Regulation of differentiation by rhogtpases in mouse embryonic stem cells

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2015

Tan, M. H. (2015). Regulation of differentiation by rhogtpases in mouse embryonic stem cells. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/65033>

<https://doi.org/10.32657/10356/65033>

NANYANG
TECHNOLOGICAL
UNIVERSITY

REGULATION OF DIFFERENTIATION BY RHOGTPASES
IN MOUSE EMBRYONIC STEM CELLS

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SCHOOL OF BIOLOGICAL SCIENCES

2015

REGULATION OF DIFFERENTIATION BY RHOGTPASES IN MOUSE EMBRYONIC STEM CELLS

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A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2015

Acknowledgements

I would like to take this opportunity to express my sincere gratitude to my supervisor Associate Professor Koh Cheng Gee for her valuable guidance and patience during my stay in School of Biological Sciences (SBS), Nanyang Technological University (NTU). This project would not have been possible without her advices and directions. I am grateful to her for allowing me to pursue PhD study in her laboratory.

I would like to thank NTU-SBS for providing a conducive environment with well-equipped facilities for my research work. I am very grateful for the generous financial supports from Ministry of Education (Singapore) and Biomedical Research Council, A*STAR (Singapore).

I would like to express my appreciation to my Thesis advisory committees: Professor Ravi Kambadur, Associate Professor Andrew Tan and Assistant Professor Zhang Li-Feng, for their constructive discussions and suggestions.

Many thanks go to my dearest friends and lab members (past and present): Xieyi, Alison, Agatha, Hong Yuen, Yiwen, Elynn, Jing Ling, Anna, Andrew, Songjing, Sirong, Weng Ting, Ishani, Kelvin, Soak Kuan, Swea Ling, Ailing and Felicia, who have created a wonderful environment for me to work and live in. I truly treasure the friendship and teamwork. Without their valuable suggestions and supports, this project would not have gone this far. In addition, I would like to thank colleagues of SBS who have helped me in one way or another.

I would like to thank Associate Professor Evelyn Yim, Mechanobiology Institute (MBI) of National University of Singapore (NUS) for sharing her knowledge and insights in mechanobiology. In addition, I would also like to thank Dr Kukumberg Marek for the tremendous help and time in providing us the resources, assistance and guidance.

At last and the most important, I thank my lovely parents for their endless love, care and faith in me. I am deeply grateful for their tremendous support and encouragement all these years. And I thank my two lovely sisters for sharing their joy, laughter and company with me. In addition, I would like to show my appreciation to my late uncle who has doted on me since young, I admire his strong will and courage. Finally, I would like to dedicate this thesis to my family.

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Abbreviation

ANOVA	Analysis of variance
ARP2/3	Actin-related protein 2/3
ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
BRY	Brachyury
BSA	Bovine serum albumin
Cdc42	Cell division cycle 42
cDNA	Complementary deoxyribonucleic acid
CR	Cysteine/histidine-Rich
CRIB	Cdc42/Rac1-interacting binding domain
DDR1	Discoidin domain receptor 1
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
Ebs	Embryoid bodies
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FH	Formin homolog
FITC	Fluorescein isothiocyanate
FOXA2	forkhead box protein A2
GAP	GTPase-activating protein
GBD	GTPases binding domain
GDI	Guanine nucleotide dissociation Inhibitor
GDP	Guanosine di-phosphate
GEF	Guanine nucleotide exchange factor
gp130	Glycoprotein 130
GSC	Goosecoid
GST	Glutathione S transferase
GTP	Guanosine tri-phosphate
hESC	Human embryonic stem cell
hMSC	Human mesenchymal stem cell
HRP	Horse radish peroxidase
HSD	Honest significant difference

ICM	Inner cell mass
IGF	insulin/insulin-like growth factor
IPTG	Isopropyl-beta-D-thiogalactopyranoside
LIF	Leukemia inhibitory factor
LIMK	LIM kinase
LPA	Lysophosphatidic acid
MAP	Microtubule associated protein 2
MARC	Multi-architectural chip
mc	mCherry
mDia	Mammalian homolog of drosophila diaphanous
MEF	Mouse embryonic fibroblast
MES	2-(N-Morpholino)ethanesulfonic acid hydrate
mESC	Mouse embryonic stem cell
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MRCK	Myotonic dystrophy kinase-related Cdc42-binding kinase
MSC	Mesenchymal stem cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NGF	Nerve growth factor
NMII	Non-muscle myosin II
NTC	No-template controls
N-WASP	Neural Wiskott-Aldrich syndrome protein
PAK	p21-activated kinase
PBD	p21-binding domain
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDMS	Polydimethylsiloxane
PE	Primitive endoderm
PH	Pleckstrin homolog
PI3K	Phosphoinositide-3-kinase
PKB	Protein kinase B
PLL	Poly-L-lysine
PMSF	Phenylmethanesulfonyl fluoride
PPPPP	Proline-rich region
RBD	Rho binding domain
ROCK	Rho-associated coiled-coil containing kinase
ROK	Rho-kinase
RT	Reverse transcription
SCC	Silica colloidal crystal
SDS-PAGE	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel

	electrophoresis
SH 2	SRC-homolog 2
Sox 2	SRY-related HMG box 2
SRF	Serum response factor
SRY	Sex determining region Y
Stat 3	Signal transducer and activator of transcription
STE	Sodium chloride-Tris-EDTA
TBP	TATA-binding protein
TBS	Tris buffered saline
TrkA	Tropomyosin receptor kinase A
VCA	Verprolin homolog, central hydrophobic and acidic region
VEGF	Vascular endothelial growth factor
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family veroprolin homologue
WH	WASP homology

Abstract

RhoGTPases, which belong to the Ras superfamily, play essential roles in the regulation of actin cytoskeleton that is vital for many cellular functions such as cell migration, cell adhesion, cell proliferation and many other cellular activities. RhoA, Cdc42 and Rac1 are the three most well studied Rho family members in mammalian cells. When expressed in fibroblast and epithelial cells, RhoA induces the formation of stress fibers, Cdc42 promotes the formation of filopodia and Rac1 stimulates lamellipodia formation. However the roles of these RhoGTPases in mouse embryonic stem cells (mESCs) are not clear. We have initially hypothesized that RhoGTPases also regulate actin cytoskeleton in mESCs. However, in this study we found that 1) unlike adherent differentiated cell types, transient expression of RhoGTPases does not induce changes in the actin cytoskeleton of mESCs, 2) RhoGTPases and their downstream effector proteins are present in mESCs, and 3) the levels of RhoA protein increase upon differentiation.

Based on our findings described above, we hypothesize that RhoA plays a role during stem cell differentiation. In order to investigate along the line of our hypothesis and to study the effects of RhoA on the differentiation of mESCs, stable mESCs expressing RhoA are generated. The cell morphology, phenotype and pluripotency of these stable RhoA expressing cells resemble that of the wild type mESCs. However, we notice reduced cell adhesion for mESCs expressing constitutively active RhoA (RhoA-V14). During differentiation, mESCs expressing RhoA-V14 preferentially differentiate into endoderm and mesoderm lineages, while inhibiting ectoderm differentiation.

Cell fate decision of ESC is also regulated by microenvironment. Cells receive extracellular signals from the microenvironment and translate them into intracellular signaling that is responsible for gene transcription and regulation. Common extrinsic factors include ECM proteins, substrate topography and stiffness. Since we have shown that active RhoA can influence the selection of cell fates during mESC differentiation and that RhoA is known to regulate signaling downstream of cell adhesion to the ECM, we hypothesize that cell fate selection which is linked to

extrinsic factors such as ECM type and topography are mediated by RhoA signaling. To study the possible roles of ECM on differentiation, we induce differentiation of mESCs on surfaces coated with different ECM proteins. Our results show that collagen and fibronectin enhance differentiation towards endoderm and mesoderm layers. Possible signaling pathways are also explored.

1 Introduction

1.1 Ras superfamily

Proteins from the Ras superfamily are important for many cellular functions such as the regulation of actin cytoskeleton dynamics, cell cycle progression, cell polarity, mobility, and differentiation. The Ras superfamily can be further classified into 5 main subgroups based on their structures and functions. The 5 main subgroups are Ras, Rho/Rac, Rab, Arf and Ran. Ras is responsible for the regulation of gene transcription. Rho/Rac regulates actin cytoskeleton dynamics and gene transcriptions. Rab and Arf are involved in the regulation of vesicular trafficking, and Ran participates in nuclear transport of the cell (Bhattacharya et al., 2004; Militello and Colombo, 2013; Sasaki and Takai, 1998).

These GTPases share the same characteristic, whereby they act as molecular switches that cycle between the active guanosine tri-phosphate (GTP)-bound and inactive guanosine di-phosphate (GDP)-bound states. The activities of the GTPases are governed by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation Inhibitors (GDIs) (Figure 1). GEFs catalyze the exchange of GDP for GTP, thus rendering the Rho proteins active. GAPs catalyze the hydrolysis of GTP to GDP, resulting in the inactivation of the RhoGTPases (Bernards, 2003; Garrett et al., 1991; Garrett et al., 1989). GDIs inhibit the dissociation of GDP from the Rho proteins, which prevent spontaneous activation of Rho proteins (Olofsson, 1999). Inactive GDP-bound Rho proteins are soluble and are localized in the cytoplasm, while active GTP-bound Rho proteins translocate to the plasma membrane (Narumiya et al., 1988) and regulate downstream effectors or targets through conformation-specific interactions.

Figure 1: Rho proteins serve as molecular switches in the regulation of actin cytoskeleton dynamics.

Rho proteins are small GTPases that cycle between the active Rho-GTP bound and inactive Rho-GDP bound state. This process is regulated by GEF, GAP and GDI. Active Rho proteins recruit and activate effectors that are required for the regulation of actin cytoskeleton dynamics and gene expressions.

1.2 RhoGTPase subfamily

RhoGTPases are classified into 8 different subfamilies based on their amino acid content. The 8 subfamilies are: Rac, Cdc42, Rho, Rif, Rho BTB, CHP and WRCH1, RND and RhoH. These subfamilies are further sub-categorized into 2 types of RhoGTPases, classical (Rac, Cdc42, Rho and Rif) and atypical RhoGTPases (Rho BTB, CHP and WRCH1, RND and RhoH) (Table 1) (Heasman and Ridley, 2008). Classical RhoGTPases cycle between the active GTP-bound form and inactive GDP-bound form, while atypical RhoGTPases are constantly present in an active state, which are likely due to a mutation that renders Rho active. RhoGTPases, such as RhoA, Cdc42 and Rac1, play roles in regulating actin cytoskeleton dynamics. In mammalian cells, RhoA induces the formation of stress fibers, which are bundles of anti-parallel actin filaments cross-linked with myosin. Cdc42 stimulates the formation of filopodia and Rac1 promotes the formation of lamellipodia (Burrige et al., 1988; Small, 1981, 1995).

	Subfamilies of RhoGTPases	Members
Classical RhoGTPases	Rac	Rac1, Rac2, Rac3 and RhoG
	Cdc42	Cdc42, TC10 and TCL
	Rho	RhoA, RhoB and RhoC
	Rif	Rif and RhoD
Atypical RhoGTPases	RhoBTB	RhoBTB1, RhoBTB2
	CHP and WRCH1	CHP and WRCH1
	RND	RND1, RND2 and RND3
	RhoH	RhoH

Table 1: Classification of RhoGTPase family.

1.2.1 RhoA signaling

All 3 isoforms of Rho protein, RhoA, RhoB and RhoC, are highly homologous in sequence and induce stress fiber formation in fibroblast (Wheeler and Ridley, 2004). However these isoforms do not share the same function in the cells. This can be due to the differences of the carboxy terminal (C-terminal) among the 3 isoforms, thus affecting their localization in the cell. RhoA and RhoC are generally found at the plasma membrane and cytoplasm, while RhoB localizes to the late endosomes, lysosomes and plasma membrane (Heasman and Ridley, 2008). RhoA has been shown to regulate actin cytoskeleton, cell adhesion and actomyosin contractility. RhoB is involved in cytokine trafficking and cell survival, and RhoC controls cell motility (Heasman and Ridley, 2008). RhoB- and RhoC-null mice are able to survive without any major developmental defects (Hakem et al., 2005; Liu et al., 2001), while knockout of RhoA in mice has been shown to be embryonic lethal (Pedersen and Brakebusch, 2012).

RhoA, the most extensively studied among the 3 isoforms, plays a role in the modulation of cell migration, cell shape and cell adhesion. The roles and functions of RhoA have been elucidated using exoenzyme C3 transferase. C3 transferases are isolated from *Clostridium botulinum*. It catalyzes the reaction to ADP-ribosylate Asparagine 41 (of Rho) and thus inhibiting the activation of Rho (Aktories et al., 1989; Machesky and Hall, 1997; Paterson et al., 1990; Ridley and Hall, 1992; Sekine et al., 1989). Treatment of fibroblast cells with C3 transferase induces cell rounding and loss of stress fibers (Chardin et al., 1989; Paterson et al., 1990; Rubin et al., 1988). On the other hand, activation of RhoA in fibroblast leads to an increase in stress fiber and focal adhesion formation (Ridley and Hall, 1992). RhoA can be activated through the use of lysophosphatidic acid (LPA), a serum derived factor, that will bind to cell surface receptor coupled with G-protein, which in turn activates RhoA through the activation of GEFs (Kumagai et al., 1993).

RhoA regulates actin cytoskeleton reorganization via the activation of its effectors Rho-kinase (ROK)/ Rho-associated coiled-coil containing kinase (ROCK) and mammalian homolog of *Drosophila* diaphanous (mDia) (Figure 2). The balance of activities from the different Rho effector pathways will determine the final outcome on the actin cytoskeletal structures and cellular morphology. Detailed discussion of the various effectors of RhoA and proteins in the effector pathways is given below.

Figure 2: Signaling Pathways of RhoGTPases.

RhoA-ROK signaling mediates stabilization of actin filament that is required for stress fiber formation. ROK/ ROCK is a serine/threonine kinase. Two isoforms, ROK α (ROCK2) and ROK β (ROCK1), have been reported. They share 62% similarity in amino acid sequences and 92% identity in their kinase domains. The protein is made up of an amino terminal (N-terminal) kinase domain, a coil-coiled region (containing Rho-binding domain) (Tu et al., 2011) and a C-terminal containing the pleckstrin homolog (PH) domain (Fujisawa et al., 1996; Ishizaki et al., 1996; Leung et al., 1996; Matsui et al., 1996; Nakagawa et al., 1996). The activity of ROK is regulated by the auto-inhibitory region located in the C-terminal region (Amano et al., 1999; Ishizaki et al., 1996). ROK can be activated either by binding of RhoA to the Rho binding domain (RBD) domain of ROK or through homologous binding of ROK, which will promote auto-phosphorylation of ROK (Garg et al., 2008; Ishizaki et al., 1997; Jacobs et al., 2006). Truncation of the C-terminal domain renders ROK active, while constructs with the N-terminal kinase domain truncated can be used as dominant negative ROK. ROK activities can be inhibited using Y27632. This small chemical inhibitor contains a pyridine moiety, which competes with adenosine triphosphate (ATP) for the ATP-binding site found in the kinase domain of ROK (Uehata et al., 1997). ROK α stabilizes actin cytoskeleton by regulating cofilin phosphorylation, while ROK β destabilizes actin cytoskeleton through the regulation of myosin light chain 2 (MLC2) phosphorylation and actomyosin contraction (Maekawa et al., 1999; Shi et al., 2013).

Activated ROK activates downstream effector LIM kinases (LIMK), a serine/threonine kinase, through phosphorylation. LIMK in turn inactivates cofilin through phosphorylation at serine 3. These phosphorylation events lead to stabilization of the actin filaments in the cells (Agnew et al., 1995; Arber et al., 1998; Bokoch, 2003; Sumi et al., 1999; Yang et al., 1998). Two highly-related isoforms of LIM kinases, LIMK1 and LIMK2, have been reported. They share 50% in overall identity and 70% identity in their kinase domains. These kinases comprise of 2 LIM domains (contain cysteine-rich residues), a PDZ domain (at N-terminal) and a serine/ threonine kinase domain (at C-terminal) (Mizuno et al., 1994; Nunoue et al., 1995; Ohashi et al., 1994). Even though these 2 isoforms share similar conserved domains, they are regulated by different regulators. For instance, PAK1 and ROK phosphorylate LIMK1 at

threonine 508 (Maekawa et al., 1999; Ohashi et al., 2000), while ROK and MRCK α activate LIMK2 through phosphorylation at threonine 505 (Kato et al., 2001; Sumi et al., 2001). Overexpression of LIMK in HeLa cells has been shown to induce stress fiber formation (Maekawa et al., 1999).

ROK has also shown to regulate MLC that is required for actomyosin assembly and contraction. Myosin is made up of myosin light and heavy chains. Phosphorylation of MLC of myosin II is regulated by both the MLC kinase (MLCK) and myosin phosphatase. Activated ROK phosphorylates and inactivates myosin phosphatase, thus abolishing its ability to dephosphorylate MLC. This leads to an increase MLC phosphorylation, which promotes contractility of smooth muscle cells, and focal adhesion assembly in non-muscle cells (Amano et al., 1996; Kimura et al., 1996). MLC can also be phosphorylated directly by ROK (Totsukawa et al., 2000). The activity of MLC phosphatase also contributes to cell rounding during mitosis (Matsumura et al., 2001).

RhoA also governs actin polymerization through regulation of mDia, a well-studied diaphanous-related formin protein. The formin proteins play a role in actin organization that is required for cell polarity and cytokinesis (Copeland and Treisman, 2002; Watanabe et al., 1997). mDia has been shown to regulate stress fiber formation and serum response factor (SRF)-mediated gene transcription (Copeland and Treisman, 2002). The mDia protein is normally in an auto-inhibited state where the C-terminal folds back to interact with the N-terminal. The binding of active RhoA to the GTPase binding domain (GBD) relieves the auto-inhibition of mDia (Li and Higgs, 2003). The well-studied formin homology 2 (FH2) domain of mDia is responsible for the nucleation and elongation of actin filaments (Pruyne et al., 2002). The FH1 domain binds to profilin and is responsible for transferring G-actin to the barbed end of actin filaments (Paul and Pollard, 2008). The function of the FH3 domain is less clear. It has been reported that they may be responsible for cellular localization (Petersen et al., 1998).

Additionally, RhoA-mediated actin reorganization is important for cell adhesions. Integrins that are present on the cell membrane connect to the actin filaments inside the cells. Upon binding of integrin to ECM/matrix, clustering of integrins occur. This clustering action in turn recruits focal adhesion proteins such as paxillin, FAK, and vinculin to the cell membrane for the formation of focal adhesion complexes and also activation of intracellular signaling pathways (Chowdhury et al., 2010b; Connelly et al., 2010; Murray et al., 2013). RhoA/ROK α phosphorylates myosin light chain, which in turn leads to formation of myosin filament and bundling of actin filament into stress fibers. These stress fibers generate tension for the aggregation of integrin and other focal adhesion proteins, which is important for the maturation of focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996; Kaibuchi et al., 1999).

1.2.2 Cdc42 signaling

Cell division cycle 42 (Cdc42) is first discovered in the budding yeast *Saccharomyces cerevisiae*. It has been shown to regulate cell polarity (Pruyne and Bretscher, 2000), whereby loss of Cdc42 inhibits budding and mating protrusion (Etienne-Manneville, 2004). In mammalian cells, Cdc42 is responsible for maintaining cell polarity during migration and reorientation of the Golgi apparatus toward the leading edge (Osmani et al., 2010). Knockout of Cdc42 in mice is found to be embryonic lethal. The embryos die before E7.5 and are generally smaller in size with disorganized filamentous actins (Chen et al., 2000). These findings suggest that Cdc42 is required for early development. Activation of Cdc42 by bradykinin resulted in the formation of microspikes and filopodia in mammalian cells (Nobes and Hall, 1995). Activated Cdc42 regulates actin cytoskeleton through 3 effectors, Neural Wiskott-Aldrich syndrome protein (N-WASP), p21-activated kinase (PAK) and myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK).

Wiskott-Aldrich syndrome protein (WASP) family of proteins is characterized by the presence of a conserved verprolin homology, central hydrophobic and acidic region (VCA) domain. The VCA domain promotes actin nucleation by actin-related protein 2/3 (ARP2/3) complex (Machesky and Insall, 1998; Machesky et al., 1999; Pollard, 2007), whereby the V domain interacts with the G-actin and the CA domain binds to

ARP2/3 complexes. WASP is commonly found in hematopoietic cells (Derry et al., 1994), while N-WASP is first identified in brain but found to be ubiquitously expressed in cells (Miki et al., 1996). The N-terminal region consists of the Cdc42/Rac1-interacting binding domain (CRIB) domain, which allows binding of Cdc42. Under normal condition, WASP/N-WASP is present in an inactive state. The binding of Cdc42 to N-WASP induces a conformational change, which disrupts the auto-inhibition. This allows the VCA domain to interact with the ARP2/3 complex leading to actin nucleation (Higgs and Pollard, 1999; Machesky and Gould, 1999). Active Cdc42 has also been shown to stimulate filopodia formation through the activation of N-WASP and ARP2/3 complex (Abdul-Manan et al., 1999).

PAK is one of the first effector proteins of RhoGTPases to be identified (Manser et al., 1994). It is a serine/threonine protein kinase that interacts with Cdc42 and Rac1. PAK has been implicated in signaling pathways regulating cell adhesion, cell motility, differentiation, cell proliferation and other cellular activities that require active remodeling of the actin cytoskeleton. PAK is further subdivided into two families. Group 1 PAK (isoform 1,2,3) consists of a C-terminal catalytic domain, CRIB or p21-binding domain (PBD), and an auto-inhibitory domain, Group II PAK (isoform 4,5,6) contains the CRIB domain but does not appear to contain the auto-inhibitory domain (Bokoch, 2003; Eswaran et al., 2008; Jaffer and Chernoff, 2002). Activation of group 1 PAK requires interaction with Cdc42 or Rac1. Even though group II PAK also interacts with active Cdc42, the binding to Cdc42 does not enhance the kinase activity (Zhao and Manser, 2005). Both PAK1 and PAK4 phosphorylate LIMK1 at threonine 508 which results in the activation of LIMK1 (Dan et al., 2001; Edwards et al., 1999). The activated LIMK1 can then phosphorylate and inactivate cofilin, leading to stabilization of actin filaments that is needed for the formation of filopodia (for Cdc42) and lamellipodia (for Rac1) (Heasman and Ridley, 2008; Knaus and Bokoch, 1998; Lei et al., 2000; Sells and Chernoff, 1997). PAK has also been shown to play a role in smooth muscle contractility. The kinase regulates MLC in smooth muscle cells through phosphorylation of MLC regulatory proteins such as MLCK and myosin phosphatase in addition to direct phosphorylation of MLC itself (Chew et al., 1998; Goeckeler et al., 2000; Sanders et al., 1999; Takizawa et al., 2002).

MRCK is a downstream effector of Cdc42. This kinase participates in the regulation of MLC phosphorylation. Phosphorylation of non-muscle MLC at serine 19 induces actin-myosin contractility. MRCK also regulates cell polarity that is necessary for directing cell migration (Gomes et al., 2005). Unlike ROK, MRCK preferentially localizes to the cell-cell junctions and cell periphery. MRCK contains a GBD, a cysteine-rich region, a PH-like domain and a kinase domain at the N-terminal. The GBD only binds to GTP-bound Cdc42. The cysteine-rich region binds to phorbol ester, and the PH-like domain is responsible for cellular localization of MRCK (Leung et al., 1998).

1.2.3 Rac1 signaling

Rac1 has been associated with cell migration, motility and phagocytosis. It induces actin polymerization at the plasma membrane to form lamellipodia (Ridley et al., 1992). Rac1 regulates actin cytoskeleton via the WASP family veroprolin homologue (WAVE) and the ARP2/3 complex to promote actin nucleation (Yamazaki et al., 2007). Rac1 activities can be initiated by the binding of external factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) or insulin to their respective cell surface receptors. Inactive Rac1 is then recruited from the cytoplasm to the cell membrane and becomes activated by GEF. Active Rac1 generally localizes to the leading edge of migrating cells. Rac1-null mice are found to be embryonic lethal. The embryos are smaller in size and their epiblasts lack lamellipodial structures, suggesting that Rac1 plays an important role in events prior to gastrulation (E6.0) (Sugihara et al., 1998).

WAVE, another member of the WASP family of proteins, participates in actin nucleation that is necessary for lamellipodia formation (Campellone and Welch, 2010). Unlike N-WASP, WAVE only contains the VCA domain, and is without the CRIB domain. WAVE is usually found in a protein complex consisting of Cyfip, Nap1, Abi and HSPC300. Upon activation by Rac1, WAVE is released from the protein complex and interacts with ARP2/3 complex to regulate lamellipodia formation (Miki et al., 1998b). There are 2 isoforms of WAVE, WAVE 1 and WAVE 2. WAVE 1 is responsible for the stabilization of lamellipodia protrusion during cell spreading

(Yamazaki et al 2005), and WAVE 2 is required for the extension of lamellipodia protrusion and regulation of cadherin-mediated cell-cell adhesion (Yamazaki et al., 2007).

1.3 Embryonic development of the mouse

Fertilization occurs when the sperm fuses with the egg, giving rise to a diploid zygote in the oviduct of female mouse (*Mus musculus*) (Figure 3). Embryonic day (also known as days post coitum) is defined as day after the detection of copulation plug. During compaction, diploid zygote (one cell stage) undergoes cell division to form 2 cells, and later 4 cells and so on to form cluster of cells known as morula (around 8 to 16-cell stage) (Lee et al., 2001). During cavity formation (16 to 32 cell stage), small cavities are formed and later fuse to form a large cavity, thus giving rise to the blastocyst (16 to 32-cell stage) (Motosugi et al., 2005). Blastocyst consists of the trophoectoderm (outer layer) and inner cell mass (ICM). The trophoectoderm develops to form trophoblast and subsequently forms the placenta. While the ICM gives rise to 2 layers of cells, the outer layer comprising of primitive endoderm (PE) that will differentiate to form visceral endoderm and extra-embryonic mesoderm. The inner layer consists of epiblast, which has the ability to differentiate into all cell types derived from the three primary germ layers (endoderm, mesoderm and ectoderm). Upon implantation of blastocyst to the uterus, cells in the ICM proliferate and occupy the cavity, which result in the formation of pre-implantation epiblast and later develop into post-implantation epiblast. Gastrulation occurs when the three primary germ layers are formed (Kojima et al., 2014), and ingression occurs at the posterior region of the epiblast (primitive streak). Subsequently, the epiblast cells undergo epithelial to mesenchymal transition (EMT), where changes in cell shape and breakdown of intercellular junctions are required for cell-matrix formation. Cells in the endodermal layer have the potential to differentiate into neuronal and epidermal cells, whereas cells in the mesodermal layer have the ability to give rise to hematopoietic, cardiac, skeletal muscle and endothelial cells, and lastly endodermal derived cells promote differentiation into pancreatic cells and hepatocytes.

Figure 3: Embryonic development of a mouse embryo.

1.3.1 Endoderm

Endoderm-derived cells have the potential to differentiate into cells of pancreas, lung, liver and prostate. Detailed studies have been made in this field of research due to its clinical potential in treating diabetes type I patients and patients with liver diseases. However research in this area of interest progresses slowly due to the low efficiency in obtaining endodermic cell type using differentiation in culture system (Keller, 2005).

1.3.2 Mesoderm

Cells in the mesoderm layers give rise to hematopoietic cells, cardiac cells, endothelial cells and skeletal muscles (Rohwedel et al., 1994). Among these mesoderm-derived lineages, hematopoietic lineage is the most extensively studied (Burkert et al., 1991; Keller et al., 1993; Nakano et al., 1994; Schmitt et al., 1991). This is because of its potential to produce transplantable hematopoietic stem cells for treatment of blood-related disorders and diseases. In addition, mesoderm-derived

cells can acquire vascular and cardiac commitments (Schroeder et al., 2003). Differentiation into hematopoietic cells is serum-induced, and can be augmented by addition of bone morphogenetic protein (BMP), vascular endothelial growth factor (VEGF) and other hematopoietic cytokines (Cerdan et al., 2004).

1.3.3 Ectoderm

Ectodermal cells can acquire neural and skin epidermal identities (Munoz-Sanjuan and Brivanlou, 2002). Ectoderm differentiation into neural cell fate using mESCs has been well characterized. Common protocols include treatment of embryoid bodies (EBs) with retinoic acids (Bain et al., 1995) with sequential removal of serum from the culture medium (Okabe et al., 1996).

1.4 Embryonic stem cells (ESCs)

ESCs are derived from the ICM of blastocyst, they possess self-renewal and pluripotent properties. Pluripotency is defined by the ability of a cell to give rise to all cell types in the body (Brivanlou et al., 2003; Hogan, 1999; Keller, 2005), and self-renewal refers to the ability to self-divide without affecting the pluripotency. Signaling pathways that govern ESC's self-renewal and pluripotent properties are highly regulated.

1.4.1 Pluripotency of ESC

The LIF/ glycoprotein 130 (gp130)/ signal transducer and activator of transcription 3 (Stat 3) pathways and several transcription factors govern the self-renewal properties of mESCs. Leukemia inhibitory factor (LIF) is a cytokine that is necessary for the proliferation and pluripotency of mESCs (Smith et al., 1988; Williams et al., 1988). It is commonly added into cell media to maintain pluripotency when culturing mESCs. Binding of LIF to the LIF receptor leads to the formation of a heterodimer, comprising of the LIF receptor and gp130. The heterodimer will then induces the activation of the tyrosine kinase Jak (Ernst et al., 1996), which in turn phosphorylates gp130. Tyrosine phosphorylated gp130 can then interact with SRC-homolog 2 (SH 2)

containing proteins, such as the Stat family of transcription factors (Ihle, 1996). Stat 3, a key downstream transcription factor of LIF/ gp130 pathway, plays an important role in maintaining the self-renewal properties of mESCs (Niwa et al., 1998). It has been reported that mutations in the tyrosine residues of gp130 inhibit the self-renewal ability of ESCs (Ernst et al., 1999; Matsuda et al., 1999). Even though LIF/ gp130/ Stat 3 pathway was first identified to regulate self-renewal properties in mESCs (Burdon et al., 2002), it is later found to be dispensable for the development of ICM and self-renewal of human embryonic stem cell (hESC) (Nichols et al., 2001), suggesting that mESCs and hESCs do not share the same signaling pathways for maintaining ESC self-renewal.

In addition, a couple of other transcription factors, Oct 4 and SRY-related HMG box 2 (Sox 2), are found to be essential for maintaining ESC pluripotency. Oct 4 (Oct 3, Oct 3/4 or Pou5f1) is expressed in the ICM. Oct 4 is found to be down-regulated upon differentiation into trophoblast (Nichols et al., 1998) or when LIF is withdrawn from the culture system (Chambers and Smith, 2004). However, expression of Oct 4 alone has been shown to be insufficient to sustain mESCs' pluripotency, it requires additional signal from LIF stimulation (Niwa et al., 2000).

Mitsui et al., (2003) has identified another transcription factor, Nanog that can maintain ESC pluripotency in the absence of the LIF/ gp130/ Stat 3 pathway. Nanog expression is mainly concentrated in the inner cells of a compacted morula and blastocyst. Decrease in Nanog levels are observed in differentiating cells (Chambers et al., 2003; Mitsui et al., 2003). Deletion of Nanog results in loss of pluripotency in both ICM and ESC. On the other hand, elevated expression of Nanog can bypass the LIF/ gp130/ Stat 3 pathway and maintain the ESC self-renewal and pluripotent properties (Mitsui et al., 2003).

In summary, both Oct 4 and Nanog transcription factors are required to inhibit differentiation from ESC into trophectoderm and primitive endoderm, respectively. However these factors are insufficient to maintain mESCs self-renewal properties for

prolonged period. An additional transcription factor, Stat 3, is required for sustaining ESC pluripotency (Matsuda et al., 1999).

1.5 Roles of RhoA in embryonic development

1.5.1 Roles of RhoA in mesodermal lineage

Detailed analysis of the role of RhoA in mesodermal differentiation from multipotent stem cells has been reported. Earlier studies mainly focus on multipotent stem cells such as mesenchymal stem cells (MSCs). Unlike ESCs, MSCs are adult stem cells that can only differentiate into limited number of defined cell types found in the body, such as adipocytes, chondrocytes, osteoblasts, and myogenic cells. MSC is first discovered to originate from bone marrow, later studies shown that it can be derived from fetal tissues, cord blood, peripheral blood, placenta and adult tissues. It has been demonstrated that RhoA can regulate MSC differentiation; several reports have revealed that RhoA/ROK α and actin cytoskeleton organization promote differentiation of hMSCs into osteogenic lineages through the use of chemical inhibitors or overexpression of constitutively active and dominant negative RhoA (Meyers et al., 2005; Xu et al., 2014). High levels of RhoA and myosin-dependent tension promote osteogenesis in hMSCs (McBeath et al., 2004). Similarly in mouse MSC model, non-muscle myosin has also shown to stimulate osteogenic differentiation, while it inhibits adipogenic differentiation (Arnsdorf et al., 2009b). Another report has suggested that non-canonical Wnt signaling is required to activate RhoA signaling pathway for the induction of osteogenic differentiation (Arnsdorf et al., 2009a; Shi et al., 2012). Wnt proteins bind and activate Dvl protein, this in turn leads to activation of RhoGTPases and their effector proteins such as ROK α and JNK (Veeman et al., 2003). In addition, RhoA has also been shown to regulate chondrogenesis in MSC, whereby activation of RhoA/ROK α inhibits chondrogenesis with concomitant reduction in chondrogenic transcriptional factor, Sox9, and also cortical actin organization, which are distinct features of differentiated chondrocytes (Woods and Beier, 2006; Woods et al., 2005). Taken together, these studies illustrate RhoA signaling play a role in regulation of cell fate determination in MSC into adipogenic, chondrogenic or osteogenic lineages (Arnsdorf et al., 2009b; McBeath et al., 2004).

Myogenic differentiation is an important process during embryonic development, as it gives rise to muscular tissues. Skeletal muscle is required for coordination of body movement through contraction and relaxation of muscle cells in the whole body (Scheunke et al., 2006). Unlike skeletal muscle, smooth muscles are generally found in the wall of blood vessels, arteries and vein, they play role in regulating blood flow, pressure, blood vessel diameter (Majesky, 2007). SRF was first discovered to promote activation of alpha-actin (for skeletal muscle cells) and gamma-actin (for smooth muscle cells) during myogenesis (Browning et al., 1998; Carson et al., 2003; Wei et al., 1998). In addition, SRF also governs myogenesis through the activation of specific promoters of myogenic transcription factors (Settleman, 2003; Sotiropoulos et al., 1999). Later studies revealed that RhoA promotes skeletal and smooth muscle cell myogenesis through SRF-mediated expression of myogenic transcription factors (Mack et al., 2001; Wei et al., 1998). In addition, RhoA signaling is required for myotube formation and expression of myogenic markers. Two downstream effectors of RhoA, ROK and mDia1, have been found to participate in myogenesis (Geneste et al., 2002; Mack et al., 2001; Staus et al., 2007).

Regulation of RhoA activities in myogenesis can be mediated by cell-cell adhesion and insulin/insulin-like growth factor (IGF) signaling pathways. Cell-cell contacts are vital for cellular development. N-cadherin, a type of cell adhesion molecule, is required for gastrulation and found to be expressed in mesoderm (Hatta and Takeichi, 1986). And N-cadherin-deficient mice die at Day 10 of gastrulation, these embryos exhibit major heart defects, without signs of proper muscle formation (Radice et al., 1997). Several reports have proposed that N-cadherin-mediated cell-cell contacts participate in myogenesis, and inhibition of N-cadherin has been shown to inhibit formation of myotubes (Goichberg and Geiger, 1998; Knudsen et al., 1990; Mege et al., 1992). Cell-cell contacts trigger the activation of N-cadherin-dependent signaling that promote upregulation of RhoA activities and myogenic-specific promoters, while inhibiting Rac and Cdc42 in mouse myoblasts (Charrasse et al., 2003; Charrasse et al., 2002). Another study has identified IGF as an upstream regulator of RhoA signaling in myogenesis. Binding of IGF to IGF receptor induces autophosphorylation of the tyrosine kinase found in the cytoplasmic tail of IGF receptor, this in turn recruits intracellular substrates (Sachev et al 2001). IGF

signaling regulates RhoGAP, a negative regulator of RhoA, during myogenesis, whereby IGF promotes the translocation of RhoGAP to the cell membrane so that RhoGAP can inhibit RhoA. Perturbation of RhoGAP in mouse cells promotes myogenesis in response to IGF induction (Sordella et al., 2003). These findings suggest a tight regulation of RhoA activity temporally and spatially for proper embryonic development.

1.5.2 Roles of RhoA in ectodermal lineage

Neurite development or initiation is dependent on reorganization of actin cytoskeleton and microtubule network. RhoA has shown to participate in neurogenesis. Initial studies have shown that treatment of neuronal cells with LPA resulted in neurite retraction and cell rounding (Jalink et al., 1994). LPA is thought to regulate RhoA activities through heterotrimeric Gi proteins, which comprises of Gα12, Gα13 and Gαq (Kato et al., 1998b). Similar studies have reported that expression of constitutively active RhoA inhibits neurite outgrowth and neurite initiation in PC12 and NIE115 neuroblastoma cells (Amano et al., 1998; Hirose et al., 1998; Kato et al., 1998a; Kranenburg et al., 1997; Sebok et al., 1999). Likewise, the inactivation of RhoA activities, using C3 transferase or expression of dominant negative RhoA, induces neurite formation. Several reports have indicated that ROK, an effector of RhoA, regulates neurogenesis, where the activation of ROK stimulates neurite retraction while inhibition of ROK either through Y27632 treatment or expression of dominant negative form of ROK in neuroblastoma cells has shown to promote neurite formation (Govek et al., 2005; Hirose et al., 1998). Similarly, RhoA has been shown to inhibit neural outgrowth in other primary systems such as chick dorsal ganglion and rat hippocampal neurons (Da Silva et al., 2003; Fournier et al., 2003; Jin and Strittmatter, 1997; Sarner et al., 2000). In addition, myosin II, a downstream effector of RhoA/ROK signaling, has demonstrated to be responsible for neurite development in neuroblastoma cells (Amano et al., 1998). In recent years, inhibition of Rho/ROK signaling in neural stem cells and mESCs has been shown to promote neural differentiation (Chang et al., 2010; Gu et al., 2013). Therefore, it is thought that RhoA/ROK signaling inhibits neurite initiation and that is dependent on actomyosin contractility, through the activation of myosin II.

Decrease in RhoA activities is often accompanied by an increase in Rac1 and Cdc42 activities, especially at the tips of protrusions (Kozma et al., 1997; Kranenburg et al., 1997; Sebok et al., 1999; Tigyi et al., 1996; Yamaguchi et al., 2001). Consistent with the above reports, Rac1 and Cdc42 have been shown to induce opposite effects from RhoA signaling on neural differentiation, where Rac1 and Cdc42 stimulate neurite formations in PC12 and NIE115 neuroblastoma cells (Aoki et al., 2004; Sarner et al., 2000). Induction of neurite outgrowth with nerve growth factor (NGF) leads to localized activation of Rac1 and Cdc42 over a large area surrounding the cell periphery (Aoki et al., 2004; Negishi and Katoh, 2002). It has been demonstrated that Rac1-induced lamellipodia and Cdc42-induced filopodia structures are prerequisite for neurite formation. Rac1 has been reported to promote neurite differentiation in other model systems like chick primary retinal neurons and rat hippocampal neuron (Albertinazzi et al., 1998; Schwamborn and Puschel, 2004). Upon NGF induction, tropomyosin receptor kinase A (TrkA)-, phosphoinositide-3-kinase (PI3K)- and Ras-mediated signaling regulate the activation of Rac1 and/or Cdc42 that is essential for neurite formation (Aoki et al., 2004; Kita et al., 1998; Kobayashi et al., 1997; Nusser et al., 2002; Sarner et al., 2000).

Rac and Cdc42 have also been shown to regulate growth cone advance and neurite outgrowth, while RhoA results in collapse of growth cone and neurite retraction (Kozma et al., 1997). Rac1 and Cdc42 regulate these events through effector protein PAK. Expression of dominant negative form of PAK inhibits neurite outgrowth (Dan et al., 2002; Daniels et al., 1998). RhoA induces growth cone collapse and neurite retraction through ROK (Gallo, 2006; Wahl et al., 2000). On the contrary, other reports have suggested opposite observations. One report has revealed that the activation of Rac1 and Cdc42 inhibits neural outgrowth in *Drosophila* neurons (Allen et al., 2000). Other studies have shown that active Rac1 inhibits neurite outgrowth in rat cortical neurons and chick dorsal ganglion (Fournier et al., 2003; Kubo et al., 2002). Another group has identified a novel FERM domain including GEF (FIR), which is a GEF for Rac1, containing tandem Dbl homology-PH domain and FERM domain. Expression of FIR in cortical neurons leads to inhibition of neurite outgrowth (Kubo et al., 2002). Contradictory findings of RhoGTPases on neurite differentiation

may be accounted for by the difference in model systems and cell types. It also implies that a tight regulation of RhoGTPases' activities is essential for neural development.

1.6 Microenvironment regulates mESC self-renewal and differentiation

Cell fate of ESC can be influenced by the microenvironment it resides in. The microenvironment, which comprises soluble factors and extracellular matrices, can impact on many cellular functions such as cell proliferation, differentiation, and migration. In the body, stem cells sense the microenvironment and send signals to the nucleus to activate gene transcriptions that are necessary for the regulation of stem cell functions such as self-renewal and differentiation. So far, extensive studies have been focusing on the effects of biochemical cues on cellular functions in mammalian cells and ESCs, while little is known about the underlying mechanisms of how biophysical cues regulate cell fate determination in ESCs. It has been suggested that mechanical cues induce changes in cell shape, which generate traction stress within the cells and hence leading to activation of signaling pathways. The *in vivo* microenvironment of the cells can be recapitulated using microfabrication technology. This development enables us to gain better understanding of the influence of microenvironment on stem cells biology, and allow the generation of homogenous population of cell types for regenerative therapy (Chai and Leong, 2007; Edalat et al., 2012). Biophysical factors include ECM proteins, topography and matrix stiffness.

1.6.1 Extracellular Matrix (ECM)

ESCs are surrounded by ECM in the body. The ECM provides scaffolds for numerous cellular functions such as cell proliferation, migration, adhesion, survival and differentiation. ECM is composed of network of fibrous proteins, polysaccharides and proteoglycans, which is important for providing mechanical and biochemical supports to the cells. There are 2 types of ECM: basement membrane and interstitial matrix. The basement membrane is a thin sheet of ECM which provides support to the epithelial cells. One main function of the basement membrane is to provide support for the epithelial cells and link them to the connective tissues. (Cooper, 2000;

Meng et al., 2014; Watt and Huck, 2013). The interstitial matrix is found mainly in the interstitial connective tissues such as bone, tendon and cartilage.

Cell surface receptors such as integrins are responsible for cell adhesion to the ECM. Integrins are heterodimers, consisting of an α -subunit and a β -subunit. Thus far, 16 α subunits and 8 β subunits have been identified (Hynes, 1992). Integrins belong to the type I transmembrane protein family. The integrin proteins have small cytoplasmic domains and large extracellular domains. Upon binding of ligand to the extracellular domain, the α - and β -subunits dimerize and the cytoplasmic tails promote recruitment of interacting proteins, leading to the activation of downstream signaling pathways and also actin reorganization in the cells (Hynes, 1992).

Collagen is the main structural protein found in the ECM. The collagen family of proteins consists of approximately 20 members, with collagen type 1 being the most abundant protein found in the ECM. Two well-studied groups of collagen are fibrillar collagens (type I, II, III, V, XI) and networking-form collagens (type IV, VIII, XIV). Fibrillar collagens form fibrils after being secreted into ECM, whereas network-forming collagens assemble to form the basement membrane (Cooper, 2000). Collagen I has been shown to regulate self-renewal of mESCs through integrin $\alpha 2\beta 1$ -mediated signaling pathways. Collagen I helps to maintain cells in undifferentiated state (through expression of Nanog, OCT 4, SSEA-1), through suppression of expression of differentiation markers (GATA4, FGF5, Cdx2) (Hayashi et al., 2007). It has been shown that the binding of collagen to integrin $\alpha 2\beta 1$ and discoidin domain receptor 1 (DDR1) promotes self-renewal of mESCs via the activation of Ras, PI3K/AKT and extracellular signal-regulated kinase (ERK) signaling pathways (Fasano et al., 2007; Hashemi et al., 2011; Nur et al., 2006; Paling et al., 2004; Park et al., 2004; Suh and Han, 2011) that regulate gene expression of pluripotency markers such as Nanog, Tbx3, Tcl1 and Esrrb (Storm et al., 2007; Storm et al., 2009). A very recent study has reported that collagen-binding integrin $\alpha 6\beta 1$ is essential for the maintenance of long term ES cell survival in *in vitro* culture, and deletion of integrin $\alpha 6\beta 1$ hinders self-renewal of ESCs (Cattavarayane et al., 2015).

Another type of ECM proteins are the adhesion proteins, they participate in connecting ECM proteins together and also to cell surfaces. Commonly found adhesion proteins are fibronectins and laminins. Fibronectins are generally found in connective tissues and laminins are located in the network-like structures of basement membrane. Both fibronectins and laminins contain binding sites for collagen, proteoglycans and cell surface receptors (Cooper, 2000). Fibronectin has been shown to induce differentiation of mESCs by upregulating FGF5 and ERK1/2 (negative regulator of self-renewal). On the other hand, it decreases alkaline phosphatase activities and expression of self-renewal markers, STAT3 and AKT/Protein kinase B (PKB) (Hayashi et al., 2007). ECM proteins such as fibronectin have been shown to induce endodermal differentiation in hESCs (Brafman et al., 2013). Moreover, endodermal differentiation leads to an increase of integrin expression, in particular integrin $\alpha 5$, αV , $\beta 1$ and $\beta 5$ (Brafman et al., 2013; Pimton et al., 2011; Wong et al., 2010). Taken together, ECM-integrin complexes are important for the regulation of differentiation and self-renewal. In a separated study, Park et al (2011) has reported that fibronectin can stimulate cell proliferation of mESCs via regulation of RhoA-PI3K and AKT-ERK1/2 pathway that is dependent on phosphorylation of cell proliferation regulator, caveolin-1 (Park et al., 2011).

1.6.2 Topography

Topography is the study of shapes and features on the surfaces. In general, all cells in the body are surrounded by ECM proteins that form complex network with pores, ridges, groove, peaks and depressions. The influence of topography on cellular behavior is first demonstrated in fibroblast cells of chicken embryo heart explants, where the fibroblast cells exhibit preferential cell migration on ridge substrates compared to groove substrates (Curtis and Varde, 1964). The type of topographies can be divided into isotropic and anisotropic patterns. Isotropic patterns comprise features that are identical in all directions, for example pillars and wells. Anisotropic patterns display different properties in different directions, for example groove or grating features that are arranged in repeated order with same dimensions. Studies have shown that fibroblasts and astroglial cells exhibit stronger cell adhesions to

pillar features compared to smooth surfaces, exhibiting an increase in organized actin filaments and focal adhesion complexes (Frey et al., 2006; Ghibaudo et al., 2009; Turner et al., 2000). Detailed investigations have demonstrated that the size, height and spacing of pillar features can influence cell morphology (Ghibaudo et al., 2009). Furthermore, fibroblast cells exhibit stellate-like cell shape with disordered actin filaments and reduced focal adhesions when cultured on pillars with wider spacing. On the other hand, fibroblast cells that are cultured on pillars with smaller spacing exhibit similar morphology as those of flat surfaces (Kolind et al., 2010).

Topography plays a role in regulation of differentiation of stem cells. A recent study has shown that mouse MSCs differentiate into osteogenic lineage more efficiently when cultured on lattice micropatterns compared to flat surfaces (Seo et al., 2011b). Furthermore, small spacings (3 μm) between lattice micropatterns support osteogenic differentiation, while larger spacings (4-8 μm) impede osteogenic differentiation. Additionally, fine-tuning of the height of micropatterns can alter matrix elasticity. Short micropillars, which exhibit stiffer matrix, promote cell spreading of hMSCs leading to bigger focal adhesions, highly organized actin cytoskeleton, and upregulation of osteogenic markers. On the other hand, hMSCs that are cultured on longer micropillars (softer matrix) remain round in shape with smaller focal adhesions and disorganized actin cytoskeleton. These cells exhibit hallmarks of adipocytes whereby lipid droplets are observed within the cells, suggesting that longer micropillars promote adipogenic differentiation in hMSCs (Fu et al., 2010; Yang et al., 2011). Grooved features can also affect cell orientation and actin cytoskeleton. Many cell types, such as fibroblasts, neuronal cells, myoblasts and endothelial cells, align along the long axis of the grooved features (Charest et al., 2007; Clark et al., 1990; Karuri et al., 2008; Li et al., 2008; Oakley et al., 1997; Walboomers et al., 1999). Actin cytoskeleton and microtubules have been demonstrated to align along the long axis of the grooves (Karuri et al., 2008; Oakley et al., 1997; Uttayarat et al., 2005).

Cells respond to mechanical cues through the activation of signaling pathways (Hynes, 2002; Nikkhah et al., 2012; Schwartz and Ginsberg, 2002). Early studies have discovered that actomyosin contractility is responsible for sensing mechanical

forces in the microenvironment, whereby differences in matrix properties result in changes in cell-matrix interaction, which eventually lead to the activation of RhoGTPase signaling pathways. Myosin II participates in sensing the mechanical forces that result in the activation of integrin signaling and regulation of cell fate decision (Friedland et al., 2009). In particular, mechanical force-induced neuronal differentiation of hESCs is mediated by MLCK (Ankam et al., 2015).

1.6.3 Stiffness

Studies have shown that by varying stiffness of the matrices, cell fate decision can be altered. Engler et al 2006 showed that hMSCs cultured on soft matrices that resemble elasticity of the brain microenvironment (0.1-1 kPa) promotes differentiation into neuronal cells. Intermediate stiffness that mimics skeletal muscle (8-17kPa) matrices gives rise to myoblast cells, whereas stiffer substrates (25-40kPa) that mimics the bone gives rise to osteogenic lineage during differentiation. Increase in matrix stiffness enhances cell spreading and cell proliferation, but this has little impact on cell adhesion (Evans et al., 2009). Studies have also shown that ESCs express higher levels of mesendoderm markers, BRY, Mix1 and Eomes, on stiffer substrates compared to softer substrates.

Apart from regulation of differentiation, matrix stiffness also influences self-renewal of mESCs and adult stem cells. mESCs that are cultured on soft substrate, which mimics the intrinsic stiffness of stem cells, can remain undifferentiated even in the absence of LIF (Chowdhury et al., 2010a; Gilbert et al., 2010). Furthermore prolonged passaging (15 passages and above) of mESCs on these soft substrates retain the pluripotency and self-renewal properties in mESCs (Chowdhury et al., 2010a). Rigid matrix leads to loss of self-renewal properties (Gilbert et al., 2010).

1.7 Specific Aims

RhoGTPases have been shown to regulate actin cytoskeleton that is required for many cellular functions such as cell migration, cell adhesion and cell polarity. Knocking out RhoA, Rac1 or Cdc42 leads to embryonic lethality in mice, indicating the importance of these RhoGTPases in embryo development. The main aim of this project is to study the roles of the RhoGTPases in mESCs. In particular, we wish to examine the roles of RhoGTPases in the regulation of actin cytoskeleton in mESCs. RhoGTPases are also reported to modulate transcription activities in the cells. Since the process of ESC differentiation involves drastic changes in cell morphology and transcription, we hypothesize that RhoGTPases can play crucial roles in the differentiation process.

In order to investigate the roles of RhoGTPases in mESC, we first constructed stable mESC lines expressing dominant positive and negative RhoA, Cdc42 and Rac1. Following the establishment of the respective stable cell lines, we proceed with investigation plans to verify the following hypothesis.

Hypothesis 1: RhoGTPases can induce similar changes of the actin cytoskeleton in mESC as observed in adherent cell types. If positive results were obtained, we will proceed to determine if the downstream effectors are also similar. If negative results were obtained, we will determine if any of the downstream effectors could be missing in mESCs.

Hypothesis 2: RhoA can influence cell fate selection during differentiation. We determine if dominant active RhoA can preferentially lead to differentiation of mESC to particular lineages.

Hypothesis 3: Cell fate of ESCs is very much dependent on the microenvironment they reside in. Changes in extrinsic factors such as ECM composition or topography can influence cell fate decision. We determine if different ECM substrates such as fibronectin or collagen can influence differentiation into particular cell lineages. We also determine if the topographies in which the mESCs are plated on can influence differentiation.

2 Methods and Materials

2.1 Cell culture and transfection

Mammalian cells (HeLa, NIH3T3, STO, and MEF) were obtained from ATCC and maintained in mammalian cell culture media in a humidified incubator at 37°C, 5% CO₂, according to ATCC® culture guides for animal cells and stem cells. D3 (derived from 129S2/SvPAS, ATCC, CRL-1934) and E14 (derived 129/Ola, ATCC, CRL-1821) mESCs were maintained in ESC culture media with 1000 U/ml ESGRO® (also known as LIF). mESCs were maintained on mitomycin-inactivated MEF cells grown on gelatin-coated tissue culture dishes. To obtain inactivated MEF cells, proliferating MEF cells were treated with 10 µg/ml of mitomycin C for 4 hours in a humidified incubator at 37°C, 5% CO₂, after which mitomycin C-treated MEF were washed thrice with 10ml of PBS. Inactivated MEF cells were then trypsinized and cultured onto gelatin-coated tissue culture dishes for 24 hours before seeding of mESCs. Inactivated MEF were removed prior to experiments. To prepare gelatin-coated tissue culture dishes, sufficient amount of gelatin solution was added to cover the surface area of dishes and dishes were incubated for 1 hour, and gelatin solution was removed prior to seeding of mESCs and MEF cells.

DNA plasmids were transfected into mESCs and other mammalian cells using Lipofectamine™ 2000 reagent together with Opti-MEM® reduced serum media. Reactions were performed according to the manufacturer's instructions. Transfection reagent was removed from cultured cells and replaced with fresh media after 5 hours of incubation.

2.2 Generating stable transgenic mESCs expressing RhoGTPases

mESCs were transfected with pBOS vector with different cDNA inserts of RhoGTPases. Transfected cells were cultured for 24 hours before treatment with blasticidin S HCL (5 µg/ml) for selection. Positive cells were selected and isolated after one month of antibiotic treatment. Positive clones were verified by the presence of mCherry (mc) (red fluorescence protein) signal under the epifluorescence microscope and immunoblot analysis.

2.3 Indirect immunofluorescence microscopy

Cells were cultured on poly-L-lysine (PLL)-coated glass coverslips (unless otherwise stated), placed in a 35mm tissue culture dishes. Cells were then fixed with fresh 4% paraformaldehyde solution for 20 minutes at room temperature, and permeabilised with 0.2% triton X-100 in phosphate buffered saline (PBS) for 10 minutes. These cells were then blocked with 4% bovine serum albumin (BSA) in PBS containing 0.1% triton X-100. Coverslips were incubated with primary antibodies overnight at 4°C in a humidified chamber, followed by secondary antibodies staining using Alexa 488/546-labelled secondary antibodies or Alexa 488/546 phalloidin for 2 hours at 25°C in a humidified chamber. Primary and secondary antibodies were diluted in 1:100 and 1:1600, respectively, with 4% bovine serum albumin (BSA) in PBS containing 0.1% triton X-100. All washing steps were performed with 0.1% triton X-100 in PBS. Coverslips were mounted onto slides and stained using Vectashield mounting medium for fluorescence with DAPI. Images were acquired with Zeiss observer inverted microscope using Zeiss Plan-Apochromat 100X/1.4 oil objective lens coupled to Cool SNAP HQ² camera (Photometrics) and analyzed using MetaMorph imaging system software (Molecular Devices).

2.4 Immunoblot assay

Cells were grown to 80-90% confluency on 35mm tissue culture dishes. Protein lysates were homogenized in 100 µl of ice-cold mammalian lysis buffer and were clarified by centrifugation at 16,000 g at 4°C for 20 minutes. The pellets were discarded. Protein concentration in the supernatant was measured at OD₅₉₅ by using Bradford protein assay. Lysates were heated at 100°C for 10 minutes and equal amount of protein samples were loaded onto the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the resolved proteins on the SDS-PAGE were transferred to nitrocellulose membrane (0.45 µm, Bio-Rad laboratories) at 80 volts for 2 hours at 4°C. The membrane was blocked with 5% skim milk or 2% BSA in tris buffered saline (TBS) for 1 hour at 25°C. The membrane was probed with primary and secondary antibodies (Horse radish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibody). The membrane

was washed with TBS containing 0.05% Tween 20. Signals were detected using enhanced chemiluminescence (ECL) substrate reagent kit and ECL advance western blot detection kit. The membrane was then exposed to Super RX Fuji medical X-ray film (Fujifilm), which was developed using Kodak X-OMAT ME-1 processor. Primary and secondary antibodies were diluted to 1:1000 and 1:4000, respectively, with 2% BSA in TBS.

2.5 RhoA and Rac/Cdc42 activation assay

RBD (of Rhotekin) and PBD (of PAK1) domains were cloned into in frame with the C-terminus of glutathione S transferase (GST) tag in pGEX 4T1 expression vector. Recombinant plasmids were transformed into chemically competent *Escherichia coli* BL-21 bacterial cells. To produce GST-RBD or GST-PBD fusion proteins, bacterial cells expressing pGEX-RBD or pGEX-PBD were cultured to an OD_{600nm} of 0.5 to 0.6, in a 37°C shaking incubator. The bacterial cultures were subjected to induction with 0.4 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 16°C for 14-16 hours, in a refrigerated shaking incubator. Bacterial cells were then lysed in ice-cold STE buffer and were sonicated and clarified by centrifugation at 16,000 g for 20 minutes at 4°C. GST-RBD/GST-PBD was purified by incubating with glutathione-sepharose 4B beads for one hour at 4°C, beads were then washed thrice with ice-cold STE buffer.

mESCs were cultured to 80-90% confluency on 60mm gelatin-coated tissue culture dishes. Cells were lysed in 300 µl lysis buffer and the cell lysates were clarified by centrifugation at 16,000 g at 4°C for 20 minutes. The supernatant was then incubated with 100 µl of GST-RBD/PBD fusion protein conjugated with glutathione-sepharose 4B beads at 4°C for 2 hours. The beads were washed thrice with 500 µl washing buffer and subjected to SDS-PAGE. Bound GTP-RhoA, GTP-Rac or GTP-Cdc42 were detected using anti-RhoA, anti-Rac and anti-Cdc42 using immunoblotting (Kimura et al., 2000; Pellegrin and Mellor, 2001; Takesono et al., 2010).

2.6 Site-directed mutagenesis

Mutation of tyrosine to glutamic acid at amino acid 256 of full length N-WASP cDNA was performed using QuikChange site-directed mutagenesis kit (Agilent Technologies) to create the single base modification. Reactions were performed according to the manufacturer's protocol. The mutation primers used were

5'GCTTAAAGACAGAGAAACATCAAAAGTTATTGAGGACTTTATTGAAAAACAG GAGGT 3' (forward) and

5'ACCTCCTGTTTTTCAATAAAGTCCTCAATAACTTTTGATGTTTCTCTGTCTTTA AGC 3' (reverse), and template used was pXJ vector carrying full length cDNA of N-WASP (HA-tagged).

2.7 Cell adhesion Assay

96-well tissue culture-grade plate was pre-coated with adhesion molecules like gelatin (0.5%)(w/v), collagen (0.4 mg/ml) and fibronectin (10 µg/ml) for overnight, at 4°C. Unbound molecules in each well were removed and washed with 200 µl of PBS. Wells were blocked with 200 µl of BSA (10 mg/ml) in PBS for 1 hour at room temperature. mESCs cultured on gelatin-coated tissue culture dishes were washed and subjected to tryptic digestion. Cells were counted using haematocytometer and 15,000 cells/100 µl were seeded into each well of a 96 well plate. After 1 hour of incubation at 37°C in a humidified CO₂ incubator, unbound cells were washed with 200 µl of warm PBS and attached cells were fixed with 100 µl of 5 % glutaraldehyde in PBS for 20 minutes at room temperature. Cells were washed thrice with 200 µl of PBS and stained with 100 µl of 0.1 % crystal violet (w/v) in 200 mM MES buffer (pH 6.0) for 1 hour at room temperature. Each well was washed thrice with 400 µl of MilliQ water, and dye was solubilized with 100 µl of 10 % acetic acid in MilliQ water. Absorbance readings were measured at 570 nm wavelength using Tecan Infinite M200 Pro quadruple monochromator microplate reader. Three technical repeats for each sample were carried out and 3 independent experiments were conducted. Changes in cell adhesion were calculated by fold change in absorbance, relative to control (Even-Ram and Artym, 2009).

2.8 RNA extraction, Reverse transcription (RT) - Polymerase chain reaction (PCR) and real time PCR

mESCs were cultured to 80-90% confluency on 35mm tissue cell culture dishes and cells were harvested using tryptic digestion. Cell pellets were snap freeze using liquid nitrogen and stored in -80°C freezer. To extract RNA from mammalian cells, RNeasy mini kit (Qiagen) and QIAshredders (Qiagen) were used, according to the manufacturer's protocol. Extracted RNA was used as template for synthesis of cDNA using VILO cDNA synthesis kit (Life technologies) according to the manufacturer's protocol. Complementary deoxyribonucleic acid (cDNA) samples were diluted and used as templates for real time PCR. Samples were run on ViiA™ 7 Real-time PCR system (Applied Biosystem) using default settings for SYBR green reaction, using SYBR® Green PCR master mix (Life Technologies). Gene expression levels were calculated by $\Delta\Delta CT$ and normalized using TATA binding protein (TBP) as endogenous control. To check for DNA contamination, no-template controls (NTCs) and no RT controls were included. To check for specificity of RT-PCR primers and contamination, melting curve analysis and DNA gel electrophoresis were conducted.

2.9 Statistical analysis

For statistical analysis, unpaired student's t-test or two-way analysis of variance (ANOVA) test were conducted to test the hypothesis (when the observed variation represent a real difference between two groups) and null hypothesis (when there is no difference between the two groups). Two-way ANOVA test was used to compare two independent variables and one dependent variable. Following ANOVA test, post-hoc analysis for multiple group comparison using Tukey's honest significant difference (HSD) test was used. All experiments were performed with at least 3 times independent and results were displayed as mean with standard deviation (SD). The P value obtained from the statistical tests was used to indicate how likely the difference observed would be obtained by chance. If the p value that is less than 0.05, observed difference was considered to be statistically significant and it does not occur by chance, thus null hypothesis was rejected. All statistical analysis were carried out using Prism 6, GraphPad software, La Jolla, USA)

2.10 Materials and Reagents

Table 2.1: List of chemicals and materials

Chemicals	Brand
Acetic acid	Merck
Aprotinin	Sigma Aldrich
Blasticidin	Life Technologies
Bradford protein assay	Bio-Rad laboratories
Bovine serum albumin (BSA)	PAA laboratories
Collagen Type 1	BD Bioscience
Crystal violet	Sigma Aldrich
Dimethyl sulfoxide (DMSO)	Sigma Aldrich
Dithiothreitol (DTT)	GE Healthcare
ECL advance western blot detection kit	GE Healthcare
ESGRO (LIF)	Chemicon
Fibronectin	Sigma Aldrich
Gelatin	Sigma Aldrich
Glutaraldehyde	Sigma Aldrich
Glutathione-sepharose 4B beads	GE Healthcare
Isopropyl-beta-D-thiogalactopyranoside (IPTG)	GE Healthcare
Lipofectamine 2000	Life Technologies
2-(N-Morpholino)ethanesulfonic acid hydrate (MES)	Sigma Aldrich
Mitomycin C	Sigma Aldrich
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma Aldrich

Novex ® Enhanced chemiluminescence (ECL) substrate reagent kit	Life Technologies
Opti-MEM® reduced serum media	Life Technologies
Paraformaldehyde	Merck
PF573228	RnD system
Phenylmethylsulfonyl fluoride (PMSF)	Sigma Aldrich
Poly-L-Lysine	Sigma Aldrich
QuikChange site-directed mutagenesis kit	Agilent Technologies
SMIFH2	RnD system
SYBR® Green PCR master mix	Life Technologies
Triton X-100	Bio-Rad laboratories
Vectashield mounting medium for fluorescence (with DAPI)	Vector laboratories Inc
Y27632	Merck

Table 2.2: List of buffer composition

Buffers and salts	Brand
<u>Mammalian cell culture media</u>	
Dulbecco's modified Eagle's medium (DMEM) - high glucose	Sigma
10 % Fetal bovine serum (FBS)	PAA laboratories
<u>ESC culture media</u>	
15 % Heat-inactivated FBS (selected batches)	PAA laboratories
2 mM Glutamax-1	Gibco
1x Penicillin/ streptomycin	PAA laboratories
0.1 mM Non-essential amino acids	Gibco
1 mM Sodium pyruvate	Gibco
0.1 mM 2 β -mercaptoethanol	Gibco
1000 U/ml ESGRO®	Merck Chemicon
<u>Gelatin solution</u>	
0.5% (w/v) gelatin	Sigma Aldrich
Top up with PBS and autoclave (121°C, 20 min) prior to use.	
<u>Mammalian cell lysis buffer</u>	
50 mM Tris-HCl (pH7.5)	Merck
100 mM Sodium chloride (NaCl)	Merck
1 mM Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich
5 mM Magnesium chloride (MgCl ₂)	Merck
10 % Glycerol	Merck
50 mM Sodium fluoride (NaF)	Merck
1 mM Sodium orthovanadate (Na ₃ VO ₄)	Sigma Aldrich
10 μ g/ml Aprotinin	Sigma Aldrich
10 % Phenylmethylsulfonyl fluoride (PMSF)	Sigma Aldrich
1 % Nonidet P-40	Sigma Aldrich
Top up with MilliQ water	

Washing buffer (for RhoA and Rac/Cdc42 activation assay)

Mammalian cell lysis buffer	Refer to recipe (above)
0.5% Triton-X100	Bio-laboratories

Tris buffered saline (TBS) (pH 7.4)

10 mM Tris HCl	Promega
150 mM NaCl	Merck

STE buffer (pH 8.0)

10 mM Tris HCL	Promega
150 mM Sodium Chloride (NaCl)	Merck
1 mM EDTA	Sigma Aldrich
1 mM PMSF	Sigma Aldrich
100 µg/ml Lysozyme	Sigma Aldrich
5 mM DTT	GE healthcare
1 % Tween-20	Bio-Rad laboratories
0.03 % SDS	Bio-Rad laboratories

Phosphate buffered saline (PBS)

137 mM Sodium chloride (NaCl)	Merck
2.7 mM Potassium chloride (KCl)	Sigma Aldrich
10 mM Sodium phosphate dibasic (Na ₂ HPO ₄)	Sigma Aldrich
1.8 mM Potassium dihydrogen phosphate (KH ₂ PO ₄)	US Biological

Trypsin solution

0.25% (w/v) trypsin	Sigma Aldrich
1mM EDTA	Sigma Aldrich

Table 2.3: List of primary antibodies

Antibody	Species	Company
Anti-RhoA	Mouse	Cytoskeleton
Anti- ROK α	Mouse	BD Transduction
Anti-actin	Mouse	Chemicon
Anti-BRY	Rabbit	Abcam
Anti-Cdc42	Mouse	ECM bioscience
Anti-RhoGDI α	Rabbit	Santa Cruz
Anti-FAK	Rabbit	Abcam
Anti-FAK (P397)	Rabbit	Cell signaling
Anti-Flag	Mouse	Sigma Aldrich
Anti-Flag	Rabbit	Sigma Aldrich
Anti-FoxA2	Rabbit	Merck
Anti-GSC	Goat	Santa Cruz
Anti-LIMK1	Rabbit	Cell signaling
Anti-Nanog	Rabbit	Chemicon
Anti-N-WASP	Rabbit	Cell signaling
Anti-Oct 4	Rabbit	Abcam
Anti-PAK1	Rabbit	Cell signaling
Anti-Rac	Mouse	Upstate
Anti-Sox17	Goat	R&D system
Anti-Sox2	Rabbit	Chemicon
Anti-Tubulin	Mouse	Sigma Aldrich

Table 2.4: List of secondary antibodies

Antibody	Species	Company
Anti-mouse IgG, HRP conjugated	Goat	Dako Cytomation
Anti-rabbit IgG, HRP conjugated	Goat	Dako Cytomation
Anti-goat IgG, HRP conjugated	Rabbit	Dako Cytomation
Anti-mouse IgG, Alexa Fluor 488	Goat	Molecular Probes®
Anti-mouse IgG, Alexa Fluor 546	Goat	Molecular Probes®
Anti-rabbit IgG, Alexa Fluor 488	Goat	Molecular Probes®
Anti-rabbit IgG, Alexa Fluor 546	Goat	Molecular Probes®

Table 2.5: List of other reagents

Name	Company
Phalloidin-Alexa 546	Molecular Probes®
Phalloidin-fluorescein isothiocyanate (FITC)	Sigma Aldrich

Table 2.6: List of plasmids used in experiments

Plasmid	Tag	Gene name/ Accession number	Mutations/Truncated
pBOS-mCherry-Flag	mCherry-Flag		
pBOS-mCherry-Flag-Cdc42 (N17)	mCherry-Flag	Cdc42 (N17)/ NM_009861.3	Threonine 17→ Asparagine 17
pBOS-mCherry-Flag-Cdc42 (V12)	mCherry-Flag	Cdc42 (V12)/ NM_009861.3	Glycine 12→ Valine 12
pBOS-mCherry-Flag-Cdc42 (WT)	mCherry-Flag	Cdc42 (WT)/ NM_009861.3	
pBOS-mCherry-Flag-Rac1 (N17)	mCherry-Flag	Rac1 (N17)/ NM_009007.2	Threonine 17→ Asparagine 17
pBOS-mCherry-Flag-Rac1 (V12)	mCherry-Flag	Rac1 (V12)/ NM_009007.2	Glycine 12→ Valine 12
pBOS-mCherry-Flag-Rac1 (WT)	mCherry-Flag	Rac1 (WT)/ NM_009007.2	
pBOS-mCherry-Flag-RhoA (N19)	mCherry-Flag	RhoA (N19)/ NM_016802.4	Threonine 19→ Asparagine 19
pBOS-mCherry-Flag-RhoA (V14)	mCherry-Flag	RhoA (V14)/ NM_016802.4	Glycine 14→ Valine 14
pBOS-mCherry-Flag-RhoA (WT)	mCherry-Flag	RhoA (WT)/ NM_016802.4	
pGEX-PBD	GST	PBD/ NM_011035.2	
pGEX-RBD	GST	RBD/ NM_001136227.1	
pXJ-Flag-MRCK α	Flag	MRCK α / NM_001033285.1	

pXJ-Flag-MRCK α -Kinase domain	Flag	MRCK α / NM_001033285.1	1-473 amino acids
pXJ-Flag-ROK α	Flag	ROK α / NM_009071.2	
pXJ-Flag-ROK α (1-432)	Flag	ROK α / NM_009071.2	1-432 amino acids
pXJ-GFP-Flag	GFP-Flag		
pXJ-GFP-Flag-Cdc42 (N17)	GFP-Flag	Cdc42 (N17)/ NM_009861.3	Threonine 17→ Asparagine 17
pXJ-GFP-Flag-Cdc42 (V12)	GFP-Flag	Cdc42 (V12)/ NM_009861.3	Glycine 12→ Valine 12
pXJ-GFP-Flag-Cdc42 (WT)	GFP-Flag	Cdc42 (WT)/ NM_009861.3	
pXJ-GFP-Flag-Rac1 (N17)	GFP-Flag	Rac1 (N17)/ NM_009007.2	Threonine 17→ Asparagine 17
pXJ-GFP-Flag-Rac1 (V12)	GFP-Flag	Rac1 (V12) / NM_009007.2	Glycine 12→ Valine 12
pXJ-GFP-Flag-Rac1 (WT)	GFP-Flag	Rac1 (WT)/ NM_009007.2	
pXJ-GFP-Flag-RhoA (N19)	GFP-Flag	RhoA (N19) / NM_016802.4	Threonine 19→ Asparagine 19
pXJ-GFP-Flag-RhoA (V14)	GFP-Flag	RhoA (V14) / NM_016802.4	Glycine 14→ Valine 14
pXJ-GFP-Flag-RhoA (WT)	GFP-Flag	RhoA (WT)/ NM_016802.4	
pXJ-HA-N-WASP	HA	N-WASP/ NM_028459.2	
pXJ-HA-N-WASP(256E)	HA	N-WASP/ NM_028459.2	Lysine 256→ Glutamic acid 256

Table 2.7: List of quantitative Real time (qRT)-PCR primers

Gene name/ Accession number	Primer name	Sequence (5' to 3')
Brachyury (BRY) / NM_009309.2	BRY-Forward BRY-Reverse	GCTGTGGCTGCGCTTCA GAACATCCTCCTGCCGTTCTT
Fibroblast growth factor 5 (FGF5) / NM_010203.4	FGF5-Forward FGF5-Reverse	AACTCCATGCAAGTGCCAAAT TCGCGGACGCATAGGTATTAT
Forkhead box protein A (FOXA2) / NM_001291065.1	FoxA2-Forward FoxA2-Reverse	GGCCCAGTCACGAACAAAG CCCAAAGTCTCCACTCAGCCTC
Goosecoid (GSC) / NM_010351.1	GSC-Forward GSC-Reverse	GAAGGTGGAGGTCTGGTTTAAGAAC CACTTCTCGGCGTTTTCTGACT
Nestin / NM_016701.3	Nestin-Forward Nestin-Reverse	CTGCAGGCCACTGAAAAGTT GACCCTGCTTCTCCTGCTC
SOX17 / NM_011441.5	Sox17-Forward Sox17-Reverse	AGCCATTTCTCCGTGGTGT AACACTGCTTCTGGCCCTCAG
TATA-Binding box (TBP) / NM_013684.3	TBP-Forward TBP-Reverse	CCACAGGGCGCCATGA TGGAGTAAGTCCTGTGCCGTAA

Table 2.8: List of commercial kits

Kits	Brand
DNA plasmid miniprep kit	Axygen
ECL advance western blot detection kit	GE Healthcare
Novex ECL Western blotting detection reagent	Life Technologies
QIAquick® Gel Extraction Kit	Qiagen
QIAquick® PCR Purification Kit	Qiagen
QIAquick® Spin Maxiprep Kit	Qiagen
QIAquick® Spin Miniprep Kit	Qiagen
QuikChange site-directed mutagenesis kit	Stratagene
Vilo cDNA synthesis kit	Life technologies

3 Results

3.1 Endogenous RhoGTPase levels are low in mESCs.

Since RhoGTPases play important roles in many cellular functions in eukaryotic cells, we investigated if RhoGTPases are also important for maintaining mESCs. First, we determined the protein levels of RhoGTPases in wild type D3 and E14 mESCs using western blot analysis. We found lower levels of RhoA, Rac1 and Cdc42 in pluripotent mESCs compared to terminally differentiated cells such as mouse fibroblast cells (NIH3T3 and STO) and mouse embryonic fibroblast (MEF) cells (Figure 4).

Figure 4: Lower levels of RhoGTPases were observed in mESCs compared to fibroblasts.

Soluble cell lysates were isolated from mouse fibroblasts (NIH3T3, STO and MEF) and mESCs (D3 and E14). Equal amount of cell lysate was loaded in each well. The cell lysates were subjected to SDS-PAGE and resolved protein bands on the gel were transferred to nitrocellulose membrane for western blot analysis. Antibodies against RhoA (Cytoskeleton), Rac1 (Upstate), Cdc42 (ECM Bioscience) and tubulin (as loading control, Sigma Aldrich) were used.

3.2 Overexpression of RhoGTPases does not induce morphological changes in the actin cytoskeleton of mESCs.

To understand the roles of RhoGTPases in the actin cytoskeleton regulation in mESCs, we introduced constitutively active forms of RhoA (V14), Rac1 (V12) and Cdc42 (V12) into two mESCs (D3 and E14), and HeLa epithelial cells (control) using transient transfections. The actin cytoskeleton was monitored by labeling the actin filament with green fluorescent dye (fluorescein)-conjugated phalloidin (Barden et al., 1987). HeLa cells were used as control because this cell type exhibits robust actin cytoskeleton in cell staining experiment.

As expected, HeLa cells transfected with active RhoA (V14) construct showed an increased in stress fibers and reduction in cell size due to increased acto-myosin contraction (Figure 5A). Unexpectedly, we did not observe any distinct changes in the actin cytoskeleton and cell size of both D3 and E14 mESCs transfected with active RhoA (V14) construct (Figure 5B and 5C). The cell morphology and actin cytoskeleton of active RhoA (V14)-transfected mESCs resembled those of the wild type mESCs. In addition, transient expression of dominant negative form of RhoA (N19) in HeLa and mESCs did not result in observable changes of the actin cytoskeleton (Figure 5A, B and C).

To study the impact of Rac1 on the actin cytoskeleton of mESCs, HeLa cells and mESCs were transiently transfected with constitutively active form of Rac1 (V12) construct. Peripheral protrusions and lamellipodia were observed at the cell edges of the active Rac1 (V12)-transfected HeLa cells. However we did not detect any lamellipodium in mESCs expressing active Rac1 (V12). No distinct changes of cell morphology and actin cytoskeleton were noted in HeLa cells and mESCs expressing dominant negative Rac1 (N17) (Figure 5A, B and C).

To explore the effect of Cdc42 in mESCs, we transiently expressed constitutively active form of Cdc42 (V12) into HeLa cells and mESCs. The appearance of filopodia was found in HeLa cells but not in mESCs expressing active Cdc42 (V12). The expression of dominant negative Cdc42 (N17) in mESCs did not yield any

observable changes. These mESCs remained round which was similar to the wild type mESCs (Figure 5A, B, and C).

Figure 5: Transient expression of constitutively active RhoA, Rac1 and Cdc42 induce morphological changes of the actin cytoskeleton of (A) HeLa cells, but not in (B) D3 and (C) E14 mESCs.

HeLa cells and mESCs (D3 and E14) were cultured on PLL-coated coverslips. Cells were transiently transfected with vectors encoding mCherry-Flag tagged alone, constitutively active RhoA (V14), Rac1 (V12) or Cdc42 (V12) and dominant negative RhoA (N19), Rac1 (N17) or Cdc42 (N17). After 24 hours of incubation, cells were fixed with fresh 4% paraformaldehyde and labeled with phalloidin conjugated with FITC (Sigma Aldrich). Red

arrows indicate stress fibers; white arrowheads indicate lamellipodia; white arrows indicate filopodia. Bar = 10 μ m.

3.3 Downstream effectors of RhoGTPases are present in mESCs and fibroblasts.

The failure to induce changes in the actin cytoskeleton of mESCs had prompted us to examine if the downstream signaling cascades of RhoGTPases are functional in mESCs. RhoGTPases regulate the actin cytoskeleton through the activation of their effector proteins. ROK α is downstream effector protein of RhoA that is involved in the regulation of stress fibers in the cells and LIMK is in turn regulated by ROK α (Agnew et al., 1995; Arber et al., 1998; Bokoch, 2003; Sumi et al., 1999; Yang et al., 1998). PAK1, a common downstream effector of Rac1 and Cdc42, regulates signaling pathways that modulates actomyosin contraction and actin polymerization (Chew et al., 1998; Goeckeler et al., 2000; Sanders et al., 1999; Takizawa et al., 2002). We observed the presence of ROK α , LIMK1 and PAK1 in both D3 and E14 mESCs although at a lower level as compared to the fibroblasts. Another downstream effector of Cdc42 is N-WASP, which regulates actin nucleation. In mESCs, N-WASP was found to be present in both mESCs and fibroblasts (Figure 6). These results suggested that the lack of morphological changes in RhoGTPase-transfected cells were not due to the absence of effector proteins. However these proteins could be in inactive states and their activities might be under the controls of alternative mechanisms.

Figure 6: Lower protein levels of ROK α , LIMK1 and PAK1 are detected in mESCs.

Soluble cell lysates were isolated from various mammalian fibroblasts (NIH3T3, STO and MEF) and mESCs (D3 and E14). Equal amount of cell lysate was loaded in each well. The cell lysates were subjected to SDS-PAGE and the resolved protein bands on the gel were transferred to nitrocellulose membrane for western blot analysis. Antibodies against ROK α (BD Transduction), LIMK1 (Cell signaling technologies), PAK1 (Cell signaling technologies), N-WASP (Cell signaling technologies) and tubulin (as loading control, Sigma Aldrich) were used.

3.4 Expressions of active forms of RhoGTPase effectors (MRCK α , ROK α and N-WASP) elicit changes in the actin cytoskeleton of mESCs.

Since both the RhoGTPases and their effectors are found to be present in mESCs, we speculated that these effector proteins could be present in inactive states, thus preventing changes in actin cytoskeleton. In order to determine if active effector proteins could induce changes to the actin cytoskeleton, we overexpressed active forms of the effector proteins (ROK α , MRCK α and N-WASP) in mESCs.

3.4.1 Constitutively active ROK α induces excessive actin condensation in mESCs.

ROK α , an effector protein of RhoA, is activated through binding of active RhoA to its RBD. Upon binding to RhoA, the interaction between the kinase and the auto-inhibitory domains of ROK α is disrupted (Ishizaki et al., 1997; Leung et al., 1996). Overexpression of full length ROK α resulted in an increase of stress fiber formation

in HeLa cells (Figure 7B) (Leung et al., 1996). However, we did not notice any distinct change in the actin cytoskeleton of D3 and E14 mESCs transfected with full length ROK α (Figure 7C and D). Therefore it could be possible that full length ROK α assumed an auto-inhibited conformation in mESCs (Amano et al., 1999; Ishizaki et al., 1996) (Garg et al., 2008; Jacobs et al., 2006). We then mimicked the active form of ROK α by introducing a C-terminal truncated form of ROK α into mESCs (Figure 7A, C and D). Excessive condensation of actin filaments was observed in the center of the mESCs. These observations were consistent with the phenotypes seen in HeLa cells expressing active ROK α (Figure 7B) (Leung et al., 1996). Our observations suggested that downstream signaling of the RhoA-ROCK pathway was functional.

A

ROK α

B

HeLa cells

Figure 7: Expression of constitutively active ROKα induces excessive actin condensation in HeLa and mESCs.

(A) Modular domain organization of ROKα: full length ROKα consists of a kinase domain, coiled coil, p21 GTPases binding domain (GBD) and Pleckstrin Homology (PH- a putative domain that contains a cysteine/histidine-rich domain) domain. C-terminally truncated ROKα consists of all domains of full length ROKα except the C-terminal.

(B) HeLa cells, (C) D3 and (D) E14 mESCs cultured on coverslips were transiently transfected with vectors encoding Flag-tagged full length and C-terminally truncated (constitutively active) ROKα. After 24 hours of incubation, cells were fixed and immunostained with anti-Flag antibody (Sigma-Aldrich), and subsequently with secondary antibodies conjugated with green fluorescence dye, Alexa fluor 488 (Molecular Probes®). Actin filament was labeled with phalloidin-conjugated with red fluorescent dye, Alexa 546 (Molecular Probes®). Arrows indicate actin condensations. Bar = 10 μm.

3.4.2 Constitutively active MRCK α induces excessive actin condensation in mESC.

MRCK α , a downstream effector protein of Cdc42, functions by regulating LIMK and cofilin that are necessary for the stabilization of actin filaments in the cells (Leung et al., 1998). To explore the effects of MRCK α on the actin cytoskeleton of mESCs, we introduced both full length MRCK α and constitutively active MRCK α (containing only the N-terminal kinase domain) into HeLa cells and mESCs (Figure 8A). We observed increased actin stress fibers in HeLa cells expressing full length MRCK α (Figure 8B), which is consistent with the results shown by Leung's group (Leung et al., 1998). However such phenotype was not observed in mESCs (Figure 8C and D). Conversely, when we introduced constitutively active MRCK α into HeLa and mESCs, excessive condensation of actin filaments was observed in the center of HeLa cells and mESCs (Figure 8B, C and D). These observations suggested that active MRCK α could induce the expected morphological and actin cytoskeletal changes in mESCs.

MRCK α

Figure 8: Expression of constitutively active MRCK α elicits actin condensation in both HeLa and mESC.

(A) Modular domain organization of MRCK α : Full length MRCK α consists of a kinase, coiled coil, cysteine/histidine-rich (CR), pleckstrin homology (PH) and p21 GTPases binding domain (GBD) domains. C-terminally truncated MRCK α only consists of kinase domain. (B) HeLa cells and (C) D3 and (D) E14 mESCs cultured on coverslips were transiently transfected with vectors encoding Flag-tagged full length and kinase domain only (constitutively active) MRCK α . After 24 hours of incubation, cells were fixed and immunostained with anti-Flag antibody (Sigma-Aldrich), and subsequently with secondary antibodies conjugated with green fluorescence dye, Alexa fluor 488 (Molecular Probes®). Actin filament was labeled with phalloidin- conjugated with red fluorescent dye, Alexa 546 (Molecular Probes®). Arrows indicate actin condensations. Bar = 10 μ m.

3.4.3 Both wild type and phosphorylated form of N-WASP induce filopodia formation in mESCs.

N-WASP, a downstream effector of Cdc42, regulates actin polymerization via the activation of ARP2/3 complex. It has been shown that N-WASP induces actin nucleation and filopodia formation in most mammalian cells (Miki et al., 1998a; Prehoda et al., 2000; Rohatgi et al., 1999). Short actin microspikes could be seen at the cell periphery of HeLa cells and mESCs transfected with full length N-WASP (Figure 9B, C and D). Since N-WASP was shown to be activated through phosphorylation of tyrosine at amino acid 256 by focal adhesion kinase (FAK) (Burton et al., 2005; Cory et al., 2002; Suetsugu et al., 2002), we constructed a phospho-mimick mutant of N-WASP using single site-directed mutagenesis, which mutated amino acid 256 from tyrosine to glutamic acid (Figure 9A). Expression of N-WASP-Y256E induced formation of longer microspikes at the cell periphery of HeLa cells and mESCs (Figure 9B, C and D).

N-WASP

Figure 9: Expression of constitutively active N-WASP (Y256E) induces formation of filopodium-like structures in HeLa and mESCs.

(A) Modular domain organization of N-WASP: Full length N-WASP consist of WASP homology (WH)-1, B-domain (a stretch of basic amino acids that mediate filamentous-actin and phosphatidylinositol (4, 5)-biphosphate binding), p21 GTPases binding domain (GBD), Proline-rich region (PPPPP-contain SRC-homology and profilin-binding sites), and Verprolin homology, Cofilin homology and Acidic domains (VCA) domains). Constitutively active N-WASP (Y256E) comprises of a full length N-WASP with mutation at amino acid 256 tyrosine (Y) to glutamic acid (E).

(B) HeLa cells and (C) D3 and (D) E14 mESCs cultured on coverslips were transiently transfected with vectors encoding HA-tagged full length and constitutively active N-WASP. After 24 hours of incubation, cells were fixed and immunostained with anti-HA antibody (Sigma-Aldrich), and subsequently with secondary antibodies conjugated with green fluorescence dye, Alexa fluor 488 (Molecular Probes®). Actin filament was labeled with phalloidin-conjugated with red fluorescent dye, Alexa 546 (Molecular Probes®). Arrows indicate filopodia structures. Bar = 10 μ m.

3.5 Generating stable transgenic mESCs expressing RhoA-, Rac1- and Cdc42.

So far, we have shown that 1) RhoGTPases and their downstream effector proteins are present in mESCs, even though RhoGTPase expression levels are lower in the mESCs relative to fibroblasts, 2) expression of constitutively active form of RhoGTPases fail to induce similar changes in the actin cytoskeleton and cell morphology in mESCs as compared to HeLa cells. However, expressions of active forms of downstream effectors of RhoGTPases are able to induce the expected changes in the actin cytoskeleton. In order to study the roles of RhoGTPases in the regulation of actin cytoskeleton and cell morphology during differentiation, it is more relevant to generate stable transgenic cell lines rather than relying on transient transfection. We constructed recombinant plasmids to express RhoGTPases and their mutants (both dominant active and dominant negative) using pBOS vector containing mCherry and FLAG epitope tags and blasticidin antibiotic selection marker. To generate stable transgenic mESCs expressing RhoGTPases, selected plasmid constructs were transfected into E14 mESCs. 24 hours after transfection, cells were diluted to obtain single and isolated cells. Isolated cells were then cultured in 96-wells plates until formation of colonies. Blasticidin S HCL (10 μ g/ml) was added to the culture media. During screening, we analyzed cells for mCherry fluorescence under epifluorescence microscope, and populations of positive cells were isolated and expanded. We have successfully generated the following stable transgenic mESCs expressing RhoGTPases: E14-mc (control, vector alone), E14-mc-RhoA-WT,

E14-mc-RhoA-V14 (active), E14-mc-RhoA-N19 (dominant negative), E14-mc-Rac1-WT, E14-mc-Rac1-V12 (active), E14-mc-Rac1-N17 (dominant negative), E14-mc-Cdc42-WT, E14-mc-Cdc42-V12 (active) and E14-mc-Cdc42-N17 (dominant negative). Characterization of these stable transgenic mESCs expressing RhoGTPases was performed by analyzing the cell morphology and protein expression. Actin filaments in these wild type E14, E14-mc (control) and E14-mc-RhoGTPases mESCs were labeled with phalloidin-tagged with FITC. These cells displayed similar actin cytoskeleton as the wild type E14 mESCs (Figure 10). The phenotypes exhibited by the stable transgenic cell lines were consistent with the phenotypes observed with transient transfection of RhoGTPases in E14 mESCs (as shown in Figure 5B and C).

Figure 10: E14-RhoGTPase expressing mESCs exhibited similar morphological phenotype as the wild type E14 mESCs.

(A) Wild type E14, (B) E14-mCherry (control), (C-E) E14-mc-RhoA (WT, V14, and N19), (F-H) E14-mc-Rac1 (WT, V12, and N17) and (I-K) E14-mc-Cdc42 (WT, V12, and N17) were cultured on PLL-coated coverslips. These cells were fixed with 4% paraformaldehyde and their actin filament was labeled with phalloidin-conjugated with FITC (Sigma Aldrich). Bar = 10 μ m.

To ensure that these GTPase mutants are functional in the respective stable transgenic mESC lines, we assay for active RhoA, Rac1 and Cdc42 proteins in the stable transgenic mESCs. Active RhoA (GTP bound form) could be assayed by binding to RBD of roktein protein, and active Cdc42/Rac1 (GTP bound form) could be monitored by binding to PBD of PAK1. Fusion proteins containing RBD/ PBD and GST were constructed and expressed in bacterial system. The fusion proteins expressed were captured on glutathione sepharose 4B beads, and these beads were then used to immunoprecipitate active RhoGTPases in the cell lysates. Active RhoA, Rac1 and Cdc42 were detected in cell lysate of E14-mc-RhoA-V14 (active), E14-mc-Rac1-V12 (active) and E14-mc-Cdc42-V12 (active) mESCs, respectively (Figure 11A, B and C, right panels). As expected, E14-RhoA-N19 (dominant negative), E14-Rac1-N17 (dominant negative) and E14-Cdc42-N17 (dominant negative) mESCs did not express any active forms of RhoA, Rac1 or Cdc42. Protein expression of both transgenic and endogenous RhoA, Rac1 and Cdc42 in both wild types and stable transgenic mESCs, and fibroblast cells was analyzed, our results showed that the protein expression of transgenic RhoA, Rac1 and Cdc42 are higher as compared to the endogenous protein levels (appendix Figure II, III, IV).

To ensure that the pluripotency of these stable transgenic mESCs expressing RhoGTPases were unaffected by the expression of RhoGTPases, we checked for the presence of common pluripotency markers: Nanog, Oct 4 and Sox 2, using immunostaining (Figure 12) and Nanog protein levels using western blot analysis (Figure 11, left panels). We confirmed the presence of these pluripotency markers in the stable transgenic mESC lines that we have generated. Our results also suggested that stable expression of RhoGTPases did not alter the pluripotency and actin cytoskeletons in mESCs.

A

B

C

Figure 11: Presence of RhoGTPases (RhoA, Cdc42 and Rac1) does not affect pluripotency marker (Nanog) expression.

Soluble cell lysates were isolated from stable transgenic E14-mCherry, (A) E14-mc-RhoA (WT, V14 and N19), (B) E14-mc-Cdc42 (WT, V12 and N17) and (C) E14-mc-Rac1 (WT, V12 and N17) mESCs. The cell lysates were subjected to SDS-PAGE and resolved protein bands on the gel were transferred to nitrocellulose membrane for western blot analysis (left panels). Active RhoA/Cdc42/Rac1 assays were carried out (right panels). Antibodies against RhoA (Cytoskeleton), Cdc42 (Cell signaling) and Rac1 (Upstate), Nanog (Chemicon) and tubulin (as loading control, Sigma Aldrich) were used.

B

Figure 12: Stable transgenic mESCs expressing RhoGTPases maintain mESC pluripotency based on immunostaining of (A) Nanog, (B) Oct 4 and (C) Sox 2.

Wild type E14, E14-mCherry (control), E14-RhoA (V14, N19), E14-Rac1 (V12, N17) and Cdc42 (V12, N17) mESCs were cultured on PLL-coated coverslips. These cells were fixed with 4% paraformaldehyde and immunostaining with antibodies against Nanog (Chemicon), Oct 4 (Abcam), Sox 2 (Chemicon), and secondary antibodies conjugated with Alexa 488 were used (Molecular Probes®). Bar = 10 μ m.

3.6 RhoA expression increases upon LIF withdrawal.

During differentiation, mESCs lose its pluripotent properties and differentiate into terminally differentiated cells with specialized cellular functions. To study the expression profile of RhoA during early differentiation, LIF was removed from the standard ESC media, and cell lysates were harvested daily for a total of 3 days. LIF is a cytokine routinely used in the culture media to prevent random differentiation of mESCs. RhoA and pluripotency marker (Oct 4 and Nanog) expression levels were analyzed. We observed a decrease in RhoA protein levels one day after LIF removal followed by an increasing expression from Day 1 to Day 3. The disappearance of Oct 4 and Nanog indicated the loss of pluripotency upon withdrawal of LIF from the culture media (Figure 13). Our observation suggested that RhoA could likely play a role during differentiation.

Figure 13: RhoA level increases upon LIF removal.

E14 mESCs were cultured in the absence of LIF, and cell lysates were harvested daily for a total of 3 days. Cell lysates were subjected to SDS-PAGE and protein bands on the gel were transferred to nitrocellulose membrane for western blot analysis. Antibodies against RhoA (Cytoskeleton), Nanog (Chemicon), Oct 4 (Abcam) and tubulin (as loading control, Sigma Aldrich) were used.

3.7 Active RhoA hinders cell attachment.

RhoA has been reported to regulate cell adhesion in most cell types such as fibroblast cells (Chrzanowska-Wodnicka and Burridge, 1996; Kaibuchi et al., 1999; Ridley and Hall, 1992), we hypothesize that RhoA also regulates cell adhesions in

mESCs. Our results indicated that active RhoA (RhoA-V14) inhibits cell adhesion on gelatin, when compared to E14-mc (control) mESCs (Figure 14A). Gelatin is commonly used to aid in cell adhesion of weakly-attached mESCs to the culture dishes. Furthermore, changes in cell shape, through different ECMs, have also shown to modulate differentiation (Hayashi et al., 2007; Hunt et al., 2012; Pimton et al., 2011). We further hypothesize that RhoA influences cell adhesion to different ECMs and hence regulating differentiation process. We first study the effect of different ECMs on cell adhesion of stable transgenic mESCs expressing RhoA. Our result showed that E14-mc (control) exhibited different cell-matrix adhesion properties, where E14-mc (control) cells showed the strongest cell adhesion on fibronectin, followed by gelatin and weakest cell adhesion was observed for collagen. We also noticed that E14-mc-RhoA-V14 (active) exhibited a marked reduction in cell attachment to gelatin- and fibronectin-coated surfaces, as compared to E14-mc (control) (Figure 14A). In general most cells were unable to attach well onto collagen-coated surfaces. Therefore we extended the incubation duration to 24 hours to help cell attachment. After 24 hours, the cells were able to attach better on the collagen-coated surfaces and we observed that E14-mc (control) cells were able to attach better than stable transgenic mESCs expressing the different RhoA constructs (Figure 14B). Consistently, E14-RhoA-V14 (active) mESCs exhibited the weakest cell adhesion to all 3 ECMs (gelatin, collagen and fibronectin) coated surfaces, among the control E14 and E14-mc-RhoA mutants.

Figure 14: Active RhoA reduces cell adhesion.

Same number of E14-mc, E14-mc-RhoA-WT, E14-mc-RhoA-V14 (active) and E14-mc-RhoA-N19 (dominant negative) mESCs were placed in wells of 96-well plate that were pre-coated with different ECM proteins: gelatin, collagen and fibronectin. 15,000 cells were seeded into each well. (A) 1 hour or (B) 24 hours after replating, the attached cells were fixed and stained with 0.2% crystal violet solution, and the absorbance readings were obtained using Tecan Infinite M200 Pro quadruple monochromator microplate reader. The absorbance corresponds to the amount of cells attached to the ECMs. The absorbance readings were normalized and expressed as a percentage of the control cells (E14-mc). Graphs were plotted using mean values. Means and standard deviations were calculated

from result obtained from 3 independent experiments with 3 technical replicates for each sample. Error bars represent standard deviations. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns >0.05 . Statistical analysis for A) 2-way ANOVA with Tukey's HSD test showed significant main effect for the ECMs, $F(2,24) = 442.6$, $p < 0.0001$; significant effect for cell types, $F(3,24) = 58.44$, $p < 0.0001$; and the interaction between ECMs and cell types was significant, $F(6,24) = 13.41$, $p < 0.0001$ (appendix Table A1), B) Student T-test, $p < 0.05$.

3.8 Active RhoA enhances differentiation towards endoderm and mesoderm layers, but inhibits differentiation towards the ectoderm layer.

Our initial data has showed that RhoA levels changes during differentiation and other studies have shown that RhoA regulates gene transcriptions that modulate differentiation (Miralles et al., 2003; Settleman, 2003). We hypothesize that RhoA regulates gene transcription leading to modulation of cell fate specification. To identify the role of RhoA in cell fate determination during mESC differentiation, we analyzed the gene expression profiles of commonly used differentiation markers from the 3 primary germ layers. The 3 primary germ layers are the ectoderm (markers: Nestin and Fibroblast growth factor 5-FGF5), mesoderm (markers: Goosecoid-GSC and Brachyury-BRY) and endoderm (markers: Sry-related HMG box 17-SOX17 and Forkhead box A2-FOXA2). We cultured E14-mc-RhoA mESCs on 3 different ECM (gelatin, fibronectin and collagen) and in the absence of LIF for 4 days. We then analyzed the gene expression of the different markers for the endoderm, mesoderm and ectoderm.

3.8.1 Active RhoA enhances differentiation towards endoderm layer.

Endodermal differentiation gives rise to hepatocytes and pancreatic cells. When cultured on gelatin-coated surface without LIF, we observed an increased gene expressions of SOX17 (9.048 ± 0.605 -fold) and FOXA2 (2.357 ± 0.129 -fold) for E14-mc-Rho-V14 (active) mESCs, as compared to control E14-mc cells (1 ± 0.444 -fold and 1 ± 0.859 -fold, respectively). While decreased gene expressions of FOXA2 (0.239 ± 0.086 -fold) and SOX17 (0.533 ± 0.192 -fold) were noted in E14-mc-RhoA-N19 (dominant negative) mESCs (Figure 16A and table 3). This observation suggested that active RhoA (V14) could enhance differentiation toward the endoderm layer.

To study how other ECM proteins influence endodermal differentiation, we cultured E14-mc (control) and E14-mc-RhoA mutants on collagen- and fibronectin-coated surfaces and determined the influence of ECM proteins on endoderm differentiation. We noticed that when E14-mc-RhoA-V14 (active) mESCs were cultured on collagen- and fibronectin-coated surfaces, they further enhanced the gene expression of the endodermal markers, FOXA2 and SOX17 (Figure 16B, C and table 3). E14-mc-RhoA-N19 cultured on collagen-coated surface exhibited similar expression levels of FOXA2 and SOX17, compared to control E14-mc (Figure 16B). However when E14-mc-RhoA-N19 (dominant negative) mESCs were cultured on fibronectin-coated surfaces, we observed a small increase in FOXA2 and SOX17 gene expression compared to control E14-mc. But their levels were lower than E14-mc-RhoA-V14 (active) mESCs (Figure 16C). In conclusion, collagen and fibronectin further enhanced differentiation towards the endoderm layer in the presence of active RhoA (V14).

Figure 15: Active RhoA enhances endodermal differentiation.

E14-mc, E14-mc-RhoA-WT, E14-mc-RhoA-V14 (active) and E14-mc-RhoA-N19 (dominant negative) mESCs were cultured on (A) gelatin-, (B) collagen- and (C) fibronectin-coated surfaces for 4 days in the absence of LIF. E14-mc cells were cultured on gelatin-coated surface and in the presence of LIF as additional control. mRNAs were extracted from the harvested cells and used as template for cDNA synthesis. RT qPCR was carried out using primers specific for endodermal markers (FOXA2 and SOX17). Data presented were calculated and expressed as mean fold change against control mc (+LIF). Means and standard deviations were computed based on results obtained from 3 independent experiments with 3 technical replicates for each sample. Error bars represent standard deviations. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns >0.05 .

Endodermal markers	Cell type	ECMs		
		Gelatin	Collagen	Fibronectin
FOXA2	mc (+LIF)	1±0.444	1±0.636	1±0.813
	RhoA-V14 (-LIF)	2.357±0.129	6.971±4.08	8.897±1.022
	RhoA-N19 (-LIF)	0.239±0.086	0.91±0.435	4.869±2.754
SOX17	mc (+LIF)	1±0.859	1±0.27	1±0.934
	RhoA-V14 (-LIF)	9.048±0.605	14.843±10.555	15.861±3.22
	RhoA-N19 (-LIF)	0.533±0.192	1.438±0.305	5.426±3.977

Table 3: Active RhoA enhances differentiation toward endoderm layer.

3.8.2 Active RhoA enhances differentiation towards mesoderm layer.

Mesodermal differentiation promotes generation of cells that belong to blood, skeletal muscles and heart. When mESCs were cultured on gelatin-coated surface in the absence of LIF, E14-mc-RhoA-V14 (active) mESCs expressed higher gene expression of mesodermal layer markers BRY (4.395 ± 0.578 -fold) and GSC (2.985 ± 0.2 -fold), relative to control E14-mc mESCs (1 ± 0.238 -fold and 1 ± 0.168 -fold, respectively). On the other hand, E14-mc-RhoA-N19 (dominant negative) mESCs did not show significant change in the gene expression of mesodermal markers (Figure 16A and table 4).

To study the effects of collagen and fibronectin on directing cell fate specification towards mesoderm layer, we cultured mESCs on collagen and fibronectin-coated surfaces. E14-mc-RhoA-V14 (active) mESCs cultured on collagen-coated surfaces showed a greater increase in the gene expression of mesodermal markers, BRY (6.778 ± 3.194 -fold) and GSC (6.51 ± 2.413 -fold) as compared to cells cultured on gelatin-coated surfaces. E14-mc-RhoA-WT and E14-mc-RhoA-N19 (dominant negative) mESCs that were cultured on collagen-coated surfaces displayed a lower gene expression of mesodermal markers (BRY and GSC) compared to E14-mc-RhoA-V14 (active) mESCs (Figure 16B and table 4). Unexpectedly, fibronectin enhances expression of BRY level, in the absence of LIF. On the other hand, their GSC level was somewhat lower than E14-mCherry control (in the absence of LIF) (Figure 16C). Our data suggested that in general, active RhoA enhanced differentiation towards mesoderm layer on gelatin, collagen and fibronectin surfaces.

Figure 16: Active RhoA promotes mesodermal differentiation.

E14-mc, E14-mc-RhoA-WT, E14-mc-RhoA-V14 (active) and E14-mc-RhoA-N19 (dominant negative) mESCs were cultured on (A) gelatin-, (B) collagen- and (C) fibronectin-coated surfaces for 4 days in the absence of LIF. E14-mc was cultured on gelatin-coated surface and in the presence of LIF. mRNAs were extracted from the harvested cell pellet and used as template for cDNA synthesis. RT qPCR was carried out using primers specific for mesodermal markers (BRY and GSC). Data presented were calculated and expressed as mean fold change against control mc (+LIF). Means and standard deviations were computed based on result obtained from 3 independent experiments with 3 technical replicates. Error bars represent standard deviations. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns >0.05 .

Mesodermal markers	Cell type	ECMs		
		Gelatin	Collagen	Fibronectin
BRY	mc (+LIF)	1±0.238	1±0.335	1±0.457
	RhoA-V14 (-LIF)	4.395±0.578	6.778±3.194	4.222±0.545
	RhoA-N19 (-LIF)	1.861±0.753	2.726±0.713	2.653±1.046
GSC	mc (+LIF)	1±0.168	1±0.157	1±0.187
	RhoA-V14 (-LIF)	2.985±0.2	6.51±2.413	2.032±0.49
	RhoA-N19 (-LIF)	0.888±0.31	2.436±0.91	2.267±0.838

Table 4: Active RhoA enhances differentiation towards mesoderm layer.

3.8.3 Active RhoA inhibits differentiation toward ectoderm layer when mESCs are cultured on gelatin- and fibronectin-coated surfaces.

Ectodermal differentiation is necessary for the generation of the neural system during development. We would like to determine if RhoA regulates ectodermal differentiation in E14 mESCs. In this study, we monitored ectodermal differentiation using two commonly used ectodermal markers, FGF5 and Nestin. In the absence of LIF, E14-mc (control) cultured on gelatin showed an increase in gene expression of ectodermal markers (FGF5 and Nestin) as compared to E14-mc cultured in presence of LIF. And E14-mc-RhoA-WT and E14-mc-RhoA-V14 (active) mESCs exhibited reduction of the gene expression of ectodermal markers (FGF5 and Nestin), while E14-mc-RhoA-N19 (dominant negative) mESCs showed a substantial increase in gene expression of ectodermal markers (Figure 17A and table 5). Our result suggested that the presence of active RhoA could inhibit ectodermal differentiation on gelatin-coated surfaces.

Notably, E14-mc-RhoA-V14 (active) mESCs cultured on fibronectin also displayed a drop in gene expression of ectodermal marker compared to E14-mc and E14-mc-RhoA-N19 (dominant negative) mESCs (Figure 17C and table 5). However, we did not observe similar trend when E14-mc-RhoA mESCs were plated on collagen surfaces. Instead, E14-mc-RhoA-WT, E14-mc-RhoA-V14 (active) and E14-mc-RhoA-N19 (dominant negative) mESCs expressed higher gene expressions of the ectodermal markers (FGF5 and Nestin) when cultured on collagen-coated surfaces (Figure 17B and table 5). In summary, we found that E14-mc-RhoA-V14 inhibits differentiation into ectodermal cell types when cultured on gelatin- and fibronectin-coated surfaces, whereas collagen appeared to influence ectodermal differentiation differently.

Figure 17: Active RhoA inhibits ectodermal differentiation when cultured on gelatin- and fibronectin-coated surfaces.

E14-mc, E14-mc-RhoA-WT, E14-mc-RhoA-V14 (active) and E14-mc-RhoA-N19 (dominant negative) mESCs were cultured on (A) gelatin-, (B) collagen- and (C) fibronectin-coated surfaces for 4 days in the absence of LIF. E14-mc mESCs were cultured on gelatin-coated surface and in the presence of LIF. mRNAs were extracted from the harvested cell pellet and used as template for cDNA synthesis. RT qPCR was carried out using primers specific for ectodermal markers (FGF5 and Nestin). Data presented were calculated and expressed as mean fold change against control mc (+LIF). Means and standard deviations were computed based on result obtained from 3 independent experiments with 3 technical replicates. Error bars represent standard deviations. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ns >0.05 .

Ectodermal markers	Cell type	ECMs		
		Gelatin	Collagen	Fibronectin
FGF5	mc (+LIF)	1±0.755	1±0.27	1±0.215
	RhoA-V14 (-LIF)	11.907±1.402	180.263±86.596	111.486±35.086
	RhoA-N19 (-LIF)	117.559±8.746	169.768±58.483	532.873±145.918
NESTIN	mc (+LIF)	1±0.601	1±0.183	1±0.107
	RhoA-V14 (-LIF)	0.756±0.075	2.858±0.868	1.719±0.374
	RhoA-N19 (-LIF)	3.55±0.753	2.749±0.812	2.538±0.526

Table 5: Active RhoA inhibits differentiation towards ectoderm layer when cultured on gelatin- and fibronectin-coated surfaces.

3.9 Mesodermal markers increase in E14-mc-RhoA-V14 (active) mESCs.

Gene expression profiles of different differentiation lineage markers provided us with an insight on the influence of RhoA on cell fate determination during differentiation of mESCs. We next confirmed the gene expression patterns observed by monitoring the protein levels of the endodermal and mesodermal markers in E14-mc (control) and E14-mc-RhoA mESCs. Western analysis showed higher levels of GSC (mesodermal marker) in E14-mc-RhoA-V14 (active) mESCs (Figure 18). This is consistent with the RT qPCR results observed (Figure 16A). However, unlike the RT qPCR study, we did not observe significant changes of the SOX17, FOXA2 and BRY protein levels in the other E14-mc-RhoA mESCs. This could be due to sensitivity of the antibodies used.

Figure 18: An increase in protein expression of mesoderm marker, GSC, is observed in E14-mc-RhoA-V14.

E14-mc (control) and E14-mc-RhoA mESCs were cultured on gelatin-coated surfaces, in the absence of LIF for 4 days. Cell lysates were harvested and subjected to SDS-PAGE and western blot analysis. Antibodies against SOX17 (R&D system), FOXA2 (Millipore), GSC (Santa Cruz), BRY (Abcam) and tubulin (loading control, Sigma Aldrich) were used.

3.10 mDia does not regulate early differentiation of mESCs.

The formin protein mDia is an effector of RhoA. It exists in an auto-inhibited state, which is activated upon the binding of active RhoA (Li and Higgs, 2003). Since cells expressing RhoA-V14 showed preference to differentiate into endodermal and mesodermal layers (gene expression results were shown in Figure 15 and 16), we went on to investigate if mDia is responsible for promoting differentiation to cells from these two germ layers. To inhibit mDia activity, 10 μ M SMIFH2 was used to treat mESCs (Pettee et al., 2014; Rizvi et al., 2009; Tien and Chang, 2014; Wyse et al., 2012).

If mDia is acting downstream of active RhoA that promotes differentiation, we should be able to block differentiation by treating the cells with SMIFH2. Treatment of cells with SMIFH2 did not result in significant changes in the protein levels of both endodermal (Sox17 and FoxA2) and mesodermal (BRY and GSC) markers, when

compared to untreated cells (Figure 19). This observation suggested that mDia might not participate in cell fate specification of mESCs towards endoderm and mesoderm.

Figure 19: Treatment of mESCs with mDia inhibitor, SMIFH2, does not affect differentiation.

E14-mc, E14-mc-RhoA-WT, E14-mc-RhoA-V14 (active) and E14-mc-RhoA-N19 (dominant negative) mESCs were cultured in the absence of LIF with or without SMIFH2 for 4 days. Fresh inhibitor and media were added daily. Soluble cell lysates were harvested and subjected to SDS-PAGE and western blot analysis. Antibodies against SOX17 (R&D system), FOXA2 (Millipore), GSC (Santa Cruz), BRY (Abcam) and tubulin (loading control, Sigma Aldrich) were used.

3.11 ROK α does not regulate endodermal and mesodermal differentiation of mESCs.

ROK α is another effector of RhoA, which we tested next. We used a small chemical inhibitor, Y27632, for the inhibition of ROK α activities. After treatment of the various E14 cell lines with 10 μ M Y27632 (Harb et al., 2008; Murray et al., 2013), we did not observe any changes in the protein levels of the endodermal (SOX17 and FOXA2) and mesodermal (BRY and GSC) markers (Figure 20) when compared to untreated cells, suggesting that ROK α might not participate in cell fate determination of mESCs.

Figure 20: Treatment of mESCs with ROK inhibitor, Y27632, does not affect differentiation.

E14-mc, E14-mc-RhoA-WT, E14-mc-RhoA-V14 (active) and E14-mc-RhoA-N19 (dominant negative) mESCs were cultured in the absence of LIF, and treated with and without Y27632 for 4 days. Fresh inhibitor and media were added daily. Soluble cell lysates were harvested and subjected to SDS-PAGE and western blot analysis. Antibodies against SOX17 (R&D system), FOXA2 (Millipore), GSC (Santa Cruz), BRY (Abcam) and tubulin (loading control, Sigma Aldrich) were used.

3.12 FAK negatively regulates endodermal and mesodermal differentiation of mESCs.

FAK is a cytoplasmic tyrosine kinase that is recruited to the plasma membrane upon the activation of integrin. Phosphorylated FAK serves as docking sites for the binding of several effectors such as SRC family proteins (Cornillon et al., 2003). FAK regulates stem cell pluripotency and cell proliferation via SRC tyrosine kinase in hESCs (Afrikanova et al., 2011; Lian et al., 2013). To inhibit FAK activities, cells were treated with 5 μ m of PF573228 (Loffek et al., 2014). Our results showed that inhibition of FAK enhanced protein expression of endodermal (Sox17 and FoxA2) and mesodermal (BRY and GSC) markers (Figure 21) for all stable mESCs. However we did not notice any differences in the protein levels of SOX17, FOXA2 and GSC between untreated and PF573228-treated E14-mc-RhoA-N19 (dominant negative) mESCs. These results suggested that activation of FAK could possibly suppress endodermal and mesodermal differentiation in mESCs.

Figure 21: Treatment of mESCs with FAK inhibitor, PF573228, affects differentiation.

E14-mc, E14-mc-RhoA-WT, E14-mc-RhoA-V14 (active) and E14-mc-RhoA-N19 (dominant negative) mESCs were cultured in the absence of LIF and with/without PF573228 for 4 days, fresh inhibitor and media were added daily. Soluble cell lysate were harvested and subjected to SDS-PAGE and western blot analysis. Antibodies against SOX17 (R&D system), FOXA2 (Millipore), GSC (Santa Cruz), BRY (Abcam), FAK (Abcam) and P397-FAK (Cell signaling), and tubulin (loading control, Sigma Aldrich) were used.

3.13 Active RhoA promotes endoderm differentiation on grating-like surfaces

Cell spreading and attachment influence differentiation through the regulation of RhoA-mediated actin reorganization. Topography has been shown to regulate the differentiation of stem cells. We hypothesize that mESCs respond to external mechanical forces via RhoA to regulate cell fate specification. Our earlier data suggest that active RhoA enhances expression of endodermal markers. Here, we make use of multi-architectural chip (MARC) to study 1) which topographical features promote endodermal differentiation, and 2) how topography and RhoA regulates mESC differentiation. MARC comprises many small fields of different topographies that vary in geometries and dimensions, thus minimizing large scale culturing (Figure 22). In this study, we differentiated E14-mc and E14-mc-RhoA-V14 (active) mESCs on MARCs, in the absence of LIF.

Our results showed that E14-mc mESCs increased SOX17 levels when cultured on nanograting-like topographies (Figure 23A, topographies 1, 2 and 3) compared to unpattern polydimethylsiloxane (PDMS) (Figure 23A, topography 17), while micrograting-like topographies (Figure 23A, topographies 4, 5 and 6) exhibited similar SOX17 level as unpattern PDMS (Figure 23A, topography 17). In addition, we did not observe any changes in SOX17 levels when E14-mc mESCs were cultured on isotropic topographies such as pillars (Figure 23A, topographies 10 and 11) and microlens (Figure 23A, topographies 13, 14, 15 and 16). However, E14-mc mESCs showed a decrease in SOX17 levels when cultured on isotropic topographies (Figure 23A, topographies 8 and 9) and hole topography (Figure 23A, topography 12).

E14-RhoA-V14 (active) mESCs elevated SOX17 levels when cultured on grating-like topographies (Figure 23B, topographies 3, 4, 5 and 6), as compared to unpattern PDMS (Figure 23B, topography 17). On the other hands, E14-RhoA-V14 (active) mESCs decreased SOX17 levels on some isotropic topographies such as 2 μ m lines perpendicular 250nm line, 2 μ m line parallel 250nm line, 2 μ m lines 250nm pillar and 1 μ m pitch 0.3 μ m sag microlens convex (Figure 23B, topographies 7, 8, 9 and 14), as compared to unpattern PDMS (Figure 23B, topography 17). In addition, isotropic topographies, such as pillars, holes and microlens, did not affect SOX17 levels of

E14-mc-RhoA-V14 (active) mESCs (Figure 23B, topographies 10, 11, 12, 13, 15 and 16).

Consistent with our previous result, expression of RhoA-V14 (active) showed enhanced SOX17 levels on MARC, when compared to control E14-mc. RhoA-V14 (active) cultured on grating-like topographies (Figure 23C, topographies 3,4 and 5) showed enhanced SOX17 level, while RhoA-V14 (active) cultured on some isotropic topographies such as 2um lines perpendicular 250nm line, 2um line parallel 250nm line, 2um lines 250nm pillar and 1um pitch 0.3um sag microlens convex (Figure 23C, topographies 7, 8, 9 and 14), (Figure 23C, topographies 7,8, 9 and 14) displayed reduced SOX17 level, when compared to control E14-mc cultured on same topographies. Together, these observations suggest that RhoA enhances endodermal differentiation of mESCs on grating-like topographies, while RhoA inhibits endodermal differentiation on isotropic topographies.

MARC layout

1	1	1	2	2	3	3
5	5	5	4	4	6	6
10	10	4	4	6	6	17
7	7	8	8	9	9	17
13	13	14	14	15	15	16
11	11	12	12	10	10	16

Figure 22: Topographies on MARC.

Images were provided by Dr Kukumberg Marek, Mechanobiology institute (National University of Singapore).

Topographies

- 1 250nm lines, 250nm space, 250nm depth
- 2 250nm lines, 250nm space, 110nm depth
- 3 460nm lines, 70nm space, 40nm height
- 4 1um lines, 2um space, 120nm height
- 5 2um lines, 2um space, 2um height
- 6 2um lines, 1um space, 80nm height
- 7 2um lines perpendicular 250nm line
- 8 2um line parallel 250nm line
- 9 2um lines 250nm pillar
- 10 250nm pillars, 400nm pitch, 250nm height
- 11 2um pillars, 12um pitch, 2um height
- 12 1um holes, 6.5um pitch, 1um depth
- 13 1um pitch 0.3um sag microlens concave
- 14 1um pitch 0.3um sag microlens convex
- 15 1.8um diameter, 2um pitch, 0.7um sag microlens concave
- 16 1.8um diameter, 2um pitch, 0.7um sag microlens convex
- Blank Unpattern PDMS

Figure 23: Active RhoA enhances endodermal differentiation on grating-like patterns

(A) E14-mc and (B) E14-mc-RhoA (V14) (active) mESCs were cultured on MARC, in the absence of LIF for 3 days. Cells were then fixed and immunostained with antibodies against SOX17 (R&D system), and subsequently with secondary antibodies conjugated with fluorescence dye, Alexa fluor 488 (Molecular Probes®). Images were viewed with epifluorescence microscope and fluorescent intensities were measured using ImageJ, background signal was subtracted. Data presented were calculated and expressed as mean fold change against blank-unpattern PDMS. Means and standard deviations were computed from 2 independent experiments with 3 technical repeats each. Error bars represent standard deviations. (C) Comparison between control E14-mc and E14-mc-RhoA (V14) were carried out, Data was calculated and expressed as mean fold-change against control E14-mc cultured on blank-unpatterned PDMS.

4 Discussion

In our study, we explore the functions of RhoGTPases in mESCs, particularly the regulation of actin cytoskeleton and embryonic development/differentiation. RhoA, Rac1 and Cdc42 have been widely documented to regulate actin cytoskeleton dynamics that are crucial for cellular functions in the cells and body. RhoA induces the formation of stress fibers and focal adhesions, Rac1 regulates the formation of lamellipodia and Cdc42 promotes the formation of filopodia (Burridge et al., 1988; Small, 1981, 1995). While there has been great progress in the understanding of RhoGTPase functions in mammalian cells and mesenchymal stem cells, the roles of RhoGTPases in mESCs have not been defined. Here, we demonstrate that the expression of RhoGTPases does not induce changes in the actin cytoskeleton of mESCs (Figure 5), which is unlike common observations made in many adherent cell types (Ridley and Hall, 1992). This unexpected observation contradicts our initial hypothesis, which we suggest that expression of RhoGTPases in mESCs induces formation of actin cytoskeleton structures similar to those in adherent cell types. Thus we proceed to determine possible explanations.

We further hypothesize that the inability of RhoGTPases to induce changes in the actin cytoskeleton may be associated with inadequate expression of downstream effector proteins in mESCs. However, our results showed that the effector proteins are present and exhibit similar protein levels in both mESCs and fibroblast cells (Figure 6), which reject our hypothesis. It is likely that the effector proteins may be present in quiescent or inactive states and require activation by regulators. Indeed, our data have revealed that introduction of constitutively active forms of the effector proteins (ROK, MRCK and N-WASP) into mESCs results in drastic changes in the actin cytoskeleton, which are similar to observations in fibroblast cells (Higgs and Pollard, 1999; Ishizaki et al., 1997; Leung et al., 1996; Leung et al., 1998; Machesky and Gould, 1999; Unbekandt and Olson, 2014). These observations suggest that even though RhoGTPases and their effector proteins are present in mESCs, they are present in inactive states, which may require activation by additional regulators. Since the activities of the Rho proteins are regulated through RhoGEF, RhoGAP and RhoGDI (Bernards, 2003; Garrett et al., 1991; Garrett et al., 1989; Olofsson, 1999), some of these RhoGTPase regulators may be modulated differentially in mESCs.

Our preliminary finding has detected similar protein expression of RhoGDI α in fibroblast cells and mESCs (appendix, Figure I) suggesting that other additional regulators of RhoGTPases may be governing the activities of RhoGTPases in mESCs both temporally and spatially.

Previous studies have suggested that inhibition of RhoA-ROK signaling in mESCs affects self-renewal properties (Chang et al., 2010; Kamishibahara et al., 2014a; Nakamura et al., 2014; Zhang et al., 2012). Recently, Kamishibahara et al., (2014) showed that inhibition of ROK may activate ERK signaling that is responsible for neuronal differentiation of mESCs (Kamishibahara et al., 2014a). This leads us to investigate if mESCs expressing RhoA will alter the self-renewal properties of mESCs. However our results do not show significant differences in self-renewal markers in RhoA overexpressing mESCs when compared to control mESCs (Figure 11 and 12). These findings further emphasize the need to identify regulators in the complex signaling pathways that modulate stem cell self-renewal and pluripotency. For future studies to further elucidate the role of RhoA in mESC, we propose to use proteomic approach for the identification of potential interacting proteins of RhoA. Interacting proteins can be isolated by immunoprecipitation with RhoA and analyzed by mass spectrometry (Hernychova et al., 2012). We will be particularly interested in the negative regulators since we do not see changes in cell morphology and self-renewal properties when active RhoA is overexpressed in mESCs.

The regulation of the actin cytoskeleton by RhoA in mammalian cells has been extensively documented (Heasman and Ridley, 2008; Ridley and Hall, 1992). The lack of distinct actin cytoskeleton structures in undifferentiated mESCs may account for its rounded cell shape. In general, undifferentiated mESCs in culture appear spherical in shape with weak cell adhesion to the culture dishes and exhibit no/low cell spreading. Staining of actin filaments in mESCs using phalloidin-tag with fluorescein has shown that actin filaments are generally found at the cell cortex (Schratt et al., 2002), which is similar to our observations (Figure 5b and 5C). Upon differentiation, these mESCs flatten out on tissue culture dishes. RhoGTPases-induced actin assembly has been reported to activate SRF, a transcription factor,

which plays a role in the regulation of transcriptional activity of genes responsible for differentiation such as c-FOS and JUNB (Miralles et al., 2003; Settleman, 2003). Knocking down SRF or its co-activator MAL in human epidermal stem cells leads to inhibition of differentiation (Connelly et al., 2010). In addition, deletion of SRF in mESCs resulted in failure to form cytoskeletal structures. These mESCs also exhibit impaired cell spreading and cell adhesion (Schratt et al., 2002). Initially, it is thought that the differentiation process may induce changes in cell shape (Gumbiner, 1996; Hu et al., 1995). However subsequent studies have shown that changes in cell spreading can also influence cell fate decision during differentiation. For instance, human mesenchymal stem cells (hMSCs) differentiate into osteoblasts when hMSCs are allowed to spread (in a low cell density environment), while hMSCs give rise to adipocytes when cell spreading is prohibited (high cell density environment) (McBeath et al., 2004). In addition, osteogenesis is inhibited in hMSCs that are restricted in cell spreading (McBeath et al., 2004). Changes in geometric shape can also influence cell fate determination of hMSCs. hMSCs undergo osteogenesis more efficiently when they are restricted to micropatterned rectangles with higher aspect ratio compared to those with lower aspect ratio. Moreover, the differentiation of these hMSC is dependent on cytoskeletal cues from the actin cytoskeleton in the cells (Kilian et al., 2010). The regulation of actin cytoskeleton in hMSC is in turn dependent on RhoA/ROK signaling. Previous studies have revealed that overexpression of dominant negative RhoA or inhibition of ROK using Y27632 blocks osteogenesis in hMSCs.(McBeath et al., 2004; Shih et al., 2011). Furthermore, spread cells express higher RhoA and higher ROK activities are observed in spread cells compared to undifferentiated cells (McBeath et al., 2004). Taken together, these studies suggest that RhoA/ROK signaling is required for the regulation of actin cytoskeleton during cell fate determination in early embryonic development. Although MSC models have been extensively studied, little is known about the role of RhoA/ROK signaling in embryonic stem cells. The RhoA mESC stable lines that we have established in this study will be helpful for investigating the roles of RhoA in the regulation of cell spreading/actin cytoskeleton and also if RhoA/ROK will impact on SRF mediated transcription.

RhoA signaling has also been shown to be important for the regulation of cell adhesion in many mammalian cells (Heasman and Ridley, 2008; Ridley and Hall, 1992). Undifferentiated mESCs generally display very weak cell adhesion as compared to adherent mammalian cell types. Increase in cell adhesion of ESCs induces differentiation. The integrin complex is the key to cell adhesion. Integrins signaling can influence mESC differentiation (Treiser et al., 2010). Strong cell adhesion to fibronectin has been shown to reduce the expression of pluripotency markers even in the presence of LIF. On the other hand, low adhesive surfaces, such as gelatin and collagen, are found to help mESCs to maintain pluripotency in the presence of LIF (Cattavarayane et al., 2015; Hayashi et al., 2007; Hunt et al., 2012; Pimton et al., 2011). Additionally, activation of integrin in mESCs plated on collagen prevents self-renewal (Hayashi et al., 2007). Both collagen and fibronectin can induce differentiation in the absence of LIF, as indicated by a downregulation of pluripotency markers (Cattavarayane et al., 2015).

Our results support earlier studies showing that fibronectin-coated surfaces further augment mesodermal and endodermal differentiation in stable transgenic mESCs expressing RhoA-V14 (Figure 15C and 16C). We noted that there are two camps of thoughts on the roles of cell-matrix adhesion on stem cell differentiation. On one hand, it has been reported that the formation of focal adhesion will help the differentiation processes, as earlier studies have shown that knocking out FAK in mice and *Xenopus* results in loss of mesoderm-derived tissues (Furuta et al., 1995; Petridou et al., 2013). Nevertheless, there are also other reports which show that inhibition of FAK promotes mesodermal differentiation to form cardiomyocytes in mESCs (Hakuno et al., 2005), and also endodermal differentiation to insulin-producing β cells in hESCs (Afrikanova et al., 2011). Our observation is consistent with the latter studies. Here, we showed that inhibition of FAK phosphorylation enhances endodermal and mesodermal differentiations (Figure 21). One possible explanation for the discrepancy could be due to the differences in the systems used, *in vivo* versus *in vitro* systems. A second possible explanation could be due to that activated FAK can lead to the phosphorylation and activation of SRC kinase, which in turn activates PI3K-AKT1, leading to increased transcription of pluripotency

markers (Cornillon et al., 2003). The involvement of FAK suggests that cell adhesion may mediate differentiation via the regulation of RhoA signaling.

Our study has, for the first time, revealed that RhoA preferentially directs cell fate specification of mESCs towards mesoderm and endoderm layers, while inhibiting ectodermal differentiation in the absence of growth factors or inducers (Figure 15A, 16A and 17A). Our results support earlier studies documenting that RhoA negatively regulates neural outgrowth/initiation in neural stem cells and neuroblastoma cells (Amano et al., 1998; Chang et al., 2010; Gu et al., 2013; Hirose et al., 1998; Jalink et al., 1994; Katoh et al., 1998a; Kranenburg et al., 1997; Sebok et al., 1999). It is thought that ROK inhibitor promotes neural differentiation through the activation of extracellular signal-regulated kinase (ERK) signaling (Kamishibahara et al., 2014b; Li et al., 2006). Furthermore, cytoskeleton tensions, generated by ROK and myosin, are required for proper organization of the germ layers. Loss of myosin II-A and II-B prevents proper formation of organized germ layers (Poh et al., 2014). Inhibition of ROK has also been shown to play an important role during mouse embryonic development. Inhibition of ROK in preimplantation embryos leads to defective implantation, cavity formation, and defects in 1st cleavage, thus suggesting that ROK participates in cleavage process and blastocyst formation during embryonic development (Duan et al., 2014; Laeno et al., 2013). A vast number of studies have demonstrated that inhibition of ROK, using Y27632, results in the loss of self-renewal properties in mESCs, which is accompanied by an increase in neural differentiation markers such as Sox1, Nestin and MAP2 (Chang et al., 2010; Kamishibahara et al., 2014a; Nakamura et al., 2014; Zhang et al., 2012). In this study, we have discovered that active RhoA inhibits lineage specification into ectodermal cell types and dominant negative RhoA promotes ectodermal differentiation in mESCs (Figure 17A).

The addition of ROK inhibitor, Y27632, to culture media of hESCs has become a common practice as it significantly enhances cell survival (Krawetz et al., 2009; Olson, 2008). Unlike hESCs, mESCs are insensitive to dissociation-induced cell death (Ohgushi et al., 2010). Treatment of mESC with Y27632 leads to an opposite effect on self-renewal. Y27632 instead promotes cell differentiation. This can be due

to the fact that mESCs and hESCs exhibit very different regulatory signaling pathways that govern self-renewal and differentiation (Schnierch et al., 2010). The difference in signaling pathways pose difficulties in making use of current knowledge acquired using mouse model for application in human model.

Unlike other studies that look at a predefined differentiation pathway, our studies have discovered how RhoA mediates or controls cell fate specification, in the absence of growth factors or inducers. Interestingly, our results show that inhibition of either ROK or mDia, using chemical inhibitors, does not affect cell fate specification during differentiation (Figure 19 and 20) suggesting that ROK and mDia1 may possibly be required at a later stage of development where actin reorganization or structures are required for more cell type-specific functions (Li et al., 1994; Stutchfield and Howell, 1984; van Obberghen et al., 1973).

Recently, many studies have focused on the study of topography that mimics the *in vivo* microenvironment. So far, this is the first study that attempt to study endodermal differentiation of mESCs on different topographical substrates. Our primary results may suggest that endodermal differentiation favors anisotropic patterns over isotropic patterns (Figure 23). In addition, we also found that mESCs possess mechanosensing mechanism that has the ability to differentiate between micrograting and nanograting (Figure 23) (Teo et al., 2013). The ability of cells to sense the spacing and shape of topographical features can be due to the spatial arrangement of the focal adhesions and actomyosin, which send out different signals that govern cell fate decision during differentiation (Friedland et al., 2009; Mitra et al., 2005; Teo et al., 2013). In order to confirm this observation, we plan to include additional endodermal markers such as FOXA2, which is essential for neural tube and somite development during embryonic development (Ang and Rossant, 1994; Weinstein et al., 1994). Up to now, most studies focus on role of RhoA in the regulation of cellular functions in differentiated endodermal cells, such as pancreatic cells and hepatocytes (Dohda et al., 2004; Kim et al., 1998; Kong et al., 2014; Liu et al., 2014), but not the differentiation process. To date, studies have only revealed that topography-induced differentiation activates RhoA signaling pathway in

mesenchymal stem cells (McBeath et al., 2004; Mitra et al., 2005; Seo et al., 2011a). Our observations support the hypothesis that the presence of active RhoA enhances topography-regulated differentiation into endoderm lineage (Figure 23). Future studies will aim to identify the downstream signaling pathways of RhoA that play a role in the regulation of topography-induced endodermal differentiation.

5 Conclusion

In this study, we uncovered novel functions of RhoA in early development, particularly in the regulation of cell fate decision. We showed that RhoA preferentially enhances mESC differentiation into mesodermal and endodermal lineages and inhibits ectodermal differentiation. We also found that the activities of the RhoGTPases are tightly regulated in mESCs. Although the downstream effectors of these RhoGTPases are present, we do not observe similar changes in the actin cytoskeleton as in the differentiated cell types when active forms of the Rho proteins are over-expressed. However, constitutively active effectors do elicit the expected changes in the actin cytoskeleton of mESCs. These observations may indicate additional levels of regulation or tighter control of downstream activities of the RhoGTPases in mESCs.

6 Reference

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7 Appendix

Analysis of Variance

ANOVA table	Sum of Square (SS)	Degree of Freedom (DF)	Mean Square (MS)	F (DFn, DFd)	P value
Interaction	12691	6	2115	F (6, 24) = 13.41	P < 0.0001
Row Factor (ECMs)	139591	2	69795	F (2, 24) = 442.6	P < 0.0001
Column Factor (Cell types)	27648	3	9216	F (3, 24) = 58.44	P < 0.0001
Residual	3785	24	157.7		

Number of families	1			
Number of comparisons per family	66			
Alpha	0.05			
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Gelatin:E14-mc (control) vs. Gelatin:E14-mc-RhoA-WT	12.06	-24.91 to 49.03	No	ns
Gelatin:E14-mc (control) vs. Gelatin:E14-mc-RhoA-V14	63.95	26.98 to 100.9	Yes	***
Gelatin:E14-mc (control) vs. Gelatin:E14-mc-RhoA-N19	-21.69	-58.66 to 15.28	No	ns
Gelatin:E14-mc (control) vs. Fibronectin:E14-mc (control)	-56.47	-93.44 to -19.50	Yes	***
Gelatin:E14-mc (control) vs. Fibronectin:E14-mc-RhoA-WT	-103.8	-140.8 to -66.87	Yes	****
Gelatin:E14-mc (control) vs. Fibronectin:E14-mc-RhoA-V14	4.971	-32.00 to 41.94	No	ns
Gelatin:E14-mc (control) vs. Fibronectin:E14-mc-RhoA-N19	-117.4	-154.4 to -80.43	Yes	****
Gelatin:E14-mc (control) vs. Collagen:E14-mc (control)	89.99	53.02 to 127.0	Yes	****
Gelatin:E14-mc (control) vs. Collagen:E14-mc-RhoA-WT	87.76	50.79 to 124.7	Yes	****
Gelatin:E14-mc (control) vs. Collagen:E14-mc-RhoA-V14	89.68	52.71 to 126.7	Yes	****
Gelatin:E14-mc (control) vs. Collagen:E14-mc-RhoA-N19	69.4	32.43 to 106.4	Yes	****
Gelatin:E14-mc-RhoA-WT vs. Gelatin:E14-mc-RhoA-V14	51.89	14.92 to 88.86	Yes	**
Gelatin:E14-mc-RhoA-WT vs. Gelatin:E14-mc-RhoA-N19	-33.75	-70.72 to 3.220	No	ns
Gelatin:E14-mc-RhoA-WT vs. Fibronectin:E14-mc (control)	-68.53	-105.5 to -31.56	Yes	****
Gelatin:E14-mc-RhoA-WT vs. Fibronectin:E14-mc-RhoA-WT	-115.9	-152.9 to -78.93	Yes	****
Gelatin:E14-mc-RhoA-WT vs. Fibronectin:E14-mc-RhoA-V14	-7.09	-44.06 to 29.88	No	ns
Gelatin:E14-mc-RhoA-WT vs. Fibronectin:E14-mc-RhoA-N19	-129.5	-166.4 to -92.49	Yes	****
Gelatin:E14-mc-RhoA-WT vs. Collagen:E14-mc (control)	77.93	40.96 to 114.9	Yes	****
Gelatin:E14-mc-RhoA-WT vs. Collagen:E14-mc-RhoA-WT	75.7	38.73 to 112.7	Yes	****

Gelatin:E14-mc-RhoA-WT vs. Collagen:E14-mc-RhoA-V14	77.62	40.65 to 114.6	Yes	****
Gelatin:E14-mc-RhoA-WT vs. Collagen:E14-mc-RhoA-N19	57.34	20.37 to 94.31	Yes	***
Gelatin:E14-mc-RhoA-V14 vs. Gelatin:E14-mc-RhoA-N19	-85.64	-122.6 to -48.67	Yes	****
Gelatin:E14-mc-RhoA-V14 vs. Fibronectin:E14-mc (control)	-120.4	-157.4 to -83.45	Yes	****
Gelatin:E14-mc-RhoA-V14 vs. Fibronectin:E14-mc-RhoA-WT	-167.8	-204.8 to -130.8	Yes	****
Gelatin:E14-mc-RhoA-V14 vs. Fibronectin:E14-mc-RhoA-V14	-58.98	-95.95 to -22.01	Yes	***
Gelatin:E14-mc-RhoA-V14 vs. Fibronectin:E14-mc-RhoA-N19	-181.4	-218.3 to -144.4	Yes	****
Gelatin:E14-mc-RhoA-V14 vs. Collagen:E14-mc (control)	26.04	-10.93 to 63.01	No	ns
Gelatin:E14-mc-RhoA-V14 vs. Collagen:E14-mc-RhoA-WT	23.81	-13.16 to 60.78	No	ns
Gelatin:E14-mc-RhoA-V14 vs. Collagen:E14-mc-RhoA-V14	25.73	-11.24 to 62.70	No	ns
Gelatin:E14-mc-RhoA-V14 vs. Collagen:E14-mc-RhoA-N19	5.445	-31.53 to 42.42	No	ns
Gelatin:E14-mc-RhoA-N19 vs. Fibronectin:E14-mc (control)	-34.78	-71.75 to 2.194	No	ns
Gelatin:E14-mc-RhoA-N19 vs. Fibronectin:E14-mc-RhoA-WT	-82.15	-119.1 to -45.18	Yes	****
Gelatin:E14-mc-RhoA-N19 vs. Fibronectin:E14-mc-RhoA-V14	26.66	-10.31 to 63.63	No	ns
Gelatin:E14-mc-RhoA-N19 vs. Fibronectin:E14-mc-RhoA-N19	-95.71	-132.7 to -58.74	Yes	****
Gelatin:E14-mc-RhoA-N19 vs. Collagen:E14-mc (control)	111.7	74.71 to 148.7	Yes	****
Gelatin:E14-mc-RhoA-N19 vs. Collagen:E14-mc-RhoA-WT	109.5	72.48 to 146.4	Yes	****
Gelatin:E14-mc-RhoA-N19 vs. Collagen:E14-mc-RhoA-V14	111.4	74.40 to 148.3	Yes	****
Gelatin:E14-mc-RhoA-N19 vs. Collagen:E14-mc-RhoA-N19	91.09	54.12 to 128.1	Yes	****
Fibronectin:E14-mc (control) vs. Fibronectin:E14-mc-RhoA-WT	-47.37	-84.34 to -10.40	Yes	**
Fibronectin:E14-mc (control) vs. Fibronectin:E14-mc-RhoA-V14	61.44	24.47 to 98.41	Yes	***
Fibronectin:E14-mc (control) vs. Fibronectin:E14-mc-RhoA-N19	-60.94	-97.91 to -23.97	Yes	***
Fibronectin:E14-mc (control) vs. Collagen:E14-mc (control)	146.5	109.5 to 183.4	Yes	****
Fibronectin:E14-mc (control) vs. Collagen:E14-mc-RhoA-WT	144.2	107.3 to 181.2	Yes	****
Fibronectin:E14-mc (control) vs. Collagen:E14-mc-RhoA-V14	146.2	109.2 to 183.1	Yes	****
Fibronectin:E14-mc (control) vs. Collagen:E14-mc-RhoA-N19	125.9	88.90 to 162.8	Yes	****
Fibronectin:E14-mc-RhoA-WT vs. Fibronectin:E14-mc-RhoA-V14	108.8	71.84 to 145.8	Yes	****
Fibronectin:E14-mc-RhoA-WT vs. Fibronectin:E14-mc-RhoA-N19	-13.57	-50.54 to 23.40	No	ns
Fibronectin:E14-mc-RhoA-WT vs. Collagen:E14-mc (control)	193.8	156.9 to 230.8	Yes	****
Fibronectin:E14-mc-RhoA-WT vs. Collagen:E14-mc-RhoA-WT	191.6	154.6 to 228.6	Yes	****
Fibronectin:E14-mc-RhoA-WT vs. Collagen:E14-mc-RhoA-V14	193.5	156.5 to 230.5	Yes	****
Fibronectin:E14-mc-RhoA-WT vs. Collagen:E14-mc-RhoA-N19	173.2	136.3 to 210.2	Yes	****
Fibronectin:E14-mc-RhoA-V14 vs. Fibronectin:E14-mc-	-122.4	-159.3 to -	Yes	****

RhoA-N19		85.40		
Fibronectin:E14-mc-RhoA-V14 vs. Collagen:E14-mc (control)	85.02	48.05 to 122.0	Yes	****
Fibronectin:E14-mc-RhoA-V14 vs. Collagen:E14-mc-RhoA-WT	82.79	45.82 to 119.8	Yes	****
Fibronectin:E14-mc-RhoA-V14 vs. Collagen:E14-mc-RhoA-V14	84.71	47.74 to 121.7	Yes	****
Fibronectin:E14-mc-RhoA-V14 vs. Collagen:E14-mc-RhoA-N19	64.43	27.46 to 101.4	Yes	****
Fibronectin:E14-mc-RhoA-N19 vs. Collagen:E14-mc (control)	207.4	170.4 to 244.4	Yes	****
Fibronectin:E14-mc-RhoA-N19 vs. Collagen:E14-mc-RhoA-WT	205.2	168.2 to 242.1	Yes	****
Fibronectin:E14-mc-RhoA-N19 vs. Collagen:E14-mc-RhoA-V14	207.1	170.1 to 244.1	Yes	****
Fibronectin:E14-mc-RhoA-N19 vs. Collagen:E14-mc-RhoA-N19	186.8	149.8 to 223.8	Yes	****
Collagen:E14-mc (control) vs. Collagen:E14-mc-RhoA-WT	-2.227	-39.20 to 34.74	No	ns
Collagen:E14-mc (control) vs. Collagen:E14-mc-RhoA-V14	-0.308	-37.28 to 36.66	No	ns
Collagen:E14-mc (control) vs. Collagen:E14-mc-RhoA-N19	-20.59	-57.56 to 16.38	No	ns
Collagen:E14-mc-RhoA-WT vs. Collagen:E14-mc-RhoA-V14	1.919	-35.05 to 38.89	No	ns
Collagen:E14-mc-RhoA-WT vs. Collagen:E14-mc-RhoA-N19	-18.36	-55.34 to 18.61	No	ns
Collagen:E14-mc-RhoA-V14 vs. Collagen:E14-mc-RhoA-N19	-20.28	-57.25 to 16.69	No	ns

Table A1: 2-way ANOVA test

Stable transgenic mESCs expressing RhoA and control cells were plated onto 96 wells that are coated with gelatin, collagen and fibronectin. Cell adhesion assay was conducted 1 hour after seeding. Results obtained were subjected to 2-way ANOVA test with Tukey's HSD test showed significant main effect for the ECMs. Statistical test was carried out using graphpad prism.

Figure 1: mESCs and stable transgenic mESCs expressing similar protein expression of RhoGDIα as the fibroblast cells.

Soluble cell lysates were isolated from mouse fibroblasts (NIH3T3, STO and MEF), mESCs (D3 and E14) and stable transgenic mESCs expressing RhoA. Equal amount of cell lysate was loaded in each well. The cell lysates were subjected to SDS-PAGE and resolved protein bands on the gel were transferred to nitrocellulose membrane for western blot analysis. Antibodies against RhoGDIα (Santa Cruz) and tubulin (as loading control, Sigma Aldrich) were used.

Figure II: Levels of endogenous RhoA and mCherry-Flag-RhoA in mESCs, fibroblast cells and stable transgenic mESCs.

Soluble cell lysates were isolated from mouse fibroblasts (NIH3T3, STO and MEF), mESCs (D3 and E14) and stable transgenic mESCs expressing RhoA. Equal amount of cell lysate was loaded in each well. The cell lysates were subjected to SDS-PAGE and resolved protein bands on the gel were transferred to nitrocellulose membrane for western blot analysis. Antibodies against RhoA (Cytoskeleton) and tubulin (as loading control, Sigma Aldrich) were used.

Figure III: Levels of endogenous Rac1 and mCherry-Flag-Rac1 in mESCs, fibroblast cells and stable transgenic mESCs.

Soluble cell lysates were isolated from mouse fibroblasts (NIH3T3, STO and MEF), mESCs (D3 and E14) and stable transgenic mESCs expressing Rac1. Equal amount of cell lysate was loaded in each well. The cell lysates were subjected to SDS-PAGE and resolved protein bands on the gel were transferred to nitrocellulose membrane for western blot analysis. Antibodies against Rac1 (Upstate) and tubulin (as loading control, Sigma Aldrich) were used.

Figure IV: Levels of endogenous Cdc42 and mCherry-Flag-Cdc42 in mESCs, fibroblast cells and stable transgenic mESCs.

Soluble cell lysates were isolated from mouse fibroblasts (NIH3T3, STO and MEF), mESCs (D3 and E14) and stable transgenic mESCs expressing Cdc42. Equal amount of cell lysate was loaded in each well. The cell lysates were subjected to SDS-PAGE and resolved protein bands on the gel were transferred to nitrocellulose membrane for western blot analysis. Antibodies against Cdc42 (ECM Bioscience) and tubulin (as loading control, Sigma Aldrich) were used.