The actin cytoskeleton during adipocyte formation

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Boston University
THE ACTIN CYTOSKELETON DURING ADIPOCYTE FORMATION

by

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B.S., Stonehill College, 2008

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2016
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DEDICATION

I would like to dedicate this work to fat. I study you, eat you, marvel over you, celebrate your appearance in the culture dish and lament your appearance on my waistline.
ACKNOWLEDGMENTS

I would like to take this opportunity to thank the entire biochemistry department, former and current members, particularly Vicki, Mary, Melissa, Erika and Dr. Schreiber. My thesis committee (Dr. Pilch, Dr. Perissi, Dr. Varelas and Dr. Aprahamian) deserves special thanks for encouraging me every step of the way.

I’d like to acknowledge the Farmer lab for supporting me through this journey. It has been an honor to work alongside Chendi, Hejiao and Hong over the entirety of my studies. Thank you for providing wisdom and smiles. I would also like to thank past and new members of the Farmer lab. We wouldn’t be rock’n and roll’n without Steve- the man who’s mentored me in the ways of science and life for five years. From the bottom of my heart, thank you.

I have been blessed with wonderful friends like Sami, Madelane and Katelyn. Sami and Madelane, being in the trenches together has bonded us for life. Katelyn, you probably felt like you were dragged into the trenches. Despite that, you provided me limitless compassion, patience, zeal, chocolate peanut butter eggs, Shaggy and leftovers. Thank you all a thousand times over.

My family also deserves tremendous thanks. This thesis wouldn’t exist without the nurture and support of my mommio Judy and my daddio Vinny. I promise you I’ll stop going to school- at least for a little while. Jasmine, thank you for giving
me periodic boosts of confidence- I needed them. I can only hope that my
munchkins, Gavin and Nessy, will start embracing the nerdiness that is their aunt
and, just maybe, be inspired. Tony, you were roped into this at the worst time,
Saw the madness and still decided to stay- thank you.
THE ACTIN CYTOSKELETON DURING ADIPOCYTE FORMATION

LYNES TORRES

Boston University School of Medicine, 2016

Major Professor: Stephen Farmer, Ph.D., Professor of Biochemistry

ABSTRACT

In addition to providing heat insulation and mechanical cushioning, adipose tissue regulates overall metabolic homeostasis and serves as an essential energy storage site. Excess adipose tissue, or obesity, is on the rise in the US among all demographics. The expansion of adipose tissue results from both adipocyte hypertrophy and hyperplasia but the mechanisms that regulate these processes are not fully understood. Destabilizing actin has been shown to promote adipogenesis while actin stabilization inhibits this process. In addition, decreased actin synthesis is known to occur. However, these studies examined total actin and did not consider that actin is in fact a family of functionally diverse isoforms and that individual isoforms may have different functions in adipogenesis. I hypothesized that actin isoforms contribute differently to adipogenic actin reorganization. To measure this, I developed a novel fractionation method that allowed for the reliable quantification of actin polymerization. I used this actin fractionation method to identify an early loss in polymerized α-smooth muscle actin (α-SMA) relative to polymerized β-actin and γ-actin and to also rule out a role for the actin severing protein gelsolin in the loss of polymerized actin. Furthermore, I showed that the loss of α-SMA expression
precedes the loss of β-actin and γ-actin expression. A known regulator of actin cytoskeleton genes is the transcription factor serum response factor (SRF) and its co-activator, myocardin related transcription factor (MRTF). I identified a role for MRTF/SRF in the downregulation of actin expression during adipogenesis, particularly α-SMA. There was an additional cAMP-responsive decrease in α-SMA expression during the initiation of adipogenesis by exposure to established inducers. Overall, my findings are consistent with growing evidence suggesting that genetic markers of smooth muscle cells, including α-SMA, help control adipogenic commitment. Understanding these early stages of adipogenesis could open new therapeutic avenues for obesity and its co-morbidities.
# TABLE OF CONTENTS

DEDICATION ........................................................................................................ iv  

ACKNOWLEDGMENTS .......................................................................................... v 

ABSTRACT ........................................................................................................... vii 

TABLE OF CONTENTS ........................................................................................... ix 

LIST OF TABLES ................................................................................................... xiv 

LIST OF FIGURES .................................................................................................. xv 

LIST OF ABBREVIATIONS ...................................................................................... xvii 

CHAPTER ONE ...................................................................................................... 1  

MATERIALS AND METHODS ............................................................................... 1  

Mice ..................................................................................................................... 1  

Isolating stromal vascular fraction cells (SVC) ...................................................... 1  

Cell culture .......................................................................................................... 2  

Treatments ........................................................................................................... 2  

Transfections ...................................................................................................... 3  

Quantitative RT-PCR RNA analysis .................................................................. 4  

Immunofluorescence staining ............................................................................ 5  

SDS-PAGE and Western analysis ....................................................................... 7  

CSK/RSB fractionation ....................................................................................... 8  

Statistical analysis ............................................................................................. 10
CHAPTER TWO .................................................................................................................. 11

INTRODUCTION .............................................................................................................. 11

The importance of adipose tissue ................................................................................. 11

Brown adipose tissue ................................................................................................. 11

Brite/beige adipose tissue ......................................................................................... 14

White adipose tissue ................................................................................................. 16

Obesity and metabolic disorder ................................................................................ 17

Hypertrophy and hyperplasia .................................................................................... 19

Models of adipogenesis ............................................................................................. 20

The process of adipocyte formation .......................................................................... 21

PPAR and adipogenesis ............................................................................................ 22

C/EBP and adipogenesis ......................................................................................... 24

ECM and adipogenesis ............................................................................................. 24

Rho/ROCK signaling ................................................................................................. 26

Actin ............................................................................................................................. 27

Actin dynamics ............................................................................................................ 28

The actin gene family ................................................................................................ 29

Mechanotransduction and adipogenesis ............................................................... 31

Taz/Yap ..................................................................................................................... 32

MRTF/SRF .................................................................................................................. 34

Significance and research goals ............................................................................... 35

CHAPTER THREE ......................................................................................................... 38
Gelsolin is not required for adipogenic actin reorganization ........................................74
Discussion ..................................................................................................................78

CHAPTER FIVE .............................................................................................................81

THE ISOFORM-SPECIFIC DYNAMICS OF ACTIN DURING EARLY ADIPOGENESIS ..................................................................................................................81

Abstract .....................................................................................................................81
Introduction ..................................................................................................................81
Results ..........................................................................................................................84
 α-SMA is enriched in the stromal vasculature of white adipose tissue ......................84
 White adipocyte precursor cells have extensive β-actin and α-SMA networks ......87
 Significant actin reorganization coincides with the period of hormonal induction ...87
 Hormonal induction decreases α-SMA protein levels in both the G-actin and F-actin compartment ........................................................................................................90
 Actin transcript levels decrease during hormonal induction ....................................92
 Actin transcript is not rendered unstable by adipogenesis .......................................94
 IBMX significantly contributes to α-SMA downregulation during hormonal induction ...........................................................................................................96
 cAMP signaling regulates α-SMA gene transcription ............................................99
 MRTF/SRF activity regulates α-SMA expression in subconfluent 3T3-L1 cells ......99
 MRTFa regulates actin expression in SVC ...............................................................103
 Discussion ..................................................................................................................105

CHAPTER SIX .............................................................................................................111
GENERAL DISCUSSION, FUTURE DIRECTIONS AND CONCLUSIONS ...... 111

DISCUSSION ........................................................................................................ 111

Potential applications for CSK/RSB fractionation ............................................. 111
Cytoplasmic actin and adipogenesis .................................................................. 114
α-SMA and adipogenesis .................................................................................... 116
Gelsolin and Taz/Yap .......................................................................................... 118
Actin in white, beige/brite and brown adipocytes ............................................. 119
Summary ............................................................................................................. 122

BIBLIOGRAPHY .................................................................................................. 123

CURRICULUM VITAE .......................................................................................... 150
LIST OF TABLES

Table 1. qPCR primer sequences ................................................................. 6
Table 2. Primary antibodies ................................................................. 9
Table 3. Actin isoforms ........................................................................ 30
LIST OF FIGURES

Figure 2.1 The regulation of MRTF/SRF activity.......................................................... 36
Figure 3.1 CSK/RSB fractionation method ................................................................. 43
Figure 3.2 Short CSK incubations extract actin ......................................................... 45
Figure 3.3 CSK extracts cytoplasmic G-actin without disrupting the F-actin network of 10T1/2 cells ...................................................................................... 47
Figure 3.4 CSK extracts cytoplasmic G-actin without disrupting the F-actin network of 3T3-L1 cells ...................................................................................... 49
Figure 3.5 CSK and RSB buffers efficiently solubilize cytoskeletal proteins ....... 51
Figure 3.6 CSK/RSB fractionation discerns actin dynamics ................................. 53
Figure 3.7 Actin depolymerization can be quantified and normalized under CSK/RSB fractionation ........................................................................................................ 56
Figure 4.1 Gelsolin is expressed by white adipocytes ............................................ 66
Figure 4.2 Gelsolin is transiently induced during early adipogenesis ............... 68
Figure 4.3 Dexamethasone induces gelsolin during hormonal induction ........ 70
Figure 4.4 Gelsolin induction is largely regulated by dexamethasone and glucocorticoid receptor ......................................................................................... 71
Figure 4.5 Gelsolin co-localizes with F-actin during early adipogenesis ........... 75
Figure 4.6 Gelsolin is not required for adipogenic actin reorganization ........... 76
Figure 5.1 Actin changes as a function of adipogenesis ........................................ 83
Figure 5.2 α-SMA is enriched in the stromal vasculature of white adipose tissue 86
Figure 5.3 White adipose SVCs have extensive β-actin and α-SMA networks... 88
Figure 5.4 The actin network is significantly reorganized during hormonal induction.............................................................. 89
Figure 5.5 Hormonal induction decreases α-SMA protein levels in both the G-actin and F-actin compartments ................................................................. 91
Figure 5.6 Actin levels decrease during hormonal induction ......................... 93
Figure 5.7 Hormonal induction does not render actin transcript unstable....... 95
Figure 5.8 IBMX significantly contributes to α-SMA downregulation during hormonal induction ................................................................. 97
Figure 5.9 cAMP signaling downregulates α-SMA gene transcription .......... 100
Figure 5.10 MRTF regulates α-SMA expression in subconfluent 3T3-L1 cells. 102
Figure 5.11 MRTF regulates actin expression in SVC................................................. 104
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>ABP</td>
<td>actin binding protein</td>
</tr>
<tr>
<td>ADF</td>
<td>actin depolymerizing factor</td>
</tr>
<tr>
<td>AF</td>
<td>adipocyte fraction</td>
</tr>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer-binding protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CD24</td>
<td>cluster of differentiation 24</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CSK</td>
<td>cytoskeleton buffer</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</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>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>Ebf2</td>
<td>early B-cell factor 2</td>
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</table>
ECM .......................................................... extracellular matrix
EMT .......................................................... epithelial-mesenchymal transition
Epac .......................................................... exchange proteins directly activated by cAMP
epiWAT .......................................................... epididymal white adipose tissue
F-actin .......................................................... filamentous actin
FBS .......................................................... fetal bovine serum
F-FDG .......................................................... F-fluorodeoxyglucose
FGF-21 .......................................................... fibroblast growth factor 21
GAP .......................................................... GTPase-activating protein
GDI .......................................................... guanine nucleotide-dissociation inhibitor
GDP .......................................................... guanine diphosphate
GEF .......................................................... guanine nucleotide-exchange factor
GFP .......................................................... green fluorescent protein
GH .......................................................... gelsolin homology
GILZ .......................................................... glucocorticoid induced leucine zipper
GR .......................................................... glucocorticoid receptor
GTP .......................................................... guanine triphosphate
HRE .......................................................... hormone response element
HRP .......................................................... horseradish peroxidase
iBAT .......................................................... intrascapular BAT
IBMX .......................................................... isobutylmethylxanthine
IGF-1 .......................................................... insulin-like growth factor 1
ingWAT ........................................................... inguinal white adipose tissue
KLF4 .............................................................. Kruppel-like factor 4
MCE .............................................................. mitotic clonal expansion
mRNA ............................................................. messenger RNA
MRTF ............................................................. myocardin related transcription factor
Myf5 .............................................................. myogenic factor 5
PAGE ............................................................ polyacrylamide gel electrophoresis
PBS ............................................................... phosphate buffered saline
PCR ............................................................... polymerase chain reaction
PDGFRβ ........................................................ platelet derived growth factor receptor β
PET/CT ........................................................ position emission tomography/computed tomography
PPAR ......................................................... peroxisome proliferator associated receptor
PPRE ............................................................ peroxisome proliferator response element
Pref1 .............................................................. preadipocyte factor 1
qPCR ............................................................. quantitative PCR
RNA ............................................................... ribonucleic acid
ROCK ........................................................... Rho kinase
RSB .............................................................. reticulocyte standard buffer
SDS .............................................................. sodium dodecyl sulfate
SEM ............................................................. standard error of mean
siRNA ............................................................ small interfering RNA
SRF ............................................................... serum response factor
SVC .........................................................................................stromal vascular cell
SVF ......................................................................................stromal vascular fraction
Taz .......................................................... transcriptional coactivator with PDZ-binding motif
TBP ..............................................................TATA binding protein
Tbx15 ..................................................................................T-Box 15
TEAD ..............................................................TEA domain family member
TGFβ ..............................................................transforming growth factor β
UCP-1 ..............................................................uncoupling protein 1
WAT ...............................................................................white adipose tissue
Yap ................................................................................Yes-associated protein
CHAPTER ONE
MATERIALS AND METHODS

Mice

Whole adipose tissue and primary stromal vascular cells were isolated from wild type C57Bl/6 mice and from MRTFA\textsuperscript{+/+} and MRTFA\textsuperscript{−/−} mice (1). All MRTFA\textsuperscript{+/+} and MRTFA\textsuperscript{−/−} mice also expressed Collagen-GFP and Smooth muscle actin-mCherry which was inbred by crossing MRTFA\textsuperscript{+/−} mice with mice with only Collagen-GFP transgene or both transgene (C57BL/6J background) (Kalajzic et al., 2005; Kalajzic et al., 2008). All mice were generously genotyped and raised by Dr. Chendi Li and Dr. Hejiao Bian (Boston University School of Medicine).

Isolating stromal vascular fraction cells (SVC)

Epididymal or inguinal fat pads were dissected from mice and minced into small pieces. Tissue was digested in 1-2 mg/mL Type 1 Collagenase (Worthington, CLS1) in DMEM containing 4.5 g/L glucose (Mediatech, Inc.) and 2% bovine serum albumin (American Bioanalytical) while shaking in a 37°C incubator. Digested tissue was filtered through a 100 μm mesh and centrifuged at 500xg for 10 min. Adipocytes and the underlying supernatant were then aspirated and the stromal vascular fraction pellet was resuspended in DMEM containing 4.5 g/L glucose (Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS) (Atlas) and plated on tissue culture-grade dishes. The resulting
stromal vascular cells (SVC) were cultured at 37°C in a 5% CO2 environment. At confluence, cells were induced to differentiate in DMEM supplemented with 10% FBS, 5 μM dexamethasone, 0.5 mM isobutylmethyl xanthine and 0.86 mM insulin. After two days of induction, cells were maintained in DMEM containing 10% FBS and 0.86 mM insulin until the time of harvest.

**Cell culture**

3T3-L1 cells were cultured in DMEM supplemented with 10% calf serum at 37 °C in a 5% CO2 environment. At confluence, cells were induced to differentiate in DMEM supplemented with 10% FBS, 5 μM dexamethasone, 0.5 mM isobutylmethyl xanthine and 0.86 mM insulin. Two days after induction, cells were maintained in DMEM containing 10% fetal bovine serum and 0.86 mM insulin until the time of harvest. C3H10T1/2 cells were cultured in DMEM supplemented with 10% FBS at 37 °C in a 5% CO2 environment. 3T3-L1 cells and C3H10T1/2 cells were purchased from the American Type Culture Collection (Manassas, VA). 3T3-L1 cells GR knockdown cells were a kind gift from the lab of Dr. Susan Fried, Boston University School of Medicine. They were created with lentivirus delivery of short hairpin RNA (shRNA) targeted to the murine GR or non-targeting shRNA (2).

**Treatments**

Dulbecco’s Modified Eagle’s Medium (DMEM) containing 4.5 g/L glucose (Mediatech, Inc; Herndon, VA) was supplemented with 10% fetal bovine serum
(Atlas Biologicals, Fort Collins, CO). Dexamethasone, indomethacin, 3-isobutylmethylxanthine and T3 were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant insulin and TRIzol reagents were purchased from ThermoFisher Scientific (Pittsburgh, PA). RNEasy Plus Kit was purchased from Qiagen (Hilden, Germany). Acti-Stain 488 Phalloidin was purchased from Cytoskeleton, Inc (Denver, CO). TGFβ1 was purchased from R&D Technologies (North Kingstown, RI). Halt protease inhibitor cocktail was purchased from Pierce (Rockford, IL). Small molecule compounds were purchased as listed: CCG1423 (Cayman Chemical Company, Ann Arbor, MI), Y27632 (Sigma Aldrich, St. Louis, MO), jasplakinolide (Tocris Bioscience, Bristol, UK), swinholide A (Enzo), latrunculin A (Cayman Chemical Company, Ann Arbor, MI)

**Transfections**

3T3-L1 cells were transfected with 10 nM small interfering RNA (siRNA) targeting gelsolin (Ambion, 4390771) or 10 nM Control siRNA-A (Santa Cruz Biotechnologies. sc-37007). They were also treated with 30 nM siRNA targeting MRTF-A (Santa Cruz Biotechnologies, sc-149641) or 30 nM eGFP siRNA (EGFP-S1 DS Positive Control, Integrated DNA Technologies). Transfections were performed using DeliverX siRNA transfection kit (Affymetrix) according to manufacturer’s instructions in subconfluent, proliferating 3T3-L1 cells.

3T3-L1 cells were transiently transfected with plasmid using Lipofectamine 3000® (Invitrogen). DNA-lipid complexes were produced per manufacturer’s instructions and cells were transfected for 24 hour before harvest or subsequent
Experimental steps. Cells were transfected with a plasmid containing full-length mouse alpha smooth muscle actin with an N-terminal mCherry tag (mCherry-Alpha-Actin2-C-18, 54973) and its empty vector (mCherry2-C1, 54563), both purchased from Addgene, and with a GFP-tagged MRTFa construct or its corresponding empty vector (provided by Dr. Matthew Layne, Boston University School of Medicine). All plasmids were purified using Pure Yield™ Plasmid Midiprep System (Promega, A2492) according to manufacturer’s instructions.

Quantitative RT-PCR RNA analysis

Total cellular RNA was isolated using TRIzol reagent (Life Technologies) or with the RNEasy Plus Kit (Qiagen). To generate cDNA, the High Capacity DNA Reverse Transcription Kit (Applied Biosystems) was utilized per the manufacturer’s instruction and with 0.5-1.0 ug of total cellular RNA. Quantitative RT-PCR was performed in 96-well plates using the Maxima SYBR Green 2x qPCR Master Mix (Thermo Fisher) in the ABI Prism 7300 sequence detector for the following program: initial denaturation at 95°C for ten minutes, followed by 40 PCR cycles, with each cycle consisting of a denaturation step for 15 seconds at 95°C, a 20 second annealing step at 60°C, and a 30 second elongation step for 30 seconds.

SYBR green fluorescence emissions were monitored at the end of each cycle. For each gene, mRNA expression was calculated relative to the expression of TATA binding protein (TBP) mRNA or cyclophilin A mRNA expression. Amplification of specific transcripts was confirmed by melting-curve
profiles (cooling the sample to 68°C and heating slowly to 95°C with measurement of fluorescence) at the end of each PCR. Primer sequences used for q-PCR analysis are listed in Table 1.

Immunofluorescence staining

Cells were fixed at room temperature in 3.75% formaldehyde for 10 minutes then rinsed with PBS briefly. Cells were then permeabilized in 0.2% TritonX-100/PBS for 5 min at room temperature. After a brief PBS rinse, cells were incubated in 3% BSA/PBS (American Bioanalytical) for 30-60 min at room temperature to block specific binding of antibodies before staining with primary antibodies in 0.02% Triton X-100/PBS overnight at 4°C. Primary antibodies used include mouse monoclonal antibodies to β-actin (1:200 dilution; A5441; Sigma-Aldrich), α-SMA (1:200 dilution; A5228; Sigma-Aldrich). Cells were rinsed three times at room temperature with 0.02% Triton X-100/PBS, 10 minutes per rinse. Immune complexes were detected with Alexa Fluor 568–conjugated goat anti-mouse IgG (1:200 dilution; A11031; Life Technologies) following 60 minute incubation at room temperature. Cells were rinsed three times at room temperature with 0.02% Triton X-100/PBS, 15 minutes per rinse. F-actin was stained with AlexaFluor 488–phalloidin (Molecular Probes, 1:200) in PBS, and G-actin was stained with Alexa Fluor 594–labelled DNaseI (Molecular Probes, 1:500) in PBS. After a brief rinse in distilled water, cells were mounted onto slides to be simultaneously counterstained with SlowFade Gold antifade reagent with DAPI (Invitrogen, S36938). Cells were observed with either a Leica SP5 point-
<table>
<thead>
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<th>Target</th>
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<td>MRTFa</td>
<td>AGGACCGAGGACTATTGAAACG</td>
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<td>CEBPβ</td>
<td>GCAAGAGCGCCGCCGAAG</td>
<td>GGCTCGGGCAGCTGCTT</td>
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<td>Acta2</td>
<td>GATCAGAAACAGGAATAAC</td>
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<td>Actb</td>
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<td>Actg1</td>
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<td>TBP</td>
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<td>GR</td>
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<td>CyclophilinA</td>
<td>TGGGAGCAGCAGACAGAGACAGA</td>
<td>TGCCGGAGTCGACAATGAT</td>
</tr>
</tbody>
</table>

**Table 1.** qPCR primer sequences
scan confocal microscope or a Nikon light phase microscope, Model TMS (120V, 50/60 Hz, 0.4 A, 50 W).

**SDS-PAGE and Western analysis**

To harvest protein lysates, cells were washed three times with cold PBS and scraped in lysis buffer containing 25 mM Tris (pH 7.4), 50 mM sodium chloride, 0.5% sodium deoxycholate, 2% NP-40, and 2% sodium dodecyl sulfate (SDS) Halt™ protease inhibitor cocktail (ThermoFischer). The lysates were incubated on ice for 20 min and centrifuged at 12000 x g for 10 min. The supernatant was collected and used for analysis.

For SDS-PAGE analysis, cell lysate extracts were quantified by the BCA method (Pierce™ BCA™ Protein Assay Kit, PI-23225, Thermo Scientific™) against a bovine serum albumin (BSA) protein standard curve. Equivalent amounts of protein (between 10 and 30 µg) were denatured in 5x sample buffer containing 10% SDS, 0.5 M DTT, 0.2 M Tris pH 6.8, 50% glycerol, and 0.5% bromophenol blue for 5 min at 95°C. The samples were then run on 10%/12% Mini-PROTEAN TGX Gels (Bio-Rad) or gels cast in-house (Ultra Pure ProtoGel, National Diagnostics) in a running buffer of 25 mM Tris, 192 mM glycine, and 0.1% SDS. Gels were run at a constant potential of 80 V for until the bromophenol blue ran off the gel. Once the samples were run on the gel, protein was transferred onto polyvinylidene fluoride membranes (Biorad) in transfer buffer containing 48 mM Tris pH 8.3, 39 mM glycine, 0.037% SDS, and 20% methanol.
Membranes were blocked in 5% milk-PBST for 1 hour at room temperature. Membranes were then washed 3 times in PBST and probed with primary antibodies (Table 2) overnight at 4°C. Following incubation with primary antibody, blots were washed 3 times in PBST, incubated with either anti-rabbit IgG-horseradish peroxidase (HRP) (Sigma, 1:5000), anti-mouse IgG-HRP (Sigma, 1:2000) or anti-goat IgG HRP (Santa Cruz, 1:10000) for 1 h at room temperature, washed 3 times in PBST, incubated with HYGLO ECL substrate kit (Denville) and developed using film or a Bio-Rad ChemiDoc imaging system. Protein quantification was measured using ImageJ software.

**CSK/RSB fractionation**

For all CSK/RSB fractionation, cells were grown to a confluent monolayer. For cells grown in 60 mm cell culture dishes, 300 μL of CSK and RSB buffers with Halt™ protease inhibitor cocktail (ThermoFischer) were used. Alternatively, cells grown in 6-well plates, 250 μL buffer was used per well. Cells were washed two or three times with cold PBS while sitting on ice. CSK buffer was added gently at the edges of the plate or well. CSK buffer was evenly distributed with gentle rocking then set on ice to rotate at ≤15 rpm for 2-15 minutes. Plates were then angled to allow for the collection of the buffer without disrupting the cell monolayer and briefly rinsed in one third the volume of the same buffer type. RSB was added to the plate and the remains of the monolayer were scraped. The scraped RSB fraction was vortexed vigorously. All fractions were then spun at 12000 x g for 10 min. The supernatant was collected and used for analysis.
<table>
<thead>
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<th>Protein specified</th>
<th>Manufacturer</th>
<th>Catalog number</th>
<th>Application (Concentration)</th>
</tr>
</thead>
<tbody>
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Table 2. Primary antibodies
Statistical analysis

Results are presented as mean ± SEM. Statistical differences were determined by a Student’s two-tailed t test with equal variance or paired. A P-value of ≤ 0.05 was considered statistically significant.
CHAPTER TWO
INTRODUCTION

The importance of adipose tissue

Adipose tissue is the most abundant tissue in the body, comprising 10-29% of body weight (3). It plays several roles in mammalian physiology, the most classic of those being energy storage. Additional roles include the regulation of glucose homeostasis, thermal insulation and hormone signaling (4). The function of adipose tissue varies by type of which there are three: brown adipose tissue (BAT), brown-in white (brite)/beige and white adipose tissue (WAT) (5-8). Below is a discussion of each type of adipose in detail. Because our studies predominately focus on WAT, it is introduced last to facilitate appropriate further discussions.

Brown adipose tissue

During the first years of human life, BAT is widely distributed across all areas of the human body (9). The regression of BAT post-infancy was thought to indicate an absence of BAT in human adults (10,11). In the early 2000’s, metabolically active BAT was identified in adults using F-fluorodeoxyglucose (F-FDG) absorption during positron emission tomography/computed tomography (PET/CT) (6,12). In adults, BAT is largely found in the supraclavicular region and neck but can also be found in the paravertebral region, mediastinum, para-aortic region and the cardiac apex (13). It should be noted that in rodents classical BAT
(i.e. interscapular) is present throughout life (14). These discoveries spurred renewed interest in brown fat biology, pushing our understanding of this unique form of adipose.

BAT catabolizes energy in the form of fatty acids and has been shown to be a powerful sink for the draining and oxidation of glucose and triglycerides from blood (14). The most common adipocyte in BAT, the brown adipocyte, can catabolize fatty acids in mass thanks to a high density of mitochondria, the site of β-oxidation (15-17). Mitochondrial abundance visibly darkens the coloring of these adipocytes, hence “brown”. These mitochondria express a protein unique to BAT, uncoupling protein-1 (UCP-1) (18). First isolated in 1980, UCP-1 is a proton transporter that uncouples electron transport from ATP synthesis (18,19). The energy that would otherwise go into ATP during electron transport is instead dissipated as heat, making BAT thermogenic (3,18,20).

BAT thermogenic activity is not constitutive but, instead must be activated. Cold exposure activates BAT to generate heat in order to maintain thermal homeostasis (21). Cold exposure leads to a pronounced increase in BAT mass via adipogenesis, an increase in total UCP-1 protein levels and an increase in mitochondrial density (22,23). Cold temperatures stimulate the sympathetic nervous system to release catecholamines, which act upon β-adrenergic receptors whose downstream effectors activate lipolysis, β-oxidation and drive UCP-1 expression (24-27). Because BAT is highly innervated and vascularized, thermogenesis can be rapidly activated and efficiently distribute heat released by
brown adipocytes for delivery to vital organs (24,28). Catecholamines may also be secreted locally by certain subtypes of macrophage and activate BAT (29).

It is generally considered that adipocytes originate from the mesoderm during embryonic development (14). BAT develops during fetal life, whereas WAT grows mainly after birth. It was once considered that brown and white adipocytes shared a common progenitor that diverges at later in development (30). A progenitor cell expressing the homeobox transcription factor Engrailed 1, though, has been shown to develop into three types of tissue, dermis, muscle, and (BAT), to imply that brown adipocytes share an origin with myocytes (31). Furthermore, intrascapular and perirenal brown adipocytes depots and skeletal muscle were found to share a progenitor expressing myogenic factor 5 (Myf5), a master transcriptional regulator of skeletal myogenesis (32).

Another proposed brown adipocyte progenitor marker is Early B-Cell Factor 2 (Ebf2), which was shown by Seale and colleagues to recruit the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) to brown fat-selective target genes (33). PPARγ and the PPAR family are later discussed in detail. It is also possible that brown adipocyte commitment has less to do with a specific progenitor profile and more to do with specific inductive signals. Tseng and colleagues isolated a progenitor cell capable of becoming WAT, BAT or skeletal muscle, where bone morphogenic protein 7 (BMP7) treatment selectively drives brown adipocyte commitment, a reminder of how mitogenic signaling helps regulate brown adipocyte fate commitment as well (34).
**Brite/beige adipose tissue**

In 1984, Ashwell and colleagues identified brown-like adipocytes expressing UCP-1 after cold acclimation in perigonadal WAT, which made up 6% of WAT pad weight (35). Countless studies since have shown brown-like, UCP-1+ islands can arise in specific WAT depots, islands which we now refer to as brite or beige adipocytes (8,36). Their presence can be induced by cold (35), PPARγ agonism (36) and β-adrenergic receptor stimulation (37). A growing list of agents including cardiac natriuretic peptides, fibroblast growth factor 21 (FGF21) and retinaldehyde dehydrogenase have been shown to induce brown adipocytes in both BAT and WAT (38). FGF21 increases UCP-1 expression in both subcutaneous WAT and BAT (39,40). Beige/brite UCP-1 protein levels increase to a thermogenically functional level making these adipocytes metabolically relevant (41). UCP-1 and T-box 15 (Tbx15), a member of the T-box family of homeodomain transcription factors are genes common to beige/brite and BAT, while the expression of other BAT-specific genes in brite/beige is mixed depending upon the approach utilized to activate and isolate beige/brite adipocytes (36,42,43).

The origins of beige/brite adipocytes are unclear. Despite shared morphological and biochemical characteristics with BAT, beige/brite adipocyte do not arise from the Myf5+ lineage of classical brown adipocytes and are thus their own form of adipose (44,45). Some speculate that beige/brite adipocytes arise from the transdifferentiation of preexisting white adipocytes (46,47). Alternatively,
beige/brite adipocytes could arise from the differentiation of an unidentified progenitor. It’s possible that these processes are not mutually exclusive. One study found that β3-adrenergic activation induces beige/brite adipocytes in epididymal WAT through the differentiation of precursors, whereas it induces transdifferentiation in inguinal WAT (48). Further complicating the delineation of adipocyte progenitors is the identification of Pax3, a transcription factor that functions upstream of Myf5, as a marker for conceivably all shades of adipocytes (49). Based on the lineage tracing study of Pax3, the authors proposed that brown, white and beige adipocytes have multiple and overlapping developmental origins.

Currently, there is increasing interest in the targeting of brown and beige/brite adipocytes as therapies for obesity and metabolic disorder (50). Correlations between these forms of adipose and obesity in have been made. When BAT is either transiently or permanently ablated via a transgenic toxigene, mice become obese even in the absence of overeating (51). BAT negatively correlates with BMI and basal metabolic indexes and is also more common among females than males (6). As a therapy, expanding these depots will not be sufficient since UCP-1 alone does not increase respiratory activity; there must be systemic activation (52). Activation has been mimicked by treating mice with β-adrenergic activators such as CL 316,243 (44,47,53). However, treatment of humans with β-AR agonists has not proven to be a viable option because of off-target effects, poor pharmacokinetic properties and poor oral bioavailability (54).
**White adipose tissue**

Compared to BAT and beige/brite adipose, WAT is abundant in the human body. WAT depots are largely either subcutaneous, i.e. in the buttocks, thighs and abdomen, or visceral, i.e. around the omentum, intestines and perirenal areas (55). Differences in fat cell size are depot-specific: in humans, the subcutaneous adipocytes are usually larger than adipocytes in visceral depots (56). WAT adiposity differences also arise between genders. Women are usually more obese as a group than men. Men tend to accumulate excess fat within the abdominal cavity and, consequently, often have significantly higher mean waist circumference (57). Intra-abdominal WAT is strongly associated with type II diabetes and with cardiovascular disease, while subcutaneous depots seem to protect against these conditions (58). Interestingly, expansion of subcutaneous WAT, what’s referred to as “pear-shape” in humans, does not carry as much risk as visceral WAT expansion (59). Such WAT differences could help explain gender-specific mortality rates.

White adipocyte progenitors are thought to reside within the stromal vasculature of adipose, the heterogeneous pool of all non-adipocyte cells (60). Within the stromal vasculature, they arise from the mural cell compartment, which generally refers to smooth muscle cells and pericytes (61). Adipocyte progenitor cell profiles vary by WAT depot. Progenitors from subcutaneous WAT differentiate more readily than those from visceral WAT, a consequence of their distinct needs in tissue culture conditions (62). General observations have been
made about white adipocyte progenitors despite this. Lineage tracing with the PPARγ promoter, specifically, has revealed that white adipocytes progenitors likely commit to the adipocyte lineage prenatally and continue to divide and maintain the progenitor pool in mature mice (61). In a fluorescence-activated cell sorting study, a CD24+ cell population in the stromal vasculature was found to proliferate and differentiate into functional adipocytes capable of reconstituting fully functional adipose in vivo (63).

**Obesity and metabolic disorder**

Excess adiposity, or obesity, is a condition quantified epidemiologically as a body mass index (BMI) greater than 30 kg/m2 (64). Obesity is an expansion particularly of WAT, which can lead to WAT can comprising >40% of body weight of in an obese human (65). For those under the age of 40, obesity produces few symptoms (66). With time, obesity frequently leads to metabolic diseases and increases the likelihood of myocardial infarction, stroke and several major cancers. Before the 1900’s, obesity was rare and malnourishment was common, driving up the incidence of infectious diseases such as tuberculosis (67). Today, roughly 35% of US adults are classified as obese (68).

As mentioned previously, adipose tissue is an energy storage site. Upon excess caloric intake, energy is largely stored by adipose tissue in the form of triglycerides (69). This biochemical process, described over 50 years ago by Kennedy and colleagues, converts glycerol-3-phosphate into triglycerides in a series of steps (70). The mobilization of adipose energy stores is achieved
through lipolysis, a sequence of actions by hydrolytic enzymes called lipases that work to convert triglycerides into free fatty acids, which fuels peripheral tissues upon metabolic demand (71).

When the storage limits of adipose tissue are exceeded, lipid begins to accumulate in peripheral tissues causing metabolic syndrome. Metabolic syndrome is a condition of intertwined metabolic abnormalities including obesity, dyslipidemia (elevated circulating lipids), hyperglycemia (elevated blood glucose), hyperinsulinemia (elevated circulating insulin), and insulin resistance (72). The causal relationship between adipose dysfunction and metabolic syndrome remains unclear. Perhaps two of the most popular theories revolve around low grade inflammation and adipokines, hormones produced largely by adipose (73). Chronic low grade inflammation, which often accompanies the accumulation of excess lipid in adipose tissue, increases the levels of pro-inflammatory cytokines that then go on to negatively affect systemic insulin sensitivity (74,75). Since the discovery of the first adipokine, leptin, adipose adopted the role of endocrine organ (76-78). The involvement of adipokines in metabolic syndrome centers around changes in their circulating levels.

With so many associated disorders, it is little wonder why obesity is a public health threat. An investigation into medical spending in 2008 found that 10% was associated with obesity and that obese patients incurred 41.5% higher costs relative to healthy weight counterparts (79). To address these issues
through public health initiatives will take time and massive social change. In the interim, we need to understand the biological origins of obesity in the hopes of identifying therapeutic targets.

**Hypertrophy and hyperplasia**

Obesity is the result of increases in adipocyte size (hypertrophy) and/or adipocyte number (hyperplasia). White adipocyte hypertrophy can occur in magnitudes that far exceed what other cell types can achieve. The adipocytes of healthy human adults can change volume 20-30 fold (80). Hypertrophy can disappear almost as quickly as it can appear. It is observed in both early onset and late onset obesity, while hyperplasia is only observed in early onset obesity (58). Hypertrophy has been cited to be a consequence of diet while hyperplasia is a consequence of genetics (81).

Because adipocytes are post-mitotic, it is accepted that hyperplasia in adipose tissue reflects *de novo* adipogenesis even in adults (82). However, fat cell numbers stay constant among lean and obese adults, even after weight loss, to suggest that adipocyte number is set during childhood (83). Instead, adipocytes experience high turnover in adults under what is likely a tightly controlled system (83). Given our limited progenitor knowledge, understanding hyperplasia and adipocyte renewal presents some challenges. What we do have is a fair understanding of the process by which progenitors become adipocytes.
Models of adipogenesis

Much of what we know about adipocyte formation, called adipogenesis, was characterized within cell culture models. These models fall into two classes: multipotent stem cell lines and preadipocyte cell lines. Of the multipotent stem cell lines, C3H10T1/2 cells are most widely known and used in the study of adipocyte, chondrocyte, osteoblast and myoblast lineage commitment. These cells were derived from C3H mouse embryos in the 1970’s and are sensitive to postconfluence inhibition of cell division (84). Additional multipotent stem cell lines include Balb/c 3T3, 1246, RCJ3.1 and CHEF/18 fibroblasts (85).

Preadipocyte cell lines are unipotent and will spontaneously differentiate into a morphological and biochemical profile fitting of white adipocytes after weeks of culture (86). The most widely used preadipocyte cell line is the 3T3-L1 cell line which was derived from murine embryonic Swiss 3T3 clones (87). While they are subconfluent and proliferative, 3T3-L1 cells are much like fibroblasts. They are homogenous and can be passaged rather indefinitely, two advantageous features of these cells.

Primary cell cultures, namely stromal vascular cells (SVC), are also used to study adipogenesis. SVC are derived from the fractionation of adipose tissue into an adipocyte pool and a non-adipocyte pool, also called the stromal vascular fraction (SVF), that each make up 50% of adipose tissue (88). SVF is a heterogenous pool of cells including endothelial cells, mural cells, monocytes, mast cells, macrophages, fibroblasts and preadipocytes (89). The process of
plating SVF to yield SVC reduces heterogeneity by selecting against the non-adherent hematopoietic stem pool (90). SVC display mutlipotency, capable of differentiating into osteoblasts, chondrocytes, myoblasts and adipocytes both *in vitro* and *in vivo* (91). When radiolabelled DNA was detected to progress from the SVF to adipocytes helped prove preadipocytes reside within adipose tissue (92). Cell sorting can reduce the heterogeneity of the SVF pool, but there is no consensus on reliable surface markers. Additionally, the propensity of SVC to go through adipogenesis varies by adipose depot. In rats, SVCs from subcutaneous WAT differentiate better than SVCs from visceral WAT (93). Despite that, the use of SVC is well accepted for investigation early steps leading to adipogenic commitment.

**The process of adipocyte formation**

For most cell culture models, adipogenesis proceeds in specific stages. First, the cells reach confluence to elicit a period of growth arrest. Studies by Pairault and Green demonstrated that growth arrest, not cell-cell contact is requisite for adipogenesis (94). Growth arrested cells can be maximally induced to become adipocytes by treatment with a mixture of “hormonal” inducers. These inducers include isobutylmethylxanthine (IBMX or MIX) which elevates intracellular cyclic AMP (cAMP), the synthetic glucocorticoid dexamethasone, insulin which interacts with insulin-like growth factor 1 (IGF-1) receptor and, lastly, fetal calf serum (85). Collectively, these agents are referred to as adipogenic cocktail or DMI and the period of time cells receive this treatment is referred to as
hormonal induction. Preadipocytes synchronously re-enter the cell cycle (G₀ to G₁) and undergo approximately two rounds of mitosis referred to as mitotic clonal expansion (95). The preadipocytes then exit the cell cycle and enter into an unusual growth arrest called Gᵈ. These now committed cells undergo terminal differentiation marked by production of lipid droplets as well as the induction of multiple metabolic programs characteristic of mature fat cells (96). The induction of these metabolic programs is regulated by two major transcription factors, PPAR and CCAAT/enhancer-binding proteins (C/EBP), which are discussed below.

**PPAR and adipogenesis**

PPAR is a member of the nuclear hormone receptor superfamily. Nuclear hormone receptors bind their respective ligand(s) with both high affinity and specificity. This superfamily includes receptors that bind steroid hormones, thyroid hormones, vitamin D, and retinoic acid among others (97). They recognize short DNA motifs, termed hormone response elements (HRE), usually located in the upstream promoter region of the target gene (98). The binding of the ligand-receptor complex to its respective HRE activates the transcription of specific genes, making nuclear hormone receptors ligand-dependent transcription factors (97).

In 1990, Issemann and Green identified a new member of the nuclear hormone receptor superfamily through cDNA cloning: PPAR (99). PPAR was found to be activated by certain hypolipidemic drugs, plasticizers, and herbicides.
that cause proliferation of peroxisomes, hence the name peroxisome proliferator activator receptor (99). There are three members of the PPAR family: PPARα, PPARγ and PPARδ. They contain highly conserved DNA-binding domains but more divergent ligand-binding domains (100). Although many tissues co-express PPAR isoforms, PPARγ is expressed at high levels in the adipose tissue and is considered the master regulator of adipogenesis (101,102).

PPARγ exists as two isoforms, PPARγ1 and PPARγ2. PPARγ1 is more broadly expressed whereas PPARγ2 expression is limited to adipose tissue (103). Although PPARγ2 genetic ablation can be compensated by PPARγ1, PPARγ2 is considered the key isoform for adipogenesis (103). In general, PPARγ overexpression in fibroblasts drives adipogenesis, as long as a synthetic ligand is available, while its absence essentially blocks adipogenesis (104,105). PPARγ has been shown to be required for the development of both white and brown adipocytes (106). When ligand bound, PPARγ heterodimerizes with the 9-cis-retinoic acid receptor (RXR) to bind a common consensus response element dubbed the PPAR response element (PPRE). PPREs consist of a direct repeat of two hexanucleotide recognition motif (PuGGTCA) spaced by one nucleotide (Direct repeat-1, DR1) and are common to hundreds of adipogenic genes thus allowing PPARγ to regulate adipogenesis (96,107). Several synthetic PPARγ ligands are known, like thiazolidinediones, but the naturally occurring ligand remains unknown.
C/EBP and adipogenesis

C/EBP is a family of ubiquitous leucine zipper transcription factors named after their ability to bind the CCAAT DNA motif (108). Its C-terminal region confers the ability to bind DNA and to form dimers, so called leucine zippers, with themselves or with other C/EBP members of the family (109). Since the cloning of the first C/EBP gene, C/EBPα, the C/EBP has grown to include C/EBPβ, C/EBPδ and C/EBPγ among others (108).

Several C/EBP isoforms play important roles in adipogenesis. The C/EBPβ and C/EBPδ isoforms work as a unit in the activation of C/EBPα (110). C/EBPα expression is sufficient to induce growth arrest and adipocyte differentiation (111,112). The genetic ablation of C/EBPα or a combined ablation of C/EBPβ and δ is sufficient to significantly attenuate adipogenesis (109). Like PPARγ, C/EBPα plays an important role in terminal differentiation (113). In fact, most PPARγ target genes are bound by both PPARγ and C/EBPα to suggest that they cooperatively orchestrate adipogenic gene expression (114). Together they form a positive feedback loop that maintains the expression of each in the mature adipocyte along with adipogenic genes (96).

ECM and adipogenesis

We have discussed the major internal events and players driving adipogenesis, but the extracellular environment also plays a role. The extracellular matrix (ECM) is the non-cellular component of tissue and among its functions it serves as scaffolding that holds cells together (115). Cells and ECM
profoundly influence one another and their interaction is influenced by ECM chemical composition, organization and mechanical properties (116). Some of the major fibrous-forming proteins of the ECM include collagens, elastin, fibronectin, laminins, glycoproteins, proteoglycans, and glycosaminoglycans (117,118).

An important subset of ECM is the basement membrane (BM). In adipose tissue, the BM is a thin layer of laminin, collagen, nidogen/entactic and proteoglycans that surrounds and supports adipocytes (119). During adipogenesis, preadipocytes locally degrade the BM and alternatively synthesize new BM as they mature (120). In culture, this begins with fibronectin that is then degraded and replaced early during differentiation with collagen types III, V, VI and lastly collagen I is laid down at late stage differentiation (120,121). Adipogenesis not only influences the ECM but is also influenced by the ECM. Treatment with ethyl-3,4-dihydroxybenzoate (EDHB), a specific inhibitor of collagen synthesis, reduces adipogenic potential as indicated by lowered triglyceride accumulation (122).

There is increasing evidence to suggest that adipocytes are mechanosensitive, much like hosts of other cell types (123-125). Preadipocytes cultured on surfaces that match the rigidity of adipose tissue were found to be more insulin responsive (126). Surfaces made rigid by fibronectin were also found to reduce lipogenic gene expression (127). Mechanical deformation of
human adipocytes was found to change gene expression, particularly inflammatory genes (128).

Mediating the communication of the ECM and cell are integrins, which bind to both ECM proteins and linkage to the actin cytoskeleton. The actin cytoskeleton will be discussed in detail in a later chapter. Integrins are found in clusters known as focal adhesions, which are typically approximately 2 µm wide and 3–10 µm long (129). ECM derived forces transmitted to integrins causes assembly and growth of focal adhesion complexes (130). This is regulated by the Rho-family of small GTPases and their downstream effectors, namely such as Rho-associated protein kinase (ROCK). The process of cell adhesion to the ECM activates Rho but in stably adhered cells Rho activity declines to a baseline (131).

**Rho/ROCK signaling**

The Rho family of GTPases (RhoA, Rac1 and Cdc42) has been primarily associated with cytoskeleton rearrangements, but we now appreciate the complex functions of this family (132). It can stimulate a variety of processes, including morphogenesis, migration, cell division and adhesion (133). The family of Rho proteins interacts with and activates downstream effector proteins when bound to GTP. When bound to GDP, Rho is inactive. The cycling between GTP/GDP is regulated by three sets of proteins, guanine nucleotide-exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide-dissociation inhibitors (GDIs) (133). All eukaryotes contain at least one Rho
GTPase. Microinjection of the Rho GTPase subfamily (RhoA/B/C), was found to induce actin stress fiber formation in Swiss 3T3 fibroblasts (134).

The first Rho downstream effector of Rho identified was ROCK (Rho-associated coiled-coil forming kinase) (Rho kinase/ROCK), it is also the mediator of Rho-induced actin cytoskeletal changes (135). The ROCK isoforms, ROCK1 and ROCK2, are serine/threonine kinases that assume open, active conformations upon interacting with GTP-bound Rho (136). These isoforms share a high amino acid sequence identity, yet the two kinases only share 65–70% sequence identity across their PH domains, which may account for their observed functional differences (137).

ROCK1 is the most ubiquitous and it phosphorylates and activates LIM kinase, which goes on to phosphorylate and inactivate cofilin (135). Cofilin is an actin binding protein that severs polymerized, filamentous actin (F-actin) (138). Thus Rho activity stops the severance of F-actin to indirectly promote actin stress fibers. This modulation of actin polymerization has profound effects on the cell; first and foremost, it causes cell spreading. In this pathway, then ECM stiffness imposes different degrees of cell spreading and, indirectly, cell fate (139). Before discussing how Rho/ROCK, actin and additional downstream effectors influence adipogenesis in particular, a discussion of actin and its dynamics is needed.

**Actin**

Actin is the largest organelle in the eukaryotic cell. Its functions are varied and essential. Actin is also the most abundant protein and is highly conserved
across species, belonging to a superfamily that includes sugar kinases, hexokinases, and Hsp70 proteins (140). The structure centers around two major domains, α and β. These domains interact very little and as a result create two clefts (141). The smaller cleft is lined with hydrophobic residues which interact well with actin binding proteins. The larger cleft binds ATP/ADP which is essential for actin dynamics.

**Actin dynamics**

Actin is very dynamic and readily responds to stimuli. Part of what allows actin to be so dynamic is its ability to polymerize and depolymerize simultaneously, a process called treadmilling. One end of the actin filament is the site of depolymerization, referred to as the minus or pointed end, and the other end is the site of polymerization, referred to as the plus or barbed end (142). ATP-bound G-actin joins the filament at the plus end. The ATP-bound state is more stable than the ADP-bound state so when ATP hydrolysis occurs, ADP-bound G-actin readily dissociates from F-actin from the minus end (141). These polar processes can occur simultaneously, mimicking a conveyor belt or treadmill.

Polymerization kinetics alone cannot account for the real-time dynamics known to occur in cells. It turns out that the dynamics of G-actin and F-actin is tightly regulated in time and space by a large number of signaling, scaffolding and actin-binding proteins (ABPs) (143). ABPs facilitate filament nucleation, elongation, severing, capping, and crosslinking and also G-actin sequestration.
By one estimate, 162 distinct and separate ABPs have been identified, not including their various isoforms (144). Some ABPs have known roles in adipogenesis. For example knockdown of Arp2/3, an ABP that promotes branching, inhibits adipocyte differentiation (145). Its expression is required for efficient GLUT4 trafficking in the mature adipocyte.

**The actin gene family**

What is broadly referred to as actin, in fact, a family of genes originally classified into two groups based upon their N-terminal amino acid sequences: the nonmuscle (also referred to as Class I or cytoplasmic) and the muscle (also referred to as Class II) isoforms (146,147). These isoforms show tissue specific enrichment and are considered functionally distinct (148). Despite this, they share no less than 93% amino acid sequence homology (149). As a general reference, Table 3 describes key attributes of all six mammalian actin isoforms (149,150). It should be noted that based on its amino acid sequence, γ-smooth a Class II based on its enrichment in smooth muscle tissues. To better describe their functional diversity, knockout and overexpression systems of Class I and Class II isoforms are discussed below.

The cytoplasmic isoforms, β-actin and γ-actin, are ubiquitous yet present in different proportions depending on cell type and stage of differentiation (146). These isoforms differ by only four biochemically similar amino acids, all of which are found in the 10 N-terminal residues (149). Despite their similarities, β-actin and γ-actin are functionally distinct. Genetic ablation of β-actin but not γ-actin
Table 3. Actin isoforms

A summary of key attributes for all six actin isoforms common to mammals

<table>
<thead>
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<th>Mouse phenotype</th>
<th>Gene symbol</th>
<th>Name</th>
<th>Class</th>
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<tbody>
<tr>
<td>Embryonic lethal</td>
<td>Actb</td>
<td>Beta actin, β-actin</td>
<td>Class I (Nonmuscle/Cytoplasmic)</td>
</tr>
<tr>
<td>Reduced viability; small size; progressive deafness</td>
<td>Actg1</td>
<td>Gamma actin, γ-actin</td>
<td>Class II (Muscle)</td>
</tr>
<tr>
<td>Viable; defects in vascular contractility and blood pressure regulation</td>
<td>Actg2</td>
<td>Smooth muscle gamma actin</td>
<td></td>
</tr>
<tr>
<td>Pups die by 9 days of age; exhibit muscle weakness</td>
<td>Acta2</td>
<td>Smooth muscle alpha actin, α-SMA</td>
<td></td>
</tr>
<tr>
<td>Embryonic/perinatal death; disorganized myofibrils</td>
<td>Acta1</td>
<td>Skeletal muscle alpha actin, β-skeletal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Actc1</td>
<td>Cardiac muscle, α-cardiac</td>
<td></td>
</tr>
</tbody>
</table>

N-terminal residue sequence (bold, underlined residues are common to the class members):

- Class I (Nonmuscle/Cytoplasmic): MDDIAALV/VDNNSGMC
- Class II (Muscle): MEEETIAALVDNNSGLC

Table rows are centered.
causes death during embryonic development, although γ-actin−/− mice die within 48 hours of birth due to respiratory failure and cannibalization by their parents (149,151). The β-actin isoform is enriched at the leading edge of migrating endothelial and 3T3 cells (152). In C2 myoblasts, overexpressed β-actin neatly integrates into the well-defined filamentous organization of actin (153). Similar observations have been made of γ-actin. Overall, the cytoplasmic isoforms are both important in cell growth, migration and survival but likely contribute differently to these processes. The muscle isoforms includes α-cardiac, α-skeletal, α-smooth muscle and γ-smooth muscle and gain their names from the tissue they are enriched within. Genetic ablation of the α-cardiac-actin is embryonic lethal owing to profound disorganization of cardiac myofibrils despite compensating expression by other muscle isoforms; genetic ablation of α-skeletal-actin is also embryonic lethal (149,154). Interestingly, genetic ablation of α-smooth muscle actin is not lethal (155). These mice are viable despite defects in vascular contractility and blood pressure homeostasis. Overall, actin isoform knockout and overexpression models across both classes demonstrate the functional diversity among the actin gene family.

Mechanotransduction and adipogenesis

The Rho/ROCK pathway has been implicated in the initiation and progression of adipogenesis. Dominant-negative RhoA commits mesenchymal stem cells to the adipocyte lineage but only under extracellular conditions that limit spreading and promote rounding (156). Overexpression of RhoA is sufficient
to block adipogenesis; Rho activity has also been observed to be downregulated during the course of adipogenesis (157). In mature, primary adipocytes both mechanical stimuli and hypertrophy activate Rho/ROCK to suggest that mechanical stretch of the plasma membrane of adipocytes in general promotes Rho/ROCK activity even at maturity (119). Rho/ROCK signaling influences cell fate and adipogenesis because it regulates the activity of transcriptional complexes, particularly Taz/Yap and MRTF/SRF.

**Taz/Yap**

The functional importance of Taz (transcriptional coactivator with PDZ-binding motif) and its paralog Yap (Yes-associated protein) is emphasized by the embryonic lethality of Yap knockout (158). Taz/Yap activity is largely regulated by the Hippo pathway. The Hippo pathway gains its name from the *Drosophila* counterpart of a primary protein kinase involved in the pathway, Hippo (Mst1/2) (159,160). The pathway is comprised of a series of phosphorylations: Mst1/2 phosphorylates Lats1/2, another pair of kinases that then phosphorylate Taz/Yap (161). Phosphorylated Taz/Yap is sequestered in the cytoplasm by its interaction with the protein 14-3-3 (162). In the absence of Lats1/2 kinase activity, unphosphorylated Taz/Yap can translocate to the nucleus where it influences transcriptional activity. Taz/Yap activity, overall, was identified as suppressing tissue overgrowth (163). Its exact transcriptional activity will be discussed following a discussion of how Taz/Yap is regulated by ECM, Rho/ROCK and actin.
As discussed previously, when ECM and integrins interact in focal adhesions and this activates Rho/ROCK which drives stress fiber formation. Picclo and colleagues demonstrated that Rho and the actin cytoskeleton are required to maintain nuclear Taz/Yap and their inhibition was sufficient to inhibit Taz/Yap transcriptional activity (164). Specifically, Taz/Yap is predominantly nuclear on stiff substrates, while soft substrates promote cytoplasmic Taz/Yap localization. Stress fiber formation, then, drives Taz/Yap nuclear localization and transcriptional activity. They went on to further show that Taz/Yap regulation serves as a link between ECM integrity and cellular function, in that applying mechanical stress through defined cell shapes and sizes controls Taz/Yap activity (165). Overall, these observations complement the observed role of Taz/Yap as major regulator of cell density control. Under low density, then, the observed cytoplasmic accumulation of Taz/Yap and the enrichment of F-actin are no longer coincidental but causal (166,167). It should be noted that in addition to Rho/ROCK signaling, Yap/Taz are regulated by Wnt signaling, G-protein coupled receptors and metabolic pathways such as mevalonate biosynthesis, energy stress, autophagy and glycolysis (168).

Active, nuclear Taz/Yap regulates transcription factors including TEADs, Pax3, MyoD, Runx2 and PPARγ (161). Given the previously discussed roles for both Pax3 and PPARγ in the development of adipocytes, Taz/Yap is undoubtedly involved in adipogenesis. Taz expression at both the protein and transcript levels is known to transiently decrease during 3T3-L1 adipogenesis due to
glucocorticoid receptor activity (169). Overexpression of Lats2 in 3T3-L1 cells has been shown to enhance both Taz/Yap phosphorylation and adipogenesis (170). Because Taz interacts with PPARγ to repress its downstream target gene expression, Taz knockdown in mesenchymal stromal cells is proadipogenic (171). Since Taz/Yap mediates levels of Wnt signaling, it could possibly influence adipogenesis by modulating the activity of the Wnt signaling pathway, which is known to modulate mesenchymal stem cell fate by the magnitude of its activity (96,161).

**MRTF/SRF**

Rho/ROCK activity has also been shown to modulate the activity of serum response factor (SRF), a MADS (Mcm1 and Arg80 in yeast, Agamous and Deficiens in plants, and SRF in animals)-domain containing transcription factor (172). SRF is essential for development since genetic ablation of SRF causes an absence of mesodermal cells in the embryo (173). SRF binds a palindromic CC(A/T)₆GG DNA sequence known as the CArG box in the promoters of hundreds of target genes (172). In addition to regulating ‘immediate early’ genes activated by mitogenic stimuli like serum, SRF also regulates tissue-specific genes (174). SRF regulates a large volume of gene subsets by interacting with over 60 different cofactors (175).

Myocardin related transcription family (MRTF) is one such family of SRF co-factors comprised of myocardin, MRTFa and MRTFb. Myocardin is exclusive to cardiac tissue and a subset of SMCs within the cardiovascular system while
MRTFa and MRTFb are ubiquitous and homologous (176). Treisman and colleagues were the first to describe the regulatory relationship between cytoplasmic G-actin and MRTF (177). MRTF binds G-actin under conditions where the G-actin pool is enriched relative to the F-actin pool. This sequesters MRTF in the cytoplasm. Actin polymerization allows MRTF to translocate to the nucleus and co-activate SRF. The Rho/ROCK-LIM kinase-cofilin axis activates MRTF/SRF transcriptional activity by driving actin polymerization (Figure 2.1).

Although global MRTFb deletion is embryonic lethal, global deletion of MRTFa is not lethal (178). Instead, MRTFa deletion causes defects in the mammary myoepithelial cells which inhibits proper offspring nursing (178). The WAT of MRTFa\textsuperscript{-/-} mice have been shown to contain more brite/beige adipocytes (179). Expression of functionally inactive MRTF was sufficient to enhance adipogenesis in C3H10T1/2 cells (179). In mouse dedifferentiated fat cells, MRTFa was found to antagonize PPAR\textgamma transcripational activity during! early adipogenesis (180).

**Significance and research goals**

Hyperplasia contributes to the expansion of adipose tissue during obesity. Targeting hyperplasia as a treatment for obesity is becoming possible as we better understand both the progenitors and commitment steps leading to hyperplasia. Mechanotransduction signaling pathways like MRTF/SRF and Taz/Yap are known regulators of cell fate. As a regulator of these pathways, actin cytoskeleton dynamics plays a major role in cell fate, and in particular adipogenic
Figure 2.1 The regulation of MRTF/SRF activity

A schematic representation depicting the relationship between Rho/ROCK, actin and MRTF/SRF. In the active, GTP-bound state Rho/ROCK promotes the polymerization of actin and MRTF can locate to the nucleus and co-activate SRF. When the G-actin pool outweighs the F-actin pool, MRTF binds G-actin which sequesters MRTF in the cytoplasm, inhibiting MRTF/SRF activity.
commitment. Although actin is a functionally diverse gene family, the contributions of the individual actin isoforms are not well understood in the context of adipogenesis.

The goal of this study was to describe the expression and dynamics of the actin gene family during adipogenesis. We initiated this study with a new approach to biochemically assessing actin dynamics. Current commercially available biochemical assays of F-actin/G-actin were not rigorous enough for our anticipated use so we developed a fractionation method that is easier to perform, takes less time to complete and is more reliable to interpret. We then used this method to explore a potential mechanism for the loss of F-actin during adipogenesis. Our observations prompted us to further investigate actin dynamic, which revealed interesting patterns in the loss of F-actin and G-actin. Lastly, we identified key differences in the expression and regulation of actin isoforms, particularly α-SMA. This study adds to a growing body of evidence suggesting major roles for actin and its distinct isoforms in cell fate determination.
CHAPTER THREE

A METHOD FOR THE BIOCHEMICAL ASSESSMENT OF ACTIN DYNAMICS

Abstract

The state of the actin cytoskeleton helps dictate the overall state of the cell, making the measurement of actin dynamics important in the study of cell biology. Current biochemical methods like G-actin/F-actin fractionation kits provide little in the way of normalizing controls and are cumbersome to conduct. The goal of this set of studies was to establish a simple, time effective, cost effective, yet accurate alternative to the commercial kits. Because G-actin and F-actin have different solubilities, we hypothesized that brief and sequential incubations of cells in distinct buffers would be sufficient to fractionate these actin pools. We demonstrated that cytoskeleton buffer (CSK) and reticulocyte standard buffer (RSB) can efficiently solubilize G-actin and F-actin, respectively. Furthermore, we were able to identify additional proteins that make for reliable fraction markers and normalizing controls. We also demonstrate that this fractionation method can indeed be used to describe actin dynamics in a number of adherent, fibroblastic cell types.

Introduction

The actin cytoskeleton contributes immensely to the fate and function of cells at all stages of development. Stem cell fate can be altered by mechanical properties such as tensile strain, extracellular stiffness and oscillatory flow (181).
As part of the scaffolding of the cell, the actin cytoskeleton mediates the communication of these mechanical properties of the cell. This mediating role of actin has been corroborated by studies where pharmacologically and mechanically alterations were shown to effect mesenchymal and embryonic stem cell fate (156,182). Even when cell fate is established, the actin cytoskeleton continues to play a role in the identity and functionality of the cell. For example, the actin cytoskeleton undergoes dramatic and requisite reorganization during the process of epithelial-mesenchymal transition (EMT) (183). Actin and its dynamics therefore contribute immensely to cell fate and to overall cell biology.

Unlike most other proteins, actin can be studied independent of the cell. In vitro studies have yielded great insight into the mechanics of actin dynamics. Without the context of the cell, though, we can only understand how actin dynamics works but not why or when it works. Actin within the cell has been studied using electron or fluorescence microscopy. Aside from spatially describing actin organization, these technologies also have the ability to describe the degree of polymerization. The equipment and training required can present a steep barrier to their use.

Alternatively, fraction kits can be used to biochemically describe actin dynamics. Cytoskeleton Inc’s G-actin/F-actin In Vivo Assay Kit (Cytoskeleton, Inc., Denver, CO) is one such kit currently on the market. The assay revolves around the use of two extraction buffers: one for the extraction of G-actin, the other for F-actin. In between, the extracts must be centrifuged at high speed for
nearly an hour to pellet cellular debris. The fractions can be probed for actin protein expression through Western blot analysis. Based on our experience with the kit, the cellular debris pellet contains significant amounts of actin (data not included). To quantifiably analyze the data, the sum of the optical densities from the G-actin and F-actin fractions is treated as total cellular actin (184). F-actin and G-actin are then expressed as a percent of that total.

Total actin protein levels can change as readily as actin polymerization. Actin gene expression is regulated by a transcriptional complex, MRTF/SRF. This complex can only drive actin expression when MRTF is within the nucleus. Whether MRTF is in the nucleus is determined by the actin cytoskeleton since G-actin binds and sequesters MRTF in the cytoplasm (185). Provoking actin depolymerization or polymerization, be that through drugs or other experimental parameters, will ultimately change actin protein levels. In the published use of the G-actin/F-actin In Vivo Assay Kit (Cytoskeleton, Inc., Denver, CO) actin protein levels are not normalized (186-188). Although some publications include a separate total protein control, it is not used as an external point for normalization and may not, in fact, be sufficient (189). Unaddressed changes in total actin protein levels may be falsely interpreted as changes in polymerization. The fractions cannot be reliably probed for additional markers, eliminating the possibility for internal normalization.

We have identified a need for a reliable and simple approach to biochemically describe F-actin/G-actin dynamics. Fractionation-based
approaches are ideal because they can be analyzed via Western blot, an accessible biochemical technique. We then needed to determine what property(ies) would distinguish the fractions. Based on their biochemical properties, cytoskeletal proteins present interesting patterns of solubility. Intermediate filament polymers are more insoluble than microfilament and microtubule polymers (190). Very little soluble intermediate filament is present in most cells whereas only half of the actin in a cell is found to be insoluble (191). Microtubules are unique in that they are temperature sensitive, depolymerizing into tubulin dimers in the cold and spontaneously reassembling in the warmth (192,193).

These distinctions in the solubility of cytoskeletal proteins present an opportunity to study F-actin and G-actin in a novel way. We hypothesized that G-actin and tubulin dimers can be isolated independent of both F-actin and intermediate filaments by using buffers with distinct stringencies. We identified CSK as a candidate for G-actin solubilization. First described in a 1963 study of polyribosomes, CSK has since been used to remove cytoplasmic proteins in a broad range of cells including fibroblasts (194,195). CSK contains Triton X-100, a non-ionic, non-denaturing detergent that does not disrupt native protein interactions. It interacts with hydrophobic regions of the plasma membrane and renders it porous. Sawasdichai and colleagues developed a method for the use of CSK in the visualization of nuclear structures (196). In their study, K562 cells incubated in CSK were void of cytoplasmic proteins yet contained DAPI-positive
nuclei. Incubating cells in cold CSK should gently pierce the plasma membrane to solubilize G-actin and newly depolymerized microtubules.

By virtue of its biochemistry, CSK does not solubilized the cytoskeletal framework, consisting largely of polymerized actin and intermediate filaments. In astrocytes, the intermediate filament protein vimentin is resistant to extraction by low salt, non-ionic detergent buffers like CSK (197). With the plasma membrane porous, we hypothesized that the low ionic strength detergent buffer RSB could solubilize the remaining framework proteins. RSB contains deoxycholate, an anionic detergent that effectively disrupts and dissociates protein interactions. RSB has long been used to fractionate cells down to the nuclear matrix. In fact, those same studies helped illustrate the close association between the nuclear matrix and intermediate filaments (198,199).

Overall, we hypothesized that CSK and RSB buffers, when used sequentially, would solubilize G-actin and F-actin separately. Tubulin and intermediate filament proteins would serve as control markers for CSK and RSB fractions, respectively. The general approach for this procedure is visually described in Figure 3.1. The proceeding is a series of experiments looking into the use of CSK/RSB as a novel method for actin fractionation. This system was tested against pharmacological modulators of actin in fibroblastic, adherent cells with great success. CSK/RSB fractionation is a simple method that allows for normalization of the actin levels for a more accurate description of actin dynamics.
Figure 3.1 CSK/RSB fractionation method

The CSK/RSB fractionation method for the isolation of G-actin and F-actin involves two major steps. First, adherent cells are incubated in cold CSK for a few minutes. CSK is gently removed from the plate and reserved. Under these conditions, CSK should now contain G-actin and depolymerized microtubules. Second, an equal volume of RSB is added to the cells. After a short incubation, RSB can be gently reserved or used to scrape the adherent cell monolayer. Either approach will extract F-actin and intermediate filament.

**CSK Buffer**
- 100 mM NaCl
- 10 mM PIPES pH 6.8
- 3 mM MgCl₂
- 1 mM EGTA
- 0.5% Triton X-100

**RSB Buffer**
- 10 mM NaCl
- 3 mM MgCl₂
- 10 mM Tris HCl pH 7.4
- 1% Deoxycholate
- 0.5% Tween 40
Results

Short CSK/RSB incubations extract detectable amounts of actin

We wanted to first gauge the feasibility of detecting actin after only brief CSK incubation. Data from the literature suggest that CSK incubations can, within the span of minutes, solubilize cytoplasmic proteins. Ornelles and colleagues extracted cytoplasmic proteins from adherent cells after just 3 minutes of incubation (195). Gilbert and colleagues measured protein loss associated with incubation in a Triton-based buffer and found protein loss plateau at the 5 minute mark for CCL-146 cells, a gerbil-derived fibroblast (200). With these time frames in mind, we incubated cells in CSK buffer for 2, 4 or 10 minutes. All CSK incubations were followed with scraping of the remaining cell monolayer in an equal volume of RSB. Actin was detected at all three incubation times, with minimal variability in CSK and RSB fraction enrichment with time (Figure 3.2). Cyclophilin A, named after its association with the immunosuppressive drug cyclosporin A, is known to be steadily expressed by fibroblastic cells including 3T3-L1 cells (201). This ubiquitous intracellular protein was detected in the CSK fraction following incubation at all time points. The intermediate filament protein vimentin was detected in the RSB fraction at all time points as well. Vimentin is the most widely distributed of intermediate filament proteins and is known to be expressed by fibroblasts (191). The data confirms that actin, along with cytoplasmic proteins are extracted by CSK incubation within
Figure 3.2 Short CSK incubations extract actin

Western blot analysis of fractions collected from 3T3-L1 cells incubated in CSK for the designated amount of time then scraped in RSB. Both the fractionation and the gel loading were conducted under equal volumes. Approximately 10% of fraction volume was loaded per lane.
minutes. CSK incubation does not solubilize a subset of proteins including vimentin.

**CSK extracts cytoplasmic G-actin without disrupting the F-actin network of 10T1/2 cells**

Although the literature indicates that CSK is only stringent enough to solubilize cytoplasmic proteins, we were not certain as to whether CSK incubation would selectively isolates cytoplasmic G-actin and not F-actin. To determine the specificity of CSK, we immunostained C3H10T1/2 mesenchymal (10T1/2) cells, for F-actin and G-actin following incubation in either PBS or CSK (Figure 3.3). F-actin was found across the cell in a vast network after both PBS and CSK incubation (Figure 3.3a). G-actin staining after CSK incubation to suggest G-actin was much lower than after PBS (Figure 3.3a). Interestingly, G-actin colocalized with the nucleus in cells whether they were incubated with CSK or PBS.

In the literature, F-actin and G-actin enrichment is analyzed as a ratio, F-actin/G-actin. We conducted a similar analysis based on the fluorescence intensity of these immunostained 10T1/2 cells (Figure 3.3b). We found that F-actin/G-actin significantly increased as a result of CSK incubation. Increases in F-actin/G-actin indicate a decrease in G-actin, an increase in F-actin or a combination therein. To determine which accounted for the observed increase F-actin/G-actin, we took the same data set and analyzed F-actin and G-actin
A.

Figure 3.3 CSK extracts cytoplasmic G-actin without disrupting the F-actin network of 10T1/2 cells

(A) 10T1/2 cells were incubated in cold PBS or cold CSK for 10 minutes on ice and fixed for immunostaining. Cells were incubated with fluorescently-tagged phalloidin (F-actin) and DNase (G-actin) and counterstained with DAPI (Nucleus). Images were taken at 20x magnification.

(B) Relative fold change in F-actin/G-actin under PBS incubation and under CSK incubation. Quantified total fluorescence from the red channel (G-actin) was normalized to the quantified total fluorescence from the green channel (F-actin). Graphs show fold change ± SEM (n=4); * p≤0.05.

(C) Fold change in F-actin and in G-actin after CSK incubation relative to PBS. Quantified total fluorescence from the green channel (F-actin) and from the red channel (G-actin) was normalized against total blue channel fluorescence (Nucleus) to account for cell number variation. Graphs show fold change ± SEM (n=4); ** p≤0.005, *** p≤0.0005.
separately as a function of incubation (Figure 3.3c). This analysis revealed that the G-actin pool experienced a greater loss than the F-actin pool following CSK incubation. A loss in G-actin, therefore, accounts for the increased F-actin/G-actin. We can conclude that CSK indeed targets cytoplasmic G-actin, with mild effects on the F-actin network and no effect on overall cell morphology.

**CSK extracts cytoplasmic G-actin without disrupting the F-actin network of 3T3-L1 cells**

To determine whether cytoplasmic G-actin is solubilized by CSK in other cell lines, we recapitulated the experiments from Figure 3.3 in 3T3-L1 cells (Figure 3.4). As with 10T1/2 cells, CSK incubation had a negligible effect on F-actin distribution and cell morphology (Figure 3.4a). Although significant loss in G-actin staining was observed, a subset was found to again co-localize with the nucleus following CSK incubation. When we analyzed F-actin/G-actin we found a small yet significant increase in F-actin/G-actin (Figure 3.4b). When the data were re-analyzed, we found that the increase in F-actin/G-actin was largely the result of cytoplasmic G-actin loss (Figure 3.4c). We concluded that CSK selectively solubilizes cytoplasmic G-actin in 3T3-L1 cells as well.

**CSK and RSB buffers efficiently solubilize cytoskeletal proteins**

With the specificity of CSK for cytoplasmic G-actin established, we sought to determine how efficient CSK and RSB incubations are at solubilizing target proteins. To test CSK/RSB efficiency, we performed a sequence of CSK and
Figure 3.4 CSK extracts cytoplasmic G-actin without disrupting the F-actin network of 3T3-L1 cells

(A) 3T3-L1 cells were incubated in cold PBS or cold CSK on ice for 10 minutes and fixed for immunostaining. Cells were incubated with fluorescently-tagged phalloidin (F-actin) and DNase (G-actin) and counterstained with DAPI (Nucleus). Images were taken at 20x magnification.

(B) Relative fold change in F-actin/G-actin under PBS incubation and under CSK incubation. Quantified total fluorescence from the red channel (G-actin) was normalized to the quantified total fluorescence from the green channel (F-actin). Graphs show fold change ± SEM (n=3); * p≤0.05.

(C) Fold change in F-actin and in G-actin after CSK incubation relative to PBS. Quantified total fluorescence from the green channel (F-actin) and from the red channel (G-actin) was normalized against total blue channel fluorescence (Nucleus) to account for cell number variation. Graphs show fold change ± SEM (n=3).
RSB incubations described in Figure 3.5a. We hypothesized that one round of CSK incubation (CSK1) would be sufficient to isolate all soluble, cytoplasmic proteins including, but not limited to, G-actin and tubulin, specifically α-tubulin. This isoform of tubulin is found in a dimeric complex with β-tubulin when microtubules are depolymerized (191). One round of CSK incubation solubilized 61% of total β-actin protein with a second round isolating only an additional 4% (Figure 3.5b and 3.5d). Both α-tubulin and cyclophilin A were detected in CSK1 and not CSK2 to confirm CSK efficiently solubilized cytoplasmic proteins.

RSB was designed to solubilize otherwise insoluble protein which we hypothesized would include F-actin and intermediate filament proteins. RSB incubation isolated vimentin and a subset of actin not solubilized by two rounds of CSK incubation (Figure 3.5b). The actin isolated by RSB1 represented 31% of total β-actin detected (Figure 3.5d). A second incubation with RSB isolated a negligible amount of vimentin and not actin. The data suggests RSB both specifically and efficiently solubilizes insoluble cellular protein including a subset of actin.

To confirm that the actin solubilized by RSB was F-actin, we also treated cells with swinholide A during the CSK/RSB series. Swinholide A is a marine toxin known to sever F-actin and stabilize actin dimers as a means of inhibiting polymerization (202). Swinholide A treatment did not alter total actin protein levels (Figure 3.5c). Instead, it caused the enrichment of β-actin in RSB1.
Figure 3.5 CSK and RSB buffers efficiently solubilize cytoskeletal proteins

(A) A scheme describing the experiment conducted

(B) Western blot analysis of sequential CSK and RSB fractions obtained from 3T3-L1 cells treated with DMSO or 0.1 μM swinholide A for 2 hours.

(C) Western blot analysis of total protein obtained from 3T3-L1 cells treated with DMSO or 0.1 μM swinholide A for 2 hours.

(D) Graph showing the relative distribution of β-actin protein levels detected across sequential CSK/RSB incubations of 3T3-L1 cells. Bars represent the percent distribution across fractions for DMSO and 0.1 μM swinholide A treatments, ± SEM (n=2).
decrease 17% and increased β-actin enrichment of CSK1 by 13% (Figure 3.5 b and 3.5d). Overall, we can conclude that both CSK and RSB efficiently at solubilize G-actin and F-actin, respectively. Actin was the only cytoskeletal protein solubilized by both CSK and RSB whereas tubulin was only solubilized by CSK and vimentin by RSB (Figure 3.5b). The data also illustrates that tubulin can be used as a marker for CSK extraction and vimentin as a marker for RSB.

**CSK/RSB fractionation discerns actin dynamics across cell types**

We wanted to determine the versatility of these buffers as a fractionation method where we incubate the cells once in CSK buffer and then harvest the remaining cell layer in RSB buffer, a process we refer to as CSK/RSB fractionation. We performed CSK/RSB fractionation on 3T3-L1 cells (Figure 3.6a), 10T1/2 cells (Figure 3.6b) and epididymal adipose-derived SVCs (Figure 3.6c). We treated the cells with swinholide A, jasplakinolide or their vehicle, DMSO (Figure 3.6). Jasplakinolide is a cyclo-depsipeptide isolated from the marine sponge that binds and stabilizes F-actin (203,204).

We identified both similarities and differences by cell type following CSK/RSB fractionation. For all cell types, vimentin was isolated by RSB buffer while cyclophilin A and/or α-tubulin were isolated by CSK buffer. The enrichment of cyclophilin A in CSK matched the enrichment of cyclophilin A in total protein lanes to suggest that CSK solubilizes all cyclophilin A. Vimentin, on the other hand, displayed variable enrichment between RSB and total. In SVC and 10T1/2 cells, vimentin is more enriched in total relative to RSB; 3T3-L1 showed the
**Figure 3.6 CSK/RSB fractionation discerns actin dynamics**

Western blot analysis of total protein and CSK/RSB fractions isolated from sets of 3T3-L1 cells (A), C3H10T1/2 cells (B) and epiWAT SVC (C) treated with DMSO, 0.1 μM swinholide A and 0.5 μM jasplakinolide for 30-45 minutes, equal volume loaded per lane.
opposite. The data suggest that some vimentin may be lost during CSK/RSB fractionation.

We probed most of the fractions for two actin isoforms, α-SMA and β-actin. In SVC and 3T3-L1 cells, α-SMA was enriched in the RSB fraction under vehicle conditions (Figure 3.6a and 3.6c). In 10T1/2 cells, both α-SMA and β-actin were evenly distributed between CSK and RSB under vehicle conditions (Figure 3.6b). In SVC, both isoforms showed significant enrichment in the RSB fraction (Figure 3.6c). The α-SMA isolated from 3T3-L1 cells was enriched in the RSB fraction (Figure 3.6a). Swinholide A treatment caused a discernable shift in enrichment from RSB to CSK for the α-SMA in 3T3-L1 cells and the β-actin in SVC. Neither swinholide A nor jasplakinolide caused significant changes in 10T1/2 cells under the conditions tested (Figure 3.6b). Overall, CSK/RSB fractionation can discern soluble and insoluble proteins in at least three types of cells. It also has the potential to trace both actin polymerization and depolymerization.

**Actin depolymerization can be quantified and normalized under CSK/RSB fractionation**

We hypothesized that CSK/RSB fractionation would allow for an accurate quantification of actin dynamics because the fractionation allows for the identification of additional markers that can serve as normalizing controls. Based upon its consistent presence in total and CSK fractions, cyclophilin A was chosen a normalizing control for CSK. RNA polymerase II was chosen as a normalizing
control for RSB for similar reasons. To test our hypothesis, we treated cells with latrunculin A, a *Red Sea Sponge*-derived drug that binds G-actin to provoke actin depolymerization (205). Latrunculin A depolymerization was sufficient to reduce α-SMA and γ-actin enrichment in the RSB and augment them in the CSK fraction of SVCs (Figure 3.7a). Similar results were obtained in 3T3-L1 cells (Figure 3.7b).

We quantified the depolymerization of actin by latrunculin A in 3T3-L1 cells with and without normalizing the data (Figure 3.7c and 3.7d). Regardless of normalization, the baseline difference in β-actin enrichment between CSK and RSB was found to be statistically significant. The increase in CSK enrichment as a result of latrunculin A was 3.9-fold while the decrease in RSB was 5.8-fold. When normalized against cyclophilin A, CSK enrichment increased 4.8-fold. When normalized against RNA polymerase II, RSB enrichment decreased 6.0-fold. Normalization of the data, overall, brought the magnitudes of CSK and RSB change closer. It also made the increase in CSK statistically significant.

Interestingly, CSK enrichment of α-SMA did not quantifiably change as a result of latrunculin A treatment. Given the error bars associated with the α-SMA CSK values, we hypothesize that α-SMA does in fact significantly change CSK enrichment following latrunculin. Based on the dynamics of β-actin, we can conclude that CSK/RSB allows for the rigorous quantification of actin depolymerization.
Figure 3.7 Actin depolymerization can be quantified and normalized under CSK/RSB fractionation

(A,B) Western blot analyses of total protein and CSK/RSB fractions isolated from SVC (A) or 3T3-L1 (B) treated with DMSO (-) or 2 uM latrunculin A (+) for 10 minutes, equal volume loaded per lane.

(C,D) Graphs of the relative distribution of α-SMA and β-actin protein detected in the CSK/RSB fractions of 3T3-L1 cells treated with DMSO (-) or 2 uM latrunculin A (+) for 10 minutes. In one set, the data was not normalized (C). In the other (D), CSK data was normalized against cyclophilin A and RSB data was normalized against RNA polymerase, ± SEM (n=3); * p≤0.05, **p≤0.005.
Discussion

In order to study actin dynamics at the level of the isoform, we needed a method to separate G-actin from F-actin simply and reliably. To address this need, we adopted the use of buffers to selectively solubilize these distinct pools of actin. Based on their detergent content, we hypothesized that the set of buffers known as CSK and RSB would effectively solubilize G-actin and F-actin, respectively.

Given the proposed use of CSK first in a sequence of incubations (Figure 3.1), we were concerned that CSK would not discern G-actin from F-actin. Immunostaining confirmed the ability of CSK to solubilize G-actin without dismantling the F-actin network (Figures 3.3a and 3.4a). When measured, a loss in F-actin was identified did not sufficient decrease F-actin/G-actin below PBS levels which indicated that the magnitude of G-actin loss exceeded the loss of F-actin (Figure 3.3b and 3.4b). Because F-actin can exist in numerous lengths, the solubilization of short actin filaments by CSK is well within the realm of possibility. The immunofluorescence images assured us, though, that the bulk of the stress fiber network comprised of long actin filaments was not solubilized by CSK and thus we concluded that CSK readily solubilizes G-actin and not F-actin stress fibers.

By incorporating Western blot analysis into the CSK/RSB protocol, two distinct compartments can be validated through the detection of tubulin and vimentin. We demonstrated the efficiency with which CSK and RSB isolated α-
tubulin and vimentin, respectively, justifying the use of these proteins as fraction control markers (Figure 3.5b). We also observed non-cytoskeletal proteins solubilized by these buffers. CSK consistently solubilized cyclophilin A and RSB, consistently solubilized RNA polymerase II. Actin was the only protein we detected consistently in both CSK and RSB fractions. The ratios of actin CSK/RSB enrichment varied by actin isoform, actin-targeting treatments and also cell type (Figure 3.6). Given the consistency of the control markers, we hypothesize that the variability observed reflects genuine differences among the systems tested. We are currently characterizing these variations with both dosage and time curves.

Although we primarily relied upon actin depolymerization to prove the efficacy of CSK/RSB fractionation, we did detect actin polymerization using CSK/RSB (Figure 3.6). Jasplakinolide did not notably alter actin polymerization in 10T1/2 cells or SVCs. It reduced actin enrichment in the CSK of 3T3-L1 cells, but it did not cause a discernable enrichment of actin in the RSB (Figure 3.6a). Fibroblasts are known to be enriched with stress fibers so it likely that CSK/RSB fractionation is not sensitive enough to detect additions to an already rich stress fiber network. Again, careful dosage and time curves with jasplakinolide will help address whether CSK/RSB can discern actin polymerization as well as it can discern actin depolymerization.

The ability to detect proteins beyond actin sets CSK/RSB fractionation apart from comparable approaches. Like any other protein, actin expression is
subject to change in response to stimuli. Comparable approaches offer no way to normalize for either the differences in actin protein content or variations in cell number. The ability to normalize actin protein to internal controls makes CSK/RSB fractionation a more reliable approach for biochemically assessing actin dynamics. Normalizing β-actin protein levels enhanced our analysis of latrunculin A treatment (Figure 3.7d). Overall, the data confirms that CSK/RSB fractionation is a rapid and reliable method for the assessment of actin dynamics in cultured cells.
CHAPTER FOUR

THE ROLE OF GELSOLIN IN ADIPOGENIC ACTIN REORGANIZATION

Abstract

During adipogenesis, the actin cytoskeleton converts from F-actin rich network to an intracellular meshwork largely localized by the plasma membrane. In general, actin reorganization is orchestrated by actin binding proteins. Little is known about the role of actin binding proteins during adipogenesis. We identified that the actin binding protein gelsolin is transiently induced by dexamethasone. The absence of gelsolin did not alter the induction of the master regulator of adipogenesis, PPARγ. Using CSK/RSB fractionation, we also discover that gelsolin is not required for the reduction of F-actin that occurs during early adipogenesis.

Introduction

The morphology of the mature adipocyte is exquisitely unique. It is spherical with, typically, a unilocular lipid droplet constituting roughly 90% of the cell's volume. Morphology is dictated in part by the cytoskeleton, a meshwork of intermediate filaments, microtubules and microfilaments. This meshwork is very dynamics and is actively involved in the spatial organization of cell content, regulating cell shape and movement along with connecting the cell to its external environment (206). During adipogenesis, the cytoskeleton is known to change dramatically.
The intermediate filament cytoskeleton of the 3T3-L1 cell is comprised of only vimentin. Vimentin organization begins with a wavy, fibrillar organization but later migrates to surround nascent lipid droplets in cage-like monolayers (207). Perturbation of vimentin intermediate filaments with nocodazole was shown to reduce the formation of lipid droplets (208). Vimentin has not been associated with any perturbations in adipose tissue, in fact, mice lacking vimentin develop and reproduce without an obvious phenotype (209).

During 3T3-L1 adipogenesis, the microtubule cytoskeleton converges to cage-like structures around nascent lipid droplets in early adipogenesis and are later found perinuclear (210). Synthesis of the monomeric unit of microtubules, tubulin, decreases during adipogenesis (182). While microtubule disruption with nocodazole is pro-adipogenic, it likely does so by promoting cell rounding. Cell shape has been shown to regulate adipogenesis by modulating Rho/ROCK and actin cytoskeletal tension (156).

Unlike the microtubule and intermediate filament networks, the microfilament network does not change to encompass lipid droplets. Instead, the actin cytoskeleton changes from a long, stress fiber organization to a series of shorter filaments found adjacent to the plasma membrane referred to as cortical actin (211). The dramatic changes that occur to the actin cytoskeleton during adipogenesis have long been observed. The mechanisms orchestrating these changes have not been clearly established and roles for actin-binding protein that modulate actin organization seem likely. We hypothesized that, through its F-
actin severing activity, gelsolin plays an important role in the reorganization of actin required for adipogenesis.

Gelsolin is the flagship member of an actin-binding protein superfamily (212). Gelsolin acquired its name from the unique effect it was found to have on rabbit lung macrophage cytoplasmic extracts. The extracts were shown to go through cycles of gelation and solation that could be manipulated with the calcium chelator EGTA. An actin-binding protein was identified as the driver of the observed cycles and as such acquired the name "gel-sol-in", readily severing F-actin in response to calcium levels (213). Most members of the gelsolin superfamily sever F-actin and include proteins such as CapG and adseverin. All members contain at least three gelsolin homology (GH) domains in their structures (214). The GH domain is similar in structure to the signature domain of the cofilin /actin depolymerizing factor (ADF) superfamily of F-actin severing proteins.

Gelsolin is a large actin-binding protein comprised of six GH domains (215). Its N-terminal region houses the capacity to sever F-actin. The actual binding to F-actin is performed by the second and third domains. When bound, gelsolin caps F-actin which stops polymerization and allows the first domain to disrupt actin-actin hydrophobic interactions leading to F-actin severance (215). Severance activity is regulated by the conformation of the C-terminal region which responds to changes in calcium, phosphatidylinositol phosphates and pH (212). F-actin severance can only occur when C-terminal region is in an open
conformation, exposing the N-terminal region. An open conformation is achieved under calcium concentrations high enough for the C-terminal region to bind. A closed conformation occurs in the presence of phosphatidylinositol phosphates, in particular PIP$_2$, and/or $>6.5$ pH which causes gelsolin to dissociate from F-actin and promote actin polymerization (216).

The gelsolin gene is expressed as two variants, a secreted, plasma variant and a cytoplasmic variant. The gelsolin originally described by Helen Yin and colleagues is the cytoplasmic variant. A plasma protein previously known as brevin was found to share identity with gelsolin and was renamed plasma gelsolin (217). Plasma gelsolin has an additional 25 amino acid residues at its N-terminus and is thought to be produced largely by muscle cells (218). Like cytoplasmic gelsolin, plasma gelsolin can sever F-actin. It is thought to play an important role in the clearance of actin released during cell death (215). Interestingly, plasma gelsolin mutation has been tied to a rare hereditary amyloid polyneuropathy (219).

The absence of gelsolin has major physiological effects. In mice, gelsolin expression is first detected at E11.5. In the mature mouse, it is predominately expressed in the heart and lungs and is otherwise considered to be ubiquitously expressed (220). Gelsolin deletion in BALB/c and C57/bl mouse backgrounds causes lethality at E17 and postnatal day 12, respectively (216). In mice not of the BALB/c and C57/bl backgrounds, the absence of gelsolin does not alter longevity. These gelsolin deficient mice instead experience notable defects in
hemostatic response, fibroblast function and platelet activation. Their platelets contain more actin filaments and cannot effectively spread upon activation, a response that is critical in wound healing (221). Dermal fibroblast size is five-fold larger due to stress fiber formation which impacts cell motility. Without gelsolin, osteoclasts cannot assemble podosomes well, which inhibits bone remodeling (222).

The expression and relevance of gelsolin in adipose tissue has not been investigated. Published microarray data available on GeoDatasets indicate an enrichment of gelsolin in murine WAT as compared to BAT and in mature white and brown adipocytes as compared to their primary progenitor cells (32,223). Gelsolin has been studied in 3T3-L1 cells by Kawaji and colleagues (224). They found that in the absence of gelsolin, 3T3-L1 adipocytes expressed less C/EBPα and PPARγ transcript and accumulated fewer lipid droplets. The authors concluded that gelsolin is required for adipogenesis and likely plays a role in mitotic clonal expansion. Interestingly, gelsolin’s role in mitotic clonal expansion or adipogenesis overall were not connected back to gelsolin’s F-actin severing capacity.

Overall, the literature on gelsolin identifies its F-actin severing capacity to play an important role in its physiological relevance. In this chapter, we set out to determine whether gelsolin is indeed expressed by adipocytes and to better characterize its role within adipose tissue. We explore the possibility that gelsolin
is required during adipogenesis for the reorganization of F-actin stress fibers in 3T3-L1 cells.

**Results**

*Gelsolin is expressed by white adipocytes*

To determine the biological relevance of gelsolin in context of adipogenesis, we first examined whether gelsolin is expressed by adipose tissue. Gene expression was analyzed in depots that represent white, brite/beige and brown adipose tissue, respectively: epididymal white adipose tissue (epiWAT), inguinal adipose tissue (ingWAT) and intrascapular brown adipose tissue (iBAT) (Figure 4.1). The epididymal adipose depot is visceral, located next to the epididymis. The inguinal adipose depot (ingWAT) is subcutaneous, running along the hindlimb and, although considered a white adipose depot, expresses a subset of brite/beige adipose markers. The intrascapular brown adipose depot is found as two lobes between the shoulder blades.

RNA isolated from the adipose tissue of 6 week old mice was analyzed for gelsolin gene expression by qPCR, as shown in Figure 4.1a. Gene expression of the adipocyte marker PPARγ is comparable across adipose depots. As expected, adiponectin gene expression was enriched in the epiWAT and ingWAT. CideA, a gene that is predominately expressed in brown fat, is significantly enriched in BAT (225). These markers collectively confirmed the white and brown identities of the depots isolated. Like adiponectin, gelsolin gene expression was enriched
Figure 4.1 Gelsolin is expressed by white adipocytes

(A) Relative mRNA levels of select genes in the epiWAT, ingWAT and iBAT of 6 week old mice as analyzed by qPCR. Graphs show fold difference ± SEM (n=3); * p≤0.05, ** p≤0.005.

(B) Relative mRNA levels of select genes in the adipocyte fraction (AF) and stromal vascular fraction (SVF) isolated from the epiWAT of 7 week old mice as analyzed by qPCR. Graphs show fold difference ± SEM (n=3); * p≤0.05.
in white adipose with a trending enrichment in epiWAT over ingWAT. There is significantly less gelsolin gene expression in iBAT.

Because adipose tissue is comprised of both adipocyte and non-adipocyte cells, we wanted to determine which subset expresses gelsolin. Whole epiWAT from 7 week old mice was digested into their respective adipocyte (AF) and stromal vascular fractions (SVF) and analyzed by qPCR (Figure 4.1b). As anticipated, PPARγ gene expression was enriched in the AF and the preadipocyte marker Pref1 in the SVF. Gelsolin was detected in both fractions suggesting it is ubiquitous. The data collectively suggests that gelsolin is expressed by adipose tissue, in particular WAT.

**Gelsolin is transiently induced during early adipogenesis**

To determine when adipocytes acquire gelsolin expression, we measured gelsolin expression in 3T3-L1 cells (Figure 4.2). Western blot analysis of gelsolin protein revealed doublet bands detectable on Days 0 and 2 (Figure 4.2a). The higher molecular weight band is likely the secreted gelsolin isoform, although this has not yet been confirmed with recombinant protein. The lower molecular weight band, gelsolin, was expressed at every time point examined. At the transcript level, there was no significant difference in gelsolin expression between Day 0 and Day 10 despite a slight drop in gelsolin protein on Day 10 (Figure 4.2). Gelsolin expression both at the protein (Figure 4.2a) and transcript (Figure 4.2b) level revealed transient induction on Day 2. No similar
Figure 4.2 Gelsolin is transiently induced during early adipogenesis

(A) Western blot analysis of total cellular protein extracted from Day 2 3T3-L1 cells treated with the labeled combination(s) of adipogenic cocktail for 48 hours.

(B) Relative mRNA levels of gelsolin from Day 2 3T3-L1 cells treated with the labeled combination(s) of adipogenic cocktail for 48 hours as analyzed by qPCR. Graphs show fold difference ± SEM (n=3); *) p≤0.05, **) p≤0.005, ***) p≤0.0005
induction of the F-actin severing protein cofilin or of destrin, also known as actin depolymerizing factor (ADF), was observed (Figure 4.2b).

**Dexamethasone induces gelsolin during hormonal induction**

The transient induction of gelsolin on Day 2 coincides with the period of hormonal induction. Interestingly, a component of the adipogenic cocktail, dexamethasone, has been shown to induce gelsolin in L929 cells (226). In those cells, gelsolin induction also corresponded with diminished F-actin content and changes in L929 morphology. We hypothesized that gelsolin’s transient induction was a direct result of the dexamethasone in the adipogenic cocktail. Dexamethasone is a synthetic glucocorticoid receptor ligand that has been shown to induce the expression of glucocorticoid-induced leucine zipper (GILZ) (227). We found that dexamethasone significantly induced GILZ protein (Figure 3A) and transcript (Figure 4.3). Gelsolin protein (Figure 4.3a) and transcript (Figure 4.3b) levels were also induced by the inclusion of dexamethasone in the adipogenic cocktail.

**Gelsolin induction is largely regulated by glucocorticoid receptor**

Since dexamethasone induced both GILZ and gelsolin during 3T3-L1 hormonal induction, we hypothesized that glucocorticoid receptor activity was responsible for the observed induction of gelsolin as it is for GILZ. To test this, we closely examined the contributions of both dexamethasone and glucocorticoid receptor to the observed gelsolin induction. First, we modified the adipogenic
Figure 4.3 Dexamethasone induces gelsolin during hormonal induction

(A) Western blot analysis of total cellular protein extracted from Day 2 3T3-L1 cells treated with the labeled combination(s) of adipogenic cocktail for 48 hours.

(B) Relative mRNA levels of gelsolin in Day 2 3T3-L1 cells treated with the labeled combination(s) of adipogenic cocktail for 48 hours as analyzed by RT-PCR. Graphs show fold difference ± SEM (n=3); * p≤0.05, ** p≤0.005, ***p≤0.0005
Figure 4.4 Gelsolin induction is largely regulated by dexamethasone and glucocorticoid receptor

(A) Western blot analysis of total cellular protein extracted from Day 2 3T3-L1 cells treated with increasing concentrations of dexamethasone within the adipogenic cocktail for 48 hours.

(B) Relative mRNA levels of gelsolin in Day 2 3T3-L1 cells treated with increasing concentrations of dexamethasone within the adipogenic cocktail for 48 hours. Graphs show fold difference ± SEM (n=3); * p≤0.05, ***p≤0.0005.

(C) Relative mRNA levels of gelsolin in Day 2 3T3-L1 cells stably expressing glucocorticoid receptor (GR) shRNA as compared to control (Ctrl) shRNA. Graphs show fold difference ± SEM (n=4); * p≤0.05, ***p≤0.0005.
cocktail to contain IBMX, insulin and a range of dexamethasone concentrations (Figure 4.4). The complete omission of adipogenic cocktail (no MI) and the omission of dexamethasone from the cocktail (MI+ethanol) were included as controls.

GILZ protein (Figure 4.4a) experienced a linear, dose-dependent increase in response to dexamethasone through the maximal dose of 20 μM (Figure 4.4a). GILZ transcript levels rose 20 fold under 0.5 μM dexamethasone and showed no dose dependence (Figure 4.4b). As a negative control for the effect of dexamethasone, we also measured the effect it had on Pref1 transcript level. Dexamethasone has been shown to repress the transcription of Pref1 in 3T3-L1 cells (228). We found that all doses of dexamethasone tested downregulated Pref1 equally (Figure 4.4b).

Gelsolin protein was again induced by dexamethasone but not in a dose-dependent pattern. Instead, gelsolin protein was significantly induced under cocktail containing 0.5 μM dexamethasone, a dose that is half the concentration normally used in adipogenic cocktail (Figure 4.4a). Treatment with 1 μM dexamethasone in the adipogenic cocktail achieved a similar level of gelsolin protein induction. Under 10 μM and 20 μM dexamethasone in the adipogenic cocktail, gelsolin protein levels were equal to the level seen under the exclusion of dexamethasone in the adipogenic cocktail (Figure 4.4a). At the transcript level, dexamethasone concentrations ranging from 0.5 μM to 10 μM in the adipogenic cocktail achieved significant yet equal gelsolin induction. Gelsolin was also
induced by cocktail containing 20 μM dexamethasone, but the induction was less than those experienced under lower dexamethasone concentrations in the adipogenic cocktail (Figure 4.4b). The data confirm that dexamethasone induces the expression of both GILZ and gelsolin, albeit with distinct dose dependencies.

With a role for dexamethasone confirmed, we next tested the role of glucocorticoid receptor in the transient induction of gelsolin. We differentiated 3T3-L1 cells expressing shRNA targeting glucocorticoid receptor gene expression and compared the results to 3T3-L1 cells expressing a control shRNA construct. The cell lines were analyzed on Day 2 where shRNA achieved a significant knockdown of glucocorticoid receptor gene expression, roughly 70% (Figure 4.4c). Because glucocorticoid receptor activity enhances adipogenesis, its knock down significantly reduced PPARγ transcript levels (229). As hypothesized, gelsolin transcript levels were was significantly reduced in the absence of glucocorticoid receptor as were the transcript levels for the known target gene GILZ (Figure 4.4c). Collectively, the data confirms that transient induction of gelsolin during 3T3-L1 differentiation is dependent upon dexamethasone-driven glucocorticoid receptor activity since the absence of either dexamethasone or glucocorticoid receptor blunts gelsolin induction.

**Gelsolin co-localizes with F-actin during early adipogenesis**

It is well established that F-actin stress fibers are lost and cortical actin is gained during adipogenesis. What distinguishes these two networks is the density and length of F-actin. Stress fibers are long actin polymers that can span
the length of a cell. Stress fibers must be shortened and spatially relocated to become the cortical actin signature to the mature adipocytes. Given gelsolin’s known F-actin severing activity and its induction during early adipogenesis, we hypothesized that gelsolin induction coincides with a loss in F-actin. We examined the relationship between gelsolin’s presence on Day 2 and the loss of F-actin by conducting an immunofluorescence time course (Figure 4.5).

Significant amounts of F-actin were lost between Day 0 and Day 2 as indicated by the diminished intensity of phalloidin-associated staining (Figure 4.5, top left panels). On both Day 0 and Day 2, gelsolin was found to strongly colocalize with F-actin (Figure 4.5, top right panels). By Day 8, both F-actin and gelsolin were largely absent relative to Day 0. The data shows that gelsolin associates with F-actin during early adipogenesis.

**Gelsolin is not required for adipogenic actin reorganization**

To determine whether the loss of F-actin observed in Figure 4.5 is a result of F-actin severing by gelsolin, we knocked down gelsolin expression with siRNA and observed the impact on both actin dynamics and adipogenic potential (Figure 4.6). Gelsolin-specific siRNA significantly downregulated gelsolin transcript levels at all concentrations tested (Figure 4.6a). The 10 nM siRNA concentration achieved the greatest difference in gelsolin transcript levels and, as such, was used in all subsequent gelsolin knock down experiments. Gelsolin protein levels were also significantly downregulated by 10 nM gelsolin siRNA.
Figure 4.5 Gelsolin co-localizes with F-actin during early adipogenesis

Immunofluorescence time course study of 3T3-L1 cells. Cells were stained with ActiStain-488 phalloidin, incubated in gelsolin antibody (Gelsolin), and counterstained with DAPI (Nuclei) two days post-confluence (Day 0), two days into adipogenic cocktail treatment (Day 2) and six days after removal of the adipogenic cocktail (Day 8). Images were taken at 20x magnification.
Figure 4.6 Gelsolin is not required for adipogenic actin reorganization

(A) Relative mRNA levels of gelsolin in 3T3-L1 cells following transfection with 1 nM, 10 nM or 30 nM control or gelsolin. Graphs show fold difference ± SEM (n=4); ** p≤0.005, ***p≤0.0005.

(B) Western blot analysis of total protein extracted from Day 2 3T3-L1’s transfected with 10 nM gelsolin or control siRNA and treated with variations of the adipogenic cocktail. Gels were loaded with 25 ug protein per lane.

(C) Western blot analysis of Day 2 3T3-L1’s transfected with 10 nM gelsolin or 10 nM control siRNA. Cells were incubated in CSK buffer for 10 minutes then scraped with an equal volume of RSB buffer. Fractions were loaded equal volume per lane.
transfection (Figure 4.6b and 4.6c). Interestingly, gelsolin protein isolated by the CSK/RSB fractionation method was detected in the CSK fraction which is in agreement with the fact that gelsolin is a cytoplasmic protein that non-covalently interacts with F-actin (Figure 4.6b).

To describe the polymerized state of actin in the absence of gelsolin, we performed CSK/RSB fractionations on gelsolin siRNA transfected, Day 2 3T3-L1 cells (Figure 4.6b). We hypothesized that the absence of gelsolin would allow for more F-actin in the cell, detected as an enrichment of actin in the RSB fraction. Under adipogenic cocktail vehicle, the absence of gelsolin did not cause an enrichment of actin isoforms in the RSB fraction (Figure 4.6b). As expected, adipogenic cocktail reduced αSMA protein levels significantly and β-actin protein levels minimally; actin was also reduced in both CSK and RSB fractions. The loss of actin in both CSK and RSB fractions was no different between control and gelsolin siRNA transfected cells (Figure 4.6b). Based on the data, we can conclude that gelsolin is not required for the observed loss of F-actin during adipogenesis.

The literature suggests gelsolin is required for adipogenesis (224,230). When we examined PPARγ expression in Day 2 3T3-L1 cells, we found no difference in PPARγ protein levels in the absence of gelsolin (Figure 4.6c). Our data suggest that, in fact, gelsolin is not required for adipogenesis.
**Discussion**

Based on the actin severing abilities of gelsolin and its known expression in 3T3-L1 cells, we wanted to determine whether gelsolin contributed to adipogenic actin reorganization. Before examining gelsolin more closely in the 3T3-L1 cells, we first wanted to determine whether gelsolin could be biologically relevant in adipose tissue. RNA analysis revealed that gelsolin is in fact expressed by adipose tissue and is likely, particularly WAT (Figure 4.1a). Adipose tissue had not been previously examined for gelsolin expression relative to other tissues and organs. We did not conducted measurements in non-adipose tissues but it would be interesting to see how gelsolin expression adipose tissue compares to tissues known to be enriched with gelsolin, namely the heart and lungs.

We found gelsolin did not experience significant net changes in expression from the preadipocyte stage to the adipocyte stage (Figure 4.2). Instead, gelsolin was observed to transiently induce concomitantly with hormonal induction. We went on to identify dexamethasone as the key trigger behind gelsolin transient induction (Figure 4.3). To confirm this, we examined the contributions of dexamethasone’s target, glucocorticoid receptor. In the absence of glucocorticoid receptor, hormonal induction no longer induced gelsolin (Figure 4.4c). The transient induction of gelsolin during adipogenesis adds to the growing list of glucocorticoid receptor functions.
We hypothesized that major role for gelsolin in its actin severing ability. Based on our CSK/RSB fractionation results, we concluded that no such role existed (Figure 4.6b). With so many actin binding proteins regulating the organization of the actin cytoskeleton, gelsolin is unlikely to be solely responsible for adipogenic actin dynamics. Although we ultimately found no role for gelsolin in adipogenic actin reorganization, we showed that dexamethasone/glucocorticoid receptor regulate a transient induction of gelsolin during 3T3-L1 adipogenesis. This may help further insight into the role(s) for gelsolin in adipogenesis.

Despite its induction by dexamethasone, we found that gelsolin is likely not required for adipogenesis. Kawaji and colleagues used shRNA to knockdown gelsolin roughly 70% in 3T3-L1 cells (224). They found decreased lipid content and PPARγ, Ap2 and C/EBPα transcript levels to suggest gelsolin is required for adipogenesis. With siRNA, we knocked down gelsolin >70% (Figure 4.6) yet no significant change in PPARγ protein levels were observed (Figure 4.6b and 4.6c) prompting us to conclude that gelsolin is not required for adipogenesis. Gelsolin could still play important adipogenic roles, though. The Kawaji study provided convincing data to suggest gelsolin plays an important role in mitotic clonal expansion (MCE). Glucocorticoids are required for MCE and we showed that glucocorticoids also induce gelsolin expression during the period of time coinciding with MCE (Figure 4.4) (229), supporting the theory of a role for gelsolin in MCE.
Gelsolin aside, this set of studies revealed interesting information on the nature of actin dynamics. Typically, the depolymerization of F-actin generates G-actin. We observed a decrease in F-actin following hormonal induction that was not accompanied by an increase in G-actin (Figure 4.6b). This is likely not an isoform specific phenomenon since α-SMA and β-actin were equally affected. Although the spatial organization of actin is regulated by actin binding proteins, it is also regulated by the abundance of actin itself. With this in mind, we studied the expression and distribution of actin isoforms to identify regulatory mechanisms beyond actin binding proteins.
CHAPTER FIVE
THE ISOFORM-SPECIFIC DYNAMICS OF ACTIN DURING EARLY ADIPOGENESIS

Abstract

It is well known that the actin cytoskeleton is downregulated and reorganized during the course of adipogenesis. These events are important for the progression of adipogenesis, but actin itself has largely been studied as a single protein when in fact it exists as a gene family. In this study, we assessed the dynamics and expression of each actin isoform during adipogenesis. We found that the α-SMA isoform experiences very dramatic changes. We also explore the mechanisms by which actin expression is downregulated, where we identify roles for both the transcriptional complex MRTF/SRF and the cAMP signaling pathway during the early stages of adipogenesis.

Introduction

Three decades ago, actin protein levels were discovered to be dramatically downregulated during the course of 3T3 adipogenesis (231). In addition, actin protein biosynthesis decreases approximately 5-fold (232). These studies describing the downregulation of actin reflect a change in pan actin. The extent to which each isoform experiences these changes is currently unknown. Furthermore, the actin isoform profiles of preadipocyte and adipocytes is currently unknown.
Actin isoform profiles can reveal much about the identity and function of a cell. For example, epithelial cells undergoing EMT experience changes in β-actin and γ-actin organization and an increase in smooth muscle actin expression as they acquire a fibroblast-like phenotype (233,234). Changes in actin reorganization, concomitant to the changes in actin expression, facilitate the migratory capacity acquired during EMT (235). In myofibroblasts, α-SMA can comprise 20% of the actin expressed yet even a mild increase in α-SMA expression is sufficient to augment stress fiber content and contractility (236,237). In the absence of α-SMA, though, myofibroblast phenotype is largely unaffected; other actin isoforms are augmented to yield no net change in total actin expression (238). These studies collectively demonstrate the complexity and importance of actin isoform expression and distribution.

In this study, we examine the adipogenic actin isoform cytoskeleton profile. Using our CSK/RSB fractionation method, we were able to track changes in actin expression and polymerization in parallel. This is significant considering that both parameters are known to change during adipogenesis yet the balance between them has not been analyzed, especially not at the isoform level. A general schematic timeline of the changes in actin during adipogenesis is depicted in Figure 5.1. Through these efforts, we characterized an interesting net loss of both G-actin and F-actin during early adipogenesis. We also identified the α-SMA isoform as one that is disproportionately downregulated and reorganized.
Figure 5.1 Actin changes as a function of adipogenesis

During 3T3-L1 differentiation, the cells must first reach confluence (Day -2). Two days post-confluence (Day 0), the cells are given an adipogenic cocktail for 48 hours. The cocktail is removed and within 24-48 hours lipid droplets begin to be visible. Actin biosynthesis is known to decrease although the starting point for this is unclear. The actin cytoskeleton is also reorganized from stress fiber rich network to a cortical network.
The MRTF/SRF transcriptional complex is a major regulator of actin cytoskeleton-specific genes. The 26 SRF-regulated actin cytoskeleton genes can be grouped into three categories by function: structural (e.g. actin), effectors of actin turnover (e.g. cofillin 1) and regulators of actin dynamics (e.g. Arp2/3, talin 1) (178,239). Recent studies into the role of MRTF in adipocyte biology have identified it as a major contributor to lineage determination, in particular beige adipocyte determination (179,240). Its inactivation, facilitated in part by G-actin-driven sequestration in the cytoplasm, is required for adipogenesis (180).

MRTF/SRF inactivation occurs during early adipogenesis and coincides with the loss in actin expression. Our studies identified a role for MRTF/SRF in the regulation of actin isoform expression, particularly of α-SMA. The early loss of α-SMA appears to be important for the progression of adipogenesis since a second round of actin attenuation, this time by cyclic AMP, occurs during hormonal induction. Hormonal induction also caused significant reductions in the expression of the cytoplasmic isoforms. Through these studies, we have established an adipogenic actin profile that will enhance our understanding of the mechanisms of adipogenic commitment that will ultimately enable us to better understand the expansion of adipose tissue and obesity.

Results

α-SMA is enriched in the stromal vasculature of white adipose tissue

We began our investigation of actin by first examining the expression levels of all relevant actin isoforms in adipose depots (Figure 5.2). We observed
1.8-fold of β-actin in epiWAT relative to iBAT. Enrichment of α-SMA in the WAT exceeded the enrichment by β-actin. It was found to be present 15-fold more in ingWAT and 26-fold more in epiWAT relative to iBAT (Figure 5.2b). Transcript levels of the cytoplasmic isoforms, coded as their gene names Actb and Actg1, show very similar patterns of expression as was observed at the protein level (Figure 5.2c). Although not statistically significant, β-actin and γ-actin trended towards enrichment in epiWAT. Transcript levels of α-SMA, coded as its gene name Acta2, showed 8-fold enrichment in epiWAT. The transcript pattern of α-SMA was similar to that of adiponection (AdipoQ), an adipokine produced largely by WAT. As anticipated, the expression of the BAT-specific gene UCP-1 was detected exclusively in iBAT (Figure 5.2c).

Adipose tissue is comprised of a milieu of cells and since actin is ubiquitous, the presence of actin is attributable to both adipocyte and non-adipocytes. To parse the actin contribution of each, we measured actin isoform transcript levels in the adipocyte fraction (AF) and stromal vascular fraction (SVF) of epiWAT. Because actin synthesis is downregulated during adipogenesis, we hypothesized that actin expression would be limited to the SVF. Transcript level differences for α-SMA in AF and SVF were enough to suggest that α-SMA expression is largely isolated to the SV; repeating the experiment with more mice will minimize error and reveal statistical significance (Figure 5.2d). Based upon these adipose tissue studies, we can conclude that
Figure 5.2 α-SMA is enriched in the stromal vasculature of white adipose tissue

(A) Western blot analysis of whole adipose tissue harvested from four 6 week old, non-littermate mice. Total protein was isolated from intrascapular brown (iBAT), inguinal white (ingWAT) and epididymal white (WAT) adipose depots. Equal protein was loaded per lane.

(B) Densiometric analysis of Western blot (A) with totals normalized to cyclophilin A. Graph shows fold difference ± SEM (n=4); * p≤0.05, ** p≤0.05.

(C) Relative mRNA levels of select genes expressed by iBAT, ingWAT and epiWAT of 7 week old mice analyzed by qPCR. Gene expression was normalized to TBP. Graphs show fold difference ± SEM (n=3); * p≤0.05, **p≤0.005, ***p≤0.0005.

(D) Relative mRNA levels of select genes in the adipocyte fraction (AF) and stromal vascular fraction (SVF) isolated from the epiWAT of 7 week old mice as analyzed by qPCR. Gene expression was normalized to TBP. Graphs show fold difference ± SEM (n=3); * p≤0.05.
overall actin expression is enriched in WAT, with α-SMA expression particularly biased towards WAT SVF.

**White adipocyte precursor cells have extensive β-actin and α-SMA networks**

Expression aside, we wanted to determine how the actin isoforms contribute to the actin cytoskeleton. To examine this, we performed immunofluorescence for β-actin and α-SMA in epiWAT SVC (Figure 5.3a) and 3T3-L1 preadipocytes (Figure 5.3b). We observed that both isoforms were present across both cell types and, more importantly, co-localizing with F-actin. Curiously, we also observed a pool of β-actin that consistently co-localizes with the nucleus of 3T3-L1 preadipocytes. This is unique to 3T3-L1 cells since β-actin was not observed in the epiWAT SVC. The immunofluorescence data shows that β-actin and α-SMA contribute equally to the F-actin network at subconfluence.

**Significant actin reorganization coincides with the period of hormonal induction**

The stress fibers found in preadipocytes are known to be significantly lost during adipogenesis. To determine whether this change is common to both α-SMA and β-actin isoforms, we observed these isoforms from subconfluence to Day 2 in 3T3L-L1 cells (Figure 5.4). As anticipated, F-actin was significantly lost by Day 2. The β-actin isoform consistently co-localized with the nucleus as well as with F-actin whereas α-SMA only co-localized with F-actin. β-actin appeared
Figure 5.3 White adipose SVCs have extensive β-actin and α-SMA networks

(A) Stromal vascular cells (SVC) were plated onto coverslips, fixed and stained for β-actin (top row) and α-SMA (bottom row) with Alexa Fluor® 568 Goat Anti-Mouse IgG2a secondary antibody. Cells were counterstained with fluorescent Acti-stain 488 phalloidin (F-actin) and DAPI for nuclear staining. Bar represents a 50 μm scale.

(B) 3T3-L1 cells were plated onto coverslips, fixed and stained for β-actin (top row) and α-SMA (bottom row) with Alexa Fluor® 568 Goat Anti-Mouse IgG2a secondary antibody. Cells were counterstained with fluorescent Acti-stain 488 phalloidin (F-actin) and DAPI for nuclear staining. Bar represents a 50 μm scale.
Figure 5.4 The actin network is significantly reorganized during hormonal induction

(A) Stromal vascular cells (SVC) were plated onto coverslips, fixed and stained for β-actin (top row) and α-SMA (bottom row) with Alexa Fluor® 568 Goat Anti-Mouse IgG2a secondary antibody. Cells were counterstained with fluorescent Acti-stain 488 phalloidin (F-actin) and DAPI for nuclear staining. Bar represents a 50 μm scale.

(B) 3T3-L1 cells were plated onto coverslips, fixed and stained for β-actin (top row) and α-SMA (bottom row) with Alexa Fluor® 568 Goat Anti-Mouse IgG2a secondary antibody. Cells were counterstained with fluorescent Acti-stain 488 phalloidin (F-actin) and DAPI for nuclear staining. Bar represents a 50 μm scale.
to co-localize with F-actin and was attenuated by Day 2 (Figure 5.4a). Interestingly, α-SMA was significantly downregulated by Day 1 (Figure 5.4b).

The data suggest a loss in both β-actin and α-SMA occurs during hormonal induction (Day 0 to Day 2). The data also suggest the β-actin and α-SMA isoforms are not equally regulated during adipogenesis.

**Hormonal induction decreases α-SMA protein levels in both the G-actin and F-actin compartment**

Although both β-actin and α-SMA were observed to contribute to the F-actin network, the rate at which these fibers are lost appeared to be different as indicated by our immunofluorescence data. This prompted us to ask whether their contributions to the F-actin and G-actin compartments are different during this period of time. To assess this, we performed a series of CSK/RSB fractionations during hormonal induction (Figure 5.5). Based on the known loss in stress fibers during adipogenesis, we anticipated that the G-actin pool would increase as the F-actin pool decreased. Instead, we observed decreases in both the G-actin and F-actin pools across all isoforms examined (Figure 5.5a). There was also an earlier loss in the α-SMA F-actin pool relative to the β-actin F-actin pool just 3 hours into hormonal induction (Figure 5.5a). Major changes in β-actin do not appear until approximately 12 hours into hormonal induction. The γ-actin pattern was more similar to that of α-SMA than β-actin with the exception of a drop in polymerized γ-actin within 6 hours of hormonal induction.
Figure 5.5 Hormonal induction decreases α-SMA protein levels in both the G-actin and F-actin compartments

(A) Western blot analysis of 3T3-L1 cells at various points (hours) during hormonal induction. Protein was extracted from cells using the CSK/RSB fractionation method. Equal volume fraction was loaded in each lane.

(B) Western blot analysis of total protein harvested from 3T3-L1 cells at various points (days) during and following hormonal induction. Equal volume protein was loaded in each lane.

(C) Western blot analysis of Day2 3T3-L1 treated for two days with either ethanol and 0.1 N NaOH (Vehicle) or adipogenic cocktail (DMI). Protein was extracted from cells using the CSK/RSB fractionation method. Equal volume fraction was loaded on each lane.

(D) A graph showing a densiometric analysis of the fold difference in α-SMA and β-actin enrichment across CSK (G-actin) and RSB (RSB) fractions in protein extracted from Day 2 3T3-L1 cells following two days ethanol and 0.1 N NaOH (Vehicle) or adipogenic cocktail (DMI). G-actin results were normalized to cyclophilin A protein and F-actin results were normalized to vimentin protein, (n=3); *p≤0.05.
To confirm the effect adipogenic cocktail has on the F-actin and G-actin pools, we performed a CSK/RSB fractionation on Day 2 3T3-L1 cells treated with either adipogenic cocktail or its vehicle (Figure 5.5b). Adipogenic cocktail was found to decrease the α-SMA G-actin pool 84% and the F-actin pool 60%, both of which were statistically significant. On the other hand, the β-actin G-actin pool fell 31% and the F-actin pool, in fact, increased 42% (Figure 5.5c). The changes in β-actin were not statistically significant, though. Overall, we can conclude that α-SMA F-actin, not β-actin stress fibers are lost during hormonal induction.

We didn’t observe net gains in G-actin in proportion with F-actin loss but instead observed what appears to be a net loss in α-SMA but not β-actin. To confirm this we measured total protein expression during and after hormonal induction (Figure 5.5d). The cytoplasmic isoform protein levels slowly and steadily decrease whereas α-SMA protein levels drop significantly within the first 24 hours of hormonal induction. After Day 1, α-SMA protein expression falls to near undetectable levels, with a late stage upregulation on Day 8. We can conclude that hormonal induction elicits a net loss of actin from both the G-actin and F-actin pools. This is not experienced equally across actin isoforms, though. The α-SMA isoform is significantly influenced by hormonal induction and its loss alone likely accounts for the majority of net actin loss, particularly F-actin loss.

**Actin transcript levels decrease during hormonal induction**

Although actin protein levels decrease during hormonal induction, it was unclear whether decreases were also occurring at the transcript level. We
A. Relative mRNA levels of actin genes and genes regulated by adipogenesis at various time points during 3T3-L1 adipogenesis. Gene expression was normalized to TBP. Graphs show fold difference ± SEM (n=4); *p≤0.05, **p≤0.005, ***p≤0.0005.

B. Western blot analysis of total protein harvested at various time points during 3T3-L1 adipogenesis. Equal amounts of protein were loaded per lane.

Figure 5.6 Actin levels decrease during hormonal induction

(A) Relative mRNA levels of actin genes and genes regulated by adipogenesis at various time points during 3T3-L1 adipogenesis. Gene expression was normalized to TBP. Graphs show fold difference ± SEM (n=4); *p≤0.05, **p≤0.005, ***p≤0.0005.

(B) Western blot analysis of total protein harvested at various time points during 3T3-L1 adipogenesis. Equal amounts of protein were loaded per lane.
measured actin transcript levels from subconfluence to Day 2 and observed that all actin isoforms fall at least 70% (Figure 5.6a). The majority of the drop occurs between Days 0 and 2. Although all isoforms drop significantly, the patterns by which they achieve the drop are unique. The majority of cytoplasmic isoform transcript loss starts at Day 0 while α-SMA transcript levels begin a steady drop on Day -2. Transcript levels of γ-actin are transiently induced before they drop on Day 0. As experimental controls, PPARγ transcript levels were observed to increase and Pref-1 to decrease. The observed transcript level patterns for each actin isoform were largely corroborated by protein level changes (Figure 5.6b).

**Actin transcript is not rendered unstable by adipogenesis**

The dramatic loss of actin transcript particularly during hormonal induction prompted us to wonder whether hormonal induction also decreases the stability of actin transcript. To gauge the stability of actin transcript during hormonal induction, we performed an actinomycin D decay curve (Figure 5.7). Actinomycin D inhibits RNA polymerase II, and to a lesser extent RNA polymerase III, which rapidly stops mRNA transcription (241). Actin transcript was measured during hormonal induction of 3T3-L1 cells treated with actinomycin D (Figures 5.7b, and 5.7d) or its vehicle DMSO (Figure 5.7a and 5.7c). If hormonal induction renders actin transcript unstable, then actin transcript levels would continue to drop during hormonal induction despite the inclusion of actinomycin D. Kruppel-Like 4 (KLF4) transcript levels served as a control since KLF4 is known to be transiently induced within 3-4 hours of hormonal induction (Figure 5.7d and 5.7e) (242).
Figure 5.7 Hormonal induction does not render actin transcript unstable

Day 0 3T3-L1 cells were pretreated with either 5 μg/mL actinomycin D (B, D, F) or DMSO (A, C, E) for 1 hour prior to having adipogenic cocktail of its vehicle, ethanol and 0.1 N NaOH overlayed. All the graphs show relative mRNA levels of select genes normalized to TBP. Graphs show fold difference ± SEM (n=4).
In the absence of actinomycin D and adipogenic cocktail, we observed differences in the stability of β-actin and α-SMA transcripts. β-actin transcript was stably expressed as indicated by the near zero slope of the curve (Figure 5.7c). Interestingly, α-SMA transcript levels were found to decrease. This inherent instability corroborates the observed early onset of α-SMA loss and suggests that this isoform is regulated in a manner that β-actin. (Figure 5.6).

During hormonal induction, β-actin and α-SMA transcript levels drop at similar rates (-0.045 and -0.037 slopes, respectively) (Figure 5.7c). The inclusion of actinomycin D nulled the effect of hormonal induction on β-actin (Figure 5.7d) and α-SMA (Figure 5.7b) transcripts. We can conclude that neither β-actin nor α-SMA transcripts are destabilized by adipogenic cocktail.

**IBMX significantly contributes to α-SMA downregulation during hormonal induction**

Although hormonal induction does not render actin transcript unstable, our time course data collectively implicate the hormonal induction in the regulation of actin. During hormonal induction, cells are treated with an adipogenic cocktail consisting of dexamethasone, isobutylmethylxanthine (IBMX) and insulin. We examined how actin transcript and protein levels respond to various combinations of the adipogenic cocktail (Figure 5.8). Complete adipogenic cocktail reduced β-actin transcript levels 70% (Figure 5.8a) and protein levels 10% (Figure 5.8c) relative to vehicle. No pattern to this downregulation was observed with respect to the individual cocktail components. In striking contrast,
Figure 5.8 IBMX significantly contributes to α-SMA downregulation during hormonal induction

An analysis of mRNA and protein expression in Day 2 3T3-L1 cells after 48 hours of hormonal induction under the specified adipogenic cocktails abbreviated as follows: dexamethasone (D), isobutylmethylxanthine (M), insulin (I). Vehicle is ethanol and 0.1 N NaOH.

(A) Relative mRNA levels of select genes expressed. Gene expression was normalized to TBP. Graphs show fold difference ± SEM (n=3); *p≤0.05.

(B) Western blot analysis of total protein extracted. Equal protein was loaded per lane.

(C) Densiometric analysis of the fold difference in α-SMA and β-actin protein normalized to cyclophilin A, (n=3); *p≤0.05,**p≤0.005, ***p≤0.0005.

(D) Relative mRNA levels of select genes expressed by confluent 3T3-L1 cells treated with DMSO and 0.1 N NaOH (Vehicle), 0.5 mM IBMX (IBMX) or 10 μM forskolin (Forskolin) for 48 hours. Gene expression was normalized to TBP. Graphs show fold difference ± SEM (n=4); *p≤0.05.
α-SMA expression was significantly affected by both cocktail combinations that included IBMX. Complete adipogenic cocktail reduced α-SMA transcript levels by ≥90% (Figure 5.8a) and protein levels by 80% (Figure 5.8c). As long as IBMX was included in the cocktail, at least 80% downregulation in α-SMA transcript was achieved (Figure 5.8c). IBMX also appeared to negatively regulate the expression of γ-actin (Figure 5.8a). The potency and specificity of IBMX was verified by its induction of C/EBPβ (Figure 5.7a) (243,244). Of the components of the adipogenic cocktail, IBMX had the most influence over actin expression particularly over α-SMA.

IBMX has two major downstream targets: cyclic nucleotide phosphodiesterase and adenosine receptor (245). Cyclic nucleotide phosphodiesterases are a large family of enzymes that cleave cAMP and/or cGMP to produce the corresponding 5'-nucleotide(246). Inhibiting these enzymes increases intracellular cAMP levels. IBMX also inhibits adenosine from binding its receptor. Given the known role for cAMP signaling in the induction of adipogenic inducers such as C/EBPβ by cAMP response element binding protein (CREB), we hypothesized that that pathway likely regulates α-SMA expression as part of an adipogenic program (244,247). To test this hypothesis, we treated 3T3-L1 preadipocytes with either IBMX or forskolin. Forskolin activates adenylate cyclase, which synthesizes cAMP from ATP. Both treatments significantly attenuated α-SMA transcript (Figure 5.8d) after 48 hour treatment. Neither of the cytoplasmic isoforms was dramatically influenced by IBMX nor forskolin.
treatment. Based on the mirrored effect of the cAMP-inducing agent forskolin, we can conclude that IBMX regulates α-SMA expression in 3T3-L1 cells at least in part through its ability to induce cAMP levels.

**cAMP signaling regulates α-SMA gene transcription**

Since IBMX and forskolin significantly reduced α-SMA transcript levels, we hypothesized that cAMP signaling was influencing α-SMA transcription. To test this hypothesis, we used α-SMA promoter activity in SVCs expressing a fluorescent mCherry construct driven by the α-SMA promoter (Figure 5.9). The SVCs were treated with IBMX and forskolin and as a control, we overlaid TGFβ treatment, which is known to induce α-SMA promoter activity. When we measured the fluorescence from individual cells in the field, we found that IBMX and forskolin treatment attenuated fluorescence intensity below that of vehicle conditions (Figures 5.9a and 5.9b). To confirm that this led to an actual loss in α-SMA, we measured α-SMA protein (Figure 5.9c) and transcript (Figure 5.9d) levels. A drop α-SMA levels in SVC treated with IBMX or forskolin. From the data we can conclude that cAMP signaling regulates α-SMA at the level of transcription.

**MRTF/SRF activity regulates α-SMA expression in subconfluent 3T3-L1 cells**

We hypothesized that an event preceeding hormonal induction was responsible for the falling levels of α-SMA transcript observed leading to
**Figure 5.9 cAMP signaling downregulates α-SMA gene transcription**

epiWAT SVC with expressing the the Acta2 promoter-mCherry construct were treated with the indicated combinations of 1 nM TGFβ, 0.5 mM IBMX and 10 μM forskolin (FSK) for a combination of their vehicles (Veh) (0.1 N NaOH, DMSO, 4 mM TRIS/HCl, 0.2% BSA) for 24 hours.

(A) A schematic of the Acta2 promoter-mCherry construct along with representative images of their mCherry fluorescence

(B) Graph showing relative mCherry fluorescence fold change in A.U. ± SEM (n=4); *p≤0.05, p≤0.005**, p≤0.0005***

(C) Western blot of total protein isolates from epiWAT SVC, equal protein loaded per lane

(D) Relative mRNA levels of select genes expressed epiWAT SVC. Gene expression was normalized to TBP. Graph shows fold difference ± SEM (n=4); *p≤0.05, p≤0.005**
confluence. Given its known role in the regulation of actin cytoskeleton-associated genes, the MRTF/SRF transcriptional complex was implicated. To determine whether MRTF/SRF regulates actin expression in 3T3-L1 cells, we first modulated the expression of MRTFa and measured the effect on actin transcript levels (Figure 5.10a and 5.10b). MRTFa knock down with siRNA did not change β-actin transcript levels and mildly attenuated γ-actin transcript. The α-SMA isoform experienced a 30% decrease in transcript levels in response to a 60% loss in MRTFa, despite a lack of statistical significance (Figure 5.10a). We observed a significant increase in α-SMA transcript and a subtle increase in γ-actin transcript levels when MRTFa was overexpressed 15-fold (Figure 5.10b). By modulating the expression levels of MRTFa, we identified MRTFa as specifically regulating the expression of α-SMA and, to a lesser extent γ-actin.

To determine the relevance of this observation in the context of adipogenesis, we modulated the activity of MRTF/SRF in 3T3-L1 cells. We pharmacologically inhibited their activity with CCG-1423, which has been shown to bind to the nuclear localization signal of MRTF to ultimately prevent MRTF nuclear accumulation and thus inhibit SRF co-activation (248). Cell-cell contact has also been reported to inhibit MRTF/SRF activity by driving MRTF out of the nucleus (249).

Extensive cell-cell contact is experienced by 3T3-L1 cells upon confluence or adipogenic Day -2. To test the impact of these two modes of MRTF/SRF inhibition on actin expression, we treated subconfluent (low density) and near
Figure 5.10 MRTF regulates α-SMA expression in subconfluent 3T3-L1 cells

3T3-L1 cells were transfected with either eGFP- or MRTFa-specific 30 nM siRNA (A) or eGFP-N1 or eGFP-MRTFa plasmid (B). Cells were then harvested for total RNA. Relative mRNA levels of select genes were measured by qPCR. Gene expression was normalized to TBP. Graphs show fold difference ± SEM (n=4); *p≤0.05, **p≤0.005, ***p≤0.0005.

(C) 3T3-L1 cells were seeded to 30% density (low) or 90% density (high) and treated with 10 μM CCG-1423 for 24 hr. Relative mRNA levels of actin genes and preadipocyte factor 1 (Pref1) at various time points during 3T3-L1 adipogenesis. Gene expression was normalized to TBP. Graphs show fold difference ± SEM (n=4); *p≤0.05, **p≤0.005.
confluent (high density) 3T3-L1 cells with CCG-1423 or its vehicle (DMSO) (Figure 5.10c). As a control, we measured the transcript levels of MRTFa, which is known to be regulated by MRTF/SRF as part of a feedback loop. We also measured the expression of Mical2 which is a speculated MRTF/SRF target gene based upon an MRTF ChIP-seq study of 3T3 fibroblasts conducted by Treisman and colleagues (250). CCG-1423 treatment attenuated Mical2, MRTFa, γ-actin and α-SMA in subconfluent 3T3-L1 cells (Figure 5.10c). High seeding density mimicked the attenuation achieved by CCG-1423 but did not potentiate the effect of CCG-1423 when combined (Figure 5.10c). Given how α-SMA expression was impacted by MRTFa overexpression, knockdown and inactivation, we can conclude that MRTFa regulates α-SMA in subconfluent, proliferating 3T3-L1 cells.

**MRTFa regulates actin expression in SVC**

To date, we have only achieved partial MRTFa knockdown or inactivation of MRTF so to confirm adipogenic actin gene regulation by MRTF/SRF, we compared the actin transcript levels of SVC isolated from epiWAT of MRTFA<sup>−/−</sup> and wild type (WT) mice (Figure 5.11)(10). The absence of MRTFa caused a 70% reduction in α-SMA transcript level, a level that nearly mirrors the level of downregulation achieved in Day 1 WT SVC (Figure 5.11a). Based on this observation, we can conclude that MRTFa regulates α-SMA during adipogenesis. Since the absence of MRTFa caused a 20-30% reduction in β-actin and γ-actin transcript levels and their patterns of downregulation in MRTFA<sup>−/−</sup> SVCs mirrored
A. Figure 5.11 MRTF regulates actin expression in SVC

(A) Relative mRNA levels of select genes at various time points epiWAT derived SVC adipogenesis. Gene expression was normalized to TBP. Graphs show fold difference ± SEM (n=4); *p≤0.05, **p≤0.005.

(B) Western blot analysis of total protein isolated from Day -2 and Day 0 epiWAT-derived SVC. Equal protein was loaded per lane.
those of WT SVCs (Figure 5.11a), we can conclude that MRTFa likely regulates these genes as well. During post-confluence (Day -2 to 0), we observed a downregulation of α-SMA in WT SVC (Figure 5.11a). As discussed previously, this likely coincides with the inactivation of MRTF/SRF in a cell density dependent mechanism. To interrogate this further, we examined the period between Day -2 and Day 0 at the protein level in WT and MRTFA−/− SVCs (Figure 5.11b). We found that, indeed, the absence of MRTFa significantly reduced α-SMA protein levels and lesser so β-actin protein levels. Interestingly, α-SMA protein levels still fall between Day -2 and Day 0 despite MRTFa genetic ablation, countering observations at the transcript level (Figure 5.11b). We are currently repeating with this sufficient mice for statitical analysis to address the disparity in RNA versus protein results.

Discussion

As discussed previously, actin cytoskeleton reorganization has been described as a loss of long actin filaments during adipogenesis in vitro. Given our development of a novel biochemical assay for F-actin/G-actin dynamics, we utilized this assay to confirm these findings. We were able to biochemically show that there is indeed a net loss in F-actin during early adipogenesis (Figure 5.5a, 5.5d and 5.5c). Interestingly, we did not observe a complementary increase in G-actin, but instead a decrease. These results are not surprising when the parallel downregulation of actin synthesis and is considered. The matter is further complicated by the facts that protein levels can fall as a consequence of
decreased transcription, decreased translation and/or increased degradation. In muscle cells, G-actin is degraded by a ubiquitin-proteasomal pathway so it is likely that actin must be in the G-actin state to be degraded (251). It is conceivable that a similar pathway is working to clear G-actin during adipogenesis as it accumulates from F-actin depolymerization.

It is also conceivable that we are undervaluing the loss of F-actin by the way we quantified the decrease. We normalized RSB-specific actin against vimentin which is also downregulated (Figure 5.5b and 5.5c). We hypothesize that normalizing against a more stably expressed protein would numerically augment the loss in F-actin. Minor discrepancies aside, the CSK/RSB method captured an intriguing aspect of actin dynamics during adipogenesis. At the very least, our data suggest that the reorganization of actin impacts both F-actin and G-actin.

Our studies identified three isoforms relevant to adipogenesis: β-actin, γ-actin and α-SMA. These isoforms were readily detected in SVC and 3T3-L1 cells compared to α-cardiac and α-skeletal muscle isoforms (data not included). It should be noted that γ-smooth muscle actin (γ-SMA) isoform was mildly detected, but was still small in magnitude relative to the enrichment of β-actin, γ-actin and α-SMA. We observed that β-actin and α-SMA localized to the polymerized actin network as indicated by immunofluorescence (Figure 5.3). We also observed a loss in α-SMA stress fibers that corresponded with F-actin loss during early adipogenesis (Figure 5.4). Curiously, we observed nuclear localization specific to
the β-actin isoform. We are currently working towards confirming this result using a fluorescently tagged β-actin construct.

As adipogenesis progressed, we observed distinctions in the expression patterns of the cytoplasmic and α-SMA isoforms. Our results showed that although the cytoplasmic isoforms are attenuated and the α-SMA isoform is significantly downregulated. These patterns were very similar in two types of cells, 3T3-L1s (Figure 5.6a) and SVCs (Figure 5.11a). Our observations were corroborated at the level of whole adipose tissue where cytoplasmic isoforms were detected in both adipocytes and SVF while α-SMA was enriched in SVF (Figure 5.2d). The data suggest that the actin present in the mature adipocyte is likely not α-SMA. Mature 3T3-L1 adipocyte cultures, though, expressed levels of α-SMA near comparable to the preadipocyte stage (Figure 5.5d). This is likely attributable to the layer of undifferentiated 3T3-L1 cells found beneath mature adipocytes, similar to a feeder layer. Although we did not explore this hypothesis further, a simple comparison of gene expression between adipocyte and feeder layer would likely reveal that the α-SMA is limited to the feeder layer.

In addition to describing patterns of expression and polymerization, we also explored the regulation of actin expression. We identified two stages of regulation. Modifying the expression and activity of MRTF/SRF and the genetic ablation of MRTFa collectively demonstrated that MRTF regulates actin isoform expression during adipogenesis (Figure 5.10 and 5.11). At high density, fibroblasts are known to have diminished microfilament polymerization (252).
This means that confluence creates a greater G-actin pool which, we hypothesize, would be sufficient to sequester MRTF in the cytoplasm. However, our data does not indicate that an increase in cytoplasmic G-actin occurs (Figure 5.5). A growing body of literature indicates a role for nuclear G-actin, not cytoplasmic G-actin, in the regulation of MRTF activity (178,253,254). The mechanisms regulating nuclear actin are not well known but one major player may be Mical2, which, as mentioned, is an MRTF/SRF target gene (254).

Our actin fractionation assay is currently not designed to isolate nuclear actin. We hypothesize that nuclear actin is isolated with RSB and could be overvaluing the magnitude of the RSB fractions. As discussed in Chapter 3, we are developing a fractionation assay that will isolate a third, nuclear fraction. If net changes in nuclear actin content are what regulate MRTF activity, then the assay will be able to detect that. On the other hand, if net changes in nuclear actin polymerization are the regulators, which appears to be the case based on the literature, then our assay may fall short. Whatever the outcome, these studies are relevant given the established role of MRTF/SRF in adipogenesis.

Our time courses pointed to a second dramatic stage in actin downregulation: hormonal induction. One particular adipogenic cocktail component, IBMX, was found to significantly attenuate α-SMA specifically (Figure 5.8). We confirmed that cAMP signaling was a potent inhibitor of α-SMA promoter activity (Figure 5.9). Cyclic AMP has been shown to inhibit α-SMA
promoter activity in embryonic stem cells (255). We have shown that this is also true in the context of adipogenic differentiation.

The distinct stages of isoform-specific regulation, particularly the loss of α-SMA, are likely to be more than just a function of adipogenesis. Our data suggest that α-SMA, and not β-actin or γ-actin, negatively correlates with adipogenesis. We hypothesize that α-SMA plays an important, functional role in adipogenesis. This hypothesis is currently being tested in an overexpression system, which we predict will have diminished adipogenic potential. If this proves to be accurate, then there is reason to believe that α-SMA helps determine the competency of a progenitor cell to proceed through adipogenesis.

It should be noted that a subtle yet important difference was observed between actin expression in 3T3-L1 cells and SVCs, namely that MRTF/SRF actin gene regulation was much more limited to α-SMA in 3T3-L1 cells. This may be the consequence of two factors. First, the methods by which we modulated MRTFa in 3T3-L1 cells were not as robust as the genetic ablation of MRTFa. Second, 3T3-L1 preadipocytes are committed to the adipogenic path while SVC represents an earlier stage of development. It is possible, then, that MRTF/SRF is intrinsically less active and/or expressed less in the preadipocyte cell line relative to SVCs. Inhibiting or inactivating MRTF/SRF in 3T3-L1 cells, then, would not elicit as dramatic changes in actin as it does in SVC.

Overall, these studies have comprehensively described adipogenic actin dynamics during early preadipocyte differentiation. We’ve shown that most of the
changes in actin are specific to the α-SMA isoform. We shed light on the complexities of adipogenic actin cytoskeletal rearrangement. Our findings point to two stages of actin regulation in what we hypothesize is a critical initiation step during adipogenesis.
CHAPTER SIX

GENERAL DISCUSSION, FUTURE DIRECTIONS AND CONCLUSIONS

DISCUSSION

The studies presented in this thesis have characterized the dynamics of actin during early adipogenesis, a period marked by morphological and transcriptional changes. Similar studies of the relationship between the actin cytoskeleton and differentiation have treated actin as a single protein when, in fact, it is a family of functionally distinct isoforms. This prompted our investigation into the changes of each actin isoform both at the level of expression and at the level of spatial organization. In the process of studying actin spatial organization, we developed a simple assay for the quantitative description of actin polymerization/depolymerization. Using this method, we were able to exclude a role for gelsolin the reduction of F-actin. Through these studies, we observed an early loss in F-actin without gains in G-actin. We interrogated this further along with changes in actin expression to identify unique changes in the α-SMA isoform specifically. The implications of these studies are broad and are prompting new and interesting directions of research.

Potential applications for CSK/RSB fractionation

Because the polymerized state of actin is influences cell fate, we were keenly interested in determining the contributions of each actin isoform to the F-actin and G-actin pools. To study this, we developed a method for the
biochemical assessment of actin dynamics, a method we refer to as CSK/RSB fractionation. CSK/RSB fractionation does not replace the use of methods like immunostaining of G-actin/F-actin or the expression fluorescently-tagged F-actin binding proteins/domains (256). Those methods yield great insight into the localization and distribution of actin and, in the case of fluorescent DNAse and/or phalloidin, are technically simple to handle for the description of actin dynamics on a pan-actin level.

Unlike those methods, we have shown that CSK/RSB fractionation can assess the polymerization/depolymerization of actin at the level of individual isoforms. Broader use of the CSK/RSB fractionation method will require further optimization and validation. One source of uncertainty lies in the observed nuclear DNAse staining faintly present after CSK incubation (Figure 3.2a and 3.3a), which suggests CSK does not solubilize all cellular G-actin. Our observation is not unorthodox, though. In their description of the use of DNAse as a marker for G-actin, Cramer and colleagues tested the sensitivity of DNAse by incubating cells in 0.1% Triton X-100 to selectively remove G-actin (257). The DNase staining pattern post-incubation revealed a faint, circular stain in the region of the nucleus. Our observations are very similar even though we use five times the amount of Triton X-100.

CSK is not stringent enough to permeabilize the nuclear membrane, so it is conceivable that CSK is not solubilizing a nuclear G-actin pool. Recent studies have confirmed the presence and relevance of nuclear actin. In the cell types
examined, nuclear actin is predominately in the G-actin form (258). An accumulation of nuclear β-actin was shown to be involved in regulating transcription during macrophage differentiation (259). There is also evidence to suggest that nuclear G-actin regulates the export and therefore activity of MRTFa (254). We have also observed the β-actin isoform selectively present in the nucleus (Figure 5.3a), although this observation requires further confirmation.

Concluding that CSK is not solubilizing a pool of nuclear G-actin assumes a high level of accuracy on the part of DNAse staining. DNAse nonspecifically cleaves DNA to release 5'-phosphorylated di-, tri-, and oligonucleotide products (260). A false positive reading within the nucleus, the storage site of DNA, is within the realm of possibility. To determine the accuracy of DNAse staining, we are currently using actin antibodies instead of DNAse/phalloidin to measure what is happening to actin during CSK incubation. We are also tracking isoform-specific solubility by expressing fluorescently tagged forms of β-actin and α-SMA.

Despite the need for further optimization, CSK/RSB fractionation has promising applicability particularly in the study of mechanotransduction. Because the state of actin polymerization dictates the activity of factors like Taz/Yap and MRTF, measuring actin polymerization is invaluable. Our method allows for the measurement of changes in both actin polymerization and expression. We were able to track the loss of actin and the changes in its polymerization simultaneously during early adipogenesis (Figure 5.4a). Our method could be used to better describe what happens to G-actin/F-actin when actin expression is
increased, too. TGFβ has been shown to induce both actin polymerization and α-SMA expression (261). These are two intrinsically related events that have not been measured simultaneously through biochemical means. Although we can anticipate an enrichment of α-SMA in the F-actin pool, it is not clear what effect TGFβ has on the G-actin pool once α-SMA levels rise. How G-actin responds would have implications on the effect of TGFβ on factors like MRTF.

We have also considered the possibility of extending the CSK/RSB fractionation to include a third, nuclear fraction. In the extended version, RSB incubation is followed by incubation in high-salt, nuclear extraction buffer. The remains of the cell monolayer are scraped in that buffer and the lysate is sonicated and spun. The supernatant, what we consider the nuclear fraction, has been found to selectively contain RNA polymerase II (data not included). We anticipate that this method will be a truly novel assay for the parallel tracking of actin dynamics and of nuclear factor dynamics, particularly those of MRTF and Taz/Yap.

**Cytoplasmic actin and adipogenesis**

Given how ubiquitous the cytoplasmic isoforms are, it was not surprising that we found preadipocytes express both β-actin and γ-actin. These isoforms also show similar patterns of expression across adipose depots (Figure 5.2c). The mere presence of these isoforms isn’t compelling but rather their loss and the divergence in their patterns of expression. In general, we observed both
isoform are downregulated with an earlier losses in γ-actin relative to β-actin during adipogenesis (Figure 5.5).

The literature suggests that the loss of the cytoplasmic isoforms drives Rho/ROCK activity (262,263). In mouse embryonic fibroblasts, the absence of β-actin leads to impaired migration, increased TGFβ production and increased expression of other actin isoforms (262). These phenotypes could be ameliorated with Rho/ROCK inactivation. The absence of γ-actin, but not of β-actin, in human epithelial cells causes them to adopt a myofibroblast phenotype and also elicits Rho/ROCK activation (263). Overall, these isoforms, specifically the lack thereof, seem to influence Rho/ROCK activity.

We are currently investigating whether similar roles exist for β-actin and γ-actin in the context of adipogenesis. The absence of the cytoplasmic isoforms in those studies caused an induction in the expression of other actin isoforms which led to the induction of Rho/ROCK activity. We observed cytoplasmic isoforms loss in 3T3-L1 and SVCS which would theoretically mean Rho/ROCK is activated. As previously discussed, Rho/ROCK activity inhibits adipogenesis which is at odds with the expected outcome of cytoplasmic isoform loss. It is probable that because all isoforms are downregulated during adipogenesis, Rho/ROCK is not activated and adipogenesis proceeds. Better still, the loss of both isoforms could help in the inhibition of Rho/ROCK, making these isoforms bona fide regulators of adipogenesis. A regulatory role for the cytoplasmic
isoforms in adipogenic commitment will be established once stable knockdown
and overexpression cells lines are established.

**α-SMA and adipogenesis**

The α-SMA isoform is one of the earliest known proteins expressed during
the differentiation of smooth muscle cells including mural cells (264). A subset of
the stromal vasculature cells contains PPARγ⁺ cells that express no additional
mature adipocyte markers yet are capable of differentiating into adipocytes in
vitro. These cells express α-SMA and platelet derived growth factor receptor β
(PDGFRβ), both markers of the mural cell lineage (61). In human bone marrow-
derived stem cells, knockdown of α-SMA promotes clonogenicity and
adipogenesis with similar results obtained with sorted α-SMA negative cells
(265). A recent study profiling WAT-derived UCP-1⁺ cells identified the
expression of smooth muscle-specific genes including α-SMA (240). This was not
true in BAT which is consistent with studies demonstrating that beige adipocytes
and not brown adipocytes are derived from a mural cell lineage (266). It appears,
then, that both white and beige adipocytes could originate from a smooth muscle
cell-like progenitor, making α-SMA expression functionally relevant.

We detected α-SMA in both SVC and 3T3-L1 cells and its expression was
downregulated to near undetectable levels in both cells. This observation was
initially considered an artifact of cell culture conditions. Growing cells on hard, flat
surfaces promotes actin polymerization and, likely, more actin expression. Given
the theories on smooth muscle cell-like origins, it is unlikely that α-SMA
expression is an artifact. We hypothesize that the loss of α-SMA expression, commits cells to an adipogenic fate. This could be another key moment in adipogenic competence. Currently, we are testing this hypothesis through α-SMA and β-actin overexpression. We hypothesize that α-SMA overexpression specifically will be sufficient to inhibit adipogenesis relative to the effect of β-actin overexpression.

In our studies, we implicated cAMP signaling in the regulation of α-SMA expression during adipogenesis. Downstream of cAMP signaling are two proteins: protein kinase A (PKA) and the exchange proteins directly activated by cAMP (Epac1 and Epac2). PKA appears to suppress Rho/ROCK activity and becomes dispensable for adipogenesis when Rho/ROCK is pharmacologically inhibited (267). The anti-adipogenic effect of Rho/ROCK activity was, at the time, associated with an inhibitory effect on insulin/insulin-like growth factor 1 signaling. We now know that Rho/ROCK inactivation is pro-adipogenic because it also helps inactivate MRTF/SRF and Taz/Yap activities.

Mack and colleagues found that SRF is phosphorylated at T159 by PKA in embryonic stem cells. This phosphorylation inhibits smooth muscle cell-specific transcription by inhibiting SRF binding to the CArG elements within the promoters of smooth muscle cell-specific genes including α-SMA (255). We are currently determining whether SRF T159 phosphorylation occurs as a result of hormonal induction and whether that is responsible for the expedited loss of α-SMA expression. Since MRTF/SRF activity regulates an initial, massive loss in α-SMA
expression (Figure 5.6 and 5.11), we hypothesize SRF continues to occupy the Acta2 promoter allowing for a low level of α-SMA transcription. Based on enhanced rate of α-SMA loss elicited by adipogenic cocktail (Figure 5.6 and Figure 5.7a), hormonal induction likely prompts the phosphorylation of SRF by PKA, which causes SRF to completely abandon the Acta2 promoter and halt all α-SMA transcription. This could help explain why hormonal induction enhances the rate of adipogenesis.

**Gelsolin and Taz/Yap**

Although we found no role for gelsolin in regulating the dynamics of actin during hormonal induction, our studies identified the mechanism by which gelsolin is induced during adipogenesis. We found that activated glucocorticoid receptor is responsible for gelsolin induction in 3T3-L1 cells (Figure 4.4). As previously discussed, Taz is transiently downregulated by glucocorticoid during hormonal induction and this coincides with gelsolin’s transient induction by dexamethasone (Figure 4.2). In immortalized human mammary epithelial cells, gelsolin knockdown has been shown to stabilize Taz expression, promote its nuclear localization and drive Taz/Yap target gene expression (165). The changes in Taz and gelsolin expression during hormonal induction are likely not coincidental.

It is believed that gelsolin inhibits Taz/Yap nuclear localization by disrupting stress fibers that mechanically prompt Taz/Yap activity. We did not observe any changes in the preponderance of stress fibers with the loss of
gelsolin (Figure 4.6b), but our measurements were taken at the end of hormonal induction. It is possible that F-actin severance by gelsolin is an early, rapid event. More likely, the absence of gelsolin and its activity is compensated for by other ABPs. The ABP coflin has been shown to drive pro-adipogenic actin depolymerization in a dedifferentiated adipocyte cell line (180). The relationship between gelsolin and Taz/Yap may explain gelsolin’s required expression for adipogenic mitotic clonal expansion (MCE). Although a role for Taz/Yap in MCE has not been established, the Hippo pathway in yeast is known to serve as a checkpoint for mitotic exit, suppressing pro-mitotic Cdk1 (163,268). It possible that gelsolin, or F-actin severance in general, is proadipogenic because it prevents Taz/Yap from inhibiting MCE. This hypothesis assumes that MCE is required for adipogenesis, which remains controversial (269,270).

**Actin in white, beige/brite and brown adipocytes**

Although 3T3-L1 cells are commonly used to study adipogenesis, they do not identify with any one type of adipose probably because they are not derived from adipose but instead from disaggregated mouse embryos (110). To address this, we also studied actin in SVC and found that the data corroborated data from the 3T3-L1 cells. We predominately studied epiWAT SVC because epiWAT experiences hyperplasia in response to high fat diet and because we found it to be most enriched with actin (Figure 5.2). As mentioned previously, SVCs harvested from visceral depots have a lower propensity for adipogenesis. Based on our findings and the growing literature, it is possible that visceral WAT SVC do
not differentiate as well as subcutaneous WAT because visceral WAT SVCs express more α-SMA.

Because we observed significant differences in actin and gelsolin expression across adipose depots, questions about the variability of the actin cytoskeleton in white, brown and beige/brite mature adipocytes is extremely relevant. Although there is abundant data about the cortical actin network of the white adipocyte no such assessment has been made in brown or beige adipocytes. In preliminary experiments, we stained whole WAT and BAT with phalloidin and observed differences in intensity and distribution (data not included). Because MRTFa drives actin expression and its absence promotes the development of beige/brite adipocytes (179), it could reasonably be hypothesized that actin is more enriched in white adipose than brown. Although our data corroborate this hypothesis (Figure 5.2), the actin cytoskeleton is molded by more than just the amount of actin present.

White adipocytes are spherical cells with ~90% of their volume comprised of a single cytoplasmic lipid droplet and a ‘squeezed’ nucleus while brown adipocytes are polygonal cells with a roundish nucleus and several cytoplasmic lipid droplets (271). From a morphological perspective, these cells are very unique. Because morphology is dictated, in part, by the actin cytoskeleton, it is also plausible that brown adipocytes have a more extensive actin cytoskeleton network by virtue of not having a volume-consuming, single lipid droplet. The hypotheses we’ve proposed need not be mutually exclusive. As our data
suggest, white adipocytes could indeed express more actin but may also selectively express a subset of ABPs that maintain a cortical actin network. Brown adipocytes, although they express less actin may lack those ABPs and/or express ABPs that promote a fibrous actin network. Our observed enrichment of gelsolin in WAT fits into this model (Figure 4.1a). A broad screen of ABP expression in white, brown and beige adipocytes would shed more light on this matter.

Differences between the actin cytoskeleton could also be the consequence of actin isoform-specific activity of ABPs, a hypothesis inspired by a 2005 study of cofilin kinetics. Equilibrium titrations demonstrated an actin isoform-dependence of cofilin binding where β-actin filaments are more cooperative than α-SMA filaments (272). What this translates into at the level of cell biology, is a greater proclivity for β-actin stress fiber severance compared to α-SMA stress fiber severance. This is dictated solely by enhanced cooperation with cofilin and not enhanced binding by cofilin. It is conceivable that cells rich in α-SMA stress fibers could be more susceptible to the actin severing activity of cofilin. The enrichment of α-SMA in visceral SVC may therefore allow cofilin to cause rapid, dramatic changes in visceral SVC actin cytoskeletons than it can achieve in subcutaneous SVCs. These SVCs would not need to express different amounts of cofilin to selectively reorganize the actin cytoskeleton and promote adipogenesis.
Summary

In this thesis, a profile of actin during adipogenesis was established at the level of the isoform. This profile centered around three actin isoforms: β-actin, γ-actin and α-SMA. Each isoform showed distinct patterns of expression and polymerization. Because actin is both downregulated and reorganized during adipogenesis, biochemically describing its dynamics becomes challenging. We developed a novel method for the assessment of actin dynamics which allows for the use of internal controls for normalization. This makes our method more reliable than current kits commercially available on the market. Using this method, we observed a rapid loss in α-SMA stress fibers in addition to an earlier overall loss in α-SMA. Our studies into the unique changes in α-SMA expression and distribution add to the growing body of evidence implicating a smooth muscle cell-like origin for adipocytes. They also help create a clearer profile of early step adipogenesis. These studies lay the foundation for future studies into whether actin directly regulates adipogenic commitment and, if so, how that plays into the development of obesity and its related co-morbidities.
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