CHARACTERIZATION OF CELLULAR ABNORMALITIES DUE TO LOSS OF TSC2

by

Alym Platinum Moosa

B.Sc., University of British Columbia, 2006

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in
The Faculty of Graduate and Postdoctoral Studies
(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

April 2014

©Alym Platinum Moosa, 2014
ABSTRACT

Tuberous sclerosis complex (TSC) is a disorder characterized by multiple benign tumours in all major organs that can result in neurological manifestations including mental retardation and autism. TSC results from mutation in TSC1 or TSC2, which together form a complex that serves as a negative regulator of protein kinase mTORC1, a key regulator of cell growth and metabolism. Thus, cells with diminished TSC1:TSC2 function display elevated mTORC1 signaling, leading to the formation of benign tumours with very large cells. Rapamycin is a potent mTORC1 inhibitor, and rapamycin derivative everolimus has been approved for treatment of TSC patients with inoperable subependymal giant cell astrocytomases. However, these drugs can have serious side effects and should not be used for extended periods of time. In a screen of approved human drugs, amiodarone, dronedarone, perhexiline, and niclosamide were found to inhibit the elevated mTORC1 signaling seen in mouse embryo fibroblast (MEF) cells lacking TSC2. The goal of this project was to determine whether the many abnormal cell phenotypes associated with loss of TSC2 are directly related to elevated mTORC1 levels, and whether these drugs can ameliorate these abnormal phenotypes.

Unlike wild type MEFs, TSC2-null MEFs showed an epithelial-like morphology with an increase in localization of actin to the cell periphery, focal adhesions, localization of β-catenin to cell-cell junctions, and localization of N-cadherin to cell-cell junctions. Exposure of TSC2-null MEFS to the mTORC1 inhibitors for seven days caused a transition from an epithelial-like to a fibroblast-like morphology in all of the aforementioned phenotypes, resembling that of wild type MEFs.
TSC2-null MEFs were shown to express E-cadherin, a cell adhesion protein not normally found in MEFs. Knocking down levels of TSC2 in wild-type MEFs did not induce expression of E-cadherin, but restoring TSC2 expression in TSC2-null MEFs slightly reduced E-cadherin expression. A tentative model was proposed to explain how TSC2 can control E-cadherin expression, which has not yet been described in literature.
This thesis is presented in six chapters. A general introductory review of the research area is presented in Chapter 1. Chapter 2 describes the Materials and Methods used in the experiments presented in this study. Chapter 3 describes my results characterizing the phenotypic differences between TSC2+/+ and TSC2−/− MEFs, and the effect of mTORC1 inhibitors on these features. The Fluorescence Recovery After Photobleaching (FRAP) work (Figure 3.11) was done in collaboration with Spencer Freeman. After I treated the cells with drugs and transfected them with N-cadherin-GFP, Spencer carried out the FRAP protocol and made the figure. All other work presented in Chapter 3 is my own. Chapter 4 describes my results in investigating the presence of E-cadherin in TSC2−/− MEFs. All the work presented in Chapter 4 is my own. Chapter 5 discusses some of the novel implications of my results. Chapter 6 concludes my findings and discusses several future experiments. To date, none of the data in any of the Chapters has been published.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td></td>
<td>PREFACE</td>
<td>iv</td>
</tr>
<tr>
<td></td>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td></td>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td></td>
<td>LIST OF ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>ACKNOWLEDGEMENTS</td>
<td>xiii</td>
</tr>
<tr>
<td>CHAPTER 1: INTRODUCTION</td>
<td>Tuberous sclerosis complex</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>The mTORC1 signaling pathway</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>mTORC2 and Akt signaling pathways</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Phenotypic differences between TSC2+/+ and TSC2−/− MEFs and underlying</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>mechanisms</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>F-Actin and cytoskeleton</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Focal adhesions</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Cell-cell adhesions</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>β-catenin</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>N-cadherin</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>E-cadherin</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Current treatments for TSC</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Discovery of new inhibitors of mTORC1 signaling</td>
<td>14</td>
</tr>
<tr>
<td>CHAPTER 2: MATERIALS AND METHODS</td>
<td>Cell lines</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Chemicals</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Long-term treatment with drugs</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Quantifying cell size and morphology</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>SDS-PAGE and immunoblotting</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Immunofluorescence microscopy</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Transfections</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Fluorescence recovery after photobleaching (FRAP)</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Video microscopy</td>
<td>22</td>
</tr>
<tr>
<td>CHAPTER 3: CHARACTERIZATION OF THE EFFECT OF LONG-TERM TREATMENT WITH mTORC1 INHIBITORS ON TSC2−/− MEF MORPHOLOGY</td>
<td>Synopsis</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>TSC2−/− MEFs display a morphological change from epithelial-like to fibroblast-like after long-term treatment with mTORC1 inhibitors</td>
<td>24</td>
</tr>
</tbody>
</table>
3.3 Long-term treatment with mTORC1 inhibitors does not significantly reduce the size of TSC2\(^{-}\) MEFs ................................................................. 34
3.4 Long-term treatment with mTORC1 inhibitors changes actin localization in TSC2\(^{-}\) MEFs ................................................................. 36
3.5 Long-term treatment with mTORC1 inhibitors reduces focal adhesions in TSC2\(^{-}\) MEFs ................................................................. 36
3.6 Long-term treatment with mTORC1 inhibitors reduces cell-cell adhesions in TSC2\(^{-}\) MEFs ................................................................. 38
3.7 Long-term treatment with mTORC1 inhibitors does not change the expression levels of full-length \(\beta\)-catenin, vinculin, or N-cadherin in TSC2\(^{+/+}\) and TSC2\(^{-}\) MEFs ..................................................................................... 45

CHAPTER 4: EXPRESSION OF E-CADHERIN BY TSC2\(^{-}\) MEFS AND ITS POTENTIAL REGULATION BY TSC2 ........................................................................ 53

4.1 Synopsis ....................................................................................... 53
4.2 E-cadherin is expressed in TSC2\(^{-}\) MEFs .......................................... 53
4.3 pEF6-TSC2 transected TSC2\(^{-}\) MEFs do not express E-cadherin .......... 57
4.4 Restoring TSC2 in TSC2\(^{-}\) MEFs reduces E-cadherin levels only modestly ...... 62
4.5 Knocking down TSC2 in TSC2\(^{+/+}\) MEFs does not induce E-cadherin expression. 72

CHAPTER 5: DISCUSSION ..................................................................... 74

5.1 Effect of long-term mTORC1 inhibition on MEF morphology and size........ 74
5.2 Actin and focal adhesions .............................................................. 80
5.3 Cell-cell adhesion ......................................................................... 83
5.4 Expression of adhesion proteins .................................................... 86
5.5 E-cadherin .................................................................................. 87

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS ......................... 96

6.1 Concluding remarks ...................................................................... 96
6.2 Determining how mTORC1 affects cell morphology .......................... 97
6.3 Determining how TSC2\(^{-}\) MEFs compensate for long-term mTORC1 inhibition . 97
6.4 Determining how TSC2 acts as a regulator of E-cadherin ...................... 98

REFERENCES ..................................................................................... 100
LIST OF FIGURES

Figure 1.1 Image of SEGA as seen in TSC patient ........................................................................ 2
Figure 1.2 Regulation of mTORC1 activity by TSC1:TSC2 .................................................................. 4
Figure 1.3 Control of protein synthesis and cell growth by mTORC1 ................................................. 6
Figure 1.4 Regulation of mTORC2 and Akt by mTORC1 .................................................................. 7
Figure 1.5 Model of cell-cell adhesion ............................................................................................... 11
Figure 3.1 Morphology of TSC2^{+/+} and TSC2^{-/-} MEFs ................................................................. 25
Figure 3.2 Change in morphology of TSC2^{-/-} MEFs induced by drugs ............................................... 26
Figure 3.3 Quantification of the change of TSC2^{-/-} MEFs from an epithelial-like to a fibroblast-like morphology .................................................................................................................. 30
Figure 3.4 mTORC1 inhibition is not sustained during long-term treatment with drugs.............. 32
Figure 3.5 Long-term treatment with niclosamide de-sensitizes TSC2^{-/-} MEFs to further morphological changes .................................................................................................................. 33
Figure 3.6 Long-term treatment with mTORC1 inhibitors does not significantly reduce TSC2^{-/-} MEF size .............................................................................................................................................. 35
Figure 3.7 Long-term treatment with mTORC1 inhibitors changes actin localization in TSC2^{-/-} MEFs .............................................................................................................................................. 37
Figure 3.8 Long-term treatment with mTORC1 inhibitors changes vinculin localization in TSC2^{-/-} MEFs .............................................................................................................................................. 39
Figure 3.9 Long-term treatment with mTORC1 inhibitors changes β-catenin localization in TSC2{-/-} MEFs.............................................................................................................................................. 41
Figure 3.10 Long-term treatment with mTORC1 inhibitors changes N-cadherin localization in TSC2−/− MEFs. .......................................................................................................................... 42
Figure 3.11 Long-term treatment with mTORC1 inhibitors increases the N-cadherin-GFP fluorescence recovery rate at cell-cell junctions in TSC2+/− MEFs after photobleaching. ...... 44
Figure 3.12 Time lapse microscopy of TSC2+/+ and TSC2−/− MEFs treated with mTORC1 inhibitors. .......................................................................................................................... 46
Figure 3.13 Effect of mTORC1 inhibitors on expression levels of β-catenin, vinculin, N-cadherin, and E-cadherin in MEFs. ........................................................................................................... 50
Figure 3.14 Effect of niclosamide on E-cadherin expression in TSC2+/− MEFs. ................. 52
Figure 4.1 TSC2+/− MEFs express E-Cadherin. ........................................................................ 54
Figure 4.2 Visualization of E-Cadherin in TSC2+/+ and TSC2−/− MEFs by
immunofluorescence microscopy. ........................................................................................................ 56
Figure 4.3 Effect of TSC2+/− MEF confluency on E-cadherin expression levels. ............... 58
Figure 4.4 pEF6-Transfected TSC2+/− MEFs do not express E-cadherin. ......................... 59
Figure 4.5 Morphology of pEF6-Transfected TSC2+/− MEFs .............................................. 61
Figure 4.6 Early and late batches of TSC2−/− MEFs appear identical. ............................... 63
Figure 4.7 Transient expression of TSC2 in TSC2−/− MEFs does not decrease E-cadherin levels after 2 days. .............................................................................................................. 64
Figure 4.8 Transient transfection with pRK7-TSC2 induces a slight reduction of E-cadherin in TSC2+/− MEFs. .............................................................................................................. 66
Figure 4.9 Visualization of TSC2 and E-cadherin in pRK7-TSC2-transfected TSC2+/− MEFs by immunofluorescence microscopy. ............................................................................................... 68
Figure 4.10 siRNA Knockdown of TSC2 in TSC2+/+ MEFs does not induce E-cadherin expression.

Figure 5.1 Model of E-cadherin regulation by TSC2.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4EBP1</td>
<td>eukaryotic translation initiation factor 4E-binding protein 1</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>deptor</td>
<td>DEP domain-containing mTOR-interacting protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>epithelial cadherin</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eIF4E</td>
<td>eukaryotic translation initiation factor 4E</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>F-Actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FKBP12</td>
<td>FK506 binding protein 12</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>G-Actin</td>
<td>globular actin</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>HIF1α</td>
<td>hypoxia-inducible factor 1 alpha</td>
</tr>
</tbody>
</table>
HRP horseradish peroxidase
IRS-1 insulin receptor substrate 1
kDa kilo Dalton
LAM lymphangioleiomyomatosis
LEF lymphoid enhancer factor
MEF mouse embryonic fibroblast
mLST8 mammalian lethal with SEC13 protein 8
mTOR mammalian target of rapamycin
mTORC1 mammalian target of rapamycin complex 1
mTORC2 mammalian target of rapamycin complex 2
MW molecular weight
N-cadherin neural-cadherin
PAGE polyacrylamide gel electrophoresis
PBS phosphate-buffered saline
PCK polycystic kidney disease
PFA paraformaldehyde
PRAS40 proline-rich Akt substrate 40
raptor regulatory-associated protein of mTOR
Rheb ras homolog enriched in brain
rictor rapamycin-insensitive companion of mTOR
RTK receptor tyrosine kinase
S6K S6 kinase
SDS sodium dodecyl sulfate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEGA</td>
<td>subependymal giant cell astrocytoma</td>
</tr>
<tr>
<td>Sin1</td>
<td>stress-activated MAPK-interacting protein 1</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCF</td>
<td>T cell factor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TP53</td>
<td>tumour protein p53</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethylrhodamine isothiocyanate</td>
</tr>
<tr>
<td>TSC</td>
<td>tuberous sclerosis complex</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to first and foremost thank my supervisor Dr. Michel Roberge for giving me the great opportunity to work in his lab, and for mentoring me throughout my graduate studies. I really appreciated all of your helpfulness, patience, encouragement, and constructive criticism of my ability as a researcher, and I am amazed at far I have grown and developed under your supervision. I would also like to thank members of the Roberge Lab, both past and present: Hilary Anderson, Aruna Balgi, Alireza Baradaran, Celia Bergeaud, Sophie Comyn, Pamela Dean, Elizabeth Donohue, Karen Lam, and Carla Zimmerman. I thoroughly enjoyed the lab dynamic, and you all were fantastic people to work with and to learn from.

I would like to thank my committee members Dr. Neal Boerkoel and Dr. Filip Van Petegem for all of your feedback and constructive criticism in all of our meetings. I would like to thank Dr. Calvin Roskelley and all the members of his lab for their assistance in understanding aspects of cell morphology, and for providing me with materials and protocols for my own use.

I would like to thank my family for being incredibly supportive of me throughout my tenure as a student. You gave me the opportunity to follow the path that I have taken, and I thank you for the many years of encouragement. To all of my fellow graduate students and friends, thank you for all the much needed entertainment and fun throughout the years. Finally, thank you to Christina for all of your love and support.
1.1 Tuberous sclerosis complex

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by multiple benign tumours (hamartomas) in all major organs, including the brain, kidneys, skin, heart, and lungs (1). The most devastating manifestation of TSC is when hamartomas are found in the brain, which can lead to neurological abnormalities such as epilepsy, mental retardation, and autism (2–5). The most common tumour type in TSC patients is a subependymal giant cell astrocytoma (SEGA) (Figure 1.1), which is benign and non-invasive, but can lead to hydrocephalus and sudden death (6). The size of these “tubers” has been shown to correlate to clinical severity, with larger tubers being associated with more severe symptoms (7). Tubers have been detected in utero and form during embryonic development, thus symptoms associated with TSC can manifest within the first year of life (2). The birth incidence of TSC is approximately 1:6000, with 30% of cases being inherited from parents and 70% of cases being de novo germ-line mutations in TSC genes (8,9). Mutations can occur in either TSC1, which encodes a 130 kDa protein (TSC1/hamartin) or TSC2 which encodes a 200 kDa protein (TSC2/tuberin) (2). TSC1 and TSC2 associate to form a heterodimeric complex (TSC1:TSC2), with TSC2 serving as a GTPase-activating protein, and TSC1 being required to stabilize TSC2 and prevent its ubiquitin-mediated degradation (1). The most conserved region in TSC1 are the N-terminal ~265 residues which contributes to TSC1 stability, and the majority of pathogenic TSC1 mutations map inside of this folded structure (10). TSC2 is known to have several domains, including a leucine zipper, two coiled-coiled domains, two transcription-activating domains, GTPase-activating
Figure 1.1 Image of SEGA as seen in TSC patient.

Figure was found from the Massachusetts General Hospital website (83) on TSC with the following legend: Axial brain MRI showing a subependymal giant cell astrocytoma (SEGA).
protein (GAP) homology, and a calmodulin-binding domain (11). Pathogenic TSC2
mutations are mostly located in the N-terminal TSC1-binding region, or the C-terminal GAP
domain (12). Pathogenic mutations in either TSC1 or TSC2 give rise to the same clinical
manifestations (2). Except for the TSC2 GAP domain, TSC1 and TSC2 have no apparent
sequence homology with other proteins (10). The TSC1:TSC2 complex acts primarily as a
GTPase-activating protein for the GTPase Rheb (Ras homolog enriched in brain), and
converts active GTP-Rheb to inactive GDP-Rheb (Figure 1.2) (2). Active GTP-Rheb is a
positive regulator of protein kinase mTORC1, which is an important controller of cell growth
(size) and metabolism (13). The TSC1:TSC2 complex thus serves primarily as a negative
regulator of mTORC1 signaling (1), and cells with impaired TSC1:TSC2 display abnormally
high mTORC1 signaling, leading to increased cellular growth and the formation of benign
tumours (1,14,15).

1.2 The mTORC1 signaling pathway

Many of the abnormal phenotypes associated with TSC have been attributed to
elevated levels of mTORC1 signaling (2,8). mTORC1 activation has also been associated
with other diseases such as polycystic kidney disease (PCK) and lymphangioleiomyomatosis
(LAM) (16,17). mTORC1 is a central hub that integrates signals from various pathways, such
as those sensing nutrients and growth factors, and then positively regulates anabolic
functions such as cell growth, increased transcription and translation, and ribosome
biogenesis (11,18). mTORC1 is composed of catalytic kinase subunit mTOR, raptor
( regulatory-associated protein of mTOR), PRAS40 (proline-rich Akt substrate 40), mLST8
(mammalian lethal with SEC13 protein 8) , and deptor (DEP domain-containing mTOR-
interacting protein) (19). The small molecule rapamycin irreversibly inhibits mTORC1 by
Figure 1.2 Regulation of mTORC1 activity by TSC1:TSC2.
TSC1 and TSC2 associate to form a heterodimeric complex with TSC2 serving as a GTPase-activating protein and TSC1 being required to stabilize TSC2 and prevent its ubiquitin-mediated degradation. TSC1:TSC2 converts active GTP-Rheb to inactive GDP-Rheb. Active GTP-Rheb is a positive regulator of mTORC1, an important controller of cell growth and protein synthesis.
complexing with FKBP12 (FK506 binding protein 12), which binds to mTOR and disrupts
the interaction of raptor with mTOR (20). One of mTORC1’s main downstream targets is
the ribosomal S6 kinase (S6K), which it phosphorylates at T389, leading to S6K activation
and downstream phosphorylation and activation of ribosomal protein S6 which controls
mRNA translation. S6K phosphorylation at T389 serves as a measurement for mTORC1
activity (Figure 1.3) (19,21). Another protein phosphorylated by mTORC1 is the eukaryotic
translation initiation factor 4E-binding protein 1 (4EBP1), that when phosphorylated releases
eIF4E, which allows for proper formation of the ribosomal machinery which in turn allows
translation to occur (19). Thus, constitutively increased mTORC1 activity directly promotes
increased protein synthesis and cell growth, and can result in the formation of SEGAs
(Figure 1.3) (1,6).

1.3 mTORC2 and Akt signaling pathways

mTORC2 is a second complex where mTOR is instead associated with rictor
(rapamycin-insensitive companion of mTOR), mLST8, deptor, and Sin1(stress-activated
MAPK-interacting protein 1) (19). While rapamycin inhibits mTORC1 signaling, mTORC2
is mostly insensitive to rapamycin (22). There is an inverse relationship between the activity
levels of mTORC1 and mTORC2, which occurs via S6K signaling (Figure 1.4).
Phosphorylated S6K can directly phosphorylate and inactivate IRS-1 (insulin receptor
substrate 1), which associates with membrane-bound RTKs (receptor tyrosine kinases)
(19,22). Active IRS-1 can cause phosphorylation and activation of mTORC2 (23). One of
mTORC2’s main downstream effects is activating phosphorylation of the kinase Akt on S473
(24). Thus, TSC1:TSC2-compromised cells with elevated levels of mTORC1 activity and
phosphorylated S6K display reduced mTORC2 signaling and thus reduced Akt
Figure 1.3 Control of protein synthesis and cell growth by mTORC1.

mTORC1 can phosphorylate and activate S6K on T389 to directly stimulate protein synthesis. mTORC1 also phosphorylates 4EBP1, causing dissociation of eIF4E, which also stimulates protein synthesis. Uncontrolled elevated mTORC1 signaling in TSC patients can thus result in cellular overgrowth and formation of SEGAs and other benign tumours.
Figure 1.4 Regulation of mTORC2 and Akt by mTORC1.

mTORC1 phosphorylates and activates S6K, which promotes IRS-1 phosphorylation and downregulation. When active, IRS-1 can phosphorylate and activate mTORC2. Thus, active mTORC1 can inactivate mTORC2. mTORC2 can phosphorylate Akt on S473 and activate it, resulting in increased phosphorylation of many downstream targets, one of which is GSK-3β, when phosphorylated at S9 is degraded. GSK-3β can phosphorylate both β-catenin and Snail, causing their degradation.
phosphorylation (1). Akt is a kinase that controls many cellular processes, including inhibiting TSC2 to allow for a feedback regulation pathway (25), as well activating several pathways that promote cancer metastasis and mesenchymal phenotypes (26). Finally, activated Akt has been shown to phosphorylate GSK-3β (glycogen synthase kinase 3 beta) on S9, which causes its degradation. GSK-3β expression causes the phosphorylation and degradation of β-catenin and Snail (27), which are discussed in later sections.

1.4 Phenotypic differences between TSC2+/+ and TSC2−/− MEFs and underlying mechanisms

One of the original aims of the project was to assess the extent to which certain drugs can reduce or normalize mTORC1 signaling in TSC2-defective cells and ameliorate the abnormal cellular phenotypes associated with TSC2 deletion. The cells used to assess the in vitro effects of the drugs were TSC2+/+ and TSC2−/− MEFs, both immortalized by TP53 deletion. I studied several phenotypic differences between TSC2+/+ and TSC2−/− MEFs. The sections below introduce the relevant cellular processes and proteins that I investigated, and current knowledge of their control by the TSC1:TSC2 complex.

1.4.1 F-Actin and cytoskeleton

Actins are structural proteins that play important roles in modulating cell polarity, morphology, and motility (28). Actin can be found either as a free monomer (globular actin (G-actin)), or polymerized in filaments (filamentous actin (F-actin)). The actin cytoskeleton is involved in cell-cell and cell-substrate interactions, as well as being a key component of tight junctions, adherens junctions, and focal adhesions (29,30). The cellular localization of actin is different depending on cell-type, and can have some bearing on the way these cells interact. For instance, epithelial cells that display more cell-cell junctions have actin
cortically localized to the cell periphery, whereas in fibroblast cells that are more motile, actin is preferentially localized to the lamellipodia (29). The GTPases Rho and Rac1 are involved in regulating actin remodeling, with Rho promoting stress fibers in focal adhesions, and Rac1 inducing formation of lamellipodia (11,31). While the effects of TSC1 and TSC2 on mTORC1 signaling have been well documented, their effects on other proteins are not well known. The TSC1:TSC2 complex has been found to regulate Rac1 and Rho activities independent of mTORC1 signaling, and thus has an effect on actin cytoskeleton dynamics and cell adhesion (11). Specifically, it was reported that TSC2 promotes Rac1 activation and inhibition of Rho, suggesting that TSC2$^{-/-}$ cells are likely to have reduced lamellipodia and less focal adhesion remodeling (11).

1.4.2 Focal adhesions

Focal adhesions are the cellular contact points with the extracellular matrix, and are composed of various proteins, including integrins, talin, paxillin, focal adhesion kinase (FAK) and vinculin (32). Vinculin is a key component of focal adhesions that is involved in stabilizing the cell adhesions in the extracellular matrix to the actin cytoskeleton. When vinculin is lost from focal adhesions, F-actin is more likely to dissociate from focal adhesion complexes, and thus destabilize adhesions (33–35). Vinculin and focal adhesions are also involved in tumor invasiveness and motility (36). Furthermore, vinculin has been shown to strengthen E-cadherin cell-cell adhesions (35). TSC1 and TSC2 have been found to be implicated in regulating the actin cytoskeleton via Rho and Rac1 pathways, which in turn affect focal adhesion remodeling, but to date no direct interactions between TSC1 or TSC2 and components of focal adhesions have been demonstrated (11,37).
1.4.3 Cell-cell adhesions

I carried out a substantial amount of work to investigate the differences in cell-cell adhesions found in TSC2$^{+/+}$ and TSC2$^{-/-}$ MEFs. Following is background on the specific cell-cell adhesion proteins that were studied over the course of this project.

1.4.3.1 β-catenin

β-catenin is a protein involved in intercellular adhesion in mammalian cells, specifically in forming the linkages between the intercellular adherens junctions and the actin cytoskeleton (Figure 1.5) (38). β-catenin complexes with both α-catenin and cadherins (including N-cadherin and E-cadherin) to form cell-cell junctions (39,40). β-catenin is primarily regulated by the Wnt pathway, with Wnt signaling resulting in increased β-catenin levels (38). The Wnt signaling pathway is prominently involved in embryonic development, as well as self-renewing tissues (41). β-catenin is thus thought to exist in 2 separate “pools”, one that is involved in cell-cell adhesions, and another that serves as an intermediate of the Wnt signaling pathway (41,42). β-catenin is involved in regulating gene regulation via the Wnt pathway, by accumulating in the cytoplasm and then interacting with lymphoid enhancer factor/T cell factor (LEF/TCF), which is then translocated into the nucleus to act as a transcription factor for a variety of target genes, including repression of E-cadherin (26,43,44). Since the Wnt pathway is highly involved in growth and development, β-catenin relocalization from the membrane to the nucleus plays a critical role in progression of cancer, and accumulation of β-catenin has been found in more than half of all cancer cases (43,45). Furthermore, nuclear β-catenin can cause epithelial tumors to become more invasive, and promotes an epithelial to mesenchymal transition (EMT), likely in part due to the repression of E-cadherin (26,41,44). Levels of β-catenin are thus tightly regulated, and its ubiquitination and degradation is controlled by a “destruction complex” composed of tumour
Figure 1.5 Model of cell-cell adhesion.

Intercellular adhesions are formed by linkages between E-cadherin or N-cadherin in the intercellular space. The cadherins are attached to β-catenin and α-catenin on the cytosolic side, which together are tethered to the actin cytoskeleton to stabilize the adherens junction.
suppressors Axin, adenomatous polyposis coli (APC), and GSK-3β, which is disrupted when Wnt signaling is activated (46). The β-catenin found in adherens junctions is relatively stable, while the β-catenin pool involved in signaling is more transient. It is currently not entirely clear whether the adhesive and signaling pools of β-catenin are interconnected (41). There has been some evidence that TSC tubers have increased levels of β-catenin (47), but also that β-catenin is preferentially localized and retained at the membrane in cells lacking TSC2 (48). There is also evidence of Wnt pathway upregulation in SEGAs (49), and thus the β-catenin/Wnt pathway should be looked into as a causative pathway, as well as a potential therapeutic target for TSC.

1.4.3.2 N-cadherin

Neural cadherin (N-cadherin) is a cell-cell adhesion protein that can be found in a variety of different cell types, and is expressed in both cells with strong cell-cell adhesions as well as highly migratory and mesenchymal cells (50). Typically, presence of N-cadherin suggests a more mesenchymal phenotype, and increase of N-cadherin is one of the hallmark features of EMT (50). N-cadherin participates in cell-cell adhesions by forming the linkages between cells, while also being attached cytoplasmically to β-catenin that is tethered to the actin cytoskeleton (50). The extracellular domain of both N-cadherin and E-cadherin are composed of 5 homologous repeats bridged by calcium ions (51). These homologous repeats form homophilic cis-interactions between molecules from the same cell, and form homophilic trans-interactions with molecules from adjacent cells (52). Additionally, it has been reported that N-cadherin can help regulate Wnt signaling by sequestering and “locking up” β-catenin to the cell-cell junctions, thus not allowing it to translocate to the nucleus to act as a transcription factor (40). To date, there is no known connection between TSC and N-cadherin.
1.4.3.3 E-cadherin

Epithelial cadherin (E-cadherin) is a very important cell-cell adhesion protein that has roles in epithelial-cell behaviour as well as tissue development (53). E-cadherin is only expressed in epithelial cells, and is not present in other cell types such as fibroblasts (54). In epithelial cell-cell adhesions, E-cadherin forms the linkages between cells, while also being attached cytoplasmically to β-catenin that is tethered to the actin cytoskeleton (53).

Epithelial cell-cell adherens junctions linked via E-cadherin are much stronger than junctions containing N-cadherin (55). Since E-cadherin is so crucial to increased adhesion in epithelial cells, the loss of E-cadherin is a hallmark feature of epithelial-mesenchymal transition (EMT) seen in malignant cancers (53,55). Due to E-cadherin’s key importance in development and EMT, the regulation of its expression and localization is crucial to understanding how these pathways are regulated. The expression of E-cadherin is strongly controlled at the transcriptional level at the promoter, which has been shown to be inactivated by hypermethylation or chromatin remodeling in many metastatic cancers (56,57). Another regulatory element that can control E-cadherin expression is the transcription factor Snail, which is a zinc finger transcriptional repressor that binds to E-boxes in the E-cadherin promoter, thus silencing E-cadherin expression (58,59). Snail has been shown to be phosphorylated and degraded by GSK-3β, which can be phosphorylated and degraded by Akt (27). Thus, cells that have increased Akt phosphorylation should display more phosphorylation and degradation of GSK-3β, and thus should have more stabilized levels of β-catenin and Snail, which represses E-cadherin expression. There have been some reports that TSC1:TSC2 promotes E-cadherin expression and membrane localization in epithelial tumour cells (60,61), but overall the link between these proteins has not been investigated thoroughly, and whether E-cadherin is involved in the pathogenesis of TSC is not known.
1.5 Current treatments for TSC

The standard treatment for SEGAs and other TSC tumours is surgical removal, but it has been found that treatment with mTORC1 inhibitors such as rapamycin and rapalogue derivatives such as everolimus can improve neurological defects and even shrink the size of SEGAs (8,62). How mTORC1 inhibition shrinks TSC tumours is not known (63).

Treatment with rapalogues is problematic however, as rapamycin is an immunosuppressant and has several adverse side-effects including skin toxicity, hyperlipidemia, hyperglycemia, and bone marrow suppression (64). Another issue with rapamycin family drugs is that many of the problems associated with TSC occur early in development, necessitating therapeutic intervention in childhood, but long-term treatment on children is to be avoided (65). There is thus a need for novel long-term treatments that reduce mTORC1 activity, to serve as alternate treatment options for individuals with TSC.

1.6 Discovery of new inhibitors of mTORC1 signaling

One of the main focuses of the Roberge lab is the use of cell-based screens to discover biologically active chemicals with therapeutic potential. A 2009 screen of approved human drugs for inducers of autophagy (a process negatively regulated by mTORC1), identified several compounds that were found to inhibit mTORC1 signaling in MEFs (66). These compounds were perhexiline (antianginal agent), niclosamide (antihelminthic agent), amiodarone (antiarrhythmic agent), and dronedarone (an alternative to amiodarone), all of distinct structural classes and with no structural similarity to rapamycin. Furthermore, these compounds inhibited elevated mTORC1 signaling in TSC2−/− MEFs, thus being potentially useful to serve as novel treatment options for TSC. For all drugs, inhibition of mTORC1 signaling was observed at concentrations close to plasma concentrations observed in humans.
during treatment (67–69). While rapamycin inhibits mTORC1 directly and irreversibly (70,71), the new compounds target unknown regulatory processes upstream of mTORC1 and have reversible effects (66). The concentration-dependent and reversible inhibition of mTORC1 signaling by these drugs indicates potential as novel treatments for TSC. Thus, one of the original aims of the project was to assess the extent to which these drugs can reduce or normalize mTORC1 signaling in TSC2-defective cells and ameliorate the abnormal cellular phenotypes associated with TSC2 deletion.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cell lines

The TSC2⁺/⁺ TP53⁻/⁻ and TSC2⁻/⁻ TP53⁻/⁻ MEF cell lines were provided by Dr. David Kwiatkowski in 2009. TSC2⁻/⁻ TP53⁻/⁻ MEFs stably transfected with pEF6-TSC2 or pEF6 alone were provided by Dr. David Kwiatkowski in 2013. The cells were maintained in DMEM (Sigma-Aldrich, #D6429) supplemented with 10% (v/v) fetal bovine serum (Gibco, Sigma-Aldrich, PAA Laboratories), 100units/mL penicillin and 100μg/mL streptomycin (Gibco) at 37°C in 5% CO₂.

2.2 Chemicals

Rapamycin was purchased from Calbiochem. Amiodarone, dronedarone, perhexiline and niclosamide were purchased from Sigma-Aldrich. All stocks were diluted in DMSO and frozen as aliquots to preserve activity.

2.3 Long-term treatment with drugs

For long-term treatments of TSC2⁺/+ and TSC2⁻/⁻ MEFs with mTORC1 inhibitors, cells were seeded at ~25% confluency in 10cm dishes. Drugs were added after cells had adhered, at final concentrations that were determined to be the maximum ones not causing cell death (15nM rapamycin, 2μM dronedarone, 5μM amiodarone, 3μM perhexiline, 1μM niclosamide). Cells were grown for the desired number of days, with medium and drugs refreshed every 3 days. In the event that cells had to be re-seeded due to reaching confluency during treatment, they were trypsinized and seeded in media without drugs and allowed to adhere overnight, and drugs were added the following day. This period of time without drugs was required, as it was found that cells would not adhere properly after trypsinization.
when drugs were present. For all protocols where cells had to be re-seeded in different plates, the treatment duration indicated includes the time spent in new plates, and thus represents the period of time from first addition of drug up until the end of the protocol.

2.4 Quantifying cell size and morphology

To obtain a quantified value for cell size and morphology, TSC2+/+ and TSC2−/− MEFs were seeded in 24-well plates at 5000 cells/mL and allowed to adhere overnight. Cells were then fixed and permeabilized with 3% paraformaldehyde (Invitrogen) in PBS with 0.3% Triton X-100 (LabChem, Inc.) for 30 minutes at room temperature in the dark. Staining was simultaneously performed both for nuclei using 500ng/mL Hoechst 33342 (Invitrogen), as well as for F-Actin using Rhodamine Phalloidin (1:250, Molecular Probes). Fixative and stain were removed and replaced with PBS for storage and imaging. Relative cell size and morphology were determined quantitatively using a Cellomics Arrayscan VTI automated fluorescence imager using the Morphology Explorer application. Cells were photographed at 50 fields/well using a 20x objective in the Hoechst and TRITC channels, and the morphology explorer algorithm was used to identify the nuclei as well as an outline of the cells determined from F-actin staining. An object was defined as being any shape containing F-actin staining. Thus an individual cell not in contact with other cells was counted as one object and multiple cells touching one another were also counted as one object. To assess cell morphology, the algorithm automatically calculated both the length (widest span) and the width (shortest span, perpendicular to the length) of each object, as well as the number of nuclei per object. For cell morphology measurements, only objects with 1 nucleus were analyzed, to select only single cells. To assess cell size, all selected objects were considered
but mean object areas were divided by total number of nuclei/object, thus representing the mean area per individual cell.

2.5 SDS-PAGE and immunoblotting

Cultured cells were washed in cold PBS and lysed on ice in lysis buffer containing 20mM Tris-HCl (Fisher Scientific), pH 7.5, 150mM NaCl (Fisher Scientific), 1% Triton X-100 (LabChem, Inc.), 1mM EDTA (Sigma-Aldrich), 1mM EGTA (Sigma-Aldrich), 2.5mM sodium pyrophosphate (Fisher Scientific), and 1x cOmplete protease inhibitor cocktail (Roche Molecular Biochemicals). Lysates were centrifuged at 18000g at 4°C, and the supernatants were collected and protein was quantified by Bradford assay (Bio-Rad). Lysates were normalized for protein content in SDS-PAGE sample buffer containing 50mM Tris-HCl, pH 6.8, 2%SDS (Fisher Scientific), 0.1% bromophenol blue (Sigma-Aldrich), 10% glycerol (Sigma-Aldrich), and 25mM DTT (Sigma-Aldrich). Samples were resolved by SDS-PAGE and electroblotted onto nitrocellulose (Bio-Rad) membrane and blocked in 5% (w/v) low-fat milk powder (Nestle) in TBS containing 0.1% Tween-20 (MP-Biomedical) (TBS-T) for 30 minutes. Membranes were incubated overnight at 4°C in the appropriate primary antibody diluted in 5% low-fat milk powder in TBS-T unless otherwise noted. Membranes were then washed 3x10 minutes with TBS-T and then incubated for 1 hour at room temperature in the secondary antibody diluted in 5% milk powder in TBS-T. Following another 3x10 minute washes in TBS-T, membranes were imaged by chemiluminescence (Millipore Immobilon Western). Deviations from this general procedure follow.
For all blots that measured phosphorylation (S6K(P), Akt(P), etc.), membranes were blocked in 5% (w/v) BSA in TBS-T for 30 minutes instead of low-fat milk. This was found to reduce the background signal in phosphorylation blots.

Primary antibodies used for immunoblotting were rabbit α-phospho-p70 S6 kinase (Thr389) (1:2500, Cell signaling cs9505s), rabbit α-TSC2 (tuberin (C-20)) (1:500, Santa Cruz Biotechnology, Inc sc-893), mouse α-E-cadherin (1:5000, BD Biosciences 610181), rabbit α-N cadherin (1:1000, Abcam ab18203), mouse α-β-catenin (1:500, BD Biosciences 610153), mouse α-vinculin (1:500, Chemicon MAB1624), rabbit α-phospho-GSK-3β (Ser9) (1:1000, Cell signaling cs9336), rabbit α-phospho-Akt (Ser 473) (1:1500, Cell signaling cs9271S), and rabbit α-β-tubulin (1:10000, Santa Cruz Biotechnology, Inc sc-9104).

Secondary antibodies used for immunoblotting were HRP conjugated goat α-rabbit IgG (1:10000, KPL 074-1506), and HRP conjugated goat α-mouse IgG (1:10000, ThermoScientific 31430).

2.6 Immunofluorescence microscopy

MEF cells were cultured on 7X detergent-treated glass coverslips in 6 or 12-well plates at 100 000 cells/mL (TSC2+/+) or 50 000 cells/mL (TSC2−/−) in normal medium. Cells reached confluency after 48 hours, after which time they were fixed with 3% paraformaldehyde in PBS for 15 minutes, permeabilized with 0.3% Triton X-100 in PBS for 15 minutes, and blocked with 3% BSA in PBS for 30 minutes. The cells were incubated with primary antibody in 3% BSA for 1 hour in a humidity box, and then washed with PBS. The cells were then incubated with the appropriate secondary antibody in 3% BSA in PBS, as well as nuclear stain DRAQ5 (1:000, eBioscience) for 1 hour in a humidity box. Cells were then washed twice with PBS, and then coverslips were mounted on glass slides using CelVol,
and images were acquired by confocal microscopy using the 63X objective of a Leica DMI6000CS laser scanning microscope. Images were analyzed using Leica Application Suite Advanced Fluorescence Lite (LAS AF Lite) 2.6.0 software.

Primary antibodies used for immunofluorescence protocols were mouse α-E-cadherin (1:500, BD Biosciences 610181), rabbit α-TSC2 (tuberin (C-20)) (1:100, Santa Cruz Biotechnology, Inc sc-893), rabbit α-N cadherin (1:250, abcam ab18203), mouse α-β-catenin (1:100, BD Biosciences 610153), mouse α-vinculin (1:100, Chemicon MAB1624).

Secondary antibodies used for immunofluorescence protocols were goat α-mouse AlexaFluor 488 (1:000, Molecular Probes), goat α-mouse AlexaFluor 568 (1:000, Molecular Probes), goat α-rabbit AlexaFluor 488 (1:1000, Molecular Probes).

Cells were stained for F-Actin by Rhodamine Phalloidin (1:250, Molecular Probes) for 30 minutes in the dark, then washed twice with PBS, and mounted on glass slides using CelVol.

2.7 Transfections

For transient plasmid transfection, cells were seeded in either 10cm dishes or 6 well plates depending on the scale of the experiment, in medium without penicillin or streptomycin, at a concentration that resulted in ~80% confluency the next day. One mixture of Lipofectamine 2000 (Invitrogen) and Opti-Mem (Gibco), (1:25) as well as one mixture of DNA and Opti-Mem (1μg/62.5μL) were incubated for 5 minutes at room temperature, after which the two mixtures were combined (1:1) and incubated for 20 minutes at room temperature. The combined mixture was then added dropwise to the culture medium so that each well had a final DNA concentration of 1.6μg/mL. Cells were incubated for 5-6 hours,
after which the medium was aspirated and replaced with fresh medium. Cells were then allowed to recover and grow for the desired number of days.

Plasmids used were pRK7-FLAG-TSC2 (Addgene plasmid 8996) as described in (14), N-cadherin-GFP provided by Dr. Shernaz Bamji (University of British Columbia, Vancouver, Canada) and pRK7-ABCA3 provided by Dr. Robert Molday (University of British Columbia, Vancouver, Canada).

For siRNA knockdowns, cells were seeded in 6-well plates in medium without penicillin or streptomycin, so that cells reached ~50% confluency the next day. Lipofectamine 2000 and Opti-Mem (1:16.7), as well as siRNA and Opti-Mem (1pmol/5μL) were incubated for 5 minutes at room temperature, and then combined (1:1) for 10 minutes at room temperature. The recommended protocol was to add 250μL of this mixture to each 2mL culture per 6-well plate and let incubate for 1-3 days at 37°C. It was found that more efficient knockdown was achieved if the cell culture medium was aspirated, and the siRNA/Lipofectamine/Opti-Mem mixture was added directly onto cells in the 6-well plate (250μL/well) and incubated for 1 hour at 37°C. During this hour, plates were swirled periodically every 10 minutes to ensure that cells did not dry out. After an hour, wells were topped off with 1mL Opti-Mem and incubated for 5 hours, after which wells were aspirated and replaced with complete medium. Cells were then allowed to recover and grow for the desired number of days.

2.8 Fluorescence recovery after photobleaching (FRAP)

Cells treated with drugs were seeded in an 8-well IDIBI chamber at low density and transfected with N-cadherin-GFP as in Section 2.7. FRAP was performed on the junction between two N-cadherin-GFP-expressing cells. The indicated regions of interest were
photobleached using a 405-nm laser (100% intensity, 0.1 seconds) on an Olympus FV1000 confocal microscope and captured using Olympus FluoView v1.6 software. Fluorescence recovery was followed at 2 second time intervals for at least 1 minute until the intensity reached a plateau. The fluorescence signal was normalized to the prebleach intensity, and single exponential fit curves of the data were generated using Prism 4 software (GraphPad).

2.9 Video microscopy

Videos of MEFs were acquired using a Leica DMI4000B inverted microscope. Cells were seeded on 7X detergent-treated glass coverslips in 12-well plates and then inserted into a Chamlide TC incubation chamber and maintained at 37°C in 5% CO₂, and imaged using bright field microscopy. Images were taken every 5 minutes for up to 8 hours and put together at 6 frames/second (i.e. 30minutes/second) using Leica Application Suite Advanced Fluorescence Lite (LAS AF Lite) 2.6.0 software.
CHAPTER 3: CHARACTERIZATION OF THE EFFECT OF LONG-TERM TREATMENT WITH MTORC1 INHIBITORS ON TSC2-/- MEF MORPHOLOGY

3.1 Synopsis

The main objective of this work was to investigate the effects of mTORC1 inhibitors on the size and morphology of TSC2+/+ and TSC2-/- MEFs. In this chapter, I describe and quantify the observation that TSC2-/- MEFs are much larger than the TSC2+/+ MEFs, and that they have an abnormal and distinct epithelial-like morphology instead of the fibroblast-like morphology typical of TSC2+/+ MEFs. I saw that during long-term treatment with mTORC1 inhibitors, the morphology of the TSC2-/- MEFs transitioned from epithelial-like to fibroblast-like, while the morphology of the TSC2+/+ MEFs did not change. However, this transition was not permanent, as all of the drug-treated TSC2-/- MEFs reverted back to their epithelial-like morphology after more than 1 week treatment, with the exception of rapamycin, which caused a permanent morphology change. The change in morphology caused by the drugs correlated with mTORC1 inhibition in TSC2-/- MEFs, with all drugs showing inhibition of mTORC1 signaling during the first week of treatment, but elevated levels of mTORC1 signaling returning during longer term treatment, except for rapamycin, which caused permanent mTORC1 inhibition. While the morphology of the TSC2-/- MEFs became more fibroblast-like following mTORC1 inhibition, they remained considerably larger than the TSC2+/+ MEFs. I also examined differences in actin localization, focal adhesions, and cell-cell adhesions during drug treatment. Overall, I found that long-term
treatment with the drugs caused the TSC2<sup>−/−</sup> MEFs to undergo a transition to the fibroblast-like phenotypes characteristic of TSC2<sup>+/+</sup> MEFs.

### 3.2 TSC2<sup>−/−</sup> MEFs display a morphological change from epithelial-like to fibroblast-like after long-term treatment with mTORC1 inhibitors

The TSC2<sup>+/+</sup> MEFs display a typical fibroblastic morphology: they are bipolar or multipolar, have an elongated shape, grow as individual cells that do not make extensive contact with neighboring cells in non-confluent cultures. By contrast, the TSC2<sup>−/−</sup> MEFs have an abnormal, more epithelial-like morphology. They are very large, flat, and round in shape, and they grow in patches with extensive cell-cell contacts. Individual TSC2<sup>−/−</sup> cells not contacting other cells are rarely seen in non-confluent cultures (Figure 3.1).

Previous experiments showed that short-term (4-8h) exposure to the mTORC1 signaling inhibitors rapamycin, dronedarone, amiodarone, perhexilene, and niclosamide did not cause any significant changes in the abnormal phenotypes of TSC2<sup>−/−</sup> cells (data not shown), so I tested lower, non-toxic, concentrations of the drugs over 1-3 weeks to determine whether long term exposure could ameliorate any abnormal phenotypes (Section 2.3). After 7 days treatment with 15nM rapamycin, 2μM dronedarone, 5μM amiodarone, or 1μM niclosamide, populations of TSC2<sup>−/−</sup> MEFs (Figure 3.2 A) changed from an epithelial-like, to a more fibroblast-like morphology resembling that of untreated TSC2<sup>+/+</sup> MEFs. Perhexiline at 3μM did not produce any visible change in TSC2<sup>−/−</sup> morphology. Further experiments showed that 5μM perhexiline induced a fibroblast-like morphology in TSC2<sup>−/−</sup> MEFs, but this concentration became toxic to the cells after several days. TSC2<sup>+/+</sup> MEFs did not display any change in morphology after 7day treatment with the drugs (Figure 3.2 B). Each drug had a slightly different effect on the morphology of TSC2<sup>−/−</sup> MEFs, with rapamycin resulting in
Figure 3.1 Morphology of TSC2+/− and TSC2−/− MEFs.

Cells were photographed at ~50% confluency using a Nikon Eclipse TS100 inverted microscope equipped with a 20x objective and Nikon Coolpix 8400 camera. Scale bar, 0.2mm
Figure 3.2 Change in morphology of TSC2\(^{−/−}\) MEFs induced by drugs.

TSC2\(^{+/+}\) and TSC2\(^{−/−}\) MEFs were left untreated or treated with 15nM rapamycin, 2µM dronedarone, 6µM amiodarone, 3µM perhexilene, or 1µM niclosamide for 7 days (Section 2.3). Pictures were taken at the indicated days at 20x magnification using a Nikon Coolpix 8400 camera attached to a Nikon Eclipse TS100 inverted microscope. Scale bar, 0.2mm. (A) Pictures of TSC2\(^{−/−}\) MEFs. (B) Pictures of TSC2\(^{+/+}\) MEFs. (C) Larger image of drug-treated cells that showed the most drastic morphological change in TSC2\(^{−/−}\) MEFs.
<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Rapamycin</th>
<th>Dronedarone</th>
<th>Amiodarone</th>
<th>Perhexiline</th>
<th>Niclosamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>0d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
flattened and highly fibrous cells, dronedarone resulting in slight elongation of the cells, amiodarone resulting in very skinny “toothpick”-like cells, and niclosamide resulting in cells that are compacted in the middle with several longer protrusions extending from the cell body. Rapamycin and niclosamide consistently showed the most drastic effect on TSC2<sup>−/−</sup> morphology, resulting in the most fibroblast-like appearance (Figure 3.2 C).

I was able to quantify this change in morphology by staining the cells for F-Actin to highlight the entire cell shape, and then using a Cellomics Arrayscan VTI automated fluorescence imager to analyze the length:width ratio of the cells (Section 2.4) (Figure 3.3B). Untreated TSC<sup>+/+</sup> MEFs showed a larger L:W ratio than untreated TSC2<sup>−/−</sup> MEFs, because of their thin elongated shape, compared to the rounder shape of TSC2<sup>−/−</sup> MEFs (Figure 3.3A). After 7 days exposure to drugs, there was an increase in the L:W ratio of TSC2<sup>−/−</sup> MEFs in conditions that induced the most obvious morphological change from epithelial-like to fibroblast-like (amiodarone, niclosamide, rapamycin). The L:W ratio of TSC2<sup>+/+</sup> MEFs did not change significantly in any treatment. This experiment provided a quantifiable measurement to the observation that the TSC2<sup>−/−</sup> MEFs were indeed becoming more fibroblast-like after long-term treatment with mTORC1 inhibitors.

Cells were treated with drugs for up to 3 weeks, and it was seen that the change in morphology in drug-treated TSC2<sup>−/−</sup> MEFs was not permanent. All of the drug-treated cells, with the sole exception of rapamycin, reverted back to their original epithelial-like morphology after more than 1 week. Lysates were collected every week to assess mTORC1 activity levels. After 1 week all of the drugs that caused the TSC2<sup>−/−</sup> MEFs to change morphology (rapamycin, dronedarone, amiodarone, niclosamide) also reduced S6K(P) levels to correlating degrees, indicating that they were indeed inhibiting mTORC1 signaling in
Figure 3.3 Quantification of the change of TSC2<sup>−/−</sup> MEFs from an epithelial-like to a fibroblast-like morphology.

TSC2<sup>+/+</sup> and TSC2<sup>−/−</sup> MEFs were treated with 15nM rapamycin, 2μM dronedarone, 6μM amiodarone, 3μM perhexiline, or 1μM niclosamide for 7 days (Section 2.3). Cells were fixed and stained for F-Actin and their length:width ratio was calculated using a Cellomics Arrayscan VTI automated fluorescence imager (Section 2.4) (A) Bar graph of L:W ratio after 7-day drug treatment (mean ± S.D., n > 3500). (B) Example of how cells are stained and measured by the Morphology Explorer program.
TSC2⁻/⁻ MEFs, which induced a morphological change (Figure 3.4). Rapamycin and niclosamide, which caused the most drastic changes in TSC2⁻/⁻ morphology, displayed the most prominent reduction of S6K(P) after 1 week. There was no significant drug-induced reduction in S6K(P) in TSC2⁺/+ MEFs after 1 week except for rapamycin and niclosamide. The basal levels of S6K(P) is much higher in TSC2⁻/⁻ MEFs than in TSC2⁺/+ MEFs and it should be noted that the TSC2⁻/⁻ MEF and TSC2⁺/+ MEF blots shown here were run separately and are thus not directly comparable. After 2 and 3 weeks, the S6K(P) levels in drug-treated TSC2⁺/⁻ MEFs were back up to untreated levels (Figure 3.4) in all treatments except for rapamycin, which is an irreversible mTORC1 inhibitor (70). This reversion of mTORC1 inhibition in TSC2⁻/⁻ MEFs correlates with the reversion in morphology seen after treatment for more than 1 week with all drugs, except for rapamycin-treated TSC2⁺/⁻ MEFs, which remain fibroblast-like.

To investigate this further, I took TSC2⁺/+ and TSC2⁻/⁻ MEFs that had been treated with niclosamide for 3 weeks, as well as untreated MEFs, and treated all of them with different concentrations of niclosamide for 24 hours (Figure 3.5). Previously untreated TSC2⁻/⁻ MEFs treated with niclosamide for 24 hours displayed a concentration-dependent change in morphology from epithelial-like to fibroblast-like, as observed previously, whereas TSC2⁻/⁻ MEFs previously treated with niclosamide for 3 weeks mostly retained their epithelial-like morphology upon re-exposure to niclosamide for 24 hours. This indicates that the TSC2⁺/⁻ MEFs that have reverted back to an epithelial-like morphology after long-term treatment with niclosamide are somehow de-sensitized to the drug. TSC2⁺/+ MEFs did not display any morphological changes at any of these niclosamide concentrations.
Figure 3.4 mTORC1 inhibition is not sustained during long-term treatment with drugs.

TSC2+/+ and TSC2−/− MEFs were treated with 15nM rapamycin, 2μM dronedarone, 6μM amiodarone, 3μM perhexiline, or 1μM niclosamide for up to 3 weeks (Section 2.3) and lysates were collected weekly. Cell lysates were immunoblotted for S6K(P) (T389) (Section 2.5).
Figure 3.5 Long-term treatment with niclosamide de-sensitizes TSC2± MEFs to further morphological changes.

TSC2+/+ and TSC2−/− MEFs that had previously been treated or not with 1μM niclosamide for 3 weeks, were exposed to different concentrations of niclosamide for 24 hours. Scale bar, 0.2mm.
3.3 Long-term treatment with mTORC1 inhibitors does not significantly reduce the size of TSC2\(^{-/-}\) MEFs

Another very prominent phenotypic difference between the TSC2\(^{+/+}\) and the TSC2\(^{-/-}\) MEFs is the much larger size of the latter. This difference is very impactful to individuals with TSC, as the increased cell size seen in SEGAs and other TSC-associated tubers is a major contributor to severe clinical symptoms (6,7). While I observed that long-term treatment with the mTORC1 inhibitors could reduce mTORC1 signaling and change the morphology of TSC2\(^{-/-}\) MEFs, I wanted to determine whether these changes were also accompanied by a reduction in cell size. To measure this, I set up a similar algorithm as used to quantify cell morphology, but set the parameters to measure cell size (Section 2.4.) According to this method of analysis, (Figure 3.6) the average cell area of the untreated TSC2\(^{-/-}\) MEFs is roughly twice that of untreated TSC2\(^{+/+}\) MEFs, in reasonable accordance with visual estimation of cell size. After 7 day treatment with drugs, the mean cell area of TSC2\(^{-/-}\) MEFs remained much larger than that of the TSC2\(^{+/+}\) MEFs in all treatments, despite their clear morphological change. There was a slight reduction in TSC2\(^{-/-}\) cell area in treatments that induced the most drastic morphological changes (amiodarone, niclosamide, rapamycin), but overall the cells still remained much larger than their TSC2\(^{+/+}\) counterparts. Visual inspection of the images of TSC2\(^{-/-}\) cells that had become more fibroblast-like as a result of drug treatment confirmed that they were much larger than TSC2\(^{+/+}\) MEFs.
Figure 3.6 Long-term treatment with mTORC1 inhibitors does not significantly reduce TSC2−/− MEF size.

TSC2+/+ and TSC2−/− MEFs were treated with 15nM rapamycin, 2μM dronedarone, 6μM amiodarone, 3μM perhexiline, or 1μM niclosamide for 7 days (Section 2.3). Cells were fixed and stained for F-Actin and average cell area was calculated using a Cellomics Arrayscan VTI automated fluorescence imager (Section 2.4) (mean ± S.D., n > 6000).
3.4 Long-term treatment with mTORC1 inhibitors changes actin localization in TSC2+/− MEFs.

The images obtained from quantification of MEF morphology and size by F-Actin staining showed another phenotypic difference between TSC2+/+ and TSC2−/− MEFs that I wished to investigate further. By looking closely at the actin localization in the cells, I noticed that TSC2+/+ MEFs display diffuse and even fluorescence throughout the entire cell, typical of fibroblasts, but TSC2−/− MEFs display actin localized more towards the periphery of the cell, typical of epithelial-like cells (11) (Figure 3.3B). After treating cells with drugs for 7 days, I visualized the actin localization in these cells by confocal immunofluorescence microscopy. In treatments that showed the most change in morphology of TSC2−/− MEFs (rapamycin, amiodarone, niclosamide), there was an accompanying change in actin localization from the cell periphery to more diffuse distribution throughout the cytoplasm (Figure 3.7). Niclosamide in particular showed the most drastic change in actin localization in TSC2−/− MEFs, causing a “crosshatch” pattern of fluorescence throughout the entire cell that is consistent with the presence of actin stress fibers (72). The TSC2+/+ MEFs in contrast, did not show any difference in actin localization after treatment with drugs.

3.5 Long-term treatment with mTORC1 inhibitors reduces focal adhesions in TSC2−/− MEFs.

It has been documented that the TSC2 gene product tuberin plays a role in the regulation of focal adhesions, specifically that it promotes Rac1 activation and Rho inhibition, resulting in increased remodeling of focal adhesions (11). Using confocal immunofluorescence microscopy of vinculin, a component of focal adhesions (32), I examined focal adhesions in TSC2+/+ and TSC2−/− MEFs. Vinculin immunofluorescence in
Figure 3.7 Long-term treatment with mTORC1 inhibitors changes actin localization in TSC2−/− MEFs.

TSC2+/+ and TSC2−/− MEFs were treated with 15nM rapamycin, 2μM dronedarone, 5μM amiodarone, 5μM perhexiline, or 1μM niclosamide for 7 days (Section 2.3) and allowed to grow on coverslips before cells were fixed, DNA was stained, and images were acquired by confocal microscopy (Section 2.6). F-actin staining is shown in red, and DNA staining is shown in blue. Scale bar, 30μm.
TSC2\(^/-\) MEFs was localized to elongated dots throughout the cell body as well as to leading edges of cells, while TSC2\(^{+/+}\) MEFs only displayed very minimal localized fluorescence on the very tips of the cell protrusions (Figure 3.8). This result suggests that there are more focal adhesions in TSC2\(^/-\) MEFs than in TSC2\(^{+/+}\) MEFs, and that TSC2\(^/-\) MEFs are likely more strongly adhered to the extracellular surface. Consistent with this observation, I have noticed that TSC2\(^/-\) MEFs take a much longer time to detach from cell culture plates during trypsinization than TSC2\(^{+/+}\) MEFs. Drugs that caused TSC2\(^/-\) MEFs to adopt a more fibroblast-like appearance after 7 days (rapamycin, amiodarone, niclosamide) reduced the amount of localized vinculin fluorescence and caused it to be only faintly visible on the very tips of the cell protrusions, similar to that seen in TSC2\(^{+/+}\) MEFs. Vinculin staining did not change in any treatment of TSC2\(^{+/+}\) MEFs. This result suggests that the drugs that cause a morphological change in TSC2\(^/-\) MEFs from epithelial-like to fibroblast-like also have the effect of reducing the amount of focal adhesions per cell and weakening the strength of attachment to the extracellular surface. I have also observed that drug-treated TSC2\(^/-\) MEFs that have become more fibroblast-like are much easier to trypsinize and detach than untreated TSC2\(^/-\) MEFs or cells treated with drugs that remained epithelial-like.

3.6 Long-term treatment with mTORC1 inhibitors reduces cell-cell adhesions in TSC2\(^/-\) MEFs.

Another very prominent phenotypic difference between the TSC2\(^{+/+}\) and TSC2\(^/-\) MEFs that I wished to investigate relates to cell-cell adhesions. TSC2\(^{+/+}\) MEFs proliferate individually with few cell-cell contacts, while TSC2\(^/-\) MEFs grow in patches with extensive cell-cell contacts. Upon long term treatment with drugs, I observed that in addition to gaining a more fibroblast-like morphology, TSC2\(^/-\) MEFs also formed less extensive cell-cell

\[38\]
Figure 3.8 Long-term treatment with mTORC1 inhibitors changes vinculin localization in TSC2+/− MEFs.

TSC2+/− and TSC2−/− MEFs were treated with 15nM rapamycin, 2μM dronedarone, 5μM amiodarone, 5μM perhexiline, or 1μM niclosamide for 7 days (Section 2.3) and allowed to grow on coverslips before cells were fixed, DNA was stained, and images were acquired by confocal microscopy (Section 2.6). Vinculin staining is shown in green, and DNA staining is shown in blue. Scale bar, 30μm.
contacts than untreated TSC2\(^{-}\) cells at a similar confluency. TSC2\(^{+/+}\) cells did not display any apparent change in cell-cell contacts upon treatment with drugs.

To assess changes in cell-cell contacts, I visualized cell-cell adhesions by confocal immunofluorescence microscopy detection of β-catenin, which is involved in forming the linkages between the intercellular cadherin junctions and the cytoskeleton (Figure 3.9). Extensive cell-cell junctions were revealed in untreated TSC2\(^{-}\) MEFs as seen by the nearly continuous β-catenin staining at the cell periphery. By contrast, TSC2\(^{+/+}\) MEFs showed much fewer regions of cell-cell contact and peripheral β-catenin. Upon treatment with drugs, the TSC2\(^{-}\) MEFs once again displayed a change from an epithelial-like to a more fibroblast-like morphology and a marked decrease in the extent of cell-cell adhesions compared to untreated TSC2\(^{-}\) MEFs at a similar confluency (Fig. 3.9). Even after exposure to 2μM dronedarone, where TSC2\(^{-}\) MEF morphology did not change very much, the cells did not form as many attachments to other cells in close proximity and β-catenin was distributed diffusely to the cytoplasm. The drugs did not affect the cell-cell contacts between TSC2\(^{+/+}\) MEFs. Therefore, long-term treatment with drugs disrupted the strong cell-cell contacts seen in TSC2\(^{-}\) MEFs, and caused a change in β-catenin localization from the cell periphery to the cytoplasm.

This observation was further pursued by performing confocal immunofluorescence microscopy of N-cadherin, another cell-cell adhesion protein which forms the linkages between cells, while also being attached cytoplasmically to β-catenin that is tethered to the actin cytoskeleton. There was prominent N-cadherin staining at cell-cell junctions in untreated TSC2\(^{-}\) MEFs illustrating extensive connections with neighbouring cells. TSC2\(^{+/+}\) MEFs only formed very small areas of N-cadherin staining, confirming much less extensive connections with neighbouring cells (Figure 3.10). Drugs that caused TSC2\(^{-}\) MEFs to
Figure 3.9 Long-term treatment with mTORC1 inhibitors changes β-catenin localization in TSC2-/- MEFs.

TSC1+/- and TSC2-/- MEFs were treated with 15nM rapamycin, 2μM dronedarone, 5μM amiodarone, 5μM perhexiline, or 1μM niclosamide for 7 days (Section 2.3) and allowed to grow on coverslips before cells were fixed, DNA was stained, and images were acquired by confocal microscopy (Section 2.6). β-catenin staining is shown in green, and DNA staining is shown in blue. Scale bar, 30μm.
Figure 3.10 Long-term treatment with mTORC1 inhibitors changes N-cadherin localization in TSC2-/- MEFs.
TSC⁺/⁺ and TSC2⁻/⁻ MEFs were treated with 15nM rapamycin, 4μM amiodarone, or 1μM niclosamide for 7 days (Section 2.3) and allowed to grow on coverslips before cells were fixed, DNA was stained, and images were acquired by confocal microscopy (Section 2.6). N-cadherin staining is shown in green, and DNA staining is shown in blue. Three different images from each coverslip are shown. Scale bar, 30μm.
become more fibroblast-like (amiodarone, niclosamide, rapamycin) greatly reduced peripheral N-cadherin distribution, even when cells were in very close proximity to one another, again indicating fewer cell-cell contacts. TSC2+/− MEFs showed no change in N-cadherin localization after drug treatment. Taken together, these results show that the drugs caused the cells to lose most of the cell-cell contacts, as well as a resulting change in localization of β-catenin and N-cadherin from the cell periphery to the cytoplasm.

While qualitatively seeing that TSC2−/− MEFs formed more cell-cell adhesions, I wished to also examine the stability of these contacts. To accomplish this, Fluorescence Recovery After Photobleaching (FRAP) was performed on MEFs transfected with N-cadherin-GFP in collaboration with Spencer Freeman. A junction area between 2 cells expressing N-cadherin-GFP was subjected to photobleaching and the fluorescence recovery rate was measured to assess the turnover of N-cadherin at the junction, which correlates to the stability of the interaction. The photobleached junctions between TSC2+/− MEFs recovered faster than those seen in TSC2−/− MEFs, indicating that the junctions between TSC2−/− MEFs are more stable than those between TSC2+/− MEFs (Figure 3.11). After treatment with rapamycin, niclosamide, or amiodarone, the recovery rate of the TSC2−/− MEFs increased, suggesting that the cell-cell junctions became less stable as a result of the treatments. This result shows that long-term treatment with drugs on TSC2−/− MEFs not only causes a decrease in the extent of cell-cell junctions, but also reduces the stability of the remaining connections to levels seen in TSC2+/− MEFs.
Figure 3.11 Long-term treatment with mTORC1 inhibitors increases the N-cadherin-GFP fluorescence recovery rate at cell-cell junctions in TSC2−/− MEFs after photobleaching.

TSC+/+ and TSC2−/− MEFs transfected with N-cadherin-GFP (Section 2.7) were treated with 15nM rapamycin, 4μM amiodarone, or 1μM niclosamide for 7 days (Section 2.3). FRAP was performed to assess the stability of the cell-cell junctions (Section 2.8). (A) Sample images of the photobleached junctions with 6 second intervals (B) Recovery rate of the junctions plotted as a function of time. (mean ± S.D., n =3).
Finally, to gain additional insight into the dynamics of cell-cell connections, I examined untreated MEFs and MEFs treated for 7 days with drugs with time-lapse microscopy over a course of five hours (Fig. 3.12). TSC2+/+ MEFs were seen to continuously make and break connections with neighbouring cells, indicating that cell-cell junctions are dynamic (Figure 3.12 A). By contrast, TSC2−/− MEFs formed much more stable connections with neighbouring cells, which did not break or reform much over this time period, typical of epithelial-like cells (Figure 3.12 B). In TSC2−/− MEFs treated with rapamycin (Figure 3.12 C) or niclosamide (Figure 3.12 D) for 7 days, time lapse microscopy showed that the cells were more likely to break connections with neighbouring cells during the observation period and that any cell-cell junctions that were formed were restricted to the newly formed protrusions between cells, similar to TSC2+/+ MEFs, rather than spanning the whole cell body as normally seen in TSC2+/− MEFs.

3.7 Long-term treatment with mTORC1 inhibitors does not change the expression levels of full-length β-catenin, vinculin, or N-cadherin in TSC2+/+ and TSC2−/− MEFs

While confocal immunofluorescence microscopy revealed much about the change in localization of vinculin, β-catenin, and N-cadherin in the MEFs, it was important to assess whether there was any change in expression levels after long-term treatment with drugs. Immunoblots of 7-day treatments revealed that the levels of full-length β-catenin, vinculin, and N-cadherin did not drastically change due to drug treatment, and that expression levels were similar between TSC2+/+ and TSC2−/− MEFs (Figure 3.13). However, there were many smaller immunoreactive bands in all blots that are likely degradation products. It would appear that untreated TSC2−/− MEFs display higher levels of the β-catenin degradation bands.
TSC2\textsuperscript{+/+} MEFs Untreated

Figure 3.12 Time lapse microscopy of TSC2\textsuperscript{+/+} and TSC2\textsuperscript{−/−} MEFs treated with mTORC1 inhibitors.

TSC\textsuperscript{+/+} and TSC2\textsuperscript{−/−} MEFs were treated with 15nM rapamycin, or 1μM niclosamide for 7 days (Section 2.3) and allowed to grow on coverslips. Movies were taken as described in Section 2.9. Images presented represent one second intervals from a sped up movie, and 30-minute intervals from real-time measurement.
Figure 3.13 Effect of mTORC1 inhibitors on expression levels of β-catenin, vinculin, N-cadherin, and E-cadherin in MEFs.

TSC2\(^{+/+}\) and TSC2\(^{-/-}\) MEFs were treated with 15nM rapamycin, 2μM dronedarone, 1μM amiodarone, or 1μM niclosamide for 7 days (Section 2.3). Cell lysates were immunoblotted for β-catenin, vinculin, N-cadherin, E-Cadherin, and β-tubulin (Section 2.5). The arrows indicate full-length protein.
than untreated TSC2+/+ MEFs, and slightly higher levels of N-cadherin degradation bands. Interestingly, rapamycin caused a clear reduction in these lower bands for all of the probed proteins in TSC2−/− MEFs, suggesting that rapamycin affects the degradation of these proteins. Interpretation of results in amiodarone-treated TSC2−/− MEFs is not possible since there was an error in the normalization of this lysate as seen in β-tubulin levels. Overall however, these results indicate that the TSC2−/− phenotypic changes observed in immunofluorescence microscopy reflect changes in localization of these proteins in TSC2−/− MEFs, without drastic changes in their expression levels.

I also took this opportunity to probe the lysates for E-cadherin, another cell-cell adhesion protein. E-cadherin is normally only expressed in epithelial cells, and is not present in other cell types such as fibroblasts (54). I was considerably surprised to find that E-cadherin was clearly expressed by the epithelial-like TSC2−/− MEFs, but not by the TSC2+/+ MEFs (Figure 3.13), and decided to investigate this observation further (Chapter 5). 7 day treatment with rapamycin and niclosamide appeared to reduce the expression of E-cadherin in the TSC2−/− MEFs. The apparent reduction of E-cadherin in amiodarone treated TSC2−/− MEFs is not interpretable, due to the error in the normalization of this lysate as seen in β-tubulin levels. As for niclosamide, I have some further data to suggest that niclosamide treatment may indeed reduce the expression of E-cadherin levels in TSC2−/+ MEFs (Figure 3.14). Unfortunately this observation was not investigated much further as I instead decided to focus more on how TSC2−/− MEFs are able to activate the expression of E-cadherin, whether TSC2 alone can regulate this process, and whether this could explain some of the abnormal morphology seen in TSC tubers.
Figure 3.14 Effect of niclosamide on E-cadherin expression in TSC2\textsuperscript{+/−} MEFs.

TSC2\textsuperscript{+/-} and TSC2\textsuperscript{-/-} MEFs were treated with the indicated gradient of niclosamide for 24 hours (Section 2.3). Cell lysates were immunoblotted for E-Cadherin, and β-tubulin (Section 2.5).
CHAPTER 4: EXPRESSION OF E-CADHERIN BY TSC2-/- MEFS AND ITS POTENTIAL REGULATION BY TSC2

4.1 Synopsis

The goal of this part of the work was to investigate the surprising observation that E-cadherin is expressed in TSC2-/- MEFs, and whether TSC2 is involved in regulating E-cadherin expression.

In this chapter, I describe my initial discovery that E-cadherin was found to be expressed in the TSC2-/- MEFs but not in TSC2+/+ MEFs, and the work that was done to determine if and how TSC2 regulated E-cadherin expression. Specifically, we received TSC2-/- MEFs stably transfected with pEF6-TSC2 and pEF6 alone, but found that neither batch of these cells expressed E-cadherin. I successfully re-introduced TSC2 into TSC2-/- MEFs by transient transfection with pRK7-TSC2 and found that with increased levels of TSC2 expression, E-cadherin expression decreased only slightly. Finally, I successfully knocked down TSC2 in TSC2+/+ MEFs using siRNA, which did not result in increased expression of E-cadherin.

4.2 E-cadherin is expressed in TSC2-/- MEFs

To assess the effect of drugs on MEF morphology (Chapter 3), I immunoblotted cell lysates for proteins involved in cell-cell adhesions, including epithelial-cadherin (E-cadherin). To confirm the surprising observation that TSC2-/- MEFs showed the presence of E-cadherin, I prepared fresh lysates from drug-treated MEFs. As seen previously, TSC2+/+ MEFs did not show any expression of E-cadherin, as expected (Figure 4.1) (73). However,
**Figure 4.1 TSC2⁺/− MEFs express E-Cadherin.**

TSC2⁺/⁺ and TSC2⁻/⁻ MEFs were exposed for 24h to 15nM rapamycin, 3μM dronedarone, 6μM amiodarone, 3μM perhexiline, 1μM niclosamide or no drug. Cell lysates were immunoblotted for E-cadherin and β-tubulin (Section 2.5).
to my great surprise once again, TSC2\(^{+/-}\) MEFs immunoblots displayed a very clear band indicating the presence of E-cadherin (Figure 4.1). While none of the drugs after 24 hours drastically altered the amount of E-cadherin expressed in TSC2\(^{-/-}\) MEFs, they did serve to confirm E-cadherin expression. The origin of the faint bands visible on western blots for E-cadherin is unknown, but they are likely degradation products. For purposes of quantifying E-cadherin expression, I decided to only consider the upper band at ~120kDa, representing the full-length form of E-cadherin.

To extend the results that I saw from western blots, I next examined E-cadherin distribution in MEFs using confocal microscopy. Cells were grown to complete confluence before fixing and staining to ensure that E-cadherin would be properly localized to cell-cell junctions. TSC2\(^{+/-}\) MEFs showed only very weak signal that was not localized at cell-cell junctions, confirming that they do not express E-cadherin (Figure 4.2). By contrast, TSC2\(^{-/-}\) MEFs displayed bright fluorescence that was localized to cell-cell junctions in areas where cells were confluent. This E-cadherin localization pattern in TSC2\(^{-/-}\) MEFs was similar to that of EpH4 cells, a mouse breast cancer epithelium cell line that served as a positive control for E-cadherin expression and localization. The results of western blots and confocal microscopy demonstrate that the TSC2\(^{-/-}\) MEFs express E-cadherin that localizes properly to intercellular junctions.

Since confluence is an important factor for detection of E-cadherin at cell-cell junctions by confocal microscopy, it was important to assess whether the degree of cell confluence affected the expression of E-cadherin. I measured this by seeding TSC2\(^{+/-}\) MEFs at varying densities, and letting them grow until the highest-density well was confluent, after which E-cadherin was measured by western blotting. The full-length E-cadherin band
Figure 4.2 Visualization of E-Cadherin in TSC2<sup>+/+</sup> and TSC2<sup>−/−</sup> MEFs by immunofluorescence microscopy.

TSC2<sup>+/+</sup> and TSC2<sup>−/−</sup> MEFs were allowed to grow to confluence before fixation, processing and acquisition of images by confocal microscopy (Section 2.6). E-Cadherin staining is shown in green and DNA staining in blue. E-cadherin-expressing EpH4 cells were used as a positive control. Three different images from each coverslip are shown. Scale bar, 30μm.
intensity was consistent across all different confluency levels (Figure 4.3), with perhaps a slight decrease at the lowest confluency. Therefore, confluency of TSC2−/− MEFs is not a determining factor for expression levels of E-cadherin.

4.3 pEF6-TSC2 transfected TSC2+/− MEFs do not express E-cadherin

The results described above indicated that TSC2 negatively controls E-cadherin expression. To test this hypothesis, we obtained TSC2+/− MEFs stably transfected with a pEF6-TSC2 expression plasmid (74) from the Kwiatkowski group, who originally provided the TSC2+/+ and TSC2−/− MEFs we received in 2009. If TSC2 indeed negatively controls E-cadherin expression, we would expect that re-expression of TSC2 in pEF6-TSC2 transfected TSC2−/− cells would reduce or eliminate E-cadherin expression.

The TSC2−/− MEFs stably transfected with pEF6-TSC2 were lysed and immunoblotted to measure a variety of diagnostic proteins. The cells indeed expressed TSC2, as expected (Figure 4.4). Additionally, TSC2 re-expression caused a considerable decrease in mTORC1 activity, as measured by a decrease in S6K phosphorylation at T389 and an increase in Akt phosphorylation at S473, showing that re-expressed TSC2 was functional as an mTORC1 inhibitor. The blot for phospho-GSK-3β showed even levels in all cell types. Moreover, there was a complete absence of E-cadherin expression in the TSC2−/− MEFs stably transfected with pEF6-TSC2. Alone, this result would have been very strong evidence that TSC2 directly controls E-cadherin levels. However, the TSC2−/− MEFs transfected with an empty pEF6 vector also did not show any E-cadherin expression. These cells were otherwise biochemically similar to their parental TSC2−/− MEFs in that they did not express TSC2 and they showed high mTORC1 activity (Figure 4.4). Their lack of E-cadherin expression was
Figure 4.3 Effect of TSC2<sup>+/−</sup> MEF confluence on E-cadherin expression levels.

TSC2<sup>+/−</sup> MEFs were seeded at different dilutions and were grown until the highest-seeded well was 100% confluent. (A) Pictures of cells immediately prior to lysis. Scale bar, 0.2mm. (B) Cell lysates were immunoblotted for E-cadherin and β-tubulin (Section 2.5).
TSC2<sup>−/−</sup> cells stably transfected with pEF6-TSC2 or empty pEF6 vector were acquired from Dr. David Kwiatkowski and grown to confluence. Cell lysates were immunoblotted for TSC2, S6K(P) (T389), Akt(P) (S473), E-cadherin, GSK-3 β(P) (S9), and β-tubulin (Section 2.5).
surprising, as we would have expected them to be essentially identical to un-transfected TSC2\(^{-/-}\) MEFs in that respect. It is unlikely that there was an error in the labeling of the new cells we received, since they displayed the right biochemical markers for the presence or absence of TSC2.

I then decided to examine in more detail the morphology of these various cell lines. The TSC2\(^{+/+}\) MEFs were of normal size and fibroblast-like appearance while the TSC2\(^{-/-}\) MEFs were abnormally large and displayed an epithelial-like morphology (Figure 4.5). The TSC2\(^{-/-}\) MEFs transfected with pEF6-TSC2 were much more fibroblast-like than their parental TSC2\(^{-/-}\) MEFs or their pEF6 empty-vector counterparts, indicating that absence of TSC2 does contribute to the abnormal epithelial-like morphology of TSC2\(^{-/-}\) MEFs (Figure 4.5). Interestingly, they still appeared to remain larger than TSC2\(^{+/+}\) MEFs, even with stable re-expression of TSC2. The TSC2\(^{-/-}\) pEF6 MEFs also had roughly the same enlarged size as the TSC2\(^{+/+}\) MEFs. However, there were some clear morphological differences between the two (Figure 4.5). The TSC2\(^{-/-}\) MEFs always displayed very smooth edges and epithelial-like connections between cells when confluent, and were most often found attached to other cells in non-confluent cultures. By contrast, the TSC2\(^{-/-}\) pEF6 MEFs did not seem to form continuous intercellular junctions, and had what appeared to be many small protrusions extending from the periphery of the cell body, similar to the ones seen in TSC2\(^{+/+}\) MEFs, albeit not as extended. Overall, the TSC2\(^{-/-}\) pEF6 MEFs which did not express E-cadherin did not seem to have as extensive an epithelial-like morphology as the TSC2\(^{-/-}\) MEFs. This was difficult to interpret as the only difference between these two cells should be the introduction of the empty pEF6 plasmid, which should not affect E-cadherin expression or drastically change other morphological properties.
Figure 4.5 Morphology of pEF6-Transfected TSC2^{+/−} MEFs.
TSC2^{+/−} MEFs stably transfected with pEF6-TSC2 or empty pEF6 vector were grown to confluence and their morphology was compared to that of parental TSC2^{−/−} MEFs and TSC2^{+/+} MEFs. Scale bar, 0.2mm.
To explain this puzzling difference, we considered the possibility that the TSC2−/− MEFs in our possession had somehow undergone a morphological change and gain of E-cadherin expression over a few years of use in our laboratory. To address this possibility, the earliest batch of TSC2−/− MEFs we had originally received from Dr. David Kwiatkowski (dated from 2009) was thawed from liquid nitrogen storage. As seen in Figure 4.6, the morphology of the TSC2−/− MEFs from 2009 was identical to that of the cells I had used, and they also showed expression of E-cadherin.

4.4 Restoring TSC2 in TSC2−/− MEFs reduces E-cadherin levels only modestly

In the previous section, I observed that the TSC2−/− MEFs stably transfected with an empty pEF6 plasmid and the parental TSC2−/+ MEFs were different with respect to morphology and E-cadherin expression, when they should not be. To resolve this issue, I decided to transfect the TSC2−/− MEFs myself with a TSC2 expression plasmid. I used a pRK7-TSC2 construct described in (14,75). Transient transfection of the TSC2−/− MEFs with pRK7-TSC2 caused a change in morphology from epithelial-like to fibroblast-like, similar to TSC2−/− cells stably transfected with pEF6-TSC2 (Figure 4.7). 48 hours after transfection, the cells expressed high levels of TSC2, even higher than seen in TSC2+/+ MEFs. However, this increase in TSC2 did not seem to inhibit mTORC1 signaling, as there was no decrease in S6K(P) levels. There was a very slight increase in Akt(P) levels, but not nearly as high as expected considering the high TSC2 levels. The levels of E-cadherin in TSC2−/− MEFs also did not decrease 48 hours after transfection with pRK7-TSC2, but it is difficult to draw conclusions from this experiment since TSC2 may not have been functional since it did not reduce mTORC1 signaling. It seemed possible that downregulation of E-cadherin might require longer than 48h transfection. Furthermore, it was possible that TSC2 expression by
**Figure 4.6 Early and late batches of TSC2<sup>−/−</sup> MEFs appear identical.**

The earliest vial of TSC2<sup>−/−</sup> cells (labelled 2009) was thawed and cells were grown to confluence to compare with TSC2<sup>+/+</sup> MEFs and TSC2<sup>−/−</sup> MEFs stably transfected with pEF6-TSC2 or empty pEF6. (A) Pictures of cells immediately prior to lysis, compared with other cells. Scale bar, 0.2mm. (B) Cell lysates were immunoblotted for E-cadherin and β-tubulin (Section 2.5).
Figure 4.7 Transient expression of TSC2 in TSC2−/− MEFs does not decrease E-cadherin levels after 2 days

TSC2−/− MEFs were transfected with pRK7-FLAG-TSC2 or pRK7-FLAG-ABCA4 as a negative control (Section 2.7) and allowed to grow for 48h post-transfection. (A) Pictures of cells immediately prior to lysis. Scale bar, 0.2mm. (B) Cell lysates were immunoblotted for TSC2, S6K(P) (T389), Akt(P) (S473), E-cadherin, and β-tubulin (Section 2.5).
pRK7-TSC2 transfection was abnormally high and exerted some kind of dominant negative effect. To address these concerns, I transfected TSC2\(^{-/-}\) MEFs with either the amount of DNA recommended by the manufacturer (16\(\mu\)g/10cm dish), or 10-fold less DNA (1.6\(\mu\)g/10cm dish), and allowed them to grow for 3 or 7 days post-transfection. In cells transfected for 3 days with either 16 or 1.6 \(\mu\)g pRK7-TSC2 plasmid, the TSC2 expression level was very similar to TSC2\(^{+/+}\) MEFs (Figure 4.8). However, TSC2 was almost completely absent 7 days after transfection with both high and low amounts of DNA, highlighting the transient nature of the transfection. 3 days after transfection, there seemed to be a decrease in S6K(P), increase in Akt(P), decreased \(\beta\)-catenin degradation, and slight decrease in E-cadherin levels compared to un-transfected TSC2\(^{-/-}\) MEFs. Moreover, 7 days after transfection, when TSC2 expression was no longer detected, all of the aforementioned proteins appeared to revert back to levels seen in un-transfected cells (Figure 4.8).

I next carried out confocal immunofluorescence microscopy to visualize TSC2, E-cadherin, and morphology in individual cells. TSC2\(^{+/+}\) MEFs all displayed diffuse green TSC2 immunofluorescence throughout the cytoplasm, as expected (Figure 4.9). TSC2\(^{-/-}\) MEFs showed a faint green cytoplasmic fluorescence, likely a result of non-specific staining by the TSC2 antibody. Observation of TSC2\(^{-/-}\) MEFs 3 and 5 days post-transfection with pRK7-TSC2 revealed that only~1-2% of the transfected cells displayed TSC2 expression (Figure 4.9 A,C). Strikingly, these cells showed incredibly bright fluorescence, indicating enormous levels of TSC2 expression. The cells overexpressing TSC2 also displayed a fibroblast-like appearance. This result might explain the western blot results where even very strong TSC2 expression in transfected TSC2\(^{-/-}\) MEFs did not cause much downstream
Figure 4.8 Transient transfection with pRK7-TSC2 induces a slight reduction of E-cadherin in TSC2\(^{+/−}\) MEFs.

TSC2\(^{+/−}\) MEFs were transfected with pRK7-FLAG-TSC2 at recommended concentration (16\(\mu\)g/10cm dish), as well as 10x less DNA (1.6\(\mu\)g/10cm dish) (Section 2.7) and allowed to grow for 3 or 7 days. Cell lysates were immunoblotted for TSC2, S6K(P) (T389), Akt(P) (S473), \(\beta\)-catenin, E-cadherin, and \(\beta\)-tubulin (Section 2.5).
reduction in S6K\(\text{P}\). If only a small percentage of cells are over-expressing TSC2, then a majority of cells would not show any change in downstream signaling.

Observation of E-cadherin immunofluorescence showed low level non-specific staining in the TSC2\(^{+/+}\) MEFs, and fluorescence localized to cell-cell junctions in TSC2\(^{-/-}\) MEFs, as seen previously (Figure 4.9 B, D). TSC2\(^{-/-}\) MEFs transfected with pRK7-TSC2 for 3 and 5 days showed patches of fibroblast-like cells, and these seemed to display less cell junction-localized E-cadherin. There were however still many patches of cells that showed localized fluorescence to cell junctions. Mock-transfected TSC2\(^{+/+}\) MEFs displayed similar E-cadherin localization to un-transfected TSC2\(^{+/+}\) MEFs. Interpretation of these images is complicated by the fact that the transfected cells were not completely confluent. Therefore, a lack of E-cadherin localization to junctions could be due to the lack of cell-cell junctions, or to a change to a fibroblast-like morphology caused by TSC2 expression. Double staining with TSC2 and E-cadherin antibodies was attempted, but unfortunately did not turn out very well due to the fact that the emission of spectrum of one of the secondary antibodies overlapped with that of the nuclear stain. Since transfection efficiency was very low, it may not even be possible to visualize E-cadherin levels via confocal microscopy with this transfection protocol, since getting a large and confluent group of transfected cells together is nearly impossible when transfection efficiencies are so low. Overall, the results of the western blots and confocal microscopy indicate that transfecting TSC2\(^{-/-}\) MEFs with pRK7-TSC2 may slightly reduce expression of E-cadherin as well as its localization to cell-cell junctions.
Figure 4.9 Visualization of TSC2 and E-cadherin in pRK7-TSC2-transfected TSC2<sup>+/−</sup> MEFs by immunofluorescence microscopy.

TSC2<sup>+/−</sup> MEFs were transfected with pRK7-TSC2 (Section 2.7) and allowed to grow to confluency for 3 or 5 days before cells were fixed, DNA was stained, and images were acquired by confocal microscopy (Section 2.6). E-Cadherin staining is shown in green, and DNA staining is shown in blue. Three different images from each coverslip are shown. Scale bar, 30μm.
B

3 Day Post-Transfection

E-Cadherin
5 Day Post-Transfection

TSC2

TSC2−/− MEFs

TSC2−/− MEFs

PRK7−/− TSC2

TSC2−/− MEFs

Mock Transfected
5 Day Post-Transfection

E-Cadherin

TSC2\(^{++}\) MEFs

TSC2\(^{--}\) MEFs

pRK7-TSC2

Mock-Transfected
4.5 Knocking down TSC2 in TSC2\(^{+/+}\) MEFs does not induce E-cadherin expression.

The work described so far was concerned with expression of TSC2 in TSC2\(^{-/-}\) cells. Next, I examined the effect of knocking down TSC2 levels on E-cadherin expression. I transfected TSC2\(^{+/+}\) MEFs with varying amounts of siRNA against TSC2 and saw excellent knockdown of TSC2 after 48 hours in all treatments (Figure 4.10). Knockdown of TSC2 was vastly improved by modifying the protocol recommended by the manufacturer as described in section 2.7. Knockdown of TSC2 in TSC2\(^{+/+}\) MEFs caused an increase in S6K(P), indicating the loss of TSC2 was sufficiently decreased to stimulate mTORC1 signaling. However, the decrease of TSC2 and accompanying increase in mTORC1 activity (S6K(P) increase) was not as high as in TSC2\(^{-/-}\) cells. There was not even the slightest trace of E-cadherin expression in any of the TSC2\(^{+/+}\) transfected cells, even after loading the gel with excess protein and over-exposing it. It is possible that expression of E-cadherin requires more than 48 hours following knockdown of TSC2, which remains to be tested. Alternatively, if very low levels of TSC2 are sufficient to suppress E-cadherin, a complete absence of TSC2 would be necessary for the expression of E-cadherin, which may not be possible using an siRNA knockdown protocol.
Figure 4.10 siRNA Knockdown of TSC2 in TSC2+/+ MEFs does not induce E-cadherin expression.

TSC2+/+ MEFs were transfected with either TSC2-siRNA at varying concentrations or scrambled RNA (SCX) as a negative control. (***) represents a modified protocol that enhanced knockdown of TSC2 (Section 2.7). Cells were allowed to grow for 48 hours post-transfection. Cell lysates were immunoblotted for TSC2, S6K(P) (T389), E-cadherin, and β-tubulin (Section 2.5). The gel for S6K(P) was loaded with less protein for TSC2+/− samples to avoid overexposure, hence the extra β-tubulin loading control.
CHAPTER 5: DISCUSSION

The observations that I have made regarding the phenotypic differences between TSC2<sup>-/-</sup> and TSC2<sup>+/+</sup> MEFs, and how these phenotypes are affected by long-term treatment with mTORC1 inhibitors gives much to be discussed, and in this chapter I touch on the implications of these observations in further understanding tuberous sclerosis and potential treatment avenues.

5.1 Effect of long-term mTORC1 inhibition on MEF morphology and size

The morphology change in TSC2<sup>-/-</sup> MEFs from epithelial-like to fibroblast-like after long-term treatment with mTORC1 inhibitors was a very drastic, and important phenotypic change, as one of the original goals of this project was to ameliorate abnormal phenotypes seen in TSC2<sup>-/-</sup> MEFs to resemble those seen in TSC2<sup>+/+</sup> MEFs. It is important to determine whether this morphology change is a downstream effect of mTORC1 inhibition, or an alternate effect of the drugs on some unrelated pathway. There are some links between TSC1:TSC2 and cell morphology, which report that the TSC1:TSC2 complex regulates the shape and distribution of the actin skeleton through Rho and Rac1 signaling, and that this effect is independent of mTORC1 signaling (11,30,60). There are no clearly known downstream targets of TSC1:TSC2 other than mTORC1, and thus how exactly TSC1:TSC2 can influence cell morphology independently of mTORC1 is not well understood. What causes TSC2-null cells to show an epithelial-like morphology has not been studied. From the experiments that I have performed, it is most likely that the epithelial-like morphology seen in TSC2<sup>-/-</sup> MEFs is in part due to elevated mTORC1 signaling, because they become fibroblast-like upon addition of the drugs that diminish mTORC1 activity. TSC2<sup>-/-</sup> MEFs
revert back to an epithelial-like morphology after more than 1 week treatment with
dronedarone, amiodarone, and niclosamide, and mTORC1 activity also goes back up to
normal levels after these treatments, further indicating that the change in morphology from
epithelial-like to fibroblast-like is an effect of mTORC1 inhibition. By contrast, rapamycin,
an irreversible mTORC1 inhibitor, is the only treatment where TSC2−/− MEFs do not revert
back to an epithelial-like morphology after more than 1 week, consistent with the complete
and permanent inhibition of mTORC1 signaling. Furthermore, perhexiline at 3μM did not
inhibit mTORC1 signaling or affect TSC2−/− MEF morphology, but 5μM inhibited mTORC1
signaling and caused a morphological change. While the specific link between mTORC1
signalling and morphology is unclear, my data suggests that mTORC1 signaling clearly plays
a role in the morphology of the MEFs. However, there are likely some other pathways that
contribute to MEF morphology that are affected, due to the fact that different treatments such
as rapamycin or niclosamide result in slightly different fibroblast-like morphologies in TSC2−/−
MEFs, which is unlikely to be due to mTORC1 signaling alone. What these pathways
could be, or how large of a role they contribute to cell morphology remains to be studied.
Overall, my results show a novel link between elevated mTORC1 signaling and an abnormal
epithelial-like morphology in TSC2−/− MEFs, and that it is possible to restore the normal
fibroblast-like morphology as seen in TSC2+/+ MEFs upon long-term inhibition of mTORC1
signaling.

While I have shown that mTORC1 signaling affects cell morphology, the fact that the
TSC2−/− MEFs revert back to their original morphology and mTORC1 signaling levels under
continued exposure to all mTORC1 inhibitors, with the exception of rapamycin, is a highly
interesting observation. This result implies that there is likely some compensatory signaling
pathway that gets activated and reverts mTORC1 signaling back to elevated levels after prolonged treatment. Such a compensatory signaling pathway is certainly possible, since mTORC1 is at the hub of a large signaling network, and there are many feedback mechanisms control mTORC1 signaling, such as those involved with energy stress (AMPK), amino acids (Rag GTPases), and hypoxia (HIF1α) (1,76). Rapamycin irreversibly inhibits mTORC1 complex formation (20), while niclosamide, dronedarone, amiodarone, and perhexiline are upstream inhibitors of mTORC1, thus allowing for mTORC1 activity levels to potentially be re-adjusted in cells under these conditions. A readjustment of mTORC1 signaling is likely the reason that the TSC2−/− MEFs re-gain their epithelial-like morphology after prolonged treatment with dronedarone, amiodarone, perhexiline, and niclosamide, and become de-sensitized to further treatment with these drugs. As seen in Figure 3.5, there were essentially two distinct populations of TSC2−/− MEFs in this experiment, those treated with niclosamide for 3 weeks (which reverted back to their normal epithelial-like morphology), and untreated TSC2−/− MEFs (which still had their normal epithelial-like morphology). While these populations of cells visually appeared the same, the fact that they responded differently to a concentration gradient of niclosamide indicates that there are some key differences in the cells and how they respond to drugs. If some compensatory signaling pathways were activated in drug-treated TSC2−/− MEFs, it should be possible to identify them by probing lysates of these cell populations with antibodies to different signaling pathways. To my knowledge, there has been no information on how mTORC1 signaling can be re-adjusted in cells that are under continuous mTORC1 inhibition, and thus my findings represent a potential novel observation on the mTORC1 signaling pathway. This could have important implications for potential treatment options in that long-term inhibition of mTORC1
signaling may require inhibiting multiple pathways. Unfortunately, I did not investigate this observation further due to changing my focus to other areas, but it is a very interesting observation that should be explored in future experiments.

Another important point to consider is that the altered morphology of drug-treated TSC2+/− MEFs may be due to more than just changes in signaling. It was seen that the mTORC1 inhibitors required up to 6 or 7 days treatment to induce a consistent morphological change in the TSC2+/− MEFs (Figure 3.2 A), suggesting that there may be other factors in play outside of changes in signaling pathways. For example, it is likely that there are changes in gene expression that occur in drug-treated TSC2+/− MEFs, thus requiring a longer time-frame to be able to have an effect on cell morphology. What these genes could be or how they change remains to be determined, but could provide valuable information on other pathways that could be targeted to have combination effects in an in vivo model.

Another factor that may be required for the cells to undergo a morphological change is the need to pass through a cell division cycle to be able to detach from the extracellular surface and to re-adhere with a more fibroblast-like morphology. It is not known whether the mTORC1 inhibitors can induce a morphological change in TSC2+/− MEFs without them going through cell division or detaching from the extracellular surface, but this should be further investigated in future experiments.

While it was exciting to see that long-term treatment with mTORC1 inhibitors caused the TSC2+/− MEFs to transition from an epithelial-like to a more normal fibroblast-like morphology, there was no accompanying reduction in cell size after 1 week, as the TSC2+/− MEFs still remained much larger than the TSC2+/+ MEFs. This was unfortunate, as the enlarged size of TSC2+/− cells due to elevated mTORC1 signaling is a large contributor to the
formation of tubers as seen in SEGAs in TSC patients (6). Rapamycin is a permanent mTORC1 inhibitor, and has been clinically shown to shrink the size of tumours in vivo (8,62), but this effect could be due to a multitude of factors not necessarily associated with reduced cell size, such as increased cell death or inhibition of proliferation. The link between cell size and mTORC1 is not entirely clear, and the cause of the enlarged size in TSC2-null cells has not been fully described. My results show that inhibiting mTORC1 signaling may not be sufficient to reduce the abnormal size of the TSC2−/− MEFs, and that there may be some other factors that determine cell size. It may be the case that 1 week-treatment is not long enough for the mTORC1 inhibitors to shrink the TSC2−/− MEF size. It may be possible that there could be a reduction in TSC2−/− MEF size after longer treatment with the drugs, if it were possible to prevent the de-sensitization to mTORC1 inhibition. While rapamycin treated TSC2−/− MEFs remained fibroblast-like for at least 3 weeks treatment, I did not quantify their size at any timepoints longer than 1 week treatment. Visually, they did not appear to shrink, although perhaps it may take a longer treatment to see any effects of rapamycin on cell size. Overall, my results show a novel quantification of the difference in cell size between TSC2++/++ and TSC2−/− MEFs, and that even upon the induction of a fibroblast-like morphology with mTORC1 inhibitors, that TSC2−/− MEFs remain considerably larger than TSC2++/++ MEFs. It is important to note that pEF6-TSC2 transfected TSC2−/− MEFs that stably re-express TSC2 (Figure 4.5), as well as pRK7-TSC2 transfected TSC2−/− MEFs that transiently express TSC2 (Figure 4.7 A) both had a fibroblast-like morphology, similar to the effect of the drugs, yet still appeared to remain larger than TSC2++/++ MEFs. This suggests that reducing cell size in the TSC2−/− MEFs cannot be rescued even by stably restoring TSC2, and that other factors may be involved in determining cell size that are
independent of mTORC1 signaling. Thus, the possible therapeutic potential of the drugs should not be discounted simply because they do not show reduction in the size of TSC2\(^{-/-}\) MEFs, as they do ameliorate many other abnormal phenotypes in TSC2\(^{+/-}\) MEFs which are more strongly correlated to mTORC1 signaling, which could have potential implications \textit{in vivo}.

A qualitative observation made upon long-term treatment of MEFs with mTORC1 inhibitors that may have potential therapeutic implications is that the drug-treated TSC2\(^{-/-}\) MEFs proliferated at a substantially slower rate than drug-treated TSC2\(^{+/+}\) MEFs or untreated MEFs. Literature states that TSC2\(^{-/-}\) cells proliferate at similar rates to TSC2\(^{+/+}\) MEFs in medium with serum (74) and I have observed this to be true during cell culture in the absence of drugs. Upon long-term treatment with mTORC1 inhibitors however, the proliferation rate of TSC2\(^{-/-}\) MEFs decreased substantially, while that of the TSC2\(^{+/+}\) MEFs only seemed to be slightly reduced. Furthermore, when the TSC2\(^{-/-}\) MEFs were de-sensitized to some of the drugs after long-term treatment, their proliferation rate seemed to be restored back to normal levels. Rapamycin treated TSC2\(^{-/-}\) MEFs had by far the slowest proliferation rate, which never changed after long-term treatment, likely due to rapamycin being a permanent mTORC1 inhibitor. This effect of rapamycin on cell proliferation has been similarly reported in literature (77). Unfortunately, this difference in proliferation rate after drug treatment was only qualitatively observed and never quantified, but could be another important effect of long-term treatment with mTORC1 inhibitors. This is an observation that needs to be tested more thoroughly in future experiments, as decreasing the proliferation rate of TSC2\(^{-/-}\) cells could decrease tuber growth \textit{in vivo}.
5.2 Actin and focal adhesions

In TSC2−/− MEFs, actin was localized predominantly towards the periphery of the cells, typical of epithelial-like cells, while the actin localization in TSC2+/+ MEFs was diffuse and even throughout the cytoplasm, typical of fibroblast-like cells (11) (Figure 3.7). There was a decrease in peripheral actin localization in drug-treated TSC2−/− MEFs, consistent with a morphological change from epithelial-like to fibroblast-like. One of the most drastic changes in actin localization in TSC2−/− MEFs occurred in niclosamide treated cells, where the actin cytoskeleton formed a “crosshatch” pattern throughout the entire cell that is consistent with the presence of actin stress fibers. Actin stress fibers have been reported to be a characteristic of mesenchymal cells (72), and it is likely that niclosamide induces a highly mesenchymal and fibroblast-like phenotype in the TSC2−/− MEFs. The differences in actin localization between TSC2+/+ and TSC2−/−, and how they are altered by drug-treatment may have significant implications with regards to TSC disease progression. The TSC1:TSC2 complex has been associated with regulation of actin localization via GTPases Rho and Rac1, thus affecting the cytoskeleton (11,30,37). Specifically, TSC2 promotes Rac1 activation and inhibition of Rho independent of mTORC1 signaling, implying that TSC2−/− cells are likely to have reduced lamellipodia and more peripheral actin localization (11). My results display a novel observation that mTORC1 inhibitors which induced a fibroblast-like morphological change in TSC2−/− MEFs all seem to alter the actin localization and cytoskeletal organization in these cells, suggesting that mTORC1 signaling may indeed play a role in these processes. The unique appearance of stress fibers in niclosamide-treated TSC2−/− MEFs is likely an effect unrelated to mTORC1 inhibition. It could be due to the fact that it has been recently seen that niclosamide can decrease β-catenin expression (78), which could result in the cells
losing the ability to anchor the cadherin junctions to the actin cytoskeleton, and thus could induce the re-localization of actin into stress fibers. Alternatively, niclosamide could also activate Rho signaling, thus promoting the formation of more stress fibers. Overall, my results display a novel phenotypic difference in actin localization between TSC2+/+ and TSC2−/− MEFs and how it is altered with the drugs. This could play a role in vivo and in TSC patients, as actin has been found to be involved in cell-cell and cell-substrate interactions, as well as being key components of tight junctions, adherens junctions, and focal adhesions (29,30). It seems plausible that by disrupting the normal actin localization in TSC2−/+ cells, mTORC1 inhibitors would alter many of the downstream effects regulated by actin with regards to cell adhesion, and thus this could potentially disrupt tumour formation in vivo.

Associated with the change in actin localization, I also observed a change in focal adhesions by observing that vinculin localization is altered in drug-treated TSC2−/+ MEFs (Figure 3.8). TSC2−/+ MEFs seem to display more focal adhesions than TSC2+/+ MEFs, suggesting that they are more strongly adhered to the extracellular matrix. Treating the cells with mTORC1 inhibitors did diminish the amount of focal adhesions seen in TSC2−/+ MEFs, corresponding to the change seen in actin localization in these cells. TSC2 has been found to be involved in regulating the Rho and Rac1 pathways, which affects cell adhesion by regulating the actin cytoskeleton, which in turn can affect focal adhesions (11,37). There is a discrepancy in the literature regarding focal adhesions in cells with disrupted TSC1:TSC2 complexes. Both Astrinidis et al. (2002) (37), and Lamb et al. (2000) (79) have reported that functional TSC1:TSC2 complex results in stronger cell adhesions to the extracellular matrix in ELT3 rat uterine leiomyoma cells, while Goncharova et al. (2004) (11) state that their results “contradict a previous paper by Astrinidis et al’’, by showing that TSC1:TSC2
activates Rac1 (increasing membrane ruffles and lamellipodia) and inhibits Rho (reducing stress fibers linked to focal adhesions), inducing more motile and less adherent ELT3 cells. My data, albeit in a different cell-line, supports the observations seen by Goncharova et al. (11), which is that TSC2−/− MEFs have more peripheral actin localization, which should promote more focal adhesions. This is also consistent with my observations that TSC2+/− MEFs have more lamellipodia and less focal adhesions than TSC2+/− MEFs. Due to the differences in actin localization between TSC2+/− and TSC2−/− MEFs, as well as the qualitative observation that TSC2−/− MEFs are more difficult to trypsinize than TSC2+/− MEFs, I am confident in my assessment that TSC2+/− MEFs have more focal adhesions than TSC2+/− MEFs and are more strongly adhered from their basal surface to the extracellular matrix. What is novel about my results is that I have shown that inhibition of mTORC1 signaling diminishes the amount of focal adhesions seen in TSC2−/− MEFs, further implying that mTORC1 signaling plays a part in not only the regulation of the actin cytoskeleton, but also in formation of focal adhesions. Furthermore, since vinculin has been shown to strengthen E-cadherin cell-cell adhesions (35), the presence of more localized vinculin in the TSC2−/− MEFs could contribute to stability of the E-cadherin junctions. Focal adhesions have been related to cancer metastasis and tumour proliferation (36), and the remodeling of focal adhesions is important for cancer invasion (80). Since tumours found in TSC patients are benign and non-invasive, it could be the case that the focal adhesions in TSC2−/− cells are strongly adherent but have less remodeling and turnover, thus explaining the characteristics of the tumours. There is not much literature concerning focal adhesions in TSC tumours however, but this is an interesting and relatively unknown pathway that may have implications on the progression and formation of tumours in TSC patients.
5.3 Cell-cell adhesion

The difference in cell-cell adhesions between TSC2<sup>+/+</sup> and TSC2<sup>−/−</sup> MEFs is another very interesting phenotypic abnormality that may have implications with regards to TSC pathology. TSC2<sup>−/−</sup> MEFs displayed much more extensive cell-cell adhesions than TSC2<sup>+/+</sup> MEFs as visualized by N-cadherin and β-catenin immunofluorescence (Figure 3.9, 3.10). β-catenin and N-cadherin both showed very extensive localization to the cell periphery in TSC2<sup>−/−</sup> MEFs, thus highlighting the extensive cell-cell adhesion complexes found in TSC2<sup>−/−</sup> MEFs which span the entire periphery of the cells. The localization of β-catenin and N-cadherin to the periphery of the TSC2<sup>−/−</sup> MEFs was greatly diminished upon long-term treatment with mTORC1 inhibitors, indicating that they are losing the tendency to form extensive cell-cell adhesions. By looking at the highly peripheral actin localization in TSC2<sup>−/−</sup> MEFs, it follows suit that they would also display extensive cell-cell connections, due to the fact that cell-cell junction proteins such as N-cadherin and E-cadherin interact with β-catenin and then are anchored to the actin cytoskeleton (50,53). Whether the peripheral actin localization promotes more cell-cell adhesions, or whether increased cell-cell adhesions causes peripheral remodeling of the actin cytoskeleton is not entirely clear. While there has been some evidence of increased β-catenin in TSC tubers (47), it is not known whether this increase is due to elevated mTORC1 levels or an alternate downstream effect of dysfunctional TSC1:TSC2. To date, there is no reported link between TSC and N-cadherin, or how TSC2 can affect cell-cell adhesions. Since the presence of N-cadherin junctions is typically a trait of mesenchymal cells (50), the presence of strong N-cadherin junctions in the TSC2<sup>−/−</sup> MEFs is somewhat unusual considering that these cells also display E-cadherin junctions. It is possible that the presence of both N-cadherin and E-cadherin junctions in
TSC2\textsuperscript{−/−} MEFs indicates that these cells have an intermediate morphology which is somewhere between fibroblast-like and epithelial-like. Overall, my results display a novel observation that TSC2\textsuperscript{−/−} MEFs have extensive cell-cell adhesions that display both β-catenin and N-cadherin strongly localized to the periphery of the cell. Furthermore, I have also shown a novel consequence of inhibiting mTORC1 signaling in TSC2\textsuperscript{−/−} MEFs, in that they are less likely to form extensive adherens junctions, and that both β and N-cadherin are less likely to localize to the periphery of the cell. However, it is unclear whether mTORC1 inhibition directly affects β-catenin and N-cadherin localization, thus affecting cell-cell adhesions, or whether mTORC1 inhibition affects cell-cell adhesions via other pathways (actin remodeling for instance), thus disrupting the localization of β-catenin and N-cadherin.

The TSC2\textsuperscript{−/−} MEFs form not only more extensive N-cadherin cell-cell adhesions than the TSC2\textsuperscript{+/+} MEFs, but these adhesions are also more stable in TSC2\textsuperscript{−/−} MEFs as revealed by FRAP experiments on N-cadherin-GFP transfected cells (Figure 3.11). The photobleached junctions between TSC2\textsuperscript{+/+} MEFs recovered faster than those seen in TSC2\textsuperscript{−/−} MEFs, indicating that the junctions between TSC2\textsuperscript{−/−} MEFs are more stable than those between TSC2\textsuperscript{+/+} MEFs. After treatment with rapamycin, niclosamide, and amiodarone, the recovery rate of the TSC2\textsuperscript{−/−} MEFs increased, suggesting that the cell-cell junctions in these treatments were less stable. This change upon treatment with mTORC1 inhibitors suggests that mTORC1 not only has an effect on the likelihood of cells to form intercellular adhesions, but also that it has an effect on the stability of these connections. This is a novel observation, as there is no known link between mTORC1 signaling and the stability of N-cadherin cell-cell adhesions, nor has there any been any reported differences in adhesion stability between TSC2\textsuperscript{+/+} and TSC2\textsuperscript{−/−} cells. It is not known whether mTORC1 signaling directly controls the
stability of N-cadherin junctions, or whether it is controlled via an alternate pathway affected by mTORC1 signaling, such as actin remodeling or localization of β-catenin away from the cell periphery.

The sped-up videos of the MEFs (Figure 3.12) further reveal novel insights into the ability of the MEFs to form and break cell-cell adhesions. The TSC2+/+ MEFs are much more likely to form and break connections with neighbouring cells as compared to the TSC2−/− MEFs, which form adhesions that do not break often, and tend to be attached to the same cells for extended periods of time. This further reinforces the fact that adhesion stability in the TSC2+/+ MEFs is lower than that of the TSC2−/− MEFs, and that they undergo more adhesion turnover and remodeling. The TSC2−/− MEFs treated with niclosamide or rapamycin to induce a morphological change tend to not form as many cell-cell junctions with neighbouring cells, and those that are formed are likely to be broken more rapidly, similar to the TSC2+/+ MEFs. While the drug-treated TSC2−/− MEFs display a similar tendency as the TSC2+/+ MEFs to have less stable cell adhesions, it should be noted that the TSC2+/+ MEFs are much more motile than the drug-treated TSC2−/− MEFs, which are as immobile as the untreated TSC2−/− MEFs. These results suggest that even though mTORC1 inhibition causes the TSC2−/− MEFs to considerably change their phenotype with regards to morphology, actin, vinculin, and cell-adhesions to resemble that of the TSC2+/+ MEFs, these changes are not sufficient to restore motility to these cells.

Even though I have shown that treatment with mTORC1 inhibitors disrupts cell-cell adhesions and alters the localization of N-cadherin, β-catenin, and actin, it remains to be seen exactly how this change is occurring. It would be useful to assess Rho and Rac1 levels in both the TSC2+/+ and TSC2−/− MEFs, and whether they change after mTORC1 inhibition.
The ability of the mTORC1 inhibitors to reduce the tendency of TSC2<sup>−/−</sup> MEFs to form extensive cell-cell adhesions could have large implications in an <i>in vivo</i> model of TSC. On one hand, inhibiting mTORC1 to disrupt the adhesiveness of TSC2-null cells could potentially inhibit the formation of the tubers by preventing cells from clustering together, thus being a new targetable pathway for treatment of TSC. On the other hand, inhibiting mTORC1 to disrupt cell adhesions could in fact have the undesirable effect of inducing the cells to be more mesenchymal, potentially causing the normally benign TSC tubers to become invasive. Overall, I have described many novel observations regarding cell-adhesions in TSC2<sup>+/+</sup> and TSC2<sup>−/−</sup> MEFs and how these change with mTORC1 inhibition that should be further examined in future experiments.

5.4 Expression of adhesion proteins

The blots displaying the expression levels of β-catenin, vinculin, and N-cadherin reveal that even though the mTORC1 inhibitors are changing the localization of these proteins, the overall expression levels seem to remain consistent (Figure 3.13). However, although the levels of full length β-catenin are similar between TSC2<sup>+/+</sup> and TSC2<sup>−/−</sup> MEFs, the TSC2<sup>−/−</sup> MEFs display more β-catenin degradation products than TSC2<sup>+/+</sup> MEFs. This may be explained by the fact that TSC2<sup>−/−</sup> MEFs have lower levels of phosphorylated Akt, which would indicate increased stabilization of GSK-3β, which is involved in degrading β-catenin (26). The increased β-catenin degradation seen in TSC2<sup>−/−</sup> MEFs may not necessarily reflect the pool of β-catenin involved in cell-cell junctions, and could just reflect increased degradation of cytosolic β-catenin. This would be consistent with the observation that the TSC2<sup>−/−</sup> MEFs display much more β-catenin localized to the cell periphery involved in stable cell-cell junctions. Rapamycin reduces the appearance of these lower bands in TSC2<sup>−/−</sup> MEFs.
suggesting that inhibition of mTORC1 signaling may inhibit the degradation and turnover of β-catenin. This would be consistent with the fact that rapamycin inhibits mTORC1 signaling, which should result in more phosphorylated Akt, and thus less stable GSK-3β and less β-catenin degradation. While the overall levels of vinculin and N-cadherin are similar in the TSC2+/+ and TSC2−/− MEFs, rapamycin once again appears to diminish the degradation products in the TSC2−/− MEFs. This could be explained by the diminished degradation of β-catenin, which would thus lead to more stable N-cadherin and vinculin adhesions, since they both interact with β-catenin to form adhesions (39,50). Overall, my results show that the phenotypic differences between the TSC2+/+ and TSC2−/− MEFs with regards to β-catenin, vinculin, and N-cadherin are primarily an issue of protein localization, and not expression. Whatever effect the drugs have on the cells primarily targets the localization of these proteins, and does not drastically alter the amount of full-length protein. The investigation into these proteins in the context of TSC has not been described, and thus my results help elucidate a novel effect of TSC2 and mTORC1 on several proteins involved in cell morphology and adhesion.

5.5 E-cadherin

The observation that TSC2−/− MEFs expressed E-cadherin was perhaps my most surprising finding because E-cadherin is normally only expressed in epithelial cells, and not in other cell types such as fibroblasts (54). Since the loss of E-cadherin is a hallmark feature of the epithelial-mesenchymal transition (EMT) seen in malignant cancers (53,55), determining whether TSC2 or mTORC1 controls E-cadherin expression thus became a main focus of this project.
The initial blots showing E-cadherin in drug-treated TSC2⁻/⁻ MEFs (Figure 3.13, 3.14) present clues into how mTORC1 signaling might affect E-cadherin expression. Similar to β-catenin, vinculin, and N-cadherin expression levels, rapamycin displays a reduction of E-cadherin degradation products in TSC2⁻/⁻ MEFs, as well as a slight reduction of full length E-cadherin. This could be explained due to the fact that rapamycin is preventing the degradation of β-catenin, which can interact with lymphoid enhancer factor/T cell factor (LEF/TCF), which is then translocated into the nucleus to repress E-cadherin (26,43,44). Thus, by stabilizing β-catenin levels, rapamycin could be diminishing the expression and turnover of E-cadherin. Niclosamide elicited a similar effect, in that it seems to reduce the levels of E-cadherin in TSC2⁻/⁻ MEFs as seen in Figure 3.13, and also Figure 3.14. Such a decrease in E-cadherin levels with niclosamide treatment is unlikely to be a result of strictly mTORC1 inhibition, as rapamycin, a much more potent mTORC1 inhibitor, did not show as much reduction of E-cadherin expression. It is likely that niclosamide affects alternate pathways to disrupt E-cadherin expression, likely via Wnt signaling. There is not much documented regarding the connection between TSC and Wnt signaling, but there is a report that there is an upregulation of the Wnt pathway in TSC SEGA tubers (49), and that Niclosamide can downregulate Wnt signaling and β-catenin levels in tumours with activated Wnt signaling (78). This is strange, as an increase in Wnt signaling in TSC tubers should elicit a more mesenchymal phenotype, which does not agree with all my observations regarding the TSC2⁻/⁻ MEFs and their epithelial-like phenotype. Nevertheless, there is likely a link between Niclosamide and Wnt signaling that controls E-cadherin regulation that should be investigated further. While understanding how the drugs might alter the
expression of E-cadherin is important, the remainder of my work instead focused on determining whether TSC2 regulates the expression of E-cadherin in MEFs.

I propose a model of how TSC2 can regulate E-cadherin expression based on my results and on known regulatory pathways of TSC1:TSC2, mTORC1, Akt(P), GSK-3β, β-catenin, and E-cadherin. These pathways have been described in literature, but have not fully been linked together to explain how TSC1:TSC2 can regulate E-cadherin (Figure 5.1). In this model, TSC2−/− MEFs display increased mTORC1 and S6K(P) activity, which then via downstream IRS-1 inhibition, decreases the activity of mTORC2 (23). Since mTORC2 phosphorylates and activates Akt (24), TSC2−/− MEFs with increased mTORC1 activity have decreased mTORC2 activity, and therefore display decreased Akt phosphorylation, which I have confirmed experimentally (Figure 4.4). Active Akt can phosphorylate and degrade GSK-3β (27). When GSK-3β is present, it causes the phosphorylation and degradation of Snail, as well as β-catenin (via the “destruction complex” composed of tumour suppressors Axin, adenomatous polyposis coli (APC), and GSK-3β (46)). Snail, can bind to E-boxes in the E-cadherin promoter, thus silencing E-cadherin expression (58,59), and β-catenin will interact with LEF/TCF and this complex is then translocated to the nucleus to repress E-cadherin expression (26,43,44). Thus, cells that have lower levels of Akt phosphorylation should display less GSK-3β degradation, and thus more β-catenin and Snail degradation. This in turn should alleviate the repression of E-cadherin, and could be a rationale for how absence of TSC2 can result in downstream E-cadherin expression. A similar mechanism has been proposed very recently (February 2014) in a different context not related to TSC2: that inhibiting mTORC1 in non-small cell lung cancer cell lines induces the loss of E-cadherin by the Akt/GSK-3β signaling pathway, by increasing the amount of Snail to repress E-cadherin
Figure 5.1 Model of E-cadherin regulation by TSC2.

Lack of TSC1:TSC2 causes an increase in Rheb-GTP, resulting in abnormally high mTORC1 activity and S6K(P). Active S6K(P) causes inhibition of IRS1 and thus also inhibition of mTORC2 and Akt(P). Since Akt(P) is diminished, there is predicted to be less phosphorylation mediated degradation of GSK-3β, which will be stabilized. Stabilized GSK-3β will increase the degradation of β-catenin and Snail, thus alleviating their repressive effects on the expression of E-cadherin.
My data suggests that what I have proposed is likely on the correct path, as I have seen that TSC2−/− MEFs display reduced levels of Akt(P) (Figure 4.7 B) and more β-catenin degradation (Figure 3.13) than TSC2+/+ MEFs, which could lead to a loss of repression of E-cadherin in the TSC2−/− MEFs. Transfecting the TSC2−/− MEFs with pRK7-TSC2 resulted in a decrease of S6K(P), increase in Akt(P), decreased β-catenin degradation, and slightly decrease E-cadherin expression, consistent with my proposed model (Figure 4.8). I only looked at GSK-3β(P) levels once (Figure 4.4), and did not see a noticeable difference between cell-types, but I would need to probe for total GSK-3β to make any definitive conclusions. Overall, I believe that there is weight to my proposed link between TSC2 and E-cadherin that reveals a novel regulatory pathway that should be further investigated in priority.

The absence of E-cadherin in the pEF6-transfected TSC2−/− MEFs we received was very confusing. These cells (described in (74)) were acquired from the same lab our original TSC2−/− MEFs, and thus should be identical save for the presence of an empty pEF6 plasmid. It should be noted however, that while the TSC2−/− MEFs transfected with pEF6-TSC were described in (74), there was never any mention PEF6-transfected TSC2−/− MEFs in this paper. However, these cells were also sent to us in addition to the PEF6-TSC2 TSC2−/− MEFs we requested. Apart from the absence of E-cadherin in the pEF6-transfected TSC2−/− MEFs, they also displayed a different morphology than our TSC2−/− MEFs (Figure 4.5). They were slightly more fibroblast-like, and did not have as continuous cell-cell adhesions. This is very strange, because in the paper by Zhang et al., they show an image of the TSC2−/− MEFs that they use ((74), Figure 5d top-right panel), which have epithelial-like morphology and continuous cell-cell adhesions and closely resembles the TSC2−/− MEFs I have been using.
Thus, the introduction of the empty pEF6 plasmid should not drastically change the morphology of these TSC2−/− MEFs. It would be useful to analyze the N-cadherin adhesions in the pEF6-TSC2−/− MEFs to determine whether they show similar N-cadherin localization to the normal TSC2−/− MEFs, or whether those adhesions are altered as well. I plan to get in contact with the authors in the future to understand this issue and what may have occurred. Regardless, the work that I performed using my own transfection method with pRK7-TSC2 ended up being quite fruitful and provided much to discuss.

Reintroducing TSC2 into TSC2−/− MEFs via transfection with pRK-TSC2 was successful, as seen from the detection of TSC2 (Figure 4.8). It was initially confusing that even though the cells displayed exceptionally high levels of TSC2, there was not a corresponding decrease in S6K(P), which should happen very rapidly upon TSC2 re-introduction. Since the TSC2 encoded by pRK7-TSC2 is of human origin instead of mouse origin, one possibility is that the TSC2 re-introduced is not fully functional. The papers describing this plasmid (14,75) both used human cells, but Zhang et al. (74) used pEF6-TSC2 also encoding human TSC2 in MEFs, and saw reduction in S6K(P) levels, so it is unlikely that this is the reason for the lack of S6K(P) reduction. A likely explanation arises from the confocal immunofluorescence results (Figure 4.9). The transfection efficiency of PRK7-TSC2 into TSC2−/− MEFs was very low (~1-2%), but those cells that had taken up the plasmid displayed incredibly bright fluorescence, indicating enormous TSC2 overexpression. This can explain the western blot results where even with very strong TSC2 expression in transfected TSC2−/− MEFs, not much downstream reduction in S6K(P) was observed. If only a small percentage of cells are over-expressing TSC2, then a majority of cells would not show any change in downstream signaling. To address this issue in the future, it would be
beneficial to either increase transfection efficiency, or to use a stable transfection protocol with selection, that would ensure that all TSC2<sup>−/−</sup> MEFs would be expressing re-introduced TSC2. It would be easier to interpret the results in this case, and there may be more noticeable reduction of S6K(P) and E-cadherin in stably-transfected TSC2<sup>−/−</sup> MEFs.

Another observation to consider is the fact that TSC2<sup>−/−</sup> MEFs transfected with TSC2 (either pEF6-TSC2, or pRK7-TSC2) all adopt a highly fibroblast-like appearance. Since introduction of TSC2 should decrease mTORC1 activity, this is consistent with my previous observations that mTORC1 inhibition similarly alters the morphology of the TSC2<sup>−/−</sup> MEFs. What is confusing in this case is that if transfection efficiency is so low in pRK7-TSC2-transfected TSC2<sup>−/−</sup> MEFs, why then do so many cells display a change in morphology? This change in morphology is likely not due to transfection reagents on their own, since TSC2<sup>−/−</sup> MEFs transfected with pRK7-ABCA4 as a negative control, or mock-transfected, did not display any change in morphology, and remained epithelial-like. Thus, this change in morphology has to be due to the introduction of TSC2 itself. It is possible that the few TSC2<sup>−/−</sup> MEFs that were overexpressing TSC2 were secreting some factor involved in regulating morphology to neighbouring cells to induce a morphological change. This may be plausible, as I have seen that conditioned media collected from TSC2<sup>+/−</sup> MEFs causes TSC2<sup>−/−</sup> MEFs to undergo a morphological change from epithelial-like to fibroblast-like similar to treatment with mTORC1 inhibitors. It is possible that TSC2 promotes secretion of some compound such as TGF-β into the surrounding media that promotes a change in morphology. Further experiments should be performed to understand this observation.

Despite all of the roadblocks described above, the fact that pRK7-TSC2 transfected TSC2<sup>−/−</sup> MEFs show what appears to be a slight decrease in E-cadherin expression 3 days
post-transfection is promising (Figure 4.8), and leads to many future questions and potential experiments. While my results show that introducing TSC2 into TSC2+/− MEFs can reduce S6K(P), increase Akt(P), and reduce β-catenin degradation, it remains to be seen whether this proposed model is fully responsible for the slight decrease in E-cadherin expression, or whether alternate pathways are involved. For example, it could be that TSC2 can promote the secretion of a factor that promotes a fibroblast-like phenotype and decreases E-cadherin expression. Alternatively, it may be the case that introduction of TSC2 promotes a fibroblast-like morphology by affecting Rho and Rac1 levels, which could influence E-cadherin expression.

siRNA knockdown of TSC2 in TSC2+/+ MEFs resulted in elevated S6K(P) levels, but did not display even a trace of E-cadherin expression in these cells (Figure 4.10). This result was disappointing, but could be explained by a few factors. Firstly, knockdown of TSC2 in TSC2+/+ MEFs was not complete, and while S6K(P) levels did increase, they were still much lower than S6K(P) levels seen in TSC2+/- MEFs. This suggests the possibility that the slight increase in S6K(P) was not enough to alleviate the repression of E-cadherin. It may be the case that 100% loss of TSC2 is required for expression of E-cadherin, and that even very small amounts of TSC2 are enough to repress this pathway. Unfortunately, other downstream signaling markers described in my model were not assessed in this experiment, but it would be useful to see what the corresponding levels of Akt(P) or GSK-3β(P) were after siRNA knockdown of TSC2 in TSC2+/+ MEFs. Another possibility is that knockdown of TSC2 perhaps requires a longer timeframe to display any E-cadherin expression. Blots shown were only 2 days post-transfection, and this may not have been enough time for the expression of E-cadherin to be turned on. Unfortunately, assessing the effects of TSC2
knockdown after more than 2 days may not be possible, as this is a transient knockdown protocol. Another possibility with regards to E-cadherin regulation is that inducing expression of E-cadherin in TSC2+/+ MEFs might be much more difficult than reducing E-cadherin expression in TSC2−/− MEFs. Since the expression of E-cadherin is so tightly regulated, more conditions may be required than a simple addition or removal of TSC2 to display any changes. The regulation of E-cadherin can be very tightly controlled and repressed by hypermethylation of the promoter (53,56,57), and it may be the case that the TSC2+/+ MEFs are hypermethylated at the E-cadherin promoter, while the TSC2−/− have somehow partially lost some of this methylation, implying that the difference between the two cell lines may have an epigenetic component that would not be altered by presence or absence of TSC2.

Overall, since the loss of E-cadherin is a hallmark feature of EMT, there is thus considerable clinical interest in inducing the expression of E-cadherin in cancer cells (82). My work describes a novel regulatory pathway for E-cadherin in MEFs that could provide further insight into E-cadherin regulation, as well as many potential therapeutic opportunities for not only TSC, but any disease that involves E-cadherin or an EMT.
CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Concluding remarks

In conclusion, the work presented in this thesis describes a novel investigation into phenotypic abnormalities due to loss of TSC2 that are not very well described in literature, and provides a basis for many future experiments. I have shown that TSC2−/− MEFs display an abnormal epithelial-like morphology, including peripheral actin localization, increased focal adhesions, more extensive as well as more stable cell-cell adhesions, and even expression and proper localization of E-cadherin. All of these phenotypes can have important bearings on an in vivo model of TSC, and should be considered as potential determinant factors for the formation of SEGAs and other TSC related tumours. I have shown that prolonged treatment with mTORC1 inhibitors causes a morphological transition in TSC2−/− MEFs from epithelial-like to fibroblast-like, including altering actin localization, focal adhesions, and cell-cell adhesions to all resemble the phenotypes seen in TSC2+/+ MEFs, suggesting that they are regulated downstream of mTORC1. I have shown that prolonged treatment of TSC2−/− MEFs with mTORC1 inhibitors other than rapamycin (i.e. ones that are not complete and irreversible inhibitors) causes a reversion in altered phenotypes. I have also shown that upon long-term treatment, the cells become de-sensitized to further inhibition, thus suggesting activation of compensatory pathways to restore the elevated mTORC1 signaling and epithelial-like phenotypes. Finally, by observing that TSC2−/− MEFs express E-cadherin that is involved in cell-cell junctions, I have potentially uncovered that TSC2 could serve as a novel regulatory protein for expression of E-cadherin.
I have provided a model to explain how TSC2 can regulate E-cadherin expression, which is in accordance with literature as well as my own observations. While I have provided a framework for potential novel therapeutic options for TSC, as well as a novel regulatory pathway for E-cadherin expression, many questions remain which should be addressed in future experiments described below.

6.2 Determining how mTORC1 affects cell morphology

While I have shown that treatment of TSC2+/− MEFs with mTORC1 inhibitors induced a morphological change from epithelial-like to fibroblast-like, it still remains to be seen exactly how this change occurs. To accomplish this, the activity levels of Rho and Rac1 in the TSC2+/− and TSC2−/− MEFs should be assessed, firstly to determine whether there is any noticeable difference between cell types, and secondly, whether there was any change in activity levels upon mTORC1 inhibition. Altered Rho and Rac1 levels could potentially explain many of the abnormal morphological phenotypes seen in TSC2−/− MEFs. It has been proposed that TSC2 promotes Rac1 activation and inhibition of Rho, suggesting that TSC2−/− cells are likely to have less lamellipodia, less focal adhesion remodeling, as well as altered actin cytoskeletal structure (11,30), which is consistent with the observations I have made. By measuring levels of Rac1 and Rho after drug treatment, it would be possible to determine how exactly the mTORC1 signaling pathway effects cell morphology.

6.3 Determining how TSC2−/− MEFs compensate for long-term mTORC1 inhibition

Another very interesting observation is the fact that during long term treatment, the TSC2−/− MEFs become insensitive to mTORC1 inhibition from all drugs except for rapamycin. Since all of the drugs target unknown pathways upstream of mTORC1, it is thus highly likely that there is some compensatory signaling occurring to re-elevate mTORC1
levels after prolonged exposure to these drugs. By treating TSC2<sup>+/−</sup> MEFs until they have gained and subsequently lost their fibroblast-like morphology, it should be possible to analyze lysates to determine what pathways were involved in the restoration of mTORC1 activity. Since mTORC1 is at the hub of a large signalling network, there are many feedback mechanisms to restore mTORC1 signaling, such as those involved with energy stress (AMPK), amino acids (Rag GTPases), and hypoxia (HIF1α) (1,76). This would provide incredibly valuable information into further understanding the mTORC1 signaling pathway and how it can be re-adjusted while under continual inhibition via compensatory signaling pathways. This could have important implications for potential treatment options, since a combination of inhibiting different pathways may be required to ensure that the inhibition of mTORC1 signaling is permanently maintained.

6.4 Determining how TSC2 acts as a regulator of E-cadherin

In my opinion, the most important and pressing future experiment to perform is to determine exactly how TSC2 can regulate E-cadherin, and whether my proposed model is correct. Accomplishing this would require further assessing the intermediates of my proposed model, including levels of Akt(P), GSK-3β, β-catenin, and Snail in both TSC2<sup>+/−</sup> and TSC2<sup>−/−</sup> MEFs with and without drugs, with pRK7-TSC2 transfection, and with siTSC2 knockdown. My data suggests that what I have proposed is likely on the correct path, as I have seen that TSC2<sup>−/−</sup> MEFs display reduced levels of Akt(P) and more β-catenin degradation than TSC2<sup>+/−</sup> MEFs (Figure 4.8), which could lead to a loss of repression of E-cadherin in the TSC2<sup>−/−</sup> MEFs. The levels of these proteins as well as GSK-3β and Snail should be more thoroughly assessed in the MEFs, as well as what effect the drugs have on this proposed pathway. Furthermore, to better assess the effect of TSC2 on E-cadherin, a
more stable transfection protocol should be used. I do see a slight decrease of E-cadherin after 3 days transient transfection with pRK7-TSC2 (Figure 4.8), but this is the maximum length of time that TSC2 is expressed in the MEFs in this protocol, and thus I would expect to see a greater decrease of E-cadherin upon prolonged expression of TSC2.

Furthermore, it would be valuable to examine the methylation state of the E-cadherin promoters in both TSC2+/+ and TSC2−/− MEFs via bisulfite sequencing to determine whether there is an epigenetic component to the differences between cell types. Regulation of E-cadherin is very tightly controlled at the transcriptional level via methylation state of the promoter (53,56,57), and it may be possible that the TSC2−/− MEFs express E-cadherin because their promoters have been de-methylated. Another option is to examine the levels of E-cadherin mRNA transcript in TSC2+/+ and TSC2−/− MEFs via RT-PCR to determine whether the expression of E-cadherin is controlled at the transcriptional or the translational level.
REFERENCES


83. How TSC Affects the Body: Brain Anatomy. at <http://www2.massgeneral.org/livingwithtscaffects/>