

The process starts with the onset of mesoderm formation during gastrulation. A subset of mesodermal cells differentiates to form angioblasts (precursors of endothelial cells). The angioblasts fuse together to form a network of simple endothelial tubes known as the primary vascular plexus. This *de novo* process of primitive vasculature formation is known as vasculogenesis (Risau and Flamme, *Annu Rev Cell Dev Biol*, 1995). The primary network of vessels then gets remodelled to form a mature and stable vasculature, comprising veins, arteries and capillaries by the process of angiogenesis (Carmeliet, *Nat Med*, 2000) (Figure 1.1).

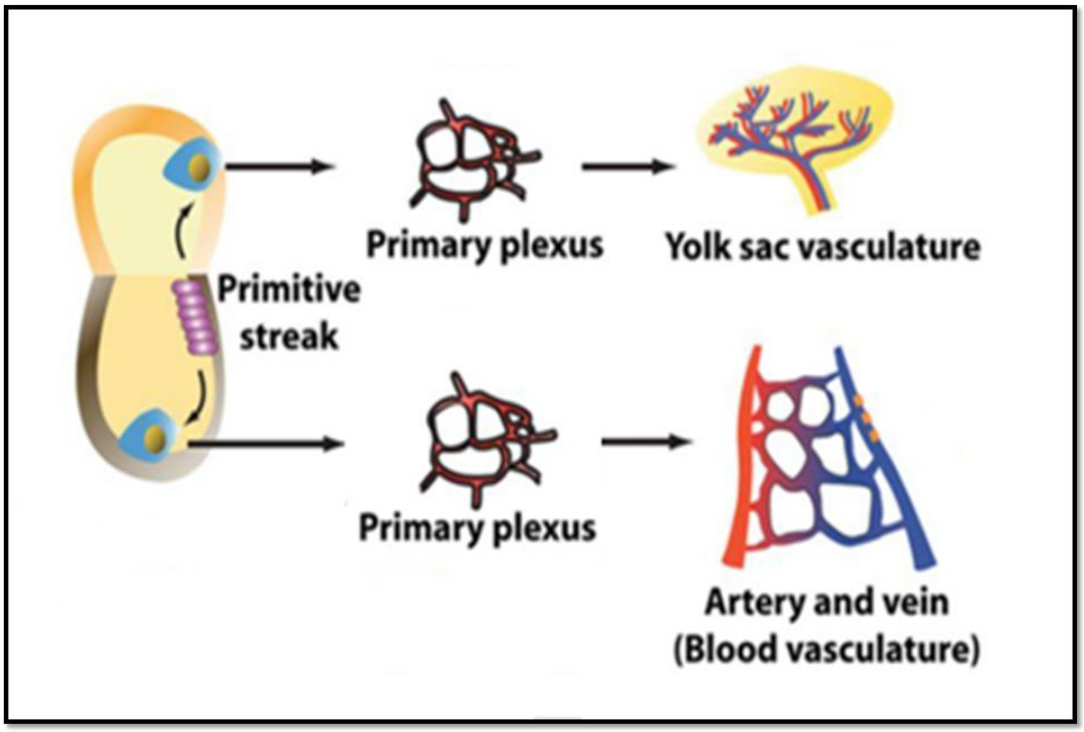

Figure 1.1: Stepwise stages of blood vessel formation *(Adapted from Park et al., Circ. Res, 2013)*

Angiogenesis occurs by sprouting or intussusception.

Sprouting angiogenesis involves growth of new vessels across gaps in the vasculature by proteolytic degradation of the extracellular matrix followed by endothelial cell proliferation, chemotactic migration, lumen formation and functional maturation of the endothelium (Carmeliet, *Nat Med*, 2000) (Figure 1.2).

Intussusceptive angiogenesis involves splitting of pre-existing vessels by joining of the opposing vessel walls, giving rise to two vessels. This enables a vast increase in the number of capillaries without a comparable increase in the number of endothelial cells (Carmeliet and Jain, *Nature*, 2011) (Figure 1.2).

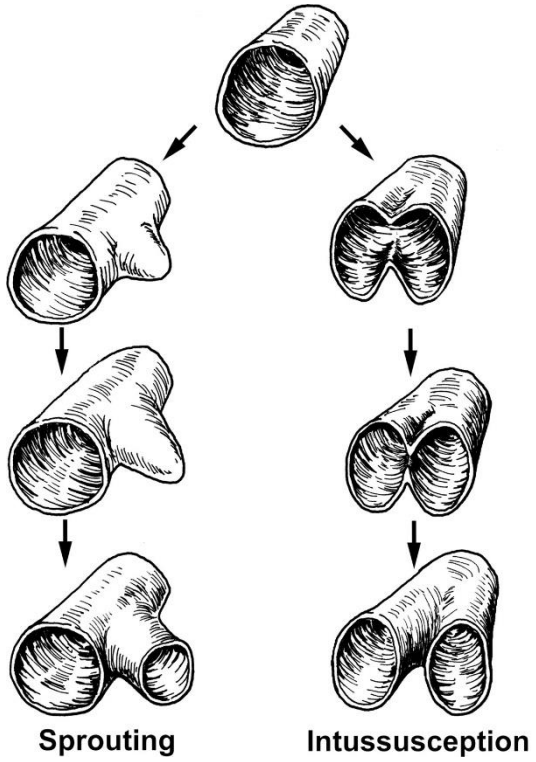

Figure 1.2: The two types of angiogenesis: sprouting and intussusceptive *(Courtesy Barry M et al., J Appl Phys, 2004)*

Blood vessel formation occurs not only during embryogenesis, but also in adults in physiological conditions like wound healing and menstrual cycle (Papetti and Herman, *Am J Physiol Cell Physiol*, 2002), and pathological conditions such as ischemia, retinopathies and tumors (Carmeliet, *Nat Med*, 2003; Chung *et al.*, *Nat Rev Cancer*, 2010). Understanding the complex molecular pathways that establish and maintain the normal vascular network may aid in designing effective therapies to overcome several cardiovascular diseases.

1.1 Model systems to study vascular development

1.1.1 *In vivo* model systems

1.1.1.1 Tumor models – As angiogenesis is crucial to tumor growth, tumors have been used as model systems to study the mechanisms behind aberrant angiogenesis. The visualization of developing vasculature *in vivo* in real time also allows us to test the efficacy of potent pro or anti-angiogenic compounds or peptides in tumor growth and invasion (Goel *et al., Physiol Rev,* 2011; Tanaka *et al., J Oncol,* 2012).

1.1.1.2 Zebrafish – Zebrafish embryo develops outside the mother and is transparent, making it easy to visualize blood vessel development. Studies on angiogenesis using these embryos have gained a lot of attention during the last couple of decades (Weinstein, *Semin Cell Dev Biol,* 2002). Angiogenesis in zebrafish is relatively simpler, primarily occurring in the head and intersomitic vessels (Isogai *et al., Dev Biol,* 2001). Zebrafish has proven to be a cost effective model system for high-throughput drug screening, since it produces a large number of offsprings and also because drugs can diffuse directly into the embryo upon addition in culture media (Taylor *et al., Cell Commun Signal,* 2010).

1.1.1.3 Mouse – With the advancement in transgenic technology, the mammalian model system, mouse, has become an attractive model to study the process of vascular development. Mouse embryos have been commonly used to study the early events of blood vessel formation either by analyzing the embryos by immunohistochemistry using respective vessel markers or by injecting the embryos with India ink or fluorescent dyes (Couffinhal *et al., Front Biosci,* 2009). Further, analyses of several transgenic mouse models harboring mutations in vascular genes have greatly enhanced our understanding of the mechanisms of formation of blood vessel networks (Ribatti, *Int Rev Cell Mol Biol,* 2008).

1.1.2 *In vitro* model systems

1.1.2.1 Embryonic stem cells – Embryonic stem cells are derived from the inner cell mass (ICM) of the blastocyst. These cells can be differentiated to form embryoid bodies (EB), which recapitulate the early events of embryo development and form vascular structures containing functional endothelial cells (Risau *et al., Development,* 1988). ES cells can also be differentiated into vascular, hematopoietic, cardiac and other lineages of interest (Itskovitz-Eldor *et al., Mol Med,* 2000). The ES/EB model has been used to screen potential angiogenic

or angiostatic compounds and to identify factors specifically involved in angioblast differentiation (Evans, *Drug Discov Today Dis Models*, 2008).

1.1.2.2 Endothelial cell lines – Endothelial cells (ECs) are currently used as *in vitro* model systems for various physiological and pathological processes, especially in angiogenesis research. Primary ECs have a limited lifespan and display characteristics that differ from batch to batch due to multidonor origin. Therefore, the need for standardized experimental conditions and reproducible results has increased the demand for immortalized, well-characterized EC lines stably presenting endothelial properties. Over the past decade, immortalized EC lines have been used to study processes like blood-endothelium interactions, vessel ageing and apoptosis in ECs.

1.2 Approaches to analyze gene function

Gene function can be studied utilizing – **Classical genetics:** to perform mutagenesis, identify phenotype of interest and determine its genetic basis; or **Reverse genetics:** to determine the function of a gene by altering its expression in a suitable host and analyzing the phenotype obtained (Argmann *et al., Curr Protoc Mol Biol,* 2006). Although the classical genetics approach is unbiased, generating several mutants using spontaneous or random mutagenesis and then screening them for the gene responsible for such a phenotype is a complex, labor intensive and time consuming process. The alternative approach, using reverse genetics may begin with predicting the function of a gene of interest by homology search with other proteins of known function, and by studying its spatial and temporal expression pattern at the cell, tissue and organism level. However, to precisely determine the function of a gene, one can utilize reverse genetics approaches, such as altering expression of the gene by the various techniques available as discussed:

1.2.1 Gene overexpression

Gain-of-function mutations are often obtained by expressing a gene in much higher amounts than normal in a particular cell. This is achieved by cloning the gene in a multicopy plasmid and driving its expression using a powerful promoter. Overexpression studies have helped in understanding the function of several genes (Helgason *et al., Blood,* 1996; Meng *et al., J Cell Mol Med,* 2012). It is important, however, to supplement the results of over-expression studies with gene silencing or disruption.

1.2.2 Gene knockdown

One of the ways to silence a gene is by reducing its expression to a level far below what is required for its function. This can be achieved by RNA interference (RNAi) technology. RNAi is based on the ability of a double stranded RNA to recognize and degrade its complementary RNA, thereby inhibiting gene expression. In mammals, a small 21bp siRNA (small interfering RNA) cleaved by Dicer is the functional RNA molecule which degrades the target mRNA with the help of RISC complex (RNA induced silencing complex) (Mittal, *Nat Rev Genet,* 2004). Small interfering RNA is a robust and fast way to identify gene function and has been successfully used in cultured mammalian cells, mouse ES cells and human ES cells. However, the major drawback of using siRNA for gene manipulation is the short term effect of silencing. In order to study loss-of-function phenotypes for a longer time, stable suppression of a gene is desired. This is achieved by short hairpin RNA (shRNA) which has been shown to function in a variety of mammalian cell types (Brummelkamp *et al., Science,* 2002).

1.2.3 Gene knockout

Another method to determine gene function is to make the gene completely inactive, a process called gene knockout. With the advances in molecular biology and stem cell biology over many years, it is possible to generate transgenic mice harboring the desired gene manipulation, using a variety of techniques, such as zinc finger nucleases, CRISPR/Cas9, TALENs, etc. One such method involves homologous recombination in embryonic stem (ES) cells. In this technique, the gene to be mutated is cloned in a suitable vector containing a selectable marker flanked by homologous sequences of the gene. The construct is then transfected into ES cells, whereby the transgene gets inserted in a specific location of the genome by homologous recombination. The positive clones are selected by antibiotic resistance and microinjected into blastocysts. The manipulated blastocyst is then implanted in a surrogate mother and the pups are screened for germline transmission by breeding for several generations (Longenecker and Kulkarni, *Curr Protoc Cell Biol,* 2009).

The Cre-loxP recombination system is generally used for generating global or tissue specific knock-out mice. It involves generating two genetically engineered mice which are intercrossed to produce the desired knock-out in the offspring. The first mouse contains the loxP sites flanking the target gene and the second one has the conventional Cre recombinase driven by tissue specific promoters. The gene would be excised out from the tissues where

Cre is active. A loxP site is a 34 bp site consisting of 8 bp core region flanked by 13 bp of palindromic sequences. Cre is a cyclization recombination enzyme encoded by bacteriophage P1. Cre activity causes deletion of the sequence flanked by the two loxP sites when the sites are placed in the same orientation, and causes an inversion when they are placed in opposite orientation (Wachsman and Heidstra, *Methods Mol Biol*, 2010) (Figure 1.3).

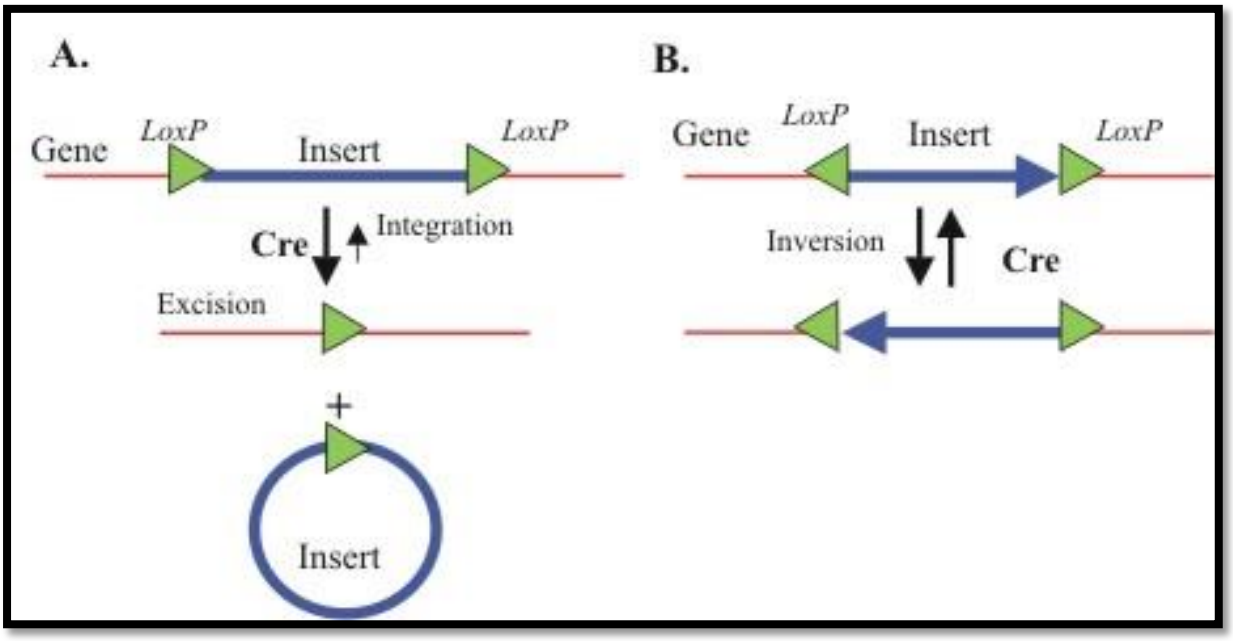

Figure 1.3: Schematic showing Cre-lox recombination system. Cre recombinase excises out the region present in between two loxP sites (floxed) by recombination and leaves one loxP site intact. Cre can either excise the transgene (A) or cause its inversion (B) (*Courtesy http://www.ruf.rice.edu/~rur/issue1_files/norman.html*)

Blood vessel formation involves multiple essential cellular processes, majorly migration, proliferation and differentiation. Cell migration, a critical process required for the formation of blood vessels, consists of a series of steps from polarity establishment and cytoskeletal rearrangements to the extension of the leading edge and retraction of the rear of a cell. Endothelial cells migrate towards the angiogenic stimulus and assemble into a tubular structure as a prelude to remodeled vasculature.

1.3 Cell migration during angiogenesis

Blood vessel formation involves multiple essential cellular processes, majorly migration, proliferation and differentiation. Cell migration, a critical process required for the formation of blood vessels, consists of a series of steps from polarity establishment and cytoskeletal rearrangements to the extension of the leading edge and retraction of the rear of a cell. Endothelial cells migrate towards the angiogenic stimulus and assemble into a tubular structure as a prelude to remodeled vasculature.

Endothelial cell (EC) migration is crucial for vascular development and occurs in both yolk sac and embryo proper. During vasculogenesis, angioblast clusters detach from each other and start moving individually in response to soluble factors or guided by cell-substrate interactions or cell-cell contacts (Schmidt *et al., Circ Res,* 2007). The angioblasts get assembled either at the region from where they originated or migrate to a location where new vessels are being formed (Coultas *et al., Nature,* 2005). Overall, EC migration is a complex process involving co-ordinated changes in the cytoskeleton, cell adhesion and signal transduction. Mutations in some of the molecules involved in cell migration, such as FAK, α4 integrin, syndecans, Rap1b and angiomotin result in defective vascular development (Lamalice *et al., Circ Res,* 2007). These and other studies show the critical involvement of cell migration in angiogenesis.

1.4 Rudhira

Establishment of a functional blood vascular system is a complex process and requires a tightly regulated interplay of various signaling molecules. Deregulation of one or more such molecules can lead to pathological conditions of the vasculature. Blood vessel formation as a result of aberrant activation of the endothelium has been found to contribute to several diseases such as ischemia, retinopathies, cardiovascular disorders and tumors (Carmeliet, *Nat Med,* 2003; Chung *et al., Nat Rev Cancer,* 2010). Even though there have been a wide range of studies addressing molecular regulation of vascular development, many of the molecules and molecular events in development of the blood vasculature remain to be elucidated. Thus, a better understanding of the complex molecular signals that establish and maintain a normal vascular network may aid in designing effective therapies to overcome several such diseases. This merits the identification of novel molecules, which can improve our existing understanding of the blood vessel formation.

Rudhira (Sanskrit: rudhira = blood) is one such molecule which was identified as a novel gene in an embryonic stem cell gene trap screen. Previous work from our laboratory has shown that *rudhira* is a highly conserved gene expressed predominantly in the embryonic vasculature (Siva and Inamdar, *Gene Expr Patterns,* 2006) and adult neo-angiogenic processes such as wound healing (Jain *et al*, *Exp Cell Res,* 2012). Rudhira protein shares 98% identity with its human ortholog, BCAS3 (Breast Cancer Amplified Sequence 3), which maps to a breakpoint of hematological neoplasms on chromosome 17q23.1 (Siva *et al*, *PLoS One,* 2007). *BCAS3* gene is reported to be a target of metastasis associated protein -1 (MTA-1) (Gururaj *et al., Proc Natl Acad Sci U S A,* 2006), and was found to be overexpressed in breast cancers (Barlund *et al., Genes Chromosomes Cancer,* 2002). Moreover, BCAS3 expression is upregulated in grade III and IV glioblastomas (Siva *et al., PLoS One,* 2007). This suggests that *rudhira/BCAS3* expression is tightly regulated and causes aberrant angiogenesis once dysregulated.

Rudhira interacts with the cytoskeletal machinery and plays a key role in directional cell migration. It is required for the activation of Cdc42 at the leading edge, thereby helping cell polarization and facilitating actin cytoskeleton reorganization to promote cell motility. Analysis of *rudhira* knockout mice revealed that it is an essential gene required for embryonic growth, survival and cardiovascular development (Shetty, Joshi *et al.,* submitted). The mutant phenotype observed in mice could be due to defective cell migration, leading to aberrant vascular patterning.

Bioinformatics analysis of Rudhira showed the presence of WD40 domains in the N-terminal half, and BCAS3 domain (Uniprot, NCBI Protein, NCBI CDD, pfam), peptidase domain (Mamta Jain thesis, 2012) and SxIP[#] motif (Jiang *et al, Current Biology,* 2012) in the C-terminal half. Moreover, most of the phosphorylation sites were observed to be located in the C-terminal half of the protein (PhosphosSitePlus) (Figure 1.4). Given the predicted structural difference between the two halves of the protein, it would be interesting to understand whether and how this difference translates at the cellular and molecular levels.

The SxIP motif was first discovered by Jiang *et al.* in BCAS3; subsequent sequence alignment showed the presence of this motif in Rudhira as well.

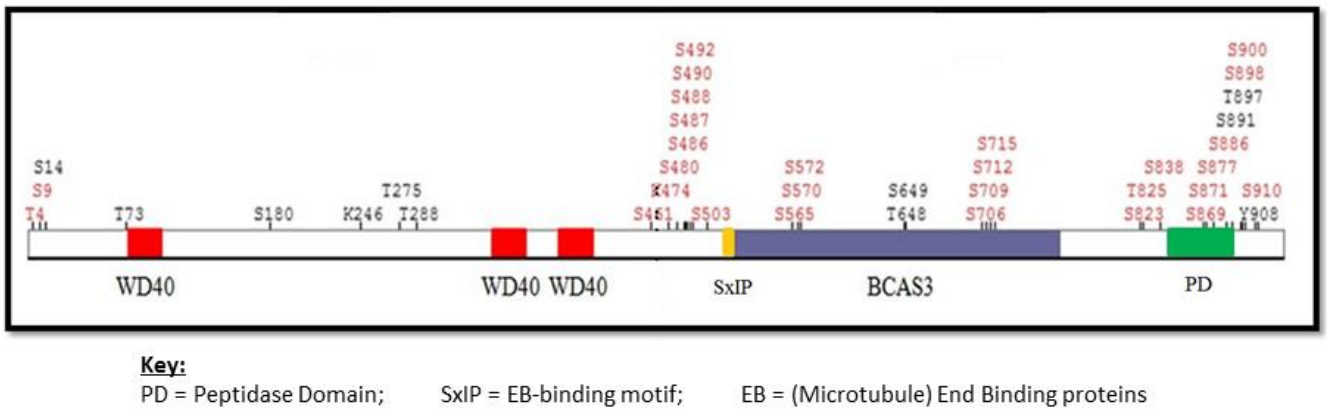

Figure 1.4: Schematic showing the phosphorylation sites and predicted domains in Rudhira (*adapted from PhosphositePlus*)

1.5 Need for a stable *rudhira* knockout endothelial cell line

In our laboratory, functional studies of Rudhira were previously performed using three model systems: *rudhira* **knockout mice**, *rudhira* **knockout primary endothelial cells** (derived from the mouse model) and *rudhira* **knockdown cell line**. Each of these model systems has its advantages and limitations. In the case of *rudhira* **knockout**, which displays embryonic lethality, isolating embryos and performing subsequent experiments with them is a much more time-consuming and expertise-based process, as compared to working with neonates and adult mice. Moreover, it is very difficult to determine and study the underlying aberrant molecular mechanisms in an *in vivo* model. Also, the cost of using whole animals has risen considerably over the years because of increased regulations for care and maintenance of the animals.

Primary endothelial cells obtained from such mouse models are also generally difficult and time-consuming to isolate and maintain. Moreover, they are limited in number, have a finite lifespan in cultures, and low-transfection efficiency. Furthermore, the isolation procedures may also affect cell viability and phenotypic homogeneity.

In case of the knockdown cell line, due to the invariably incomplete silencing efficiency, some functional RNA always remains and is translated at lower levels, reducing, but not eliminating the gene function. This may result in certain important phenotypes being missed. Moreover, the possibility of off-target effects still remains, and needs to be eliminated using appropriate controls.

Considering the advantages and limitations of various model systems to study the function of Rudhira, we set out to generate a stable ***rudhira* knockout endothelial cell line** from *rudhira* knockout embryos. Such a cell line would help resolve a number of limitations of the previous model systems. Since the cells have already undergone all the aberrant processes that occurred from the beginning of development of the embryo till the lethality sets in (as a result of *rudhira* being absent), they would show true pathological phenotype. To generate the cell line, these cells were immortalized, allowing us to perform long-term *in vitro* assays on them, as compared to primary cells that have a limited proliferative poteintial. Also, the cells can be easily transduced with high efficiency using viral vectors. Lastly, due to the complete absence of Rudhira protein in these cells, we may discover novel phenotypes that were not apparent in the knockdown cell line.

1.6 Aims of the present study

Keeping in mind the limitations of the model systems already present and the advantages of a knockout cell line, I proposed the following objectives that would help me answer the question of whether the two halves of Rudhira are involved in regulating different aspects of cell migration:

1) To isolate and generate *rudhira* KO and WT endothelial cell lines
2) To determine the role of different regions of Rudhira protein in cell migration

2. Generation of *rudhira* KO and WT endothelial cell lines

2.1 Introduction

Several studies have used gene targeting to generate "knock-out" mice to understand the role of key vascular molecules. A major limitation with generating conventional knock-out mice is that the gene deletion could lead to lethality at an early stage. This hinders further studies of the role of the gene in various processes. This limitation could be overcome by: a) generating a tissue-specific knockout, b) isolating primary cells from the knockout embryo, c) immortalizing cells from the knockout embryo. In case of *rudhira*, endothelial-specific knockout mice were generated using Tek-Cre recombination, and the effect of deletion of the gene on the vasculature was analyzed. However, even in case of endothelial-specific knockout, lethality sets in at the embryonic stage, albeit at a delayed time (11.5dpc) (Mamta Jain thesis, 2012). Culturing and studying primary cells from the knockout embryos is a costly and labour-intensive option. Thus, to overcome such limitations, a stable endothelial cell line was generated from the knockout embryos, and used to study the role of Rudhira in cell migration.

One of the ways to immortalize endothelial cells is to transduce them with PyMT antigen-containing viral particles. PyMT antigen, which is the Polyoma Middle T antigen, when expressed by a virus, causes it to specifically transduce endothelial cells. GgP+E, a fibroblast cell line, packages viral particles expressing the PyMT antigen. It was generated from the GP+E cell line initially developed as a means for retroviral delivery and expression of the gene of interest in a suitable host (Markowitz *et al, J Virol,* 1988). Thus, viral particles from the GgP+E cell line were used to immortalize endothelial cells obtained from *rudhira* KO and WT embryos. Once transduced by the virus, the cells start proliferating at a much higher rate compared to untransduced cells, and subsequently form colonies (islands) of immortalized cells called endotheliomas (Burek *et al, J Vis Exp,* 2012). These immortalized cells can then be cultured as a cell line.

2.2 Materials and Methods

2.2.1 Generation of *rudhira* floxed mice

The heterozygous floxed *rudhira* (*rudh*^{lox}/^{lox}) mice were available earlier in the laboratory. In these mice, the *rudhira* locus (Chromosome location: 11:85166669-85639560:1) has two loxP sites flanking the sixth exon - the floxed *rudhira* allele (Figure 2.1). *rudh*^{lox}/*rudh*^{lox} mice were mated to B6.C-Tg(CMV-cre)1Cgn/J (The Jackson Laboratories, Bar Harbor, USA) to generate heterozygous *rudhira*-null mice (+/Δ). Heterozygous null mice were crossed to obtain homozygous *rudhira* null mutant embryos (Δ/Δ). Cre-mediated deletion of exon 6 would lead to a frame-shift mutation resulting in a truncated peptide (~90 aa) lacking the majority of Rudhira sequence (Appendix A2.1). All mouse experiments in our laboratory were performed in accordance with the guidelines of CPCSEA (Committee for the purpose of Control and Supervision of Experiments on animals).

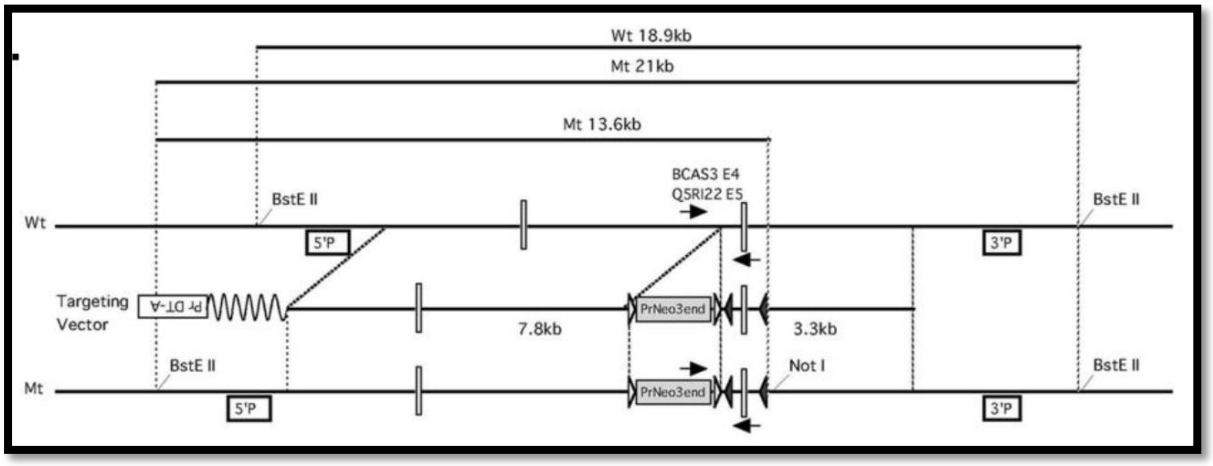

Figure 2.1: Schematic showing strategy for floxing *rudhira* allele at exon 6 *(from Shetty, Joshi et al., submitted)*

2.2.2 Culturing of primary endothelial cells

E9.5 embryos and yolk sacs were isolated from pregnant female mice, washed with 1X PBS, and incubated with 1.5mg/ml collagenase type IV (cat. no. 17104-019, GIBCO/BRL, Invitrogen, Carlsbad, USA) at 37˚C for 5 min (with intermittent tapping) to dissociate the cells. The collagenase was neutralized using several volumes of culture medium. Cells were pelleted, resuspended in fresh yolk sac culture medium (Appendix A2.2) containing 100µg/ml Endothelial Cell Growth Supplement (ECGS, cat. no. E2759, SIGMA Chemical

Co., USA) and plated onto 0.1% gelatin (cat. no. 07903, STEMCELL Technologies Inc., Canada) coated dishes.

2.2.3 Culturing of GgP+E cell line and collection of viral particles

The viral packaging cell line, GgP+E (kindly provided by Dr. Elisabetta Dejana (IFOM, Milano, Italy)) was thawed in DMEM (cat. no. 41965062, Gibco-BRL, USA) containing 15% FBS (cat. no. 10270, Gibco-BRL, USA) and 2 mM Glutamax (cat. no. 35050, Invitrogen, Carlsbad, USA). The cells were seeded on 0.1% gelatin-coated dishes. After two subsequent passages, conditioned medium was collected from cells, centrifuged, filtered through 0.45µ filter (cat. no. 16555-K, Sartorius, Germany) and used immediately or stored at -80ºC.

2.2.4 Transduction of primary cells with viral particales

The conditioned medium collected from GgP+E cell line was mixed with Polybrene (10 µg/ml final concentration) (Hexadimethrine bromide, cat. no. H9268, Sigma Chemical Co.) and added to the primary cells in culture. The medium was replaced after 4 hours with fresh yolk sac culture medium, and the cells were incubated for 20hrs. This procedure was repeated thrice, followed by changing culture medium every day, till islands of cells, called endotheliomas were observed. Once the culture dish became confluent with these endotheliomas, the cells were passaged till most of the fibroblasts were removed from culture.

2.2.5 Genotyping

Cells from the tail of E9.5 embryos were used to extract genomic DNA using genotyping kit (cat. no. G1N70, SIGMA, Chemical Co., USA). The primers used to identify wild type allele of *rudhira* were as follows:

RudGNWTF forward primer:

5’- TCATGGTGTAGCTAGTGTGG-3’

RudGNWTR reverse primer:

5’- CCTGCATTCCGTCACTGTAG-3’

The primers used to identify complete excision of the floxed allele of *rudhira* were as follows:

RudGNMUTF forward primer:

5'-AATGGAAGGATTGGAGCTACG-3'

RudLoxR2 reverse primer:

5'- AATCCCACAACTGCTGCTAC-3'

The PCR conditions were as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 60s (for wildtype PCR)/90s (for excision PCR).

2.2.6 RT PCR

RNA was isolated from the transduced cells using TRIzol reagent (cat. no. 15596-026, Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Reverse transcription was performed using Superscript II (cat. no. 18064-014, Invitrogen, Carlsbad, CA). Specific primers used for reverse transcription PCR are provided in the Appendix A2.3.

2.2.7 Western Blot

The transduced cells were pelleted, washed with 1X PBS and lysed in buffer A (Appendix A2.4) on ice and clarified by centrifugation. Protein was estimated using Bradford reagent and 100 μg of lysate was loaded on SDS-PAGE gel. Gel was electroblotted on nitrocellulose membrane, probed with specific antibody and developed using ECL chemiluminescence (cat. no. 34077, Thermo Scientific, Rockford, IL, USA).

2.2.8 Acetylated LDL uptake assay

Primary cultures were grown to confluence, stained with 5μg/ml DiI-Ac-LDL (cat. no. L3584, Invitrogen, Carlsbad, USA) for 4hrs (to mark endothelial cells), and imaged using a fluorescence microscope.

2.2.9 Immunostaining

Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% TritonX-100 for 5min and blocked with 4% FBS for 1 hr. Cells were then incubated with appropriate primary antibody overnight at 4˚C, washed and incubated with respective secondary antibody for 1 hr at room temperature. Cells were then imaged.

2.2.10 Antibodies and Reagents

Primary antibodies used were directed against: PECAM (cat. no. 557355) - 1:100 (BD Biosciences, San Jose, CA), BCAS3 (cat. no. A300-916A) - 1:2000 (Bethyl Labs, Texas, USA) and GAPDH (cat. no. G9295) - 1:4000 (SIGMA Chemical Co., USA). Secondary antibodies used were goat anti-rabbit (cat. no. 62114038001A) - 1:10,000 (Bangalore Genei, Bangalore, India) and goat anti-rat Alexa 568 (cat. no. A11077) - 1:400 (Molecular Probes, Carlsbad, CA, USA).

2.2.11 Fluorescence Microscopy

Brightfield and fluorescence microscopy was performed using an inverted microscope (IX70, Olympus, Tokyo, Japan). Images were captured using a CCD camera (CoolSNAP, Roper Scientific, Inc, Trenton, NJ).

2.3 Results

2.3.1 Generation of *Rudhira* knockout and wildtype endothelial cell lines

Timed matings were set up between heterozygous *rudh*^{flox}/+ mice. The pregnant female was sacrificed at E9.5, and embryos and yolk sacs isolated. Primary cells from the embryos and yolk sacs were dissociated with collagenase type IV and seeded in culture dishes. Genotyping was performed to determine whether the embryos were *rudh*+/+, *rudh*^{flox}/+ or *rudh*^{flox}/^{flox}. Simultaneously, the viral packaging cell line, GgP+E, was cultured. The conditioned medium was collected, filtered and used to transduce primary cells from all *rudh*^{flox}/^{flox} embryos (and yolk sacs), and a few of *rudh*+/+ and *rudh*^{flox}/+ embryos (and yolk sacs). Cells were passaged once the dish became confluent with endotheliomas. Most of the fibroblasts were removed in the subsequent passages (Figure 2.2). The immortalized endothelial cells can now be used as a cell line.

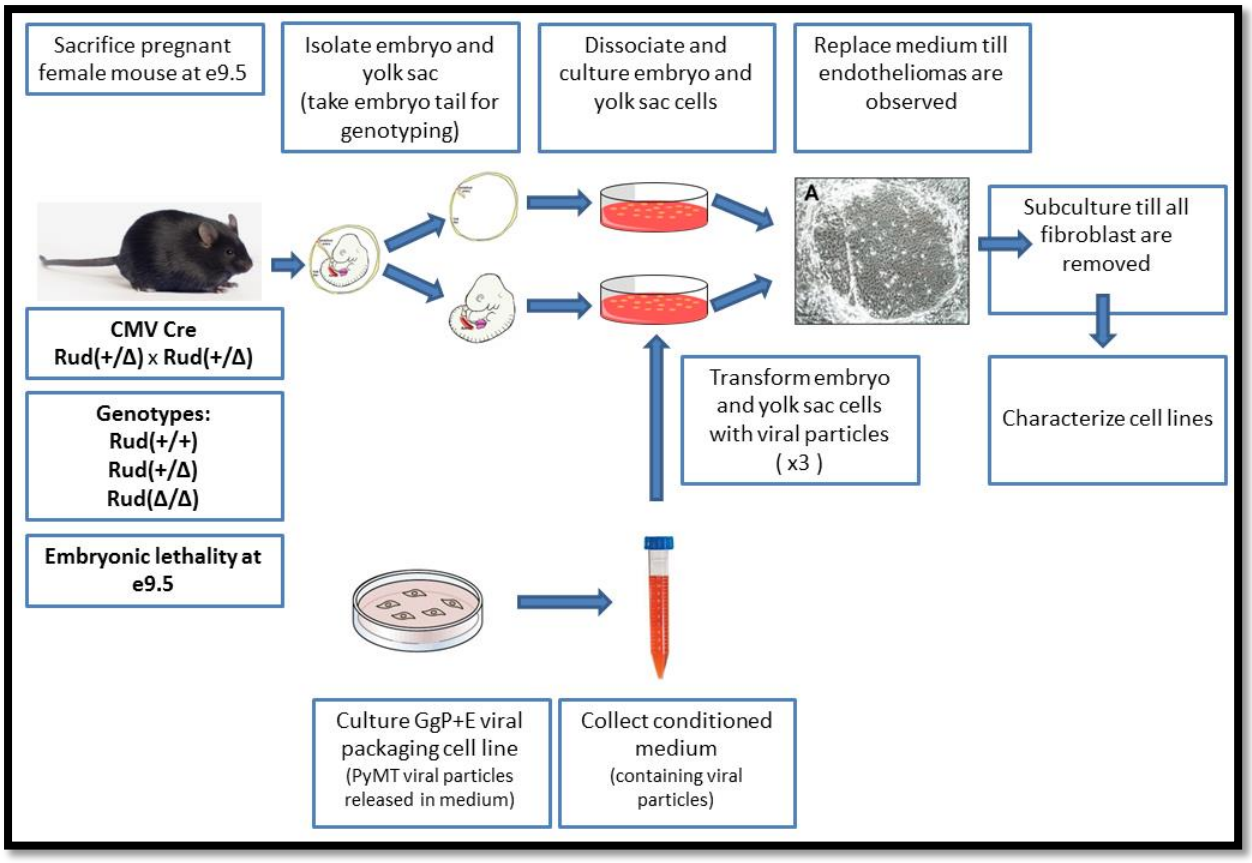

Figure 2.2: Strategy for generating *rudhira* KO and WT endothelial cell lines.

2.3.2 Validation of *Rudhira* knockout and wildtype endothelial cell lines

The morphology of the cell lines appeared cobblestone-like, indicative of endothelial cells (Figure 2.3). The cell lines were validated for the presence or absence of *rudhira* at the DNA level by genotyping, RNA level by RT-PCR and protein level by Western Blot (Figure 2.4). Several markers (PECAM1, eNOS, VE-cadherin, and von Willebrand Factor) and assays exist for validation of the endothelial nature of specific cell lines. One such assay is the acetylated LDL uptake assay based on the observation that endothelial cells specifically take up acetylated form of LDL (Voyta *et al*, *J Cell Biol*, 1984). Presence of PECAM at the intercellular junctions and uptake of acetylated LDL by the cells confirmed that the *rudhira* knockout and wildtype cell lines are indeed endothelial in nature (Figure 2.5).

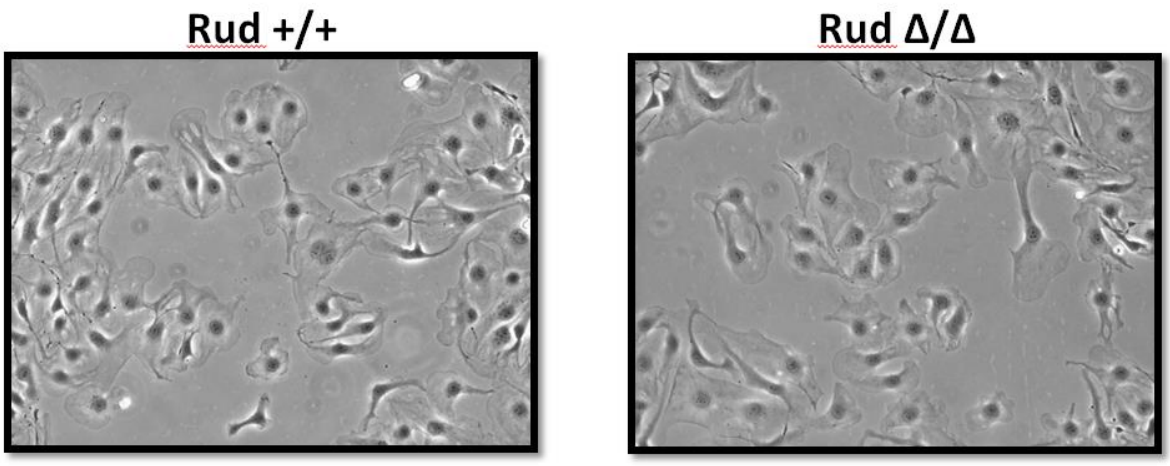

Figure 2.3: Cobblestone morphology of cells, indicative of endothelial nature.

2.4 Discussion

Owing to the limitations of the *rudhira* KO mouse model and *rudhira* KD cell line, there was a need to generate a *rudhira* KO cell line, which could recapitulate the aberrant developmental changes occurring in the *rudhira* KO embryo, and would provide an easy resource for performing long-term *in vitro* assays. Thus, *rudhira* KO and WT endothelial cell lines were generated by transduction with PyMT antigen-containing viral particles. Presence or absence of *rudhira* was confirmed in these lines at all the three levels: DNA, RNA and protein. The cell lines were validated as endothelial in nature by acetylated LDL uptake assay and presence of PECAM at the intercellular junctions.

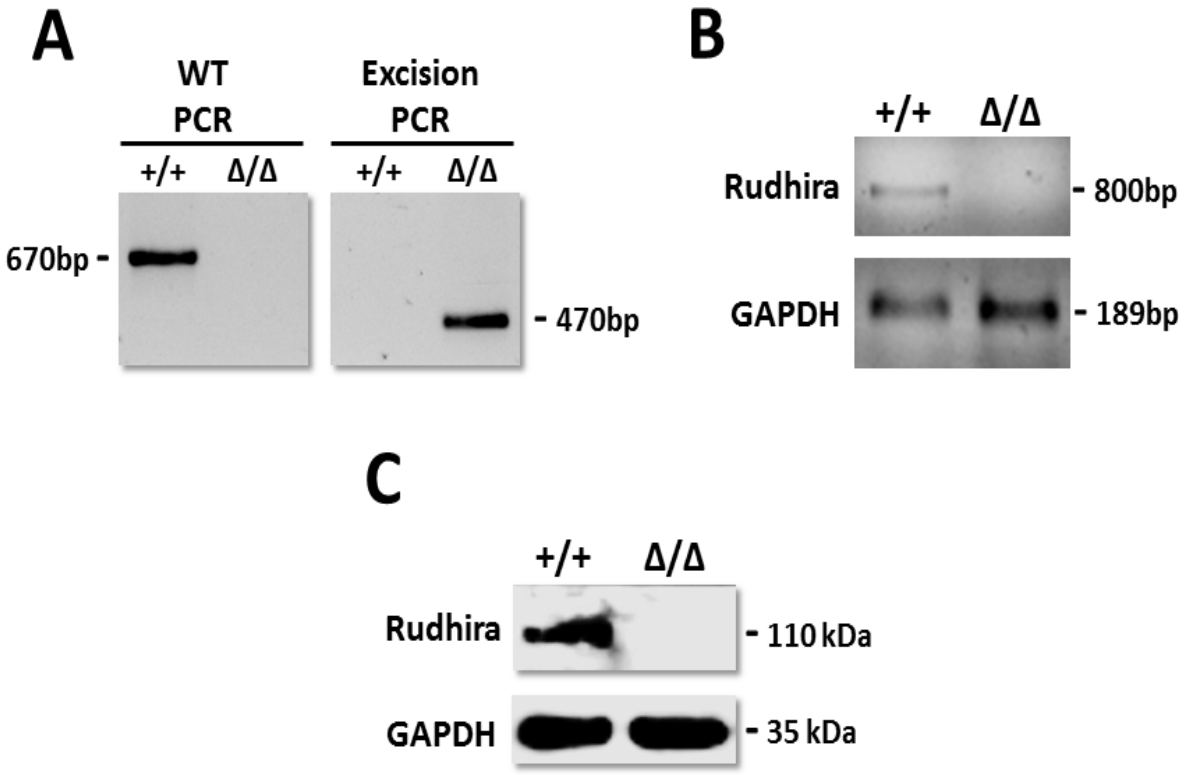

Figure 2.4: Validation of *rudhira* knockout (Δ/Δ) and wildtype (+/+) endothelial cell lines at the DNA (A), RNA (B) and protein (C) levels.[*Genotyping and Figure 2.4A courtesy Aksah Sam*]

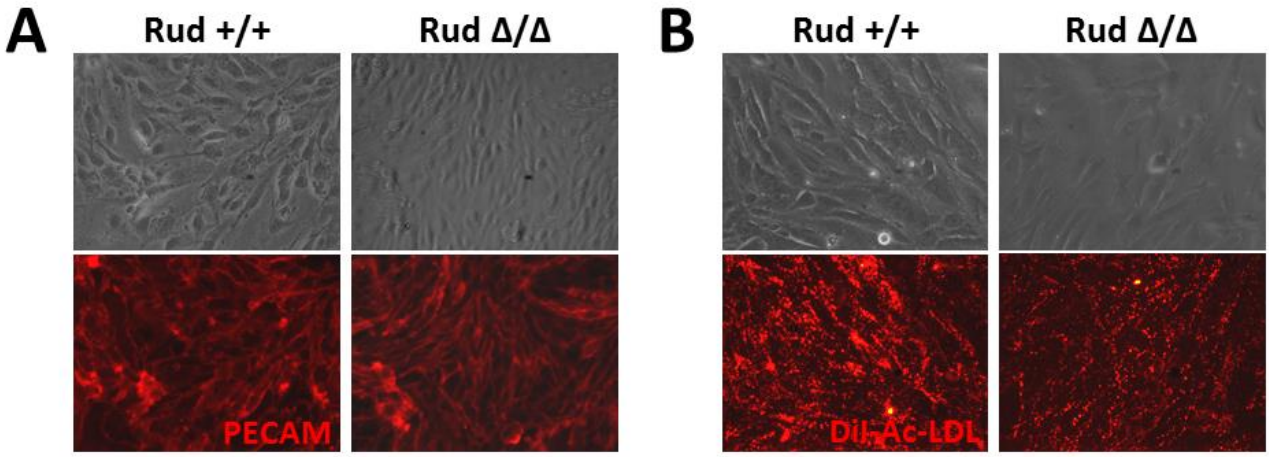

Figure 2.5: Validation of endothelial nature of *rudhira* knockout (Δ/Δ) and wildtype (+/+) cell lines by (A) PECAM immunostaining and (B) DiI-acetylated LDL uptake.

3. Effect of RudA and RudB on cell migration

3.1 Introduction

Neo-angiogenesis, i.e., establishment of a functional vascular network into the injured area is of paramount importance for wound healing. Previous studies showed that Rudhira is expressed in angiogenic precursors during embryonic vasculature development and adult angiogenesis (Siva and Inamdar, *Gene Expr Patterns*, 2006). These studies suggest a role for Rudhira in processes that require angiogenesis, such as wound healing. Rudhira was subsequently shown to be required for cell migration, a critical process in wound healing, using both *in vivo* assays (such as *in vivo* wound healing) and *in vitro* assays (such as endothelial tube formation, *in vitro* wound healing assay).

Bioinformatics analysis of Rudhira showed the presence of WD40 domains in the N-terminal half, and BCAS3 domain (Uniprot, NCBI Protein, NCBI CDD, pfam) and peptidase domain (Mamta Jain thesis, 2012) in the C-terminal half. Moreover, most of the phosphorylation sites were observed to be located in the C-terminal half of the protein (PhosphosSitePlus). This difference in the predicted domain architecture of the two halves of Rudhira indicated a difference in their molecular functions, primarily cell migration. Therefore, to understand which region of Rudhira was involved in regulating cell migration, the two fragments, RudA and RudB, were independently overexpressed in HEK293 cells and the relative distance covered by these cells was determined by performing *in vitro* wound healing assay.

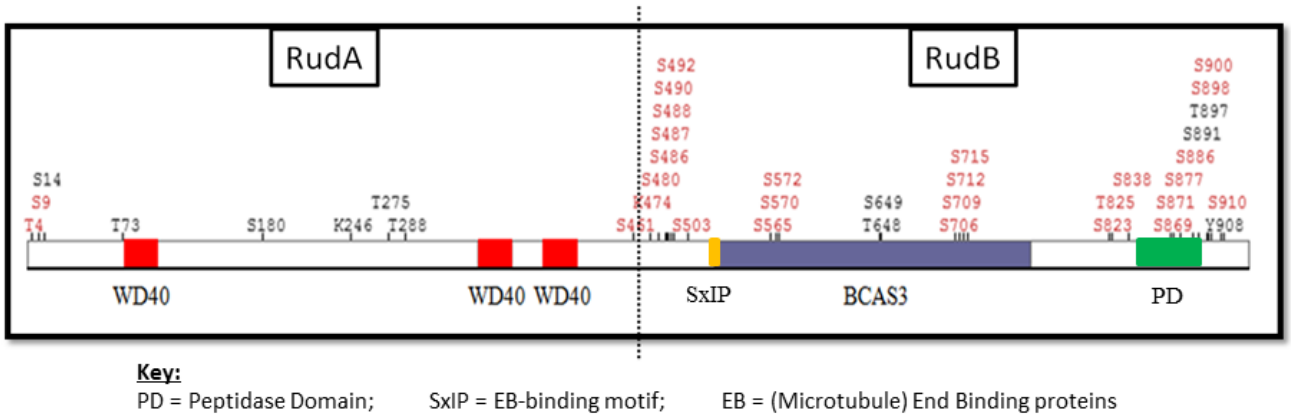

Figure 3.1: Schematic showing division of Rudhira protein into two halves (adapted from PhosphositePlus)

3.2 Materials and Methods

3.2.1 Cell culture

HEK293 cell line (American Type Culture Collection, Rockville, MD) was cultured in DMEM containing 10% FBS (cat. no. 10270, Gibco-BRL, USA) and 2 mM Glutamax (cat. no. 35050, Invitrogen, Carlsbad, USA).

3.2.2 Generation of RudA and RudB overexpressing HEK293 stable cell lines

The two regions (RudA and RudB) were previously cloned into the pCMV-FLAG vector (Divyesh Joshi). To remove the possibility of a fusion tag interfering with the structure and/or localization of the protein, and also to have an indicator of transfection in the cells, the two regions, RudA (460 residues) and RudB (469 residues), were subsequently cloned into EcoRI-SalI sites of pCMV-IRES2-EGFP (Clontech Laboratories, Inc., Palo Alto, USA), wherein, Rudhira and EGFP would be expressed as separate proteins. HEK293 cells were transfected with the four constructs: pIRES2-EGFP, RudA-IRES2-EGFP, RudB-IRES2-EGFP and Rudh-IRES2-EGFP, using calcium phosphate transfection method. Transfected (GFP positive) cells were selected by 1mg/ml G418 and sorted by FACS. Repeated selection and sorting led to the generation of GFP positive cell lines stably expressing the constructs. The cell lines were validated by Western blot and RT-PCR.

3.2.3 *In vitro* wound healing assay

HEK293 cell lines were grown to 100% confluence. Two scratches were made per cell line, using a 200 µl pipette tip. The cells were gently washed with PBS and incubated with fresh culture medium. Cells were monitored using IX70 inverted fluorescence microscope (Olympus, Tokyo, Japan) and imaged at t=0hrs and t=7hrs (two images per scratch) using a CCD camera (CoolSNAP; Roper Scientific, 30 Inc, Trenton, NJ). For quantification, scratch width was measured from the upper, middle and lower regions of each image using ImageJ (NIH, Bethesda, MD). The scratch width at the two time points was converted to distance travelled by cells in 7hrs (Distance traveled at 0hrs - Distance traveled at 7hrs). This distance was then converted to fold change. Statistical significance analysis was done using one sample t test (GraphPad) for N=3, n=1. P values < 0.05 were considered significant.

3.3 Results

3.3.1 Both RudA and RudB promote cell migration

RudA and RudB were overexpressed in HEK293 cells to check the effects of the two regions of Rudhira on cell migration. *In vitro* wound healing assay was performed with the overexpression cell lines, and both RudA and RudB overexpression was observed to significantly increase rate of cell migration as compared to vector control (Figure 3.1). These results indicate that RudA and RudB can independently promote cell migration in HEK293 cells. Moreover, the increase in migration rate caused by RudA was more compared to that by RudB or RudFL.

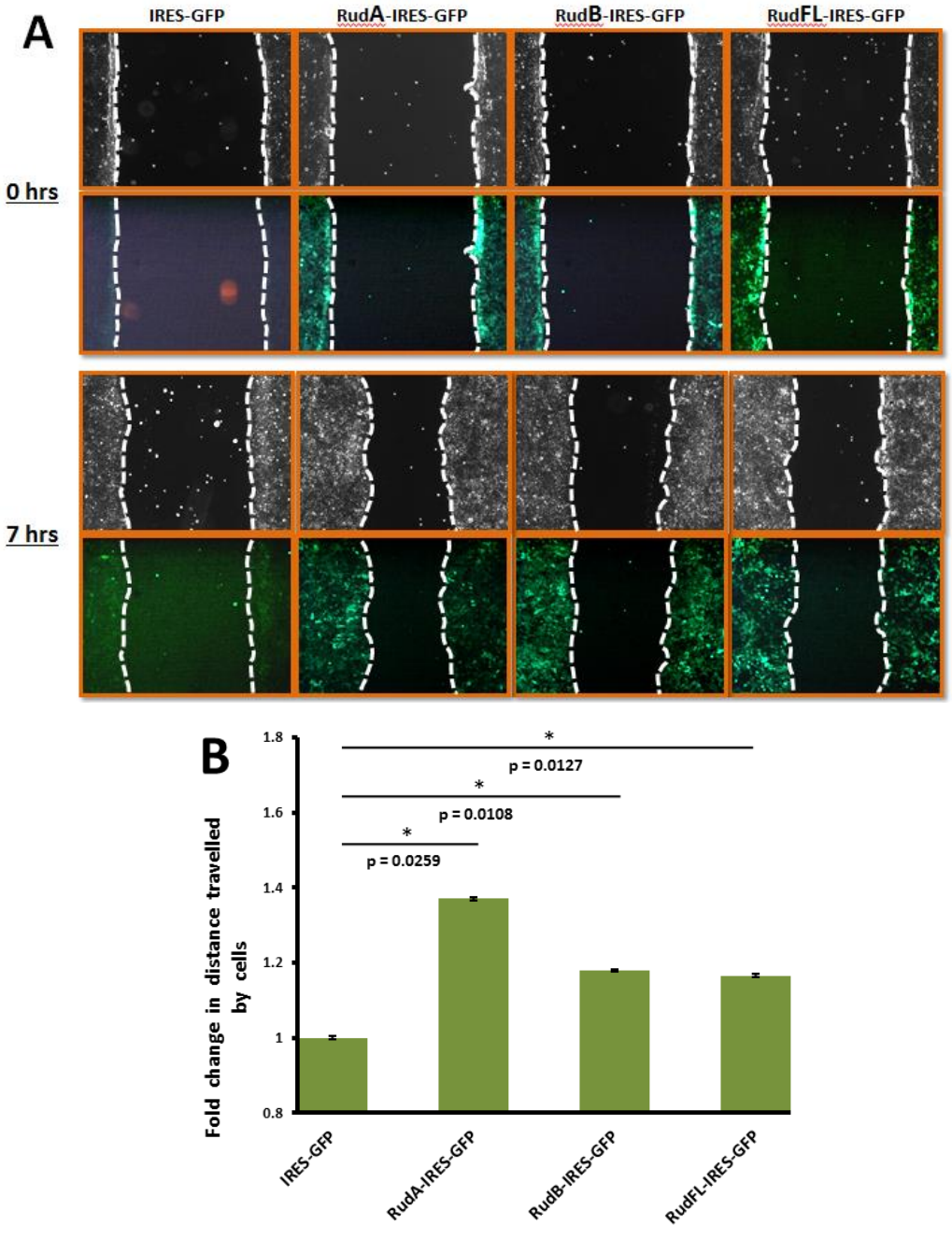

Figure 3.2: Overexpression of RudA or RudB induces increased cell migration. (A) HEK293 cell monolayers overexpressing IRES2-EGFP, RudA-IRES2-EGFP, RudB-IRES2-EGFP or RudFL-IRES2-EGFP were scratched and monitored for healing. Wounded monolayers were imaged at 0 hr and 7 hr. (B) Graph showing relative distance travelled by cells overexpressing the four constructs. Data represent the average of three independent experiments. Error bars indicate means ± SD. *, $p < 0.05$ by one sample t test.

	IRES-GFP	**RudA-IRES-GFP**	**RudB-IRES-GFP**	**RudFL-IRES-GFP**
Biological Replicate 1	1	1.319773	1.21602	1.186692
Biological Replicate 2	1	1.491837	1.166122	1.127755
Biological Replicate 3	1	1.300544	1.154991	1.181125
Average fold change	**1**	**1.370718**	**1.179045**	**1.165191**

Table 3.1: Fold change values for distance traveled by HEK293 cells overexpressing the four IRES-GFP constructs (N=3, n=1)

3.4 *Discussion*

Rudhira is expressed in several normal and pathological processes where angiogenesis takes place such as embryonic development, wound healing, tumorigenesis, etc. Angiogenesis plays a critical role in wound healing by re-establishing the vasculature of injured tissues.

Rudhira has an essential role to play during cell migration, a crucial process required for angiogenesis (Jain *et al, Exp Cell Res,* 2012). Rudhira may, thus, contribute to angiogenesis by guiding the movement of the initial pathfinding/pioneer endothelial cells for the formation of the primitive vascular tubes. The transient expression of Rudhira in angiogenic precursors (Siva and Inamdar, *Gene Expr Patterns,* 2006) supports this view. The hypothesis is also strengthened by the fact that exogenous Rudhira can induce cell migration in stationary/slow moving cells (HEK293).

To determine the region of Rudhira involved in promoting cell migration, we next investigated the effect of overexpression of the two regions - RudA (N-terminal half) and

RudB (C-terminal half) on the rate of migration of HEK293 cells that have low endogenous expression of Rudhira. Using *in vitro* wound healing assay, we show that both RudA and RudB are able to promote cell migration independent of the other. Interestingly, RudA overexpression was observed to increase migration rate much more than RudB or RudFL overexpression. To determine the molecular basis of this difference, the effect of RudA and RudB overexpression was checked on the sub-cellular machinery (markers of migration) required for cell migration (data shown in the next chapter).

4. Effect of RudA and RudB on microtubule stabilization, actin reorganization and focal adhesion turnover

4.1 Introduction

Over the past few years, immense progress has been made in understanding cell migration, allowing us to assemble the information at hand into an emerging model for how cells polarize and migrate (Figure 4.1).

4.1.1 A Molecular Model for Cell Migration

Cells migrate directionally in response to a variety of cues, including gradients of chemokines, growth factors, or ECM molecules. These factors engage cell surface receptors, initiating a cascade of events, including the local activation and recruitment of Rho GTPase family proteins - Cdc42, Rac1 and RhoA. Cdc42 and Rac1 regulate formation of membrane protrusions, i.e., filopodia and lamellipodia, respectively, at the leading edge (Nobes and Hall, *Cell*, 1995; Ridley and Hall, *Cell*, 1992). Cdc42 also contributes to cell polarization by mediating reorientation of the MTOC toward the cell front, leading to an increased growth of microtubules at the leading edge as compared to the trailing edge. Moreover, microtubules at the leading edge get stabilized, and this helps in transport of cargo to and from the cell membrane (Kaverina and Straube, *Semin Cell Dev Biol*, 2011). The propulsive forces required for migration are provided by focal adhesions (FAs), which are large macromolecular complexes that connect the cell to the extracellular matrix (ECM). Proper assembly and disassembly of FAs is crucial for cell migration. Cell contraction and rear retraction occurs via the activity of RhoA, by formation of actin stress fibers (Raftopoulou and Hall, *Dev Biol*, 2004; Spiering and Hodgson, *Cell Adh Migr*, 2011). Thus, the migration cycle is completed as adhesions disassemble at the rear and the rear retracts.

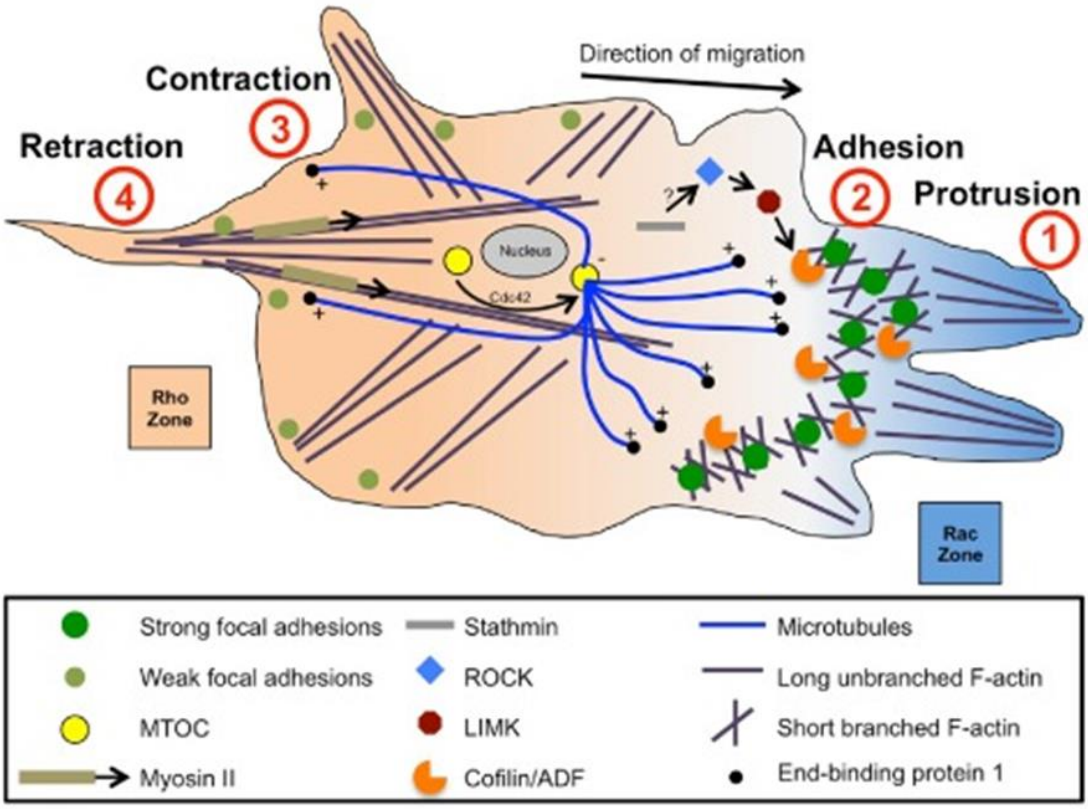

Figure 4.1: The four steps of cell migration. A cartoon schematic shows a migrating cell (direction of migration is indicated by the arrow). Cytoskeletal regulatory and associated proteins are also shown (myosin II, cofilin/ADF, LIMK, ROCK, stathmin). *(Courtesy Fife et at., British Journal of Pharmacology, 2014)*

The model explained above describes specific characteristics of cell migration, which can be used as tools to infer whether the process of cell migration is in progress. Such specific characteristics will henceforth be referred to as markers of cell migration. A few such common markers include:

1. Microtubule pattern

2. Microtubule stabilization

3. Focal adhesion turnover

4. Filopodia and lamellipodia formation

5. Stress fiber levels

The role of each of these markers is briefly explained below:

4.1.2 Microtubule pattern

In order to support asymmetry of cellular activities, the microtubule network itself has to be asymmetric. Microtubule asymmetry in a motile cell includes both asymmetric microtubule distribution and microtubule dynamics. In most cells, more microtubules extend to the cell front than to the cell rear. Such difference is mainly due to the reorientation of the MTOC (microtubule-organizing centre) toward the cell front brought about by Cdc42 signaling. The microtubule plus end generally grows persistently in the cytoplasm and tends to ultimately come into contact with the cell edges (Komarova *et al, J Cell Sci*, 2002). This results in most microtubules reaching the cell edge, thus ensuring efficient cargo transport in both directions. In addition, microtubules actively slide along each other to support the formation of parallel microtubule arrangements in cellular protrusions (Jolly *et al, Proc. Natl. Acad. Sci. U.S.A.*, 2010).

4.1.3 Microtubule stabilization

During cell migration, microtubules are selectively captured and stabilized at the cell front. Such long-lived microtubules that extend to the cell front are often post-translationally modified. It is thought that stable microtubules accumulate tubulin modifications such as acetylation and detyrosination over time (Gundersen and Bulinski, *Proc. Natl. Acad. Sci. U. S. A.*, 1988). Tubulin modifications selectively increase the affinity of certain molecular motors to their tracks (e.g. kinesin-1) and could thereby serve as signposts to facilitate directional transport to and from the cell front (Reed *et al, Curr Biol,* 2006).

4.1.4 Focal adhesion turnover

Another important process that modulates cell migration relates to focal adhesion turnover. Focal adhesions are large macromolecular structures that ensure proper communication between the cell and the ECM during adhesion and migration. The dynamic assembly and disassembly of FAs during migration is performed by a coordinated action of actin filaments and microtubules. Actin filaments are involved in the assembly of FAs, whereas the disassembly occurs via microtubules. During migration, microtubules directionally grow towards FAs, target them and promote their disassembly by dynamin and clathrin-mediated endocytosis (Ezratty *et al, J Cell Biol*, 2009).

4.1.5 Filopodia and lamellipodia formation

During cell migration, lamellipodia (sheet like structures in the form of ruffles) and filopodia (long, thin, needle like projections) are the two major membrane protrusions that form at the leading edge (Friedl and Wolf, *Cancer Metastasis Rev*, 2009; Lauffenburger and Horwitz, *Cell*, 1996), and their formation is regulated by the Rho GTPase family members, Rac1 and Cdc42, respectively. Cdc42 and Rac1 perform this function by activating the WASp and WAVE family proteins, respectively, which in turn activate Arp 2/3 complex, which ultimately causes actin polymerization. The two actin structures are highly dynamic and protrude in the direction of movement in response to a gradient of chemoattractants and growth factors (Raftopoulou and Hall, *Dev Biol*, 2004; Spiering and Hodgson, *Cell Adh Migr*, 2011).

4.1.6 Stress fiber levels

Assembly of actin filaments into larger structures and their functioning at the cell rear depends on myosin II contractility regulated by RhoA signalling. The important downstream targets for RhoA are mDia and p160ROCK which stabilize actin structures and generate contractile forces in the form of stress fibers (Raftopoulou and Hall, *Dev Biol*, 2004; Spiering and Hodgson, *Cell Adh Migr*, 2011). Actin stress fibers are often connected to focal adhesions, and thus appear to play an important role in cell adhesion and migration (Geiger *et al., Nat. Rev. Mol. Cell Biol*, 2009; Parsons *et al., Nat. Rev. Mol. Cell Biol.*, 2010). The assembly, growth and maintenance of focal adhesions depend on mechanical stress, provided by stress fibers. Stress fibers are absent from many highly motile cells, such as leukocytes (Valerius *et al., Cell*, 1981) and Dictyostelium discoideum amoeba (Rubino *et al., J. Cell Biol.*, 1984). It is thus possible that under many conditions, stress fibers inhibit cell motility because the reorganization of stable actin bundles and focal adhesions can be a relatively slow process (Castella *et al., J. Cell Sci.*, 2010).

Rudhira plays an essential role in direction cell migration by activating and recruiting Cdc42 to the leading edge, thereby causing actin reorganization (by inducing filopodia formation and reducing stress fibers) and reorientation of MTOC towards the leading edge. Moreover, Rudhira also mediates stabilization of microtubules towards the leading edge (Jain *et al*, *Exp Cell Res*, 2012) and turnover of focal adhesions (Divyesh Joshi, unpublished work) (both established features of cell migration), demonstrating that it is an active player in establishing polarity for directional cell migration.

To narrow down the region of Rudhira responsible for these processes, we checked for rescue of phenotype by overexpressing the two regions, RudA and RudB, in *rudhira* knockout endothelial cells.

4.2 Materials and Methods

4.2.1 Culturing Rudhira knockout endothelial cell line

Rudhira knockout endothelial cell line was grown in DMEM containing 20% FBS (cat. no. 10270, Gibco-BRL, USA) and 2 mM Glutamax (cat. no. 35050, Invitrogen, Carlsbad, USA), on 0.1% gelatin coated dishes.

4.2.2 Transfection

Rudhira knockout endothelial cells were transfected with the four constructs (IRES-GFP, RudA-IRES-GFP, RudB-IRES-GFP, RudFL-IRES-GFP) using Lipofectamine 2000 (cat. no. 11668019, Invitrogen, Carlsbad, CA) as per the manufacturer's protocols. Briefly, complete medium used to culture the cells was replaced with DMEM. DNA and Lipofectamine 2000 (both dissolved in DMEM) were mixed in a ratio of 1:2 (1µg DNA: 2µl Lipofectamine) and incubated at RT for 20 minutes. This mixture was gently added to the cells, and incubated at 37ºC for 6hrs. The DMEM was then replaced with complete medium, and the cells were cultured for 24hrs. Cells were then trypsinized and seeded onto coverslips coated with 0.1% gelatin. Cells were fixed after 24hrs.

4.2.3 Immunostaining

Cells on coverslips were fixed with 4% paraformaldehyde for 15min, permeabilized with 0.1% TritonX-100 for 5min and blocked with 4% FBS for 1 hr. Cells were then incubated with appropriate primary antibody overnight at 4˚C, washed and incubated with respective secondary antibody for 1 hr at room temperature. Cells were then stained with DAPI and imaged.

4.2.4 Antibodies and Reagents

Primary antibodies used were directed against: GFP (cat. no. A6455) - 1:500 (Invitrogen, Carlsbad, USA), acetylated tubulin (cat. no. T6793) - 1:300 (SIGMA Chemical Co., USA), paxillin (clone 5H11, cat. no. 05-417) - 1:300 (Merck, NJ, USA), β-tubulin (clone no. E7) - 1:100 (DSHB, Iowa, IA, USA) and Alexa Fluor 568 phalloidin (cat. no.A12380). Secondary antibodies used were Alexa Fluor 488 goat anti rabbit IgG (cat. no. A-11008) – 1:400 and

Alexa Fluor 568 goat anti-mouse IgG, (cat. no. A-11004) – 1:400 (both from Molecular Probes, Carlsbad, CA, USA).

4.2.5 Fluorescence Microscopy

Brightfield and fluorescence microscopy was performed using a confocal microscope (LSM 880, Zeiss, Germany).

4.2.6 Quantifications and statistical analyses

For the quantitation of stress fibers and localized acetylated tubulin, a fixed number of GFP positive cells were scored for either presence or absence of the corresponding phenotype. The data was then represented as percentage of cells showing the phenotype. Focal adhesions were quantified on the basis of their size, using ImageJ (NIH, Bethesda, MD). Statistical significance analysis was done using t-test (Graph Pad). P values < 0.05 were considered significant.

4.3 Results

4.3.1 Both RudA and RudB can independently increase levels of acetylated tubulin

One of the hallmarks of cell polarity is the stabilization of microtubules towards the leading edge during directional cell migration. Acetylation of α-tubulin of microtubules is considered as a marker of microtubule stability (Gundersen and Bulinski, *Proc. Natl. Acad. Sci. U. S. A.*, 1988). Rudhira modulation (silencing or overexpression) affects microtubule stabilization. Depletion of Rudhira in endothelial cells causes a dramatic reduction (~80%) in Ac-tubulin expression at the leading edge. Conversely, Rudhira overexpression increases Ac-tubulin expression. Moreover, the expression of Ac-tubulin was concentrated towards one side of the cell (Jain *et al., Exp Cell Res,* 2012), which suggests that Rudhira overexpression induces the phenotype of a polarized cell. In case of *rudhira* knockout endothelial cells as well, we observed a reduction in the level of acetylated tubulin, which increased significantly upon overexpression of full length Rudhira, as expected. In order to narrow down the region of Rudhira responsible for this phenotype, we independently overexpressed the two halves of the protein in these cells. Interestingly, overexpression of both RudA and RudB caused an increase in the Ac-tubulin level, indicating that both the fragments are individually able to rescue the loss of tubulin acetylation (Table 3.1; Figure 4.2). This suggests that both halves of Rudhira are independently involved in promoting stabilization of microtubules.

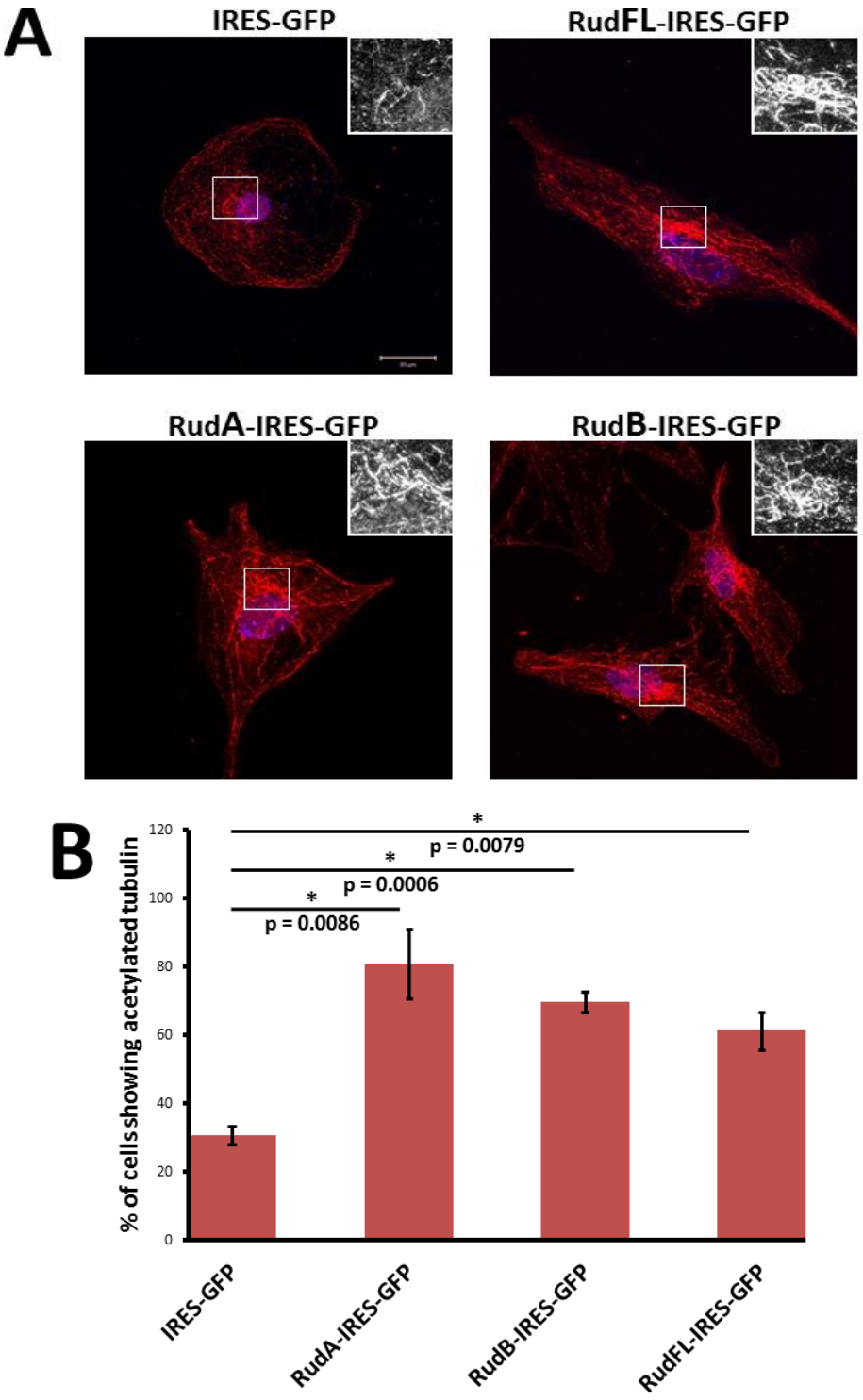

Figure 4.2: Both RudA and RudB can independently increase the stabilization of microtubules. (A) Representative images of *rudhira* knockout cells transfected with IRES-EGFP, RudA-IRES-EGFP, RudB-IRES-EGFP and RudFL-IRES-EGFP, and stained with anti-acetylated tubulin antibody. Insets indicate acetylated tubulin. (B) Graph representing rescue of acetylated tubulin loss phenotype upon overexpression of RudA, RudB or RudFL as compared to empty vector (* = $p < 0.01$). Error bar indicates mean ± SEM of a total of 10 cells from three independent experiments. Scale bar: 20µm.

		IRES-GFP (%)	RudA-IRES-GFP (%)	RudB-IRES-GFP (%)	RudFL-IRES-GFP (%)
Number (percentage) of GFP positive cells showing acetylated tubulin	**Biological Replicate 1**	4 (25)	3 (100)	4 (75)	3 (66.67)
	Biological Replicate 2	3 (33.33)	4 (75)	3 (66.67)	3 (66.67)
	Biological Replicate 3	3 (33.33)	3 (66.67)	3 (66.67)	4 (50)
	Average	**(30.56)**	**(80.56)**	**(69.44)**	**(61.11)**

Table 4.1: Number (percentage given in brackets) of GFP positive cells (i.e., cells transfected with the four IRES-GFP constructs) showing increased acetylated tubulin (N=3, n=3 or 4)

4.3.2 Both RudA and RudB are independently able to decrease the level of stress fibers

One of the characteristics of a migratory cell is the reduced level of stress fibers (contractile actin bundles) (Jimenez et al., 2000. Overexpression of Rudhira causes a significant reduction in stress fibers (Jain *et al.*, 2012). Such a change in the actin organization by Rudhira futher supports the role of this protein in cell migration. We observed increased stress fibers in *rudhira* knockout cells, and this phenotype was rescued by overexpression of full length Rudhira in these cells. The latter observation is in concordance with the Rudhira overexpression phenotype observed previously. Also, overexpressing RudA or RudB was also able to decrease the level of stress fibers (Table 3.2; Figure 4.3), suggesting that both halves of the protein are independently able to cause a decrease in the level of stress fibers.

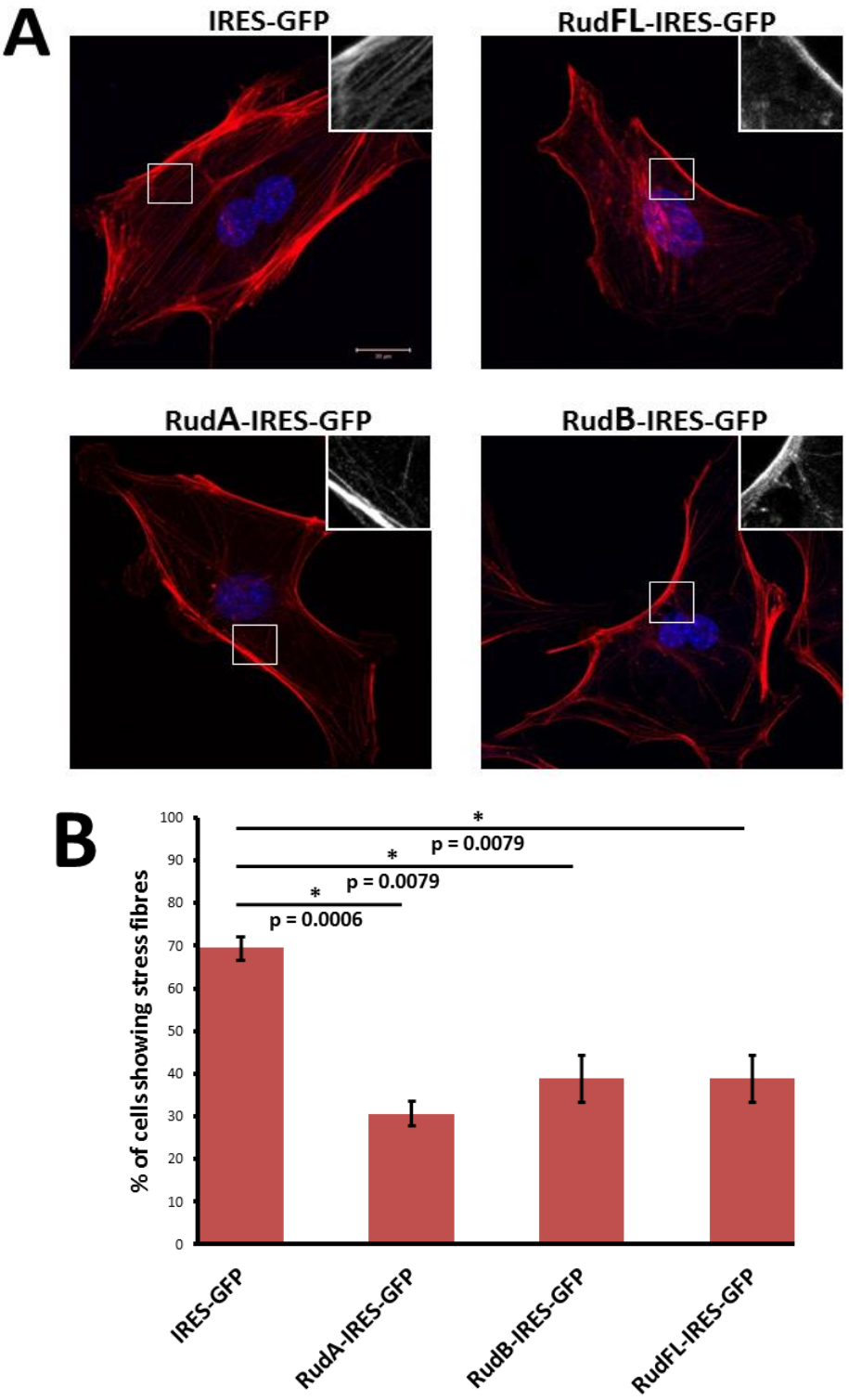

Figure 4.3: Both RudA and RudB can independently decrease the level of stress fibers. (A) Representative images of *rudhira* knockout cells transfected with IRES-EGFP, RudA-IRES-EGFP, RudB-IRES-EGFP and RudFL-IRES-EGFP, and stained with phalloidin (to indicate F-actin). Insets indicate stress fibers. (B) Graph representing rescue of increased stress fiber phenotype upon overexpression of RudA, RudB or RudFL as compared to empty vector (* = $p < 0.01$). Error bar indicates mean ± SEM of a total of 10 cells from three independent experiments. Scale bar: 20µm.

		IRES-GFP (%)	RudA-IRES-GFP (%)	RudB-IRES-GFP (%)	RudFL-IRES-GFP (%)
Number (percentage) of GFP positive cells showing stress fibers	**Biological Replicate 1**	4 (75)	3 (33.33)	3 (33.33)	3 (33.33)
	Biological Replicate 2	3 (66.67)	3 (33.33)	4 (50)	3 (33.33)
	Biological Replicate 3	3 (66.67)	4 (25)	3 (33.33)	4 (50)
	Average	**(69.44)**	**(30.56)**	**(38.89)**	**(38.89)**

Table 4.2: Number (percentage given in brackets) of GFP positive cells (i.e., cells transfected with the four IRES-GFP constructs) showing increased stress fibers (N=3, n=3 or 4)

4.3.3 Both RudA and RudB are required together for focal adhesion turnover

In stationary cells, focal adhesions are quite stable under normal conditions, while in moving cells their stability is diminished. This is because in motile cells, focal adhesions are being constantly assembled and disassembled as the cell establishes new contacts at the leading edge, and breaks old contacts at the trailing edge of the cell. Thus, the dynamic assembly and disassembly of focal adhesions plays a central role in cell migration.

Depletion of Rudhira in endothelial cells causes a significant increase in the size of focal adhesions. Moreover, overexpressing Rudhira in these cells was able to reduce FA size, and hence, rescue the mutant phenotype. Conversely, overexpressing Rudhira in wildtype endothelial cells causes a reduction in FA size. Later, it was shown that Rudhira is able to mediate a decrease in FA size by promoting FA turnover (Divyesh Joshi, unpublished data). Thus, regulating FA turnover might be one of the critical steps by which Rudhira is able to promote cell migration.

Increased FA size (and rescue of the phenotype upon overexpression of Rudhira) was also observed in *rudhira* knockout endothelial cells, as expected. However, overexpression of neither RudA nor RudB was able to reduce the size of FAs (Table 3.3; Figure 4.4), suggesting that both the halves of Rudhira are probably required together to mediate a decrease in FA size, and thus regulate FA turnover.

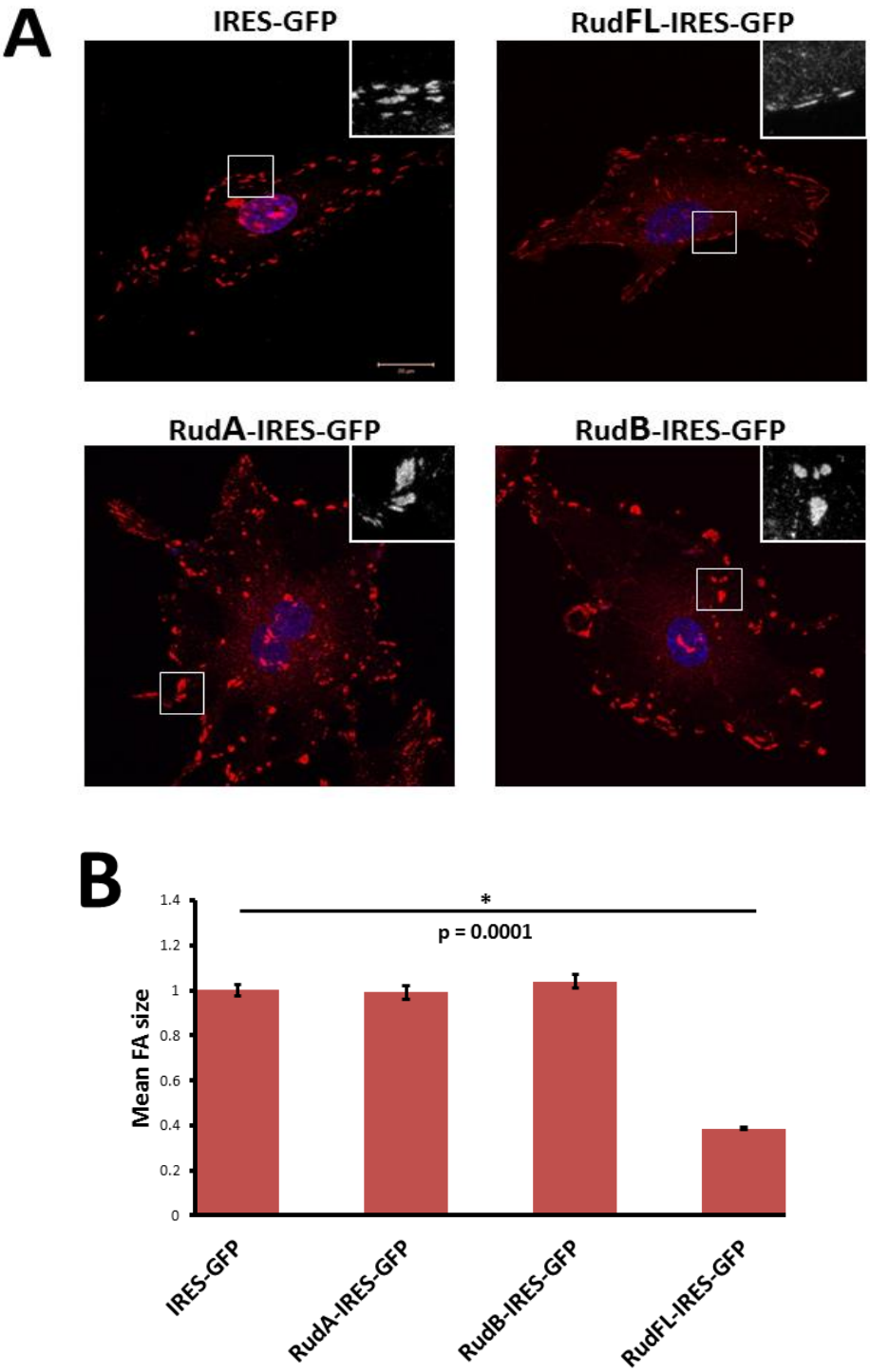

Figure 4.4: Both RudA and RudB are required together for focal adhesion turnover. (A) Representative images of *rudhira* knockout cells transfected with IRES-EGFP, RudA-IRES-EGFP, RudB-IRES-EGFP and RudFL-IRES-EGFP. Images of GFP positive transfected cells stained with paxillin. Insets indicate focal adhesions. (B) Graph representing fold change in FA size upon overexpression of RudA, RudB or RudFL as compared to empty vector (* = $p < 0.001$). Error bar indicates mean ± SEM of a total of 11 cells from three independent experiments. Scale bar: 20µm.

	IRES-GFP	RudA-IRES-GFP	RudB-IRES-GFP	RudFL-IRES-GFP
Biological Replicate 1	1	0.994	1.07	0.393
Biological Replicate 2	1	0.994	1.07	0.393
Biological Replicate 3	1	0.982	0.976	0.374
Average fold change	**1**	**0.99**	**1.038**	**0.386**

Table 4.3: Fold change values for FA size in cells overexpressing the four IRES-GFP constructs (N=3, n=4)

4.4 Discussion

Rudhira regulates cell polarity during directional cell migration by mediating a number of steps of the process, including activation and recruitment of Cdc42 to the leading edge, reorganization of the actin network (by inducing filopodia formation and reducing level of stress fibers) (Jain *et al*, *Exp Cell Res,* 2012), directing microtubules towards focal adhesions, causing focal adhesion turnover (Divyesh Joshi, unpublished data), etc.

To determine if the two regions of Rudhira mediate different subsets of its functions during cell migration, we overexpressed the two regions in the *rudhira* knockout background, and studied their effects on various steps of cell migration. We observed that overexpression of both RudA and RudB was able to increase the level of acetylated tubulin and decrease the level of stress fibers, indicating that both the regions can independently mediate increase in stabilization of microtubules and decrease in the level of acetylated tubulin. On the other hand, neither of the two regions was able to rescue the large size of focal adhesions, suggesting that perhaps both the regions are required together, as a whole, to regulate focal adhesion turnover. Given the difference in the predicted domain structure of the two halves of Rudhira, it is unusual that both halves of the protein seem to be involved in the same steps of cell migration (microtubule stabilization, FA turnover, stress fiber reduction). The effect of the two regions on microtubule pattern at the leading edge and induction of filopodia remains to be seen.

5. Discussion and Future Work

Blood vessel formation comprises an indispensable part of the cardiovascular development, required for growth, nourishment and repair of tissues and organs throughout the life of a vertebrate. Dysregulation of such a vital process contributes to several diseases like ischemia, cardiovascular disorders and tumors. In order to provide better and more effective ways of treatment of such diseases, a detailed understanding of the processes involved in cardiovascular development is required.

The formation of blood vessels involves multiple cellular processes, primarily migration, proliferation and differentiation. Cell migration involves a series of steps from polarity establishment and cytoskeletal rearrangements to the extension of the leading edge and retraction of the rear of a cell. Previous reports from our laboratory have shown that Rudhira is a cytoskeletal protein essential for directional cell migration. Further, *in vivo* studies have shown that *rudhira* is an essential gene, predominantly expressed in the developing vasculature. Both global and endothelial knockout of *rudhira* are embryonic lethal with severe cardiovascular defects, arising likely from defective cell migration (Jain *et al, Exp Cell Res,* 2012). Rudhira promotes cell migration by reorienting the MTOC and stabilizing microtubules towards the leading edge, causing a reduction in actin stress fibers, increasing the number of filopodia (Jain *et al, Exp Cell Res,* 2012), and mediating focal adhesion turnover (Divyesh Joshi, unpublished data) (Figure 5.1A, B). Hence, a better understanding of the molecular functions of Rudhira, due to its restricted expression pattern, may provide a novel target for therapy in aberrant development and disease.

Identification of functional domains aids in understanding the mechanism of action of the protein. Given that Rudhira regulates cytoskeleton architecture and cell migration, assigning these functions to specific regions of the protein would help in better understanding of its molecular function.

Bioinformatics analysis shows that Rudhira contains predicted WD40 domains in its N-terminal half, and a predicted BCAS3 domain and majority of the phosphorylation sites in the C-terminal half. This interesting inherent difference between the predicted domain architecture of the two halves of the protein was suggestive of differences in molecular function, primarily cell migration.

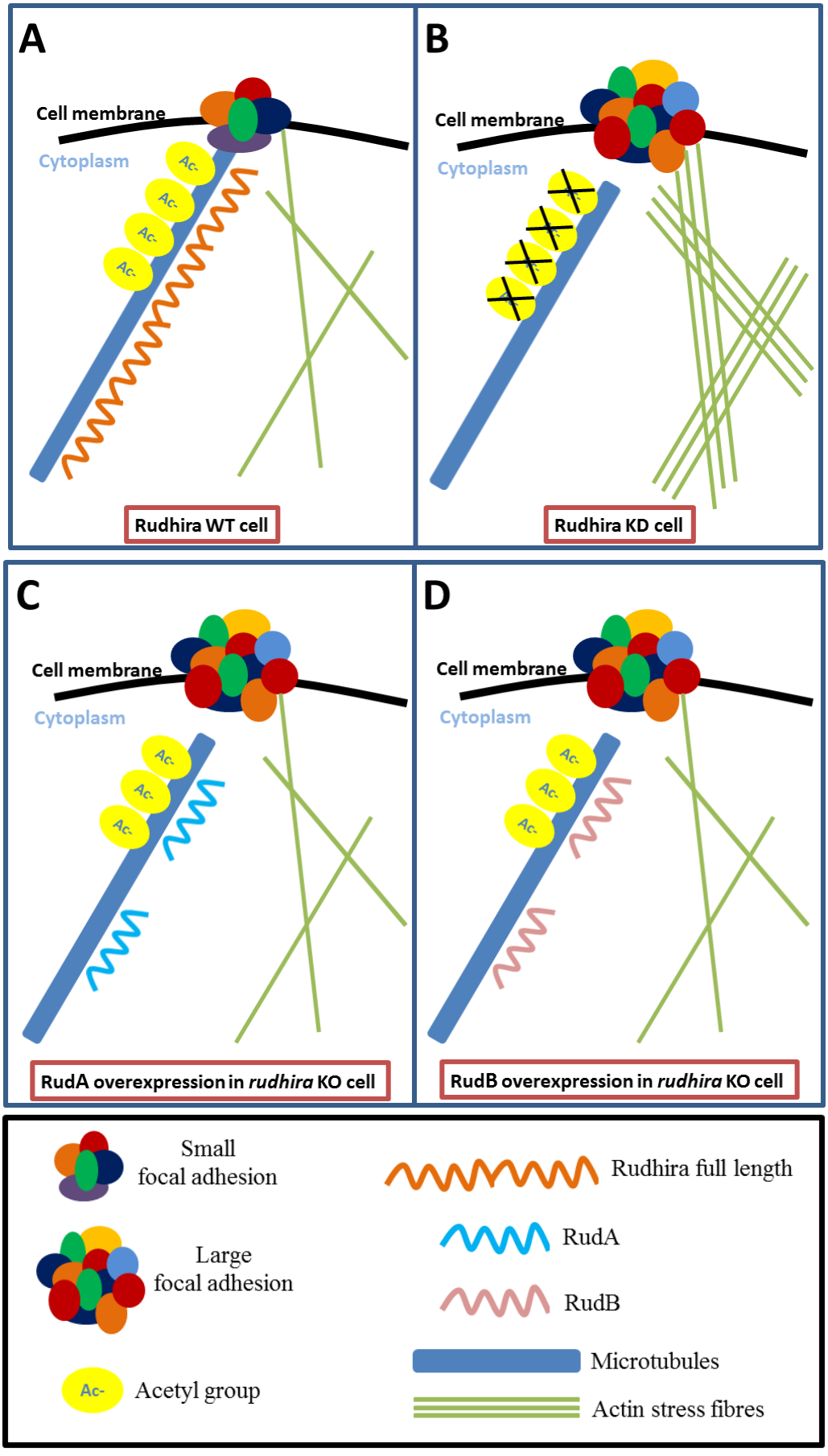
A
Cell membrane
Cytoplasm
Ac-
Ac-
Ac-
Ac-
Rudhira WT cell
B
Cell membrane
Cytoplasm
Rudhira KD cell
C
Cell membrane
Cytoplasm
Ac-
Ac-
Ac-
RudA overexpression in rudhira KO cell
D
Cell membrane
Cytoplasm
Ac-
Ac-
Ac-
RudB overexpression in rudhira KO cell
Small focal adhesion
Large focal adhesion
Ac-
Acetyl group
Rudhira full length
RudA
RudB
Microtubules
Actin stress fibres

Figure 5.1: Effect of Rudhira full length (FL), RudA and RudB on cell migration: (A) Rudhira FL promotes microtubule stabilization, focal adhesion turnover and reduction of stress fibers; (B) Absence of Rudhira FL causes loss of function of these phenotypes. Overexpression of RudA (C) or RudB (D) rescues microtubule stabilization and stress fiber reduction, but not focal adhesion turnover.

My first aim was to generate a *rudhira* knockout cell line which could serve as a resource and also overcome some of the limitations of the previous models. Thus, endothelial cell lines were generated from *rudhira* knockout and wildtype embryos using PyMT virus mediated immortalization of endothelial cells. These lines were validated for the presence or absence of Rudhira and also for their endothelial nature. These lines were then used to determine the roles of the two halves of Rudhira in cell migration.

Using *in vitro* wound healing assay, we observed that both RudA and RudB can independently promote cell migration in non-migratory cells. Interestingly, RudA was able to increase the rate of cell migration substantially more compared to RudB or RudFL, suggesting an inherent difference between the two halves with respect to their contribution to cell migration. It is possible that RudB is dominant over RudA, such that when the two regions are present together as the full length protein, cell migration is promoted only to the extent allowed by RudB. To understand the molecular basis of this difference, the two regions were overexpressed in the *rudhira* knockout cell line, and their ability to rescue the molecular phenotypes of *rudhira* knockout was checked.

It was observed that both the regions could independently mediate increase in stabilization of microtubules. They could also independently cause a decrease in the level of stress fibers (Figure 5.1 C, D). This points to the possibility of both the halves performing similar functions that help them in stabilizing microtubules independently.

Neither RudA nor RudB was able to reduce the size of focal adhesions (Figure 5.1C, D), suggesting that perhaps both the regions are required together to mediate focal adhesion turnover. One possible reason for this could be that the two regions may facilitate different steps of FA disassembly, depending on what they can interact with. This possibility can be assessed by determining if the two regions differentially interact with molecules involved in FA disassembly, using co-immunoprecipitation. Another possibility is that perhaps both the halves are structurally required together for interaction with specific molecule(s) involved in FA disassembly. Furthermore, to determine whether the two halves are capable of association

and rescue of function in the absence of any covalent bonds, we can co-express them within the same cell, and check whether they restore function to wildtype level.

Future work in this direction includes determining the effects of the two regions on the alignment of microtubules at the leading edge and on the induction of filopodia, since the full length protein has previously been shown to help microtubules reach focal adhesion (Divyesh Joshi, unpublished data) (Figure 5.1A, B) and promote formation of filopodia (Jain *et al*, *Exp Cell Res*, 2012). To correlate the phenotypes at the subcellular level with the ones at the molecular level, the interaction of the two regions with the already known interactors of Rudhira (tubulin, vimentin, cytokeratin, EB1, IQGAP1) needs to be determined using co-immunoprecipitation studies. This will help us ascertain the molecular pathway through which each half performs its functions.

Once we delineate the list of processes regulated by the two halves of Rudhira and the molecular pathways involved, deletion mutants can be generated to narrow down the potential domains responsible for each of the functions. Moreover, in mutants that cause loss of function, we can test whether a lone half can interact with the corresponding half of the full length protein, by co-expressing the appropriate half with the full length protein containing mutation in the corresponding half, and check for rescue. This may give us a clue on the ability of Rudhira to form higher order structures. However, the possibility of recombination between the two corresponding halves needs to be eliminated. For this, the cell expressing both plasmids may be cured of each one separately. The subsequent loss of function would mean that the observed rescue was not due to recombination.

Although there have been a wide range of studies done in order to understand the molecular regulation of vascular development, what has always remained a challenge is to find novel targets for therapeutic intervention. Identifying novel molecules (such as Rudhira) playing roles in vascular development will provide several avenues for potential targets for therapy.

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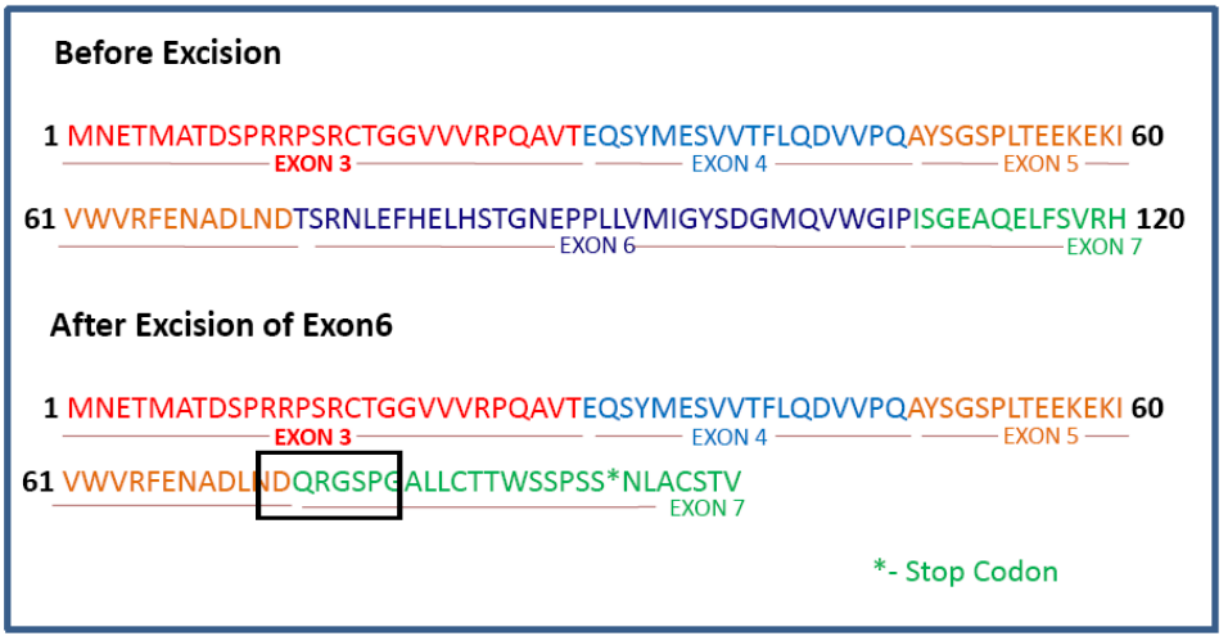
Appendix

A2. Chapter 2

A2.1 Schematic showing truncated Rudhira protein formed after excision of exon 6



(Courtesy Mamta Jain thesis, 2012)

A2.2 Yolk sac culture medium

	For 100ml
DMEM	78ml
FBS	20ml
100X Glutamax	1ml
100X Penstrep	1ml
ECGS (5mg/ml)	0.2 ml

A2.3 List of primers for RT-PCR

Rudhira:

mR272 F: 5’-TGCAGCCATGGACGACACATCACAGAATCTAG-3’

mR272 R: 5’-GAAGTGCTCGAGAATGCCATCACTGTCCGAG-3’

GAPDH:

GAPDH F: 5’-TGCCCCCATGTTTGTGATG-3’

GAPDH R: 5’-TGTGGTCATGAGCCCTTCC-3’

A2.4 Buffer A for protein lysate

HEPES-KOH (pH 7.4)	25mM
NaCl	150mM
$MgCl_2$	5mM
Triton-X 100	0.5%
Glycerol	10%
PMSF	100mM
Sodium orthovanadate	1mM
PI cocktail (SIGMA, cat. no. P8340)	10µg/ml