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Myocardin-related transcription factor A regulates conversion of progenitors to beige adipocytes

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MYOCARDIN-RELATED TRANSCRIPTION FACTOR A REGULATES
CONVERSION OF PROGENITORS TO BEIGE ADIPOCYTES

by

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ABSTRACT

Thermogenic brown adipose tissue generates heat via mitochondrial uncoupling protein-1 (UCP-1), increases whole-body energy expenditure and may protects against obesity and metabolic disorders. White adipocytes store excess energy in the form of triglycerides. UCP-1 positive adipocytes develop within white adipose tissue (beige or brite adipocytes) in response to cold exposure or β3 adrenergic agonists. It was known that beige adipocytes arise from a distinct lineage compared with brown adipocytes, but the developmental origin of the beige adipocytes is still unclear. Signaling pathways that control beige adipocyte determination and formation are essentially unknown. Here, we identified a novel signaling pathway that regulates the lineage specification of beige adipocytes. Bone morphogenetic protein 7 (BMP7), a known brown adipogenesis inducer, suppresses Rho-GTPase kinase (ROCK) and depolymerizes F-actin (filamentous actin) into G-actin (globular actin) in mesenchymal stem cells. G-actin regulates myocardin-related transcription factor A (MRTFA) that co-transactivates serum response
factor (SRF) and promotes smooth muscle cell differentiation in various organs. Subcutaneous white adipose tissue from $MRTFA^{-/}$ mice had enhanced accumulation of UCP-1$^{+}$ adipocytes and elevated levels of brown-selective proteins. Compared with wild type (WT) controls, $MRTFA^{-/}$ mice exhibited improved metabolic profiles and were protected from diet-induced obesity and insulin resistance, suggesting that the beige adipocytes are physiologically functional. Compared to WT mice, stromal vascular cells from $MRTFA^{-/}$ mice expressed higher levels of distinct beige progenitor markers and reduced levels of smooth muscle markers. Our studies demonstrate a novel ROCK-actin-MRTFA/SRF pathway that contributes to the development of beige adipocytes.
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<tr>
<td>18FDG-PET</td>
<td>18fluorodeoxyglucose-Positron emission tomography</td>
</tr>
<tr>
<td>Agt</td>
<td>Angiotensinogen</td>
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<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
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<tr>
<td>BMP7</td>
<td>bone morphogenetic protein 7</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer-binding protein</td>
</tr>
<tr>
<td>CD137</td>
<td>tumor necrosis factor receptor superfamily member 9</td>
</tr>
<tr>
<td>Cdh5</td>
<td>Cadherin 5</td>
</tr>
<tr>
<td>Cidea</td>
<td>cell death-inducing DFFA-like effector a</td>
</tr>
<tr>
<td>Cox7a1</td>
<td>Cytochrome c oxidase polypeptide 7A1</td>
</tr>
<tr>
<td>CRP2/smLim</td>
<td>Cysteine rich protein 2/smooth muscle LIM</td>
</tr>
<tr>
<td>CycA</td>
<td>Cyclophilin A</td>
</tr>
<tr>
<td>Dex</td>
<td>dexamethasone</td>
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<tr>
<td>DIO</td>
<td>diet induced obesity</td>
</tr>
<tr>
<td>Elovl3</td>
<td>elongation of very long chain fatty acids 3</td>
</tr>
<tr>
<td>EPI</td>
<td>epididymal</td>
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<tr>
<td>F-actin</td>
<td>filamentous actin</td>
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<tr>
<td>Fabp3</td>
<td>fatty acid binding protein 3</td>
</tr>
<tr>
<td>FSK</td>
<td>forskolin</td>
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<tr>
<td>G-actin</td>
<td>globular actin</td>
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<td>GTT</td>
<td>glucose tolerance test</td>
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<tr>
<td>ING</td>
<td>Inguinal</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>LacZ</td>
<td>lactose operon Z</td>
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<tr>
<td>LAP</td>
<td>liver-enriched activator protein</td>
</tr>
<tr>
<td>LIP</td>
<td>liver-enriched inhibitory protein</td>
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<td>Mix</td>
<td>isobutylmethylxanthine</td>
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<td>MRTFA</td>
<td>myocardin-related transcription factor A</td>
</tr>
<tr>
<td>Myf-5</td>
<td>myogenic factor 5</td>
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<td>Myh11</td>
<td>Myosin-11</td>
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<tr>
<td>NDG2/chchd10</td>
<td>coiled-coil-helix-coiled-coil-helix domain containing 10</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline Tween-20</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>Platelet-derived growth factor receptors α</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor γ coactivator 1-α</td>
</tr>
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<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor γ</td>
</tr>
<tr>
<td>PRDM16</td>
<td>PR [PRDI-BF1 and RIZ] domain containing 16</td>
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<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
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<td>ROCK</td>
<td>Rho-GTPase kinase</td>
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<td>SMA</td>
<td>smooth muscle actin</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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<td>SRF</td>
<td>serum response factor</td>
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<tr>
<td>SVC</td>
<td>stromal vascular cell</td>
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<tr>
<td>T3</td>
<td>3,3,5-triiodo-L-thyronine</td>
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<td>Tbx1</td>
<td>T-box protein 1</td>
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<td>Tmem26</td>
<td>Transmembrane protein 26</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>TRE</td>
<td>tet-response element</td>
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<tr>
<td>tTA</td>
<td>tetracycline transactivator</td>
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<tr>
<td>UCP-1</td>
<td>uncoupling protein-1</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
</tr>
<tr>
<td>Zfp423</td>
<td>zinc-finger protein 423</td>
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<tr>
<td>Zic1</td>
<td>zinc finger protein 1</td>
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CHAPTER ONE

INTRODUCTION

Obesity and insulin resistance

The prevalence of obesity has risen dramatically throughout the world over the last three decades (Ogden et al., 2006). This epidemic was first observed in developed countries, and then spread across all the developing countries (Doria et al., 2008; Guilherme et al., 2008; Saltiel, 2012). Obesity has become a public health concern, particularly because it is a risk factor for a many co-morbidities, including type II diabetes, cardiovascular diseases and many types of cancer (WHO, 2013) The American Medical Association has recently classified obesity as a disease, leading to an expanding population of chronically ill people with shorter life span. Novel weight-loss treatments that prevent and reduce obesity are critically in need (Cai et al., 2010).

Among obesity-associated co-morbidities, type II diabetes is a particularly destructive one. The global incidence of type II diabetes is predicted to reach 350 million cases by the year of 2030, costing $132 billion in the United States alone (Hogan et al., 2003). Insulin resistance is a leading cause of type II diabetes (Reaven, 2005a) (Reaven, 2005b). Insulin resistance is defined by impaired insulin sensitivity in adipose tissue, liver, and muscle (Zeyda and Stulnig, 2009). Insulin resistance also leads to increased circulating free fatty acid levels and ectopic fat accumulation in muscle and liver, which prevents insulin-mediated glucose uptake in skeletal muscle and elevated glucose production in the liver (Zeyda and Stulnig, 2009). Numerous studies have detected a strong correlation between obesity and insulin resistance (Reaven, 2005a; Reaven,
2005b). Over-nutrition and excess energy accumulation in adipocytes, myocytes, and hepatocytes triggers signals that lead to insulin resistance and, finally, decreased pancreatic insulin secretion (Saltiel, 2012).

Several hypotheses have emerged to explain the increased prevalence of obesity including increased food availability, adaptation to a sedentary lifestyle, changes in food content or nutritional value, intestinal microbial symbiosis (microorganisms living in the intestine), viral infection, low or high birth weight, evolutionary pressure (Saltiel, 2012). One aspect of obesity is certain: the increased occurrence of metabolic disease is due to a positive energy balance in individuals (Saltiel, 2012).

**White adipocytes**

A chronic positive energy balance leads to the expansion of white adipose tissue (WAT) due to the excess energy storage as triglycerides. WAT is characterized by adipocytes containing large unilocular lipid droplets (Cristancho and Lazar, 2011). As an active endocrine organ, WAT regulates diverse metabolic activities, such as insulin sensitivity, lipid metabolism and satiety (Galic et al., 2010). WAT is distributed in several locations in humans including subcutaneous regions and surrounding visceral organs. WAT in subcutaneous and visceral depots is histologically similar but has distinct metabolic properties (Ibrahim, 2010). In fact, accumulation of visceral WAT during the development of obesity correlates with pathologic inflammation and insulin resistance (Kintscher et al., 2008), whereas subcutaneous WAT may improve glucose tolerance and insulin sensitivity (Tran et al., 2008). In response to excess energy, WAT undergoes
hypertrophy and hyperplasia during the development of obesity in mice (Wang et al., 2013) and humans (Drolet et al., 2008).

**Brown adipocytes**

In contrast to WAT, brown adipose tissue (BAT) is an adipose organ that dissipates energy by thermogenesis (heat production) (Frontini and Cinti, 2010). In rodents and infant humans, BAT primarily localizes in interscapular and perirenal regions (English et al., 1973; Frontini and Cinti, 2010). In adult humans, active BAT was identified in patients with cancer by using $^{18}$FDG-PET (Nedergaard et al., 2007) and then in healthy individuals (Cypess et al., 2009; Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). In contrast with rodents and newborn humans, BAT in adult humans is mainly located in cervical, supraclavicular, axillary and paravertebral regions (Sidossis and Kajimura, 2015). Compared to the unilocular white adipocytes, brown adipocytes feature multilocular lipid droplets and are packed densely with mitochondria that express uncoupling protein-1 (UCP-1) (Cannon and Nedergaard, 2004).

In BAT, UCP-1 can generate heat from the respiratory chain by creating a “proton leak”. In mammalian cells, oxidation of fuels (lipid, sugar and amino acids) leads to formation of NADH and FADH$_2$ in the mitochondria where they donate electrons to the electron transport chain. During respiration, the movement of electrons along the electron transport chain is coupled to the transfer of protons from the matrix into the inner membrane space of the mitochondrion forming a proton gradient (Krauss et al., 2005). The potential energy of the proton gradient is used by the ATP synthase complex to
synthesize ATP through oxidative phosphorylation. UCP-1, which resides in the inner membrane of mitochondria, creates a proton leak resulting in diminished ATP synthase activity (Krauss et al., 2005). As a consequence, energy generated through collapse of the proton gradient is released as heat (thermogenesis) (Krauss et al., 2005).

**Beige adipocytes**

Clusters of UCP-1 positive (UCP-1+) adipocytes can be observed within inguinal subcutaneous WAT (ING WAT) of young mice (7-9 weeks old) that are maintained at ambient temperature (Wu et al., 2012). This UCP-1+ population of adipocytes becomes more prominent following cold exposure mediated by β3-adrenergic receptor activation (Collins et al., 1997; Gorbani and Himms-Hagen, 1997; Granneman et al., 2005; Guerra et al., 1998; Nagase et al., 1996). In addition, UCP-1+ cells in WAT increase in response to PPARγ agonist administration (Petrovic et al., 2010; Vernochet et al., 2009; Wilson-Fritch et al., 2004; Xue et al., 2005), exposure to FGF21, irisin, cardiac natriuretic peptides (Bordicchia et al., 2012; Boström et al., 2012; Fisher et al., 2012), or pharmacological activation of A2A receptors (Gnad et al., 2014). These brown-like adipocytes in WAT were originally named brite adipocytes (Loncar, 1988; Young et al., 1984), and then were later referred to as beige adipocytes (Wu et al., 2012). Beige adipocytes and brown adipocytes share a similar multilocular morphology and a gene expression pattern consistent with a thermogenic role. However, beige adipocytes also have distinctive gene expression signatures compared to brown adipocytes, suggesting that they are a distinct thermogenic cell type (Petrovic et al., 2010; Sharp et al., 2012; Walden et al., 2012; Wu et al., 2012).
Beige adipocytes contribute to metabolism in rodents

Some investigators argue that BAT is the predominant source for thermogenic energy expenditure over brite/beige adipocytes (Shabalina et al., 2013). This claim is based on the low thermogenic density (UCP-1-dependent oxygen consumption per gram of tissue) of beige adipocytes and the inefficient thermogenic capacity of beige mitochondria (Shabalina et al., 2013). However, there are several lines of evidence suggesting beige adipocytes contribute to whole-body metabolism.

Overexpression of Forkhead box protein C2 (FOXC2), a transcriptional factor preferentially expressed in adipose tissue, results in hypertrophic BAT and brown-like adipocytes in WAT (Cederberg et al., 2001). UCP-1 mRNA is increased in the ING WAT of these transgenic mice compared to WT mice without major differences observed in BAT. The FOXC2 transgenic mice are surprisingly resistant to high-fat feeding induced insulin resistance (Cederberg et al., 2001), suggesting that the brown-like adipocytes in subcutaneous WAT might contribute to the enhanced insulin sensitivity.

Fat-specific overexpression of PRDM16 (PRD1-BF-1-RIZ1 homologous domain-containing protein, a key regulator of brown adipogenesis) (Cristancho and Lazar, 2011) promotes the recruitment of beige adipocytes to subcutaneous WAT without any change in UCP-1 expression in BAT (Seale et al., 2011). In response to a high-fat diet, PRDM16 transgenic mice display increased energy expenditure, limited weight gain, and improved glucose tolerance (Seale et al., 2011).

WAT-specific knockdown of retinaldehyde dehydrogenase (Aldh1a1, also known as Raldh1) induces beige adipocyte formation in WAT without any change in BAT.
These Aldh knockdown mice have improved glucose homeostasis compared to WT littermates when challenged with a high-fat diet (Kiefer et al., 2012).

Interestingly, Myf-5 driven deletion of BMP receptor 1A in BAT precursors results in a paucity of BAT but activation of beige adipocyte formation in both subcutaneous and visceral WAT. These mice maintained their body temperature and thermogenic capacity upon cold exposure, indicating that beige adipocytes have the ability to contribute to thermogenesis (Schulz et al., 2013). Taken together, these studies support a model where beige adipocytes contribute to energy homeostasis and whole-body metabolism.

**Characterization of Thermogenic Adipocytes in Adult Humans and Mice**

As discussed above, there are two distinct types of thermogenic cells in rodents: beige adipocytes in WAT and brown adipocytes in the interscapular BAT depot. Each one has a distinct program of gene expression and can be stimulated by different effectors. It is, therefore, important to determine which type of thermogenic adipocytes function in humans. This will be crucial when using mice for the identification of targets in thermogenic adipocytes as a potential therapeutic strategy to treat obesity-associated co-morbidities (Rosen and Spiegelman, 2014).

*Anatomical deposition of BAT in infants and adult humans.*

In infants, BAT primarily localizes to the interscapular region (English et al., 1973), is composed of multilocular UCP-1⁺ adipocytes, and is separated from surrounding WAT by connective tissue. These features are also true of the interscapular BAT in rodents (Lidell et al., 2013). UCP-1⁺ adipocytes in the neck and upper thoracic
BAT in adult humans form clusters surrounded by many white adipocytes (Cypess et al., 2009; Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). This structural arrangement is similar to beige adipocytes in murine WAT.

*Adult human BAT and murine beige adipocytes share similar molecular signatures*

Several molecular markers for distinguishing rodent brown, beige and white adipocytes were identified, at least at the messenger RNA level (Seale et al., 2007; Sharp et al., 2012; Timmons et al., 2007; Walden et al., 2012; Wu et al., 2012). The mRNA expression of these molecular markers was also analyzed in BAT of infant and adult humans (Cypess et al., 2009; Jespersen et al., 2013; Lidell et al., 2013; Sharp et al., 2012; Wu et al., 2012). Interestingly, interscapular BAT of infant humans expresses ZIC1 (zinc-finger protein 1), a brown adipocyte marker in rodents (Lidell et al., 2013). However, infant BAT expresses significantly lower levels of TBX1 (T-box transcription factor 1), a murine beige-precursor marker, than supraclavicular BAT of adult humans (Lidell et al., 2013). Strikingly, BAT in various locations of adult humans preferentially express murine beige markers (whose messenger RNA are enriched in murine beige adipocytes): Supraclavicular BAT is enriched for CD137 (tumor necrosis factor receptor superfamily member 9), CITED1 (cbp/p300-interacting transactivator 1), SHOX2 (short stature homeobox 2), TBX1 and TMEM26 (transmembrane protein 26) and intermediate neck BAT expresses high levels of CD137, SHOX2 and TMEM26 (Cypess et al., 2009; Jespersen et al., 2013; Lidell et al., 2013; Sharp et al., 2012; Wu et al., 2012). Although it was suggested that the deep neck BAT of adult humans express some murine BAT markers e.g. ZIC1 and LHX8 (LIM-homeodomain Protein 8), the volume of the deep
neck BAT is relatively small compared to the BAT in supraclavicular and intermediate neck region. Thus, the majority of BAT in humans has the molecular signature of murine beige adipocytes.

Taken together, primary human BAT has the molecular signature of murine beige adipocytes, suggesting that identification of signaling pathways that regulate beige adipocyte formation might provide relevant targets for development of therapeutics that enhances energy expenditure in obese individuals.

**Physiological stimuli controlling beige/brite adipocyte formation**

Numerous secreted molecules have been identified that regulate brown and beige adipocyte activity through systemic, autocrine and paracrine mechanisms. Specific examples are described below.

*Activation of β3-adrenergic signaling by norepinephrine*

Sympathetic nerves releases norepinephrine that binds to β-adrenoreceptors (AR) and increases the cellular cyclic AMP levels, leading to the phosphorylation of protein kinase A (PKA) and p38 MAPK (Cao et al., 2004). P38 MAPK can directly phosphorylate ATF2 and PGC-1α, leading to the transcriptional expression of UCP-1 and PGC-1α itself (Cao et al., 2004). β3-adrenoreceptor signaling seems to be exclusively regulating beige adipocyte formation. In β3-AR knockout mice, cold-induced beige adipocyte formation is severely abolished, but brown adipocytes appear normal. In addition, β3-adrenergic agonists selectively induce beige adipocyte formation in WAT (Barbatelli et al., 2010; Jimenez et al., 2003). Interestingly, activation of β-adrenergic signaling induces Cox2 activation in white adipocytes, which secrete Prostaglandin E2/I2
(PGE E₂/I₂) and shift the differentiation of defined mesenchymal progenitors toward beige/brite adipocytes in WAT (Vegiopoulos et al., 2010).

**Fibroblast growth factors 21 (FGF21)**

FGF21 is an endocrine hormone that regulates the fasting response in the liver (Badman et al., 2007; Inagaki et al., 2007; Kharitonenkov et al., 2005). FGF21 plays a physiologic role in beige adipocyte formation upon cold exposure. In fact, FGF21 deficient mice display an impaired ability to adapt to chronic cold exposure, with diminished browning of WAT. Adipose-derived FGF21 acts in an autocrine manner and increases expression of UCP-1 and other thermogenic genes in WAT at least in part, by enhancing the PGC-1α protein levels (Fisher et al., 2012).

**Irisin/Fibronectin type III domain-containing 5 (FNDC5)**

Endurance training and PGC-1α overexpression in muscle can stimulate the expression of the myokine FNDC5, leading to an increased circulating level of irisin. Irisin can induce a thermogenic program in primary adipocytes (Boström et al., 2012). It was also suggested that beige adipocytes preferentially respond to this hormone (Wu et al., 2012). The molecular mechanism of irisin in browning of WAT remains elusive and several lines of evidence suggest that irisin may not promote beige adipocyte formation (Crujeiras et al., 2014)

**Atrial natriuretic peptides (ANP) and brain NP (BNP)**

Atrial natriuretic peptides (ANP) and brain NP (BNP) are released from heart and are crucial for fluid and hemodynamic homeostasis (Bordicchia et al., 2012). Bordicchia et al. have shown that BNP treatment can activate BAT-selective thermogenic gene
program, mitochondria biogenesis and uncoupling respiration in mice or human adipocytes in a p38 MAPK dependent manner (Bordicchia et al., 2012).

*Methionine-enkephalin peptides by group 2 innate lymphoid cells (ILC2s)*

ILC2s, which are present in lymphoid and non-lymphoid tissues (Moro et al., 2010; Neill et al., 2010), orchestrate type 2 innate and adaptive immune responses in the setting of tissue damage, infection, and allergen exposure (Koyasu and Moro, 2013; McKenzie et al., 2014; Walker et al., 2013). ILC2 activation results in the proliferation of bi-potential adipocyte precursors and their subsequent commitment to the beige adipocyte lineage (Lee et al., 2015). In addition, ILC2 were reported to produce methionine-enkephalin peptides that induce UCP-1 expression in adipocytes (Brestoff et al., 2014).

*Developmental lineages of brown, white and beige adipocytes*

*Brown adipocytes arise from Myf5+ myogenic lineage*

As described above, brown adipocytes are mainly distributed in the interscapular region of mice. Using *in vivo* fate mapping, Seale et al. have shown that brown, but not white adipocytes arise from precursor cells that express the myogenic transcription factor Myf-5 (Seale et al., 2008). By using the Engrailed-1 (En1, a homeobox transcriptional factor expressed in central dermomyotome, also referred to as “dermatome” (Scaal and Christ, 2004)) *in vivo* fate mapping, Atit et al. showed that En1 expressing cells give rise to interscapular brown fat (Atit et al., 2006). En1-expressing progenitors can also give rise to skeletal muscle (Atit et al., 2006). A first indication that brown adipocytes might share a common origin with muscle came from the studies of Cannon, Nedergaard and collaborators who showed that primary brown precursors contain a myogenic
transcriptional signature of gene expression (Timmons et al., 2007). Consistently, mitochondrial proteomics of BAT and muscle have shown that the brown fat mitochondria are more similar to their counterparts in muscle (Forner et al., 2009).

To be discussed in more detail below, PRDM16 and C/EBPβ (CCAAT/ enhancer-binding protein β) are key regulators of brown adipogenesis (Rosen and Spiegelman, 2014). They also control a switch that determines the fate of progenitors to progress to brown adipocytes as opposed to skeletal myocytes. Myf-5-driven knockout of PRDM16 or C/EBPβ in brown precursor cells results in muscle-like brown adipocytes expressing myogenic-selective markers (Kajimura et al., 2009; Seale et al., 2008). These findings confirm a common lineage between brown fat and muscle (Scheme 1).

_White adipocytes arise from both Myf5⁻ and Myf5⁺ lineages_

In contrast with brown adipocytes, the origin of white adipocytes is less defined due in part to the existence of multiple progenitors (Berry and Rodeheffer, 2013). Early attempts to identify adipose lineages used fluorescence-activated cell sorting (FACS) to isolate stem cells expressing specific surface markers, which could be converted to adipocytes in vitro (Cawthorn et al., 2012; Rodeheffer et al., 2008). Later genetic fate mapping traced white adipocyte precursors to mural cells (SMA⁺, PDGFRβ⁺, NG2⁺) (Tang et al., 2008) and endothelium (Gupta et al., 2012; Tran et al., 2012), though the endothelial origin has been challenged by other studies (Berry and Rodeheffer, 2013; Kanda et al., 2009). Recent studies have also suggested perivascular mural cells are not the only origin of white precursors (Berry and Rodeheffer, 2013) (Jiang et al., 2014).
White adipocytes were initially suggested to arise exclusively from a Myf5<sup>-</sup> lineage given the fact that the myogenic gene signature was absent in white adipocytes and their precursors (Seale et al., 2008; Timmons et al., 2007; Walden et al., 2012). However, recent studies challenged this idea: myf5-Cre-driven deletion of PTEN, a negative regulator of insulin signaling and adipogenesis, resulted in an adipose tissue redistribution in which interscapular BAT and WAT had expanded several fold compared to WT littermates. In these mice, other depots including inguinal and visceral were absent (Sanchez-Gurmaches et al., 2012). This lipodystrophy of subcutaneous and abdominal WAT is caused by the selective expansion of Myf5<sup>+</sup> precursors (Sanchez-Gurmaches et al., 2012). Subsequently, lineage-tracing analysis demonstrated that both Myf5<sup>+</sup> and Myf5<sup>-</sup> give rise to white adipocytes (Sanchez-Gurmaches et al., 2012; Shan et al., 2013) (Scheme 1).

*Beige adipocytes derive from both transdifferentiation of white adipocytes and de novo recruitment of progenitors*

Beige adipocytes are not derived from a Myf5<sup>+</sup> lineage (Seale et al., 2008). Two major hypotheses regarding the origin of beige adipocytes exist: transdifferentiation from pre-existing white adipocytes or de novo recruitment of distinct beige progenitors. The former hypothesis was presented based on the absence of cell proliferation following activation of β-adrenergic signaling in adipose tissue, and identification of a morphological transition between white and brite adipocytes (Himms-Hagen et al., 2000; Vitali et al., 2012). The latter hypothesis, however, proposed that beige adipocytes arise from recruitment and expansion of distinct progenitors that could be identified by both
sorting and single cell- cloning (Lee et al., 2012; Schulz et al., 2011; Vegiopoulos et al., 2010; Wu et al., 2012). Two lineage-tracing studies support the latter hypothesis: new adipogenesis is required for beige adipocyte formation upon cold exposure (Wang et al., 2013). The second study traced UCP-1 in an indelible fashion, and suggested that beige adipocytes recruited by initial cold exposure transform into inactive beige adipocytes (with a white adipocyte morphology) upon warm adaption, and then regain beige adipocyte signatures after additional cold exposure (Rosenwald et al., 2013). Thus, the plasticity of beige adipocytes might cause a false observation of conversion between pre-existing pure white adipocytes and active beige adipocytes. Taken together, beige adipocytes likely derive from distinct precursors within WAT (Scheme 1).

Transcriptional regulators of the development of brown and beige adipocytes

Many studies have investigated the regulation of brown adipogenesis, but fewer have examined beige adipocyte formation. For both cell types, the core regulators of adipogenesis, PPARγ and C/EBPβ, are involved as well as the transcriptional cofactors, PGC-1α and PRDM16 (Cristancho and Lazar, 2011).

PPARγ and C/EBPs in adipogenesis

PPARγ and C/EBPβ are the core conserved transcription factors that regulate white and brown adipocyte formation (Cristancho and Lazar, 2011). PPARγ is the master regulator of adipogenesis (Hu et al., 1995; Tontonoz et al., 1994). Ectopic expression of PPARγ in non-adipogenic mouse fibroblasts initiates the entire adipogenic program, giving rise to adipocytes that have many of the functions of mature adipocytes (Tontonoz
et al., 1994). Genetic deletion of PPARγ results in embryonic lethality (Barak et al., 1999; Rosen et al., 1999). WAT-hypomorphic PPARγ mutation in mice resulted in animals that were severely lipodystrophic (Koutnikova et al., 2003), authenticating PPARγ as a master regulator of adipogenesis.

The C/EBP family members, C/EBPα, C/EBPβ and C/EBPδ are also important inducers of adipogenesis whereby C/EBPβ and C/EBPδ regulate early steps and C/EBPα, in cooperation with PPARγ regulates terminal adipogenesis (Cao et al., 1991; Yeh et al., 1995). Ectopic expression of C/EBPβ and C/EBPδ can induce C/EBPα expression and the adipogenic program without adipogenic cocktails (Cao et al., 1991; Yeh et al., 1995). Ectopic expression of C/EBPβ in NIH-3T3 fibroblasts can induce the expression of PPARγ2 and facilitate the conversion of cells into adipocytes (Wu et al., 1996; Wu et al., 1995). A positive feedback loop between C/EBPα and PPARγ reinforces terminal differentiation (Park et al., 2012; Rosen et al., 2002; Wu et al., 1999).

*C/EBPβ in brown and beige adipocyte formation:

Other than its regulatory role in white adipogenesis as outlined above, C/EBPβ regulates programs that are specific to brown and beige adipocyte development. C/EBPβ is enriched in BAT compared to WAT (Kajimura et al., 2009). Overexpression of C/EBPβ reprograms white 3T3-L1 adipocytes into brown-like adipocytes with expression of BAT-selective genes (Karamanlidis et al., 2007). Inactivation of C/EBPβ in mice still permits the formation of white and brown adipocytes, but the functional efficiency of BAT is compromised due to loss of UCP-1 expression (Tanaka et al., 1997). Thus, it
Scheme 1. Brown, white and beige adipocyte lineages. Brown adipocytes arise from a Myf5$^+$ lineage. White adipocytes derive from both Myf5$^+$ and Myf5$^-$ lineages. Beige adipocytes arise from Myf5$^-$ lineage and can be transformed in to inactive form that represents white adipocyte morphology.
appears that C/EBPβ regulates the thermogenic gene program. Consistently, disruption of one of the C/EBPβ upstream transcriptional activator, Plac8 (placenta-specific 8), results in absence to cold-induced browning in WAT (Jimenez-Preitner et al., 2011). Additional regulators for brown adipocyte formation other than PPARγ and C/EBPs are PGC-1α and PRDM16 that will be described below:

**PGC-1α**

PGC-1α was first identified as a transcriptional cofactor of PPARγ that is dramatically induced upon cold exposure in BAT and skeletal muscle (Uldry et al., 2006). PGC-1α is suggested to be the major regulator of the adrenergic activation of the thermogenesis in adipocytes: ectopic expression of PGC-1 in white adipocytes activates expression of UCP-1 and key mitochondrial enzymes of the respiratory chain, and increases the cellular content of mitochondrial DNA (Puigserver et al., 1998). PGC-1α−/− mice are unable to maintain core body temperature following cold exposure and develop hepatic steatosis and insulin resistance due to a combination of reduced mitochondrial respiratory capacity and an increased expression of lipogenic genes (Kleiner et al., 2012; Leone et al., 2005; Uldry et al., 2006). The expression and activity of PGC-1α is directly controlled by the β-adrenergic pathway. Upon β-adrenergic stimulation, p38 MAPK is phosphorylated and in turn phosphorylates and activates PGC-1α to enhance UCP-1 gene expression (Cao et al., 2004; Cao et al., 2001). PGC-1α greatly increases the transcriptional activity of PPARγ and the thyroid hormone receptor on the uncoupling protein (UCP-1) promoter (Puigserver et al., 1998).
PRDM16

PRDM16 is a zinc-finger transcriptional factor that is expressed preferentially in BAT compared to WAT in both mice and humans (Lee et al., 2012; Seale et al., 2007). Ectopic expression of PRDM16 in myoblasts induces their differentiation into UCP-1 expressing brown adipocytes (Seale et al., 2008). PRDM16 directly interacts with transcriptional factors C/EBPβ, PPARγ and PGC-1α to regulate their activities (Hondares et al., 2011; Kajimura et al., 2009; Seale et al., 2008; Seale et al., 2007). Knockdown of PRDM16 in brown precursors can switch the gene expression profile of the precursors into a white-specific and muscle-specific pattern (Seale et al., 2008; Seale et al., 2007), indicating the role of PRDM16 in regulating the lineage determination of brown adipocytes.

PRDM16 is also selectively expressed in subcutaneous WAT relative to visceral WAT in mice (Seale et al., 2011). Overexpression of PRDM16 in all adipose depots by using the FABP4 promoter robustly induces the development of beige adipocytes in subcutaneous WAT, but not in epididymal WAT (Seale et al., 2011). PRDM16 knockdown in isolated subcutaneous adipocytes causes a sharp decrease in expression of thermogenic genes and a reduction in uncoupled cellular respiration (Seale et al., 2011). Finally, PRDM16 haploinsufficiency reduces the appearance of beige adipocytes in WAT in response to β-adrenergic stimulation (Seale et al., 2011). These results demonstrate that PRDM16 is a cell-autonomous determinant of the beige gene program in subcutaneous WAT (Seale et al., 2011).
**Determination of adipocyte lineages by TGFβ superfamily.**

Numerous studies have identified pathways that are involved in adipogenic lineage determination, e.g. Wnt and hedgehog pathways, non-canonical Wnt5A and 10B pathway and TAZ/YAP pathway (Cristancho and Lazar, 2011). Most of the pathways converge onto PPARγ and C/EBPβ (Okamura et al., 2009; Xu et al., 2008). Among the many adipogenic effectors, members of the TGFβ superfamily stand out as regulators of white, brown and beige adipocyte formation:

**TGFβ**

TGFβ levels positively correlate with obesity in humans and rodents (Zamani and Brown, 2011). However, TGFβ inhibits in vitro adipogenesis in 3T3-L1 preadipocytes (Choy et al., 2000) by signaling through phosphorylation of Smad3. Interestingly, Smad3 KO mice are resistant to diet-induced obesity and have an elevated level of beige adipocyte formation in ING WAT (Yadav et al., 2011). Interestingly, Smad3 can directly bind to C/EBPβ and repress its activity in 3T3-F442A cells (Choy and Derynck, 2003). These data suggest that TGFβ-Smad3 pathway might negatively regulate beige adipogenesis.

**BMPs**

Bone morphogenetic proteins (BMPs) regulate white, brown and beige adipocyte formation (Zamani and Brown, 2011). BMPs can induce adipogenesis by activating different Smads or p38 map kinase (MAPK) (Hata et al., 2003; Huang et al., 2009; Tseng et al., 2008).
BMP2 can enhance both adipogenic and osteogenic commitment and differentiation in C3H10T1/2 cells (Wang et al., 1993). In fact, BMP2 promotes both adipogenesis and osteogenesis when multipotent cells are provided with a PPARγ agonist or retinoid acid, respectively (Wang et al., 1993) (Hata et al., 2003; Skillington et al., 2002; Sottile and Seuwen, 2000). BMP2 activates Smad1 and stimulates PPARγ expression during early adipogenic differentiation in MEFs (Jin et al., 2006). In addition, BMP2 can induce commitment of C3H10T1/2 mesenchymal cells into adipocytes by activating Smad1/5/8 and p38-MAPK (Huang et al., 2009).

Similar to BMP2, BMP4 can prime C3H10T1/2 mesenchymal cells to respond more robustly to an adipogenic cocktail (Tseng et al., 2008) by stimulating Smad 1/5/8 and Smad4 (Bowers and Lane, 2007). BMP4 transgenic mice have reduced white adipocyte size and enhanced beige adipocyte formation, while BMP4 deficient mice exhibit enlarged white adipocytes and impaired insulin sensitivity (Qian et al., 2013).

BMP7 induces brown adipogenesis (Tseng et al., 2008) by activating p38 MAPK and PGC-1α dependent mitochondria biogenesis. In addition, BMP7 can induce brown adipogenesis in primary human adipose stem cells (Elsen et al., 2014; Neumann et al., 2007). BMP7 administration in subcutaneous WAT results in browning of WAT in mice (Boon et al., 2013).

Taken together, the literature supported the idea that members of the TGFβ superfamily likely play a critical role in formation of beige, brown and white adipocytes
**Determination of adipocyte lineages by the Actin-RhoGTPase pathway**

*Actin dynamics in regulating adipocyte formation.*

In 1982 Farmer and Spiegelman reported that actin and tubulin synthesis decreases by 90% during differentiation of 3T3-F442A preadipocytes, (Spiegelman and Farmer, 1982) suggesting a negative correlation between adipocyte formation and actin/tubulin synthesis. Actin dynamics are regulated by specific actin-binding proteins including coflin and profilin (Olson and Nordheim, 2010). During 3T3-L1 adipocyte formation, the actin cytoskeleton undergoes a major remodeling by converting actin stress fibers to thick cortical actin filaments after adipogenic induction (Yang et al., 2014). Disruption of stress fibers by the actin-severing protein coflin is a requisite step for adipogenesis of 3T3-L1 preadipocytes (Yang et al., 2014). Profilin, an actin-nucleating factor has a positive correlation with obesity in both humans (Alfadda et al., 2013) and mice (Romeo et al., 2013). Haploinsufficiency of profilin-1 protects against obesity-associated glucose intolerance and preserves adipose tissue immune homeostasis by significantly reducing the size of white adipocytes and inhibiting macrophage infiltration (Romeo et al., 2013). These results indicate that the regulation of actin dynamics contribute to adipocyte formation.

*Rho-GTPase-Rho associated kinase (ROCK) signaling in adipogenic lineage determination*

Cofilin, profilin and other actin binding proteins are effector proteins of the Rho-GTPase family, the master modulators of the polymerization equilibrium of G-actin and
F-actin (Olson and Nordheim, 2010). Rho-GTPase and its regulator, p190-B RhoGAP, determine the fate of murine embryo-developed fibroblasts (MEFs) toward myogenesis rather than adipogenesis (Sordella et al., 2003). Rho-GTPase A mediated actin tension commits human mesenchymal stem cells to osteoblasts instead of adipocytes (McBeath et al., 2004). These findings suggest a role for Rho-GTPase in regulating commitment of mesenchymal stem cells to different lineages.

**MRTFs-SRF signaling is controlled by actin-Rho-GTPase dynamics**

**SRF as the downstream transcriptional factor of Rho-GTPase**

Given the contribution of actin-RhoGTPase in adipocyte formation, it is important to investigate their downstream transcription factors to gain more insight into the mechanisms by which they negatively regulate adipocyte formation. The link between the actin cyto-architecture and downstream transcriptional activity was first identified by Treisman and collaborators who showed that Rho-GTPase and changes in actin dynamics regulate serum response factor (SRF) activity (Hill et al., 1995; Posern et al., 2002; Sotiropoulos et al., 1999). SRF is a versatile transcriptional factor that regulates many genes that support a broad range of biological process including production of cytoskeletal and extracellular matrix proteins (Olson and Nordheim, 2010).

Interestingly, by mapping histone modification and transcriptional motifs in preadipocytes versus adipocytes, Rosen and associates identified SRF as a potential negative regulator of adipogenesis in mice and humans (Mikkelsen et al., 2010).
**MRTFs are co-transactivators of SRF**

SRF can be activated by cofactors, e.g. TCFs (Treisman, 1994; Treisman, 1995), Smad3 (Qiu et al., 2005). Identification of the SRF co-transactivators, myocardin related transcription factors (MRTFs) family revealed the direct mechanism enabling G-actin to control the nuclear transcriptional activity of SRF (Miralles et al., 2003; Wang et al., 2002). Monomeric G-actin regulates cytoplasm-nucleus shuttling of MRTFs and thereby influence the expression of SRF target genes (Olson and Nordheim, 2010) (Scheme 2). Specifically, high levels of G-actins retain MRTFs in the cytoplasm (Miralles et al., 2003; Posern et al., 2004; Posern et al., 2002). Rho-ROCK induces the incorporation of G-actin into F-actin that liberates MRTFs to enter the nucleus and interact with SRF (Miralles et al., 2003; Posern et al., 2004; Posern et al., 2002). This triggers the expression of a subset of SRF target genes (Olson and Nordheim, 2010) (Scheme 2).

**MRTF family**

MRTF family is composed of three members: myocardin, MRTFA and MRTFB. While myocardin is expressed exclusively in cardiovascular tissues (Wang et al., 2002), MRTFA (MAL, MKL1) and MRTFB (MKL2) are more widely expressed (Parmacek, 2007; Pipes et al., 2006; Wang et al., 2002). MRTFs have three RPEL domains that bind to G-actin (Guettler et al., 2008; Miralles et al., 2003; Mouilleron et al., 2008; Posern et al., 2004). Myocardin, does not bind G-actin efficiently and is not controlled by changes in actin dynamics (Kuwahara et al., 2005; Wang et al., 2002). Myocardin and MRTFB deletion in mice results in embryonic lethality (Wang et al., 2002) (Oh et al., 2005). MRTFA KO mice are viable, but females have impaired myoepithelial development in
mammary gland (Li et al., 2006; Sun et al., 2006). Most MRTF-SRF target genes have two major functions: 1. regulating muscle-specific and contractile genes, 2. controlling actin dynamics and cell motility. (Detailed functions will be discussed in Chapter 3)

**Cross-talk between TGFβ superfamily and Rho-GTPase-MRTF/SRF**

TGFβ superfamily of cytokines TGFβ, BMPs and activins stimulate different signaling pathways and modulate Rho-GTPase mediated actin dynamics (Moustakas and Heldin, 2008). TGFβ induces epithelial-mesenchymal transition (EMT) by altering cellular structure and motility (Moustakas and Heldin, 2008). MRTFs-SRF activity was observed in TGFβ-induced EMT (Fan et al., 2007; Hinson et al., 2007; Morita et al., 2007). BMPs (2, 4 and 7) induce the nuclear localization of MRTFA and MRTFB and in 10T1/2 cells, thereby promoting vascular smooth muscle cell differentiation (Lagna et al., 2007). However, our lab has shown that BMP7, a brown adipogenesis promoter (Tseng et al., 2008) disrupts the actin cytoskeleton and inhibits Rho kinase that would hypothetically deactivate MRTF-SRF transcriptional activity (Mcdonald et al., 2015). The discrepancy in BMPs’ action might be a result of different inducers (smooth muscle or adipogenic inducers) used later to program the cells into different cell types.

**Summary**

From what we discussed previously, members of the TGFβ superfamily regulate the lineage determination of beige adipocyte formation: specifically, TGFβ-Smad3 negatively regulate beige adipogenesis. BMP4 or BMP7 can enhance beige adipocyte formation in vivo and in vitro. In addition, Rho-GTPase mediated actin tension is a negative regulator of adipogenic lineage determination. BMP7 can inhibit Rho kinase and
Scheme 2. Actin-RhoGTPase-MRTFA signaling. Monomeric G-actin regulates cytoplasm-nucleus shuttling of MRTFs and thereby influence the expression of SRF target genes. BMP7 can inhibit Rho kinase and disrupt the actin cytoskeleton.
disrupt the actin cytoskeleton. TGFβ and Rho-GTPase signaling converge onto MRTF-SRF transcriptional activity. However, the potential role of MRTFs in regulating adipocyte formation is not clear (Scheme 2).

We hypothesized that MRTFA negatively regulates adipocyte formation. We initiated the study by characterizing the phenotype of MRTFA-deficient mice. To understand the role of MRTFA in adipose tissue development, we examined the morphology and molecular characteristic of different fat pads as well as body composition, whole body glucose homeostasis and metabolic profile of the mice. In addition, we challenged the MRTFA−/− mice with different stimuli, such as β3-adrenergic stimulation and high-fat feeding to understand the potential role of MRTFA in adipose tissue in response to different physiological situations, as outlined in Chapter 3.

Using stromal vascular cells as a model, we continued to follow the finding in Chapter 3 and studied the potential role of MRTFA in the determination of beige versus smooth muscle cell, as outlined in Chapter 4.

We demonstrated the MRTFA-SRF’s role in negatively regulating beige adipocyte formation in mice. MRTFA−/− stromal vascular cells exhibit more beige adipocyte but less smooth muscle features, implicating an MRTFA-SRF circuit’s role in shifting progenitor populations towards the beige phenotype from the smooth muscle lineage.
CHAPTER TWO
MATERIAL AND METHODS

Animal

Housing

MRTFA+/− mice (mixed C57BL/6J and 129 genetic background) were a gift from Dr. Eric Olson, UT Southwestern Medical Center (Li et al., 2006). Collagen-GFP and Smooth muscle actin-mCherry were inbred into MRTFA WT or KO mice by crossing MRTFA+/- mice with mice with only Collagen-GFP transgene or both transgene (C57BL/6J background) (Kalajzic et al., 2005; Kalajzic et al., 2008). All littermates were generated by breeding MRTFA+/− female with MRTFA +/− male mice. Mice were housed in a pathogen-free environment and housed in groups of 2-5 in filter-top cages (23°C in a 12 hours light/dark cycle). All experiments used matched littermate controls. The Boston University Laboratory Animal Science Center supervised all breeding and experimental procedures following protocols approved by the Institutional Animal Care and Use Committee.

Genotyping

For genotyping, 1-2 mm snips were cut from mouse tails. Tails were incubated in 300 μL of 50 mM NaOH at 95°C for 1.5 h with occasional vortexing. To neutralize the samples, 30 μL of 1 M Tris-HCl (pH 8.0) was added. Two hundred and fifty (250) μL of phenol:chloroform:isoamyl alcohol (Fisher et al.) was then added to the sample and mixed vigorously. The mixture were then centrifuged 5 min at maximum speed. Upper phase were pipetted into a fresh tube. Three hundred (300) μL of isopropanol and 60 μL
of 3 M sodium acetate (pH 5.2) were added to the upper phase. After vortex the mixture vigorously, samples were centrifuged at maximum speed for 20 min. Supernatants were decanted on paper towels and the DNA pellets were air-dried for 5 min. DNA pellets were then washed by 500 µL of 70% ethanol twice and re-suspended in 250 µL of T-low E buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) (Luchsinger et al., 2011).

PCR was used to screen for GFP Topaz, mCherry or MRTFA (Kalajzic et al., 2005; Kalajzic et al., 2008). For PCR, 1 µL DNA was added to 10 µL of GoTaq Green Master Mix (Promega), nuclease-free H2O (Promega) and sense and antisense primers (Table 2) (Luchsinger et al., 2011). PCR cycling conditions are listed in Table 1. PCR reaction products were run on a 1% agarose gels at 120V for 15 min. mCherry, GFP Topaz and MRTFA bands can be observed at 591 bp, 525 bp, 600 bp (WT allele) and 350 bp (KO allele), respectively (Luchsinger et al., 2011).

High-fat and low-fat feeding

Animals were fed standard rodent chow. 4-6 weeks-old male WT and MRTFΔ/Δ mice were switched to diet with 10% kcal% fat (low fat diet, Research Diet Inc., D12450B) or diet with 60% kcal% fat (high fat diet, Research Diet Inc., D12492) for 7 weeks.

β-adrenergic stimulation of adipose tissue

WT and MRTFΔ/Δ mice (14 weeks) were injected intraperitoneally (IP) daily for 7 days with 1mg/kg body weight of CL316,243 (Sigma-Aldrich) or saline.
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<td><strong>Stage 3</strong>&lt;br&gt;30 cycles&lt;br&gt;72°C&lt;br&gt;4°C</td>
<td><strong>Stage 3</strong>&lt;br&gt;30 cycles&lt;br&gt;72°C&lt;br&gt;4°C</td>
<td><strong>Stage 3</strong>&lt;br&gt;30 cycles&lt;br&gt;72°C&lt;br&gt;4°C</td>
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<td><strong>Stage 4</strong>&lt;br&gt;72°C&lt;br&gt;4°C</td>
<td><strong>Stage 4</strong>&lt;br&gt;72°C&lt;br&gt;4°C</td>
<td><strong>Stage 4</strong>&lt;br&gt;72°C&lt;br&gt;4°C</td>
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<td>5 min&lt;br&gt;Hold&lt;br&gt;1 cycle</td>
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</table>

Table 1. PCR cycling conditions for genotyping.
**Histology**

*Tissue preparation and embedding*

Inguinal, epididymal and brown fat pads were dissected from male mice and fixed in 4% paraformaldehyde (Fisher et al.) in PBS for overnight. Tissue were embedded in paraffin and sectioned for thickness of 7 µm.

*Hematoxylin & Eosin staining*

Slides were heated in oven with the temperature of 57.5°C for 15 min and then rehydrated in xylene, 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol and distilled water. Slides were then stained with Hematoxylin (Fisher et al.) for 5 min. After rinsing in tap water for 2 min, slides were dipped in acid alcohol for 2-3 dips. After rinsing in tap water for another 2 min, slides were immersed in Scott’s Tap Water for 5 min. Slides were then stained with Eosin (Sigma-Aldrich) for 5 min. After dehydration, slides were cleared by xylene and mounted by Cytoseal (Thermo Scientific). Bright-field images were obtained by using a Nikon Eclipse E400 microscope.

*Immunohistochemistry*

Slides were heated in oven with the temperature of 57.5°C for 15 min and then rehydrated in xylene (Fisher et al.), 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol and distilled water. Slides were then immersed into antigen-retrieval buffer (10 mM citric acid, 0.05% Tween20, pH 6.0) in a pressure cooker with the temperature of 100°C. The pressure cooker was then allowed to rock for 2 min. Slides were taken out from the pressure cooker and washed in Tris-buffered saline (TBS) twice for 5 min. TBS with 3% H₂O₂ were then used to block the endogenous peroxidase activity. After washing
in TBS twice for 5 min, slides were blocked in 5% normal serum in TBS. Slides were then incubated with UCP-1 primary antibody (Table 4) at room temperature for 1 h and then at 4°C for overnight. After washing in TBS, slides were incubated in 1:200 biotinylated anti goat IgG (Vector Lab) at room temperature for 1 h. Slides were then incubated in Elite ABC solution (Vector Lab) at room temperature for 1 h. After washing with TBS, slides were incubated in 3,3′-diaminobenzidine (DAB) substrate (Vector Lab) solution for 5 min. Slides were rinsed in water for 5 min and counter-stained with hematoxylin. After dehydration, slides were cleared by xylene and mounted by Cytoseal (Thermo Scientific). Bright field images were obtained by using a Nikon Eclipse E400 microscope.

**Whole body metabolism**

**Body composition**

Body composition of the mice was measured by non-invasive quantitative magnetic resonance (EchoMRI700) (BUMC Metabolic Phenotyping Core). Live mice were placed into the machine and fat, free water, total water and lean tissue values were obtained.

**Indirect Calorimetry**

Animals were individually housed in metabolic chambers maintained at 20-24°C on a 12-hour light/12 dark cycle with lights on at 7 am. Metabolic parameters (Heat, VO₂, VCO₂ and RER) were obtained continuously using Comprehensive Laboratory Animal Monitoring System (CLAMS) consisting of open circuit calorimeter and motion detectors (BUMC Metabolic Phenotyping Core). Mice were fed with low fat diet (Research Diet
Inc., D12450B) or high fat diet (Research Diet Inc., D12492) and tap water and food intake were measured by subtracting the weight of food left in the cage from the weight of food that were put into the cage. Mice were allowed 1 day of adaption to the cage before collecting data.

*Blood Glucose and GTT*

Mice were fasted overnight for 14 hours followed by intraperitoneal D-glucose (Sigma-Aldrich) injection (2 g/kg body weight). Blood glucose was measured by tail bleeding at 0, 15, 30, 60, and 120 min after the injection (Bayer Contour).

*ELISA*

After euthanization of the mice, blood was collected from heart. Serum was obtained by using serum separation tubes (Alfadda et al.). Serum leptin, adiponectin and insulin were measured by Mouse Leptin Elisa Kit (Millipore EMD), Mouse adiponectin ELISA Kit (ALPCO) and Mouse Insulin Kit (ALPCO), respectively.

*Stromal Vascular Cell Isolation and Culture*

*Stromal Vascular Cell Isolation*

Inguinal fat pads were dissected from male mice and were minced into small pieces. Tissue were then digested in 2 mg/mL Type 1 Collagenase (Worthington, CLS1) in DMEM containing 4.5 g/L glucose (Mediatech, Inc.) with 2% BSA (American Bioanalytical) while shaking in a 37°C incubator. Digested tissue was filtered through a 100 µm mesh and centrifuged at 500 rcf for 10 min. Adipocytes and the underlying supernatant was removed by aspirating and the stromal vascular fraction pellet was re-suspended in DMEM containing 4.5 g/L glucose (Mediatech, Inc.) supplemented with
10% FBS (Atlas) and plated on tissue culture plastic. The resulting stromal vascular cells (SVCs) were cultured at 37°C in a 5% CO₂ environment.

Cell Culture

Sub-confluent SVCs were treated with 6.3 nM human recombinant BMP4 (R&D Systems) for 3 days. The confluent cells were induced to differentiate in 10% FBS (Gibco), 5 mM dexamethasone (Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), 860 nM insulin (Life Technologies), 1 nM 3,3,5-triiodo-L-thyronine (T3) (Sigma-Aldrich), and 125 mM indomethacin. Two days after induction, cells were maintained in 10% FBS (Atlas), insulin (Life Technologies), and T3 (Sigma-Aldrich) for 6 days (Tseng et al., 2008).

Quantitative RT-PCR RNA Analysis

Whole adipose tissues were homogenized and total RNA was extracted using QIAzol (QIAGEN) reagent. Similarly, SVCs were scraped into QIAzol reagent and total RNA was isolated. Total RNA was used as a template to generate cDNA with the Applied Bio systems High Capacity DNA Reverse Transcription Kit. Quantitative RT-PCR was performed in 96-well plates using the Fermentas Maxima SYBR Green QPCR Master Mix (Fermentas Life Sciences) 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 at 72°C. 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 adiponectin mRNA expression. Melting curve profiles (cooling the sample to 68°C and heating slowly to 95°C with measurement of fluorescence) were also performed. Data were analyzed by the ddCT method (Livak and Schmittgen, 2001). Primer sequences used for RT-PCR analysis are listed in Table 2.

**Western blotting**

Total cellular proteins were extracted from cells or tissue, and the concentration of protein was assessed using the BCA (bicinchoninic acid) Protein Assay Reagent (Pierce). Equal protein amounts were mixed with 5x reducing sample buffer (200 mM Tris pH 6.8, 8% SDS, 0.4% bromophenol blue, 40% glycerol, 400 mM DTT) and samples was fractionated by 8-12.5% SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membranes (BioRad). Following transfer, PVDF membranes were blocked in 5% nonfat dry milk in PBST and probed overnight with primary antibodies corresponding to the various target proteins (Table 3). Horseradish peroxidase-conjugated secondary antibodies (Sigma) and an ECL substrate kit (Denville) were used for detection of specific proteins.

**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. Significance was considered as p ≤ 0.05.
## Table 2. Primer sequences

### For RT-PCR

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### For Genotyping

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Table 3. Antibodies. MRTFA antibodies were diluted in PBST with 2% BSA with the dilution factor of 1:200. All other antibodies were diluted in PBST with 2% BSA with the dilution factor of 1:1000.
CHAPTER THREE
MRTFA/SRF SIGNALING CONTRIBUTES TO THE DEVELOPMENT OF
BEIGE ADIPOCYTES IN MICE

Introduction

As discussed in Chapter One, actin-RhoGTPase and the TGFβ superfamily might contribute to adipose lineage determination and adipocyte formation. One possible convergence of the two pathways is MRTF-SRF transcriptional signaling. We hypothesized that the MRTF-SRF signaling pathway negatively regulates adipocyte formation.

Molecular and Biological Functions of MRTF-SRF target genes

Muscle specific and contractile genes

Many studies have shown that the MRTF-SRF circuit regulates expression of muscle specific and contractile genes: First, MRTF-SRF target genes can regulate the contractile function of muscle cells (Lockman et al., 2004; Long et al., 2007). Second, in cells other than muscle cells, forced expression of MRTFs activates the expression of smooth muscle-specific genes (Du et al., 2004; Hinson et al., 2007; Jeon et al., 2008; Li et al., 2005; Lockman et al., 2004; Selvaraj and Prywes, 2004; Wang et al., 2002). Third, MRTFs are required for skeletal muscle differentiation (Li et al., 2005; Selvaraj and Prywes, 2004).
Cytoskeleton and cell motility genes

MRTF-SRF target genes are involved in cytoskeleton regulation and cell motility. Using CHIP-Seq and GO analysis, Treisman and associates identified 921 out of 960 SRF target genes that are directly controlled by MRTFs (Esnault et al., 2014), 112 among those genes have biological functions that regulate the cytoskeleton. Other studies have shown the function of the MRTF-SRF circuit in promoting cell motility in epithelial/endothelial-mesenchymal transition (EMT/EnMT) (Gilles et al., 2009; Kametaka et al., 2007; Medjkane et al., 2009; Mihira et al., 2012; O'Connor and Gomez, 2013).

MRTFA and MRTFB

As discussed in Chapter one, MRTFB deletion in mice results in embryonic lethality due to failure in cardiovascular development (Oh et al., 2005; Wang et al., 2002). MRTFA KO mice are viable, but females have impaired myoepithelial development in the mammary gland (Li et al., 2006; Sun et al., 2006). While both MRTFA and MRTFB contribute to smooth muscle cell differentiation the redundancy of MRTFA and MRTFB is only partially understood. However, recent studies have shown the exclusive role of MRTFB in circadian control (Esnault et al., 2014; Gerber et al., 2013). MRTFA seems to be actively involved in modulating cell motility and migration in epithelial-mesenchymal transition (EMT) of various cell systems (Gomez et al., 2010; Hayashi et al., 2014; Mihira et al., 2012; O’Connor and Gomez, 2013). MRTFA is also involved in pro-inflammatory programs in vascular smooth muscle cells (Yang et al., 2014) and induces insulin resistance in muscle (Jin et al., 2011; Small et al., 2010).
To investigate the role of MRTFA in adipocyte formation, we initiated the study by characterizing the phenotype of $MRTFA^{-/-}$ mice. We examined adipose tissue morphology, gene/protein expression and metabolic profile of $MRTFA^{-/-}$ mice. We also examined the effect of β-adrenergic pathway stimulation and high-fat feeding in these mice.

**Results**

1. **Increased induction of beige adipocytes in WAT of $MRTFA^{-/-}$ mice**

   We started by examining the different adipose tissues of MRTFA deficient mice. We observed massive accumulation of multilocular adipocytes within inguinal (ING) WAT compared to WT mice (Figure 1A). We did not observe any multilocular cells in epididymal (EPI) WAT of $MRTFA^{-/-}$ mice. However, the size of adipocytes in $MRTFA^{-/-}$ EPI WAT was much smaller than the corresponding cells in WT littermates. Immunohistochemical staining of UCP-1 in ING WAT of $MRTFA^{-/-}$ mice demonstrated that the multilocular cells were positive for UCP-1 (Figure 1A). No detectable morphological alterations or changes in UCP-1 expression were found in $MRTFA^{-/-}$ BAT. With maturation, ING WAT from WT mice underwent hypertrophy leading to an increase in the size of individual unilocular adipocytes (Figure 1B). In contrast to WT mice, the ING WAT of $MRTFA^{-/-}$ mice consisted of more multilocular cells and the extent of hypertrophy with age was less pronounced (Figure 1B). The body weight,
average weight gain and overall fat mass relative to lean mass were lower in $MRTFA^{-/}$ mice compared to WT littermates. (Figure 1C, 1D and 1E).

Since activation of the browning of white fat has been shown to improve glucose homeostasis in vivo (Cederberg et al., 2001; Seale et al., 2011), we measured fasting glucose and glucose tolerance in $MRTFA^{-/}$ mice. These mice had lower fasting levels of glucose than WT mice but there were no differences in glucose measured during a glucose tolerance test (GTT) (Figures 2A and 2B).

Leptin is a cytokine that characterizes white adipocyte development and is down-regulated by browning of WAT (Boström et al., 2012). Similarly, we showed that $MRTFA^{-/}$ mice produced lower circulating levels of leptin (Figure 2C). Adiponectin is an adipokine that improves insulin sensitivity by decreasing triglyceride content in muscle and liver in obese mice (Yamauchi et al., 2001). Browning of WAT increases the adiponectin transcription (Boström et al., 2012). As expected, higher levels of circulating adiponectin were observed in $MRTFA^{-/}$ mice compared to WT littermates (Figure 2C).

To gain insight into the molecular characteristics of different fat depots in $MRTFA^{-/}$ mice, mRNA expression of brown, beige, smooth muscle and white specific genes were analyzed by RT-PCR. ING WAT from $MRTFA^{-/}$ mice expressed 30-fold more UCP-1 mRNA relative to WT littermates (Figure 3A). BAT expressed much higher levels of Ucp1 mRNA than other WAT depots. Similar to the morphology and UCP-1 immunostaining of BAT (Figure 1A), no significant difference in Ucp1 mRNA levels in
Figure 1. Accumulation of beige adipocytes in ING WAT of MRTFA−/− mice
Figure 1. Accumulation of beige adipocytes in ING WAT of $MRTFA^{−/−}$ mice
(A) H&E and UCP-1 staining of representative sections (n = 7) of ING (Inguinal) WAT, EPI (Epididymal) WAT and BAT from 11- to 13-week-old WT and $MRTFA^{−/−}$ mice on rodent diet with 10% kcal% fat.
(B) H&E staining of representative sections (n = 7) of ING (Inguinal) from 8-21-week-old WT and $MRTFA^{−/−}$ mice on rodent chow diet.
(C) Gross morphology of representative ING and EPI fat pads of mice from (A).
(D) Body weight and average weight gain per week of WT and MRTFA−/− mice on rodent diet with 10% kcal% fat (n = 5/group, *p < 0.05).
(E) Body composition determined by NMR (described in Experimental Procedures) analysis of mice from (B). Data presented mean ± SEM, n = 5/group, *p < 0.05.
Adapted from McDonald et al 2015 with permission.
Figure 2. MRTFA\textsuperscript{-/-} mice have lower fasting glucose and an improved adipokine profile.
(A) Mice were fasted overnight and glucose of whole blood from the tail vein was measured using a standard glucose monitor.
(B) Mice were IP injected with glucose at 2 mg/kg of body weight after fasting overnight and whole-blood glucose was measured at the indicated times.
(C) Circulating amounts of leptin, HMW adiponectin and insulin were measured as outlined in Material and Methods.
Adapted from McDonald et al 2015 with permission.
BAT of WT and \textit{MRTFA}\textsuperscript{+/−} mice was observed. Expression of other BAT-selective mRNAs (Fabp3, Cox7a1, Elov13, and Cox8b) was similarly higher in the ING WAT of \textit{MRTFA}\textsuperscript{−/−} mice compared to WT littermates. Angiotensinogen (Agt) is a marker for WAT and its expression is much higher in WAT compared to BAT (Massiéra et al., 2001; Vernochet et al., 2009) (Figure 3). Importantly, Agt was expressed at much lower levels in \textit{MRTFA}\textsuperscript{−/−} ING WAT relative to WT ING WAT (Figure 3), consistent with the enhanced development of brown like adipocytes within ING WAT of KO mice. Expression of SMA as well as MRTFA was also reduced in \textit{MRTFA}\textsuperscript{−/−} ING WAT compared to the WT depot. T-box transcription factor (Tbx1) was recently shown to be a beige precursor marker (Wu et al., 2012). Importantly, Tbx1 expression was significantly higher in \textit{MRTFA}\textsuperscript{−/−} ING WAT compared to WT ING WAT (Figure 3) suggesting that the appearance of UCP-1 positive, multilocular adipocytes in \textit{MRTFA}\textsuperscript{−/−} mice are likely due to recruitment of developmental beige progenitors.

\textbf{2. Stromal vascular cells from \textit{MRTFA}\textsuperscript{−/−} ING WAT undergo beige adipogenesis more extensively than WT stromal vascular cells.}

Because the mice used in this study were a total body KO, we examined whether MRTFA represses beige adipocyte formation directly. Stromal vascular cells (SVCs) of WT and KO ING WAT converted into adipocytes \textit{in vitro} to approximately the same extent as judged by comparable expression of adiponectin, adipsin, and FABP4 (Figure 4A and 4B). \textit{MRTFA}\textsuperscript{−/−} SVCs, however, expressed higher amounts of select BAT proteins including UCP-1 and Chchd10/NDG2 (Figure 4B). Smooth muscle marker SMA was down-regulated in KO SVCs (Figure 4).
Figure 3. *MRTFA*−/− mice express higher mRNA of brown and beige-selective genes while lower white and smooth muscle-specific genes
Relative mRNA levels of BAT- and WAT-enriched genes as well as other genes in WAT and BAT depots of 15-week-old WT and *MRTFA*−/− mice on chow diet was analyzed by RT-PCR. Data presented mean ± SEM, n = 4/group, *p < 0.05, **p < 0.01. Adapted from McDonald et al 2015 with permission.
Forskolin is an activator of cyclic AMP accumulation (Litosch et al., 1982) and can largely induce the expression of UCP-1 (Cao et al., 2001). Treatment of the adipocytes with FSK for 4 hours dramatically induced expression of UCP-1 mRNA in both WT and MRTFA−/− cells when normalized to basal untreated levels (Figure 4C). The FSK-dependent induction of UCP-1, Fabp3, Cidea, Cox7a1, and Elovl3 was significantly higher in MRTFA−/− versus WT adipocytes (Figure 4C). The higher induction of BAT selective genes in KO SVCs derived adipocytes indicate that MRTFA is regulating beige adipocyte formation in a cell-autonomous manner.

3. Both WT and MRTFA−/− mice respond to β-adrenergic receptor agonist and generate browning in ING WAT

Brown-like, multilocular adipocytes respond to β-adrenergic receptor stimulation by inducing expression of thermogenic genes most notably UCP-1 (Barbatelli et al., 2010; Cousin et al., 1992; Wu et al., 2012). To determine whether the MRTFA−/− mice respond differently than WT mice, both groups of mice were IP injected daily for 2 weeks with a β-adrenergic agonist, CL316,243. We showed that WT mice exhibited “browning” (beige and/or brite cells) of WAT depots based on more intense UCP-1 immunohistochemical staining in response to CL316,243. (Figure 5A). Importantly, MRTFA−/− mice, however, responded many fold more dramatically to CL316,243 than their WT littermates by producing larger regions of UCP-1 positive multilocular adipocytes in ING WAT depots as well as producing higher amounts of select brown genes Elovl3, Fabp3, Cidea, Cox7a1 and Dio2 (Figure 5B) in addition to UCP-1 mRNA and UCP-1 protein (Fig 5C).
Figure 4. Stromal vascular cells from MRTFA−/− ING WAT undergo beige adipogenesis more extensively than WT stromal vascular cells.
(A) Phase contrast imaging of SV-adipocytes from WT and MRTFA−/− inguinal depots.
(B) Western blot analysis on BAT- and WAT-enriched proteins of adipocytes arising from hormonal induction of stromal vascular fractions (SVCs) isolated from WT and MRTFA−/− inguinal depots.
(C) WT and MRTFA−/− SVC adipocytes were treated with or without forskolin (Lee et al.) for 4 hours prior to isolation of total RNA for analysis. Values are presented as fold change in response to treatment with FSK in WT and MRTFA−/− adipocytes. Adapted from McDonald et al 2015 with permission.
Figure 5. Both WT and MRTFA−/− mice are highly responsive to β-adrenergic signaling, which enhances beige adipocyte formation in ING WAT.
Figure 5. Both WT and MRTFA<sup>−/−</sup> mice are highly responsive to β-adrenergic signaling, which enhances beige adipocyte formation in ING WAT. (A) UCP-1 immunohistochemistry of representative sections of BAT, ING, and EPI depots of WT and MRTFA<sup>−/−</sup> mice following daily intraperitoneal (IP) injections of 1 mg/kg CL316,243 or saline for 7 days. (B and C) mRNA and protein levels of BAT- and WAT-enriched genes as well as other genes in ING WAT of WT and MRTFA<sup>−/−</sup> mice treated with or without CL316,243 were determined by RT-PCR (B) and western blot (C), respectively. Data presented mean ± SEM, n = 4/group, *p < 0.05, **p < 0.01. Adapted from McDonald et al 2015 with permission.
4. MRTFA-deficient mice are protected from diet-induced obesity and insulin resistance

The enhanced beige adipocyte development in MRTFA−/− mice led us to examine the physiological significance of this finding. We fed the mice with a HFD (high fat diet, 60% kcal) for 6-7 weeks. MRTFA−/− mice gained significantly less body weight compared to their WT littermates, without any difference in food intake (Figure 6A, 6D). The MRTFA−/− mice had significantly less fat mass while no differences in lean mass were observed compared to their WT littermates (Figure 6C). Consistent with fat mass, the weights of white fat pads (ING and EPI) were lower in MRTFA−/− mice compared to WT mice, while there were no differences in the weight of BAT depot (Fig 6B). Interestingly, the weight of liver was also lower in MRTFA−/− mice (Figure 6E). The observation of potentially less lipid storage in MRTFA−/− versus WT mice is consistent with enhanced energy expenditure in MRTFA−/− mice.

Diet-induced obesity is frequently associated with glucose intolerance and progressive metabolic dysfunction (Surwit et al., 1988). MRTFA−/− mice had significantly lower fasting glucose levels and exhibited enhanced glucose tolerance compared to WT littermates (Figures 7A and 7B). The level of serum leptin is increased during DIO(Lin et al., 2000). MRTFA−/− mice had significantly less serum leptin than WT mice (Figure 7C), consistent with the mice being less obese compared to their WT littermates. Adiponectin is a adipokine that improves insulin sensitivity by decreasing triglyceride content in muscle and liver in obese mice (Yamauchi et al., 2001). MRTFA−/− mice had higher levels
of serum HMW (High molecular weight) adiponectin (Figure 7C) compared to WT littermates.

H&E staining of fat depots of $MRTFA^{-/-}$ and WT mice showed that HFD significantly enhanced the lipid influx into each fat depot (Figure 7D). No observation of multilocular adipocytes was observed in ING WAT of WT mice after high-fat feeding. $MRTFA^{-/-}$ mice however, retained numerous UCP-1 positive beige adipocytes in ING. Crown-like structures were observed in EPI of WT mice, indicating that the HFD caused inflammation of the depot. These crown-like structures were not observed in EPI of $MRTFA^{-/-}$ mice (Figure 7D). Consistent with the morphology of the adipocytes, protein levels of BAT related genes (UCP-1, FABP3) were significantly higher in the ING of $MRTFA^{-/-}$ compare to their WT littermates after HFD (Figure 7E). Additionally, the level of smooth muscle markers were decreased in ING of $MRTFA^{-/-}$ mice, which indicates that there might be less development of smooth muscle cells in the ING of $MRTFA^{-/-}$ mice (Figure 7E).

Taken together, these results indicate that MRTFA deficiency reduced HFD-induced obesity, insulin resistance, EPI WAT inflammation, and hepatic steatosis (lipid accumulation in liver).
Figure 6. MRTFA deficiency reduces high-fat diet induced weight gain, fat depot hypertrophy and hepatic steatosis.
Figure 6. MRTFA deficiency reduces high-fat diet induced weight gain, fat depot hypertrophy and hepatic steatosis.

(A and B) WT and MRTFA\textsuperscript{−/−} mice, starting from 4- to 5-week-old, were fed a rodent HFD (60% kcal% fat) for 6 weeks. Body weight and weight gain were measured in weekly intervals (A), while weights of ING WAT, EPI WAT, BAT, and liver were measured at end of study (B).

(C and D) Body composition analysis by NMR (grams) and food intake of WT (red) and KO (green) mice. (n = 5/group, *p < 0.05).

(E). H&E stained sections of liver from WT and KO mice following a 6 weeks of HFD. Adapted from McDonald \textit{et al} 2015 with permission.
Figure 7. MRTFA deficiency reduces high-fat feeding induced insulin resistance and EPI WAT inflammation.
Figure 7. MRTFA deficiency reduces high-fat feeding induced insulin resistance and EPI WAT inflammation.

(A) After 5 weeks on the HFD, mice were fasted overnight and glucose of whole blood from the tail vein was measured using a standard glucose monitor.

(B) After 5 weeks on the HFD, mice were then IP injected with glucose at 2 mg/kg of body weight after fasting overnight and whole-blood glucose was measured at 15, 30, 60, and 120 min.

(C) Circulating amounts of leptin, HMW adiponectin and insulin from WT and MRTFA\(^{−/−}\) following 6 weeks on a HFD.

(D) H&E and UCP-1 staining of sections of ING, EPI, and BAT depots of MRTFA\(^{−/−}\) and WT mice after 6 weeks of HFD.

(E) Western blot analysis of BAT- and WAT-enriched genes as well as other genes in WAT and BAT depots of WT and MRTFA\(^{−/−}\) mice were conducted as in Figure 6. Arrows point to crown-like structures. Data presented are mean ± SEM, n = 6–7/group, *p < 0.05, **p < 0.01.

Adapted from *McDonald et al 2015* with permission.
**MRTFA regulates whole-body energy expenditure**

Beige adipocyte formation in WAT is associated with an improved whole-body metabolic profile in mice (Bi et al., 2014; Chatterjee et al., 2014). Indirect calorimetry analysis of mice following 6 weeks on high or low fat diets (HFD or LFD) demonstrated that KO mice produced more heat and consumed more oxygen (O$_2$) (Figure 8A) without any change in food intake (data not shown) or physical activity compared to WT animals (Figure 8B). HFD decreased the cumulative CO$_2$ in both WT and KO mice (Figure 8A), this is due to lower content of carbohydrates in the HFD compared to LFD (Research Inc.). Scan of Heat and VCO$_2$ measures demonstrated that KO mice metabolized more carbohydrate and generated more heat throughout the entire night-day-night period (Figure 7C).

Respiratory exchange ratio (RER) calculates the ratio between the amount of CO$_2$ produced and O$_2$ consumed. An RER of 0.70 indicates that fat is the predominant fuel source, RER of 0.85 suggests a mix of fat and carbohydrates, and a value of 1.00 or above is indicative of carbohydrate being the predominant fuel source (Seidell et al., 1992). Measurement of RER following the 6 weeks on LFD revealed that KO mice metabolized significantly larger amounts of carbohydrate than WT mice during the dark period with both sets of mice switching equally to a mix of fat and carbohydrate expenditure during the day (Figure 7C).

These results indicate that MRTFA$^{-/-}$ mice have overall improvement in metabolic efficiency compared to WT mice, correlating with the accumulation of beige adipocytes.
Figure 8. MRTFA regulates whole-body energy expenditure. 
(A and B) WT and MRTFA−/− mice on LFD or HFD for 6 weeks were housed individually in metabolic chambers for 3 days and 2 nights. Cumulative O$_2$ consumption and CO$_2$ production were measured by CLAMs (C). Traces of heat production, CO$_2$ production, and respiratory exchange ratio (RER) during 12 hours dark and light cycles. Respiratory exchange ratio (RER) were calculated by volume of carbon dioxide produced (exhaled)/volume of oxygen consumed (inhaled). Data presented are mean ± SEM, n = 5–7/group, *p < 0.05. 
Adapted from McDonald et al 2015 with permission.
Summary

In this chapter we investigated the role of MRTFA in adipocyte formation. We conclude that ING WAT from MRTFA\(^{-/-}\) mice contained more multilocular adipocytes that express higher amounts of UCP-1 and other BAT-selective genes, including Tbx1 mRNA (a marker of beige precursor) relative to ING WAT from WT littermates. SVCs from ING WAT of MRTFA\(^{-/-}\) mice developed into beige adipocytes that respond more robustly to a forskolin stimulus than WT SVF cultures in vitro, indicating a cell-autonomous role of MRTFA in beige adipocyte formation. MRTFA\(^{-/-}\) mice mount a robust response to a β-adrenergic stimulus expressing enhanced levels of BAT genes in ING WAT. The accumulation of beige adipocytes in MRTFA\(^{-/-}\) mice correlated with improved whole-body energy expenditure. MRTFA\(^{-/-}\) mice were also partially protected from against diet-induced obesity and exhibited improved glucose homeostasis and secretion of an improved adipokine profile. We demonstrated that MRTFA contributes to the development of beige adipocytes in mice.
CHAPTER FOUR
MRTFA REGULATES THE BALANCE BETWEEN BEIGE ADIPOCYTES AND SMOOTH MUSCLE-LIKE CELLS

Introduction

The appearance of physiologically functional beige adipocytes by genetic manipulation of MRTFA has led us to further investigate the molecular mechanisms by which MRTFA deficiency enhances beige adipocyte formation. Beige adipocytes can readily be observed within ING WAT of young mice that are maintained at ambient temperature (Wu et al., 2012) and become more prominent upon different physiological stimulation (Bordicchia et al., 2012; Boström et al., 2012; Brestoff et al., 2014; Collins et al., 1997; Fisher et al., 2012; Ghorbani and Himms-Hagen, 1997; Gnad et al., 2014; Granneman et al., 2005; Guerra et al., 1998; Himms-Hagen et al., 2000; Kataoka et al., 2014; Nagase et al., 1996; Petrovic et al., 2010; Vernochet et al., 2009; Wilson-Fritch et al., 2004; Xue et al., 2005). The accumulation of beige adipocytes in ING WAT of MRTFA−/− mice occurred without exposure to any physiological stimuli, suggesting that MRTFA might be regulate early events in beige adipocyte development.

1. Common adipocyte precursors in vivo

As we discussed earlier, brown adipocytes in mice arise from Myf5+ positive precursors that reside in the dermomyotome (Atit et al., 2006; Seale et al., 2008; Timmons et al., 2007). Unlike BAT progenitors, the developmental origin of beige progenitors and white progenitors is less clear.
**Isolation of adipose precursors by fluorescence-activated cell sorting**

Adipose precursor cells have been characterized in detail by fluorescence-activated cell sorting (FACs) to isolate sub-populations of adipose progenitor cells. Using this approach, the SVCs were characterized and isolated based on expression of cell surface proteins (Rodeheffer et al., 2008; Schulz et al., 2011). One subpopulation of SVCs was first isolated using antibodies to cell surface markers. This population of cells is negative for hematopoietic (CD45, Ter119 (Ly76, lymphocyte antigen 76)) and endothelial (CD31) specific markers and positive for mesenchymal stem cell markers (CD29, CD34, Sca-1 (stem cell antigen-1)). The isolated progenitors had a high adipogenic capacity *in vitro* and formed fat pads after being transplanted into WAT depots of lipodystrophic mice (Rodeheffer et al., 2008). Adipose precursors were also identified in humans by FACs: Sca1⁺; CD45⁻; Mac1 (Macrophage-1 antigen)⁻ progenitors from human subcutaneous WAT are highly inducible by BMP7 to form brown adipocytes (Schulz et al., 2011), indicating that progenitor cells exist in human fat pads and can be induced to brown adipocytes. The limitation of FACs identification of adipose precursors is that these cell surface markers do not have known functions in adipogenesis, thus making it impossible to bridge the gap between the localization of these markers with developmental processes of adipocyte formation.

*Identification of adipose progenitors by lineage tracing in vivo*

*In vivo* lineage tracing provides also has been used to identify adipose progenitors. Several genetic mouse models were established to trace adipose progenitors *in vivo*. PPARγ is believed to be a master regulator of adipogenesis (Hu et al., 1995; Tontonoz et
al., 1994). To trace PPARγ positive precursor cells in adult mice, Tang et al. established the Adipotrak mouse model that combines endogenous PPARγ driven tetracycline transactivator (tTA) with reporter lines under control of tet-response element (TRE). They showed that PPARγ-marking cells resemble at least one subset of mural cells/pericytes that are localized on the perivascular region and express SMA, NG2 (neural/glial antigen 2) and PDGFRβ (Tang et al., 2008).

Although PPARγ is a promising marker for adipose progenitor lineage tracing, it is turned on at a late stage of in vitro adipogenesis based on former studies. A new early preadipose transcription factor Zfp423 (zinc finger protein 423) was identified by quantitative analysis of transcriptional components of fibroblasts (Gupta et al., 2010). Zfp423 is enriched in preadipose versus non-preadipose fibroblasts. Forced expression of Zfp423 in non-adipogenic NIH3T3 fibroblasts promoted adipogenesis by enhancing the expression of PPARγ (Gupta et al., 2012). Zfp423 deficiency impaired the formation of both WAT and BAT in mice (Gupta et al., 2010). To identify adipose progenitors in vivo, Gupta et al. labeled the committed murine adipose progenitors through Zfp423 promoter-driven GFP. GFP positive fibroblasts isolated from SVCs undergo robust adipogenesis. Immunostaining of GFP and other mural cell markers in vivo confirms the perivascular origin of GFP positive cells in both WAT and BAT. However, a subset of endothelial cells expressing GFP was also observed in both WAT and BAT, suggesting a contribution of specialized endothelial cells to the adipose lineage (Gupta et al., 2012).

Since the observation of Zfp423 positive precursors in the endothelial compartment suggested an endothelial origin of adipocytes, endothelial marker derived
fate-mapping would be of great importance for understanding if the endothelium also contains adipose progenitors. VE (vascular endothelial)-cadherin is required for vascular formation and is expressed specifically in endothelial cells of fetal and adult mice. A Cdh5 (VE-Cadherin)-Cre driven LacZ mouse model was created to trace endothelial cells during adipose tissue development (Tran et al., 2012). X-gal staining was found in endothelial cells, preadipocytes and mature adipocytes of WAT and BAT, suggesting that adipose precursors arose from Cdh5 expressing endothelial cells. Capillary sprouts from human adipose tissue, which have endothelial characteristics, also express Zfp423 (Tran et al., 2012). However, another Cdh5-Cre driven mT/mG (fluorescence membrane dTomato/membrane eGFP) reporter mouse displayed different results. In these animals all mature adipocytes, WAT or BAT, remained dTomato positive and eGFP negative. The CD31+ endothelial cells however, expressed eGFP, suggesting that the adipose progenitors were not from the vascular endothelium (Berry and Rodeheffer, 2013). The former study using LacZ reporter line for adipose tissue is confounded by the paucity of cytoplasm in mature adipocytes and the high vascularity of adipose tissue (Berry and Rodeheffer, 2013). The latter study however, permits a clear distinction of Cre-expressing cells (eGFP positive) and non-cre-expressing cells (dTomato positive).

In the same study with the Cdh5-Cre driven mT/mG mice, the authors also created PDGFRα-Cre: mT/mG mice and showed that all adipocytes in WAT depots were derived from PDGFRα-expressing precursors (Berry and Rodeheffer, 2013). In addition, PDGFRα-expressing precursors were shown to form beige adipocytes in response to β3-adrenergic agonist treatment in abdominal WAT. These cells also give rise to white
adipocytes upon high-fat feeding (Lee et al., 2012; Lee et al., 2013). However, the bi-
potential PDGFRα-expressing progenitors were not specifically located in the vasculature
and did not co-localize with mural cells that express SMA and PDGFRβ (Berry and
Rodeheffer, 2013; Lee et al., 2012). Together with the PPARγ lineage tracing study, the
adipose progenitors might arise from vasculature and non-vasculature compartments.
Whether different compartments may affect the fate determination of adipose precursors
into beige/brown/white adipocytes remains to be determined.

2. Smooth muscle origin of beige adipocytes

As we discussed in Chapter One, beige adipocytes are not derived from a Myf5+ line-
age (Seale et al., 2008). Two major theories exist regarding the origin of beige
adipocytes: transdifferentiation from pre-existing white adipocytes (Himms-Hagen et al.,
2000; Vitali et al., 2012) and de novo recruitment of distinct beige progenitors (Lee et al.,
2012; Schulz et al., 2011; Vegiopoulos et al., 2010; Wu et al., 2012). Several recent
studies have provided new insight into the lineage of beige precursors.

Using a 3T3 immortalization protocol (Todaro, 1963) and limited dilution of
SVCs from mice under ambient conditions, Wu et al., isolated a population of beige
precursor cells that have a distinct gene expression profile compared to white precursor
cells (Wu et al., 2012), featuring the expression of Tbx1, CD137 and Tmem26. This
study led to the hypothesis that developmental beige adipocytes arise from a different
origin than white adipocytes.

Using translating ribosome affinity purification (TRAP) technology, the
polysomes of UCP-1-positive cells were selectively isolated from mouse adipose tissues.
Analysis of the UCP-1⁺ cell polysomes also showed striking differences between brown and beige cells in vivo, including a smooth muscle-like gene expression signature in beige, but not in brown cells. In vivo fate mapping using Myh11 (smooth muscle marker) driven GFP/tdTomato in mice showed a co-localization of dTomato with UCP-1 and perilipin, providing evidence for a smooth muscle-like origin of a subset of beige adipocytes (Long et al., 2014).

Another study suggested a similar idea: estrogen receptor α (ERα) is an inhibitor of TGFβ induced smooth muscle cell differentiation (Farhat et al., 1996; Iafrati et al., 1997; Mendelsohn and Karas, 2005). Deletion of ERα specifically in PPARγ-marked adipose precursor cells reprograms the cells to adopt both smooth muscle and beige adipogenic fates instead of a white adipogenic fate. Both female and male adipose-lineage ERα-mutant mice are lean, have improved glucose sensitivity and are resistant to weight gain on a high-fat diet (Lapid et al., 2014). Further, they are hypermetabolic, hyperphagic and hyperthermic (Lapid et al., 2014). These findings are consistent with a common developmental origin for beige and smooth muscle cells.

3. Summary and hypothesis

Taken together, beige adipocytes appear to originate from a lineage that is different from the white adipocyte lineage. In addition, beige precursors might share the same origin with smooth muscle cells. Given the role of MRTFA-SRF in smooth muscle differentiation, we therefore questioned whether enhanced beige adipocyte formation in
*MRTFA*<sup>−/−</sup> mice results from the development of beige precursors versus smooth muscle-like cells (Scheme 3).

In this chapter, we test this hypothesis by examining the potential of WT and *MRTFA*<sup>−/−</sup>SVCs to differentiate into beige versus smooth muscle-like cells. The effect of physiological/pharmacologic stimuli (BMP and CCGs) was also examined on WT and MRTFA SVCs.
Scheme 3. Potential role for MRTFA in beige adipocyte and smooth muscle differentiation
MRTFA regulation stromal vascular cells differentiation into beige or smooth muscle cells.
Results

1. Whole tissue or SVCs of MRTFA<sup>−/−</sup> ING WAT express higher beige-precursor markers.

To test the hypothesis that MRTFA<sup>−/−</sup> mice might have enhanced expression of beige precursor markers in WAT, RNA expression of beige markers in whole ING WAT of MRTA<sup>−/−</sup> and WT mice was analyzed by RT-PCR. mRNA expression of the beige-precursor marker Tbx1 was higher in ING WAT versus EPI WAT or BAT (Figure 9). CD137 mRNA was also significantly higher in MRTFA<sup>−/−</sup> ING compared to WT littermates, while the mRNA expression of a general adipose precursor marker Pref1 (a protein that marks all adipose precursor (Sul, 2009)) and Zfp423 was similar in ING WAT of each of the littermates (Figure 9). Adipose progenitors reside in a stromal vascular niche (Tang et al., 2008), thus we isolated the SVCs and expanded them in vitro to test if these SVCs exhibited differences in beige precursor markers. Expectedly, Mrtfa messenger level is lower in KO SVCs. MRTFA<sup>−/−</sup> SVCs exhibited increased expression of Tbx1, Tmem26 and decreased expression of the smooth muscle marker Sma SMA mRNAs compared to SVCs of WT littermates (Figure 9). Taken together, SVCs from KO mice express lower smooth muscle markers and higher beige precursor markers at the messenger RNA level.

2. MRTFA<sup>−/−</sup> SVCs express lower smooth muscle markers and higher adipogenic markers before and after adipogenic induction

To determine the effect of MRTFA deficiency on smooth muscle specific genes, MRTFA<sup>−/−</sup> and WT SVCs were allowed to undergo expansion and adipogenesis in culture.
Figure 9. Whole tissue or SVCs of $MRTFA^{-/-}$ ING WAT express elevated beige-precursor markers

Upper panel: mRNA expression of beige-precursor markers was analyzed in whole ING WAT from WT and $MRTFA^{-/-}$ mice (12 weeks, n=4/group, *p ≤ 0.05). Left panel: mRNA expression of Tbx1 was normalized to WT ING. Right panel: ING WAT was isolated and analyzed from a separate group of mice. (n=4/group, *p ≤ 0.05)

Lower panel: Stromal vascular cells were isolated from WT and $MRTFA^{-/-}$ mice and differentiated into adipocytes. mRNA expression of markers enriched in beige precursors were analyzed by RT-PCR.

TBP was used as a control for ddCT method.
As expected, expression of MRTFA protein was absent in KO SVCs. Smooth muscle markers CRP2 (Yet et al., 1998) and SMA were initially up-regulated as both populations of SVCs became confluent at Day 0, although the level of expression of each was much lower in the KO versus WT SVCs at this stage. As the SVCs undergo adipogenesis (day 0 to day 3) expression of the smooth muscle genes dropped extensively and more prominently in the KO cells. Interestingly, induction of C/EBPβ (LAP and LIP) and PPARγ were higher in KO SVCs. Importantly, C/EBPβ protein expression was already more abundant in KO SVFs even before adipogenic induction (Figure 10).

3. BMP4 can reduce smooth muscle genes and induce adipogenic genes in WT SVCs

BMPs promote brown adipogenesis in MSCs (Tseng et al., 2008). Treatment with BMP4 during the expansion stage of differentiation of WT SVCs induced PPARγ1 and 2. C/EBPβ LIP expression was also slightly induced by BMP4 before adipogenic induction while αSMA expression was slightly reduced by BMP4 (Figure 11B), indicating that BMP4 might prime one subset of SVF progenitors to a beige adipocyte lineage rather than a smooth muscle lineage.

To test if MRTFA deficiency altered PPARγ and C/EBPβ in response to BMP4, we treated WT and MRTFA−/− SVCs with BMP4 prior to adipogenic induction. Consistent with Figure 10, KO SVCs had higher expression of C/EBPβ LIP, LAP* and lower expression of αSMA and CRP2 compared to WT SVCs. BMP4 induced PPARγ expression in both WT and KO SVCs. In KO SVCs, BMP4 had higher induction of PPARγ. Interestingly, while BMP4 enhanced the expression of both LIP and LAP* C/EBPβ in WT SVCs, it had no additional effect on expression of LIP and LAP*
Figure 10. *MRTFA*−/− SVCs express lower smooth muscle markers and higher adipogenic markers before and after adipogenic induction.

Stromal vascular cells were isolated from ING WAT of WT and *MRTFA*−/− mice and plated in culture. SVCs were differentiated by adipogenic media DMIIT (Material and Methods). Protein samples were harvested at day-1, 0, 0.5, 1, 2 and 3. Protein samples from different stage of adipogenesis were probed for smooth muscle-selective genes and adipogenic genes. Equal amount of protein were loaded to each lane.
C/EBPβ in KO SVCs (Figure 11C), suggesting that the induction of C/EBPβ by BMP4 is at least in part through inhibition of MRTFA-SRF transcriptional activity.

4. **CCG1423 and CCG203971 induces beige adipogenesis in SVCs at least in part by inhibiting MRTFA**

CCGs are small molecules identified in a screen for inhibitors of ROCK signaling and were discovered to pharmacologically inhibit MRTF-SRF transcriptional activity (Evelyn et al., 2007; Haak et al., 2014). A recent study identified Mical-2, a regulator of nuclear actin, to be a target of CCG1423 [N-[2-(4-chloroanilino)-1-methyl-2-oxoethoxy]-3,5-bis(trifluoromethyl)benzamide] (Lundquist et al, 2014). We asked if another CCG molecule CCG203971 [N-(4-chlorophenyl)-1-[[3-[[furan-2-yl]phenyl]carbonyl]piperidine-3-carboxamide] reduced SMA-driven mCherry (a red fluorescence protein) fluorescence during expansion of WT SVCs prior to adipogenic induction (Figure 12B). Importantly, after adipocyte differentiation UCP-1 induction by FSK was higher in SVCs pre-treated with CCG 203971 than in vehicle treated SVCs (Figure 12B), although the overall extent of adipocyte formation is similar between CCG203971 and vehicle groups.

To assess whether MRTFA deficiency can yield additional effect on brown adipogenesis than inhibition of SRF activity (e.g. CCG203971), WT and *MRTFA*−/− SVCs were exposed to CCG1423 prior to initiation of adipogenesis. The extent of morphological conversion was similar in KO cells to that in WT cells (Figure 12C), whereas the conversion was higher for WT cells treated with CCG1423. Importantly, CCG1423 had no additional effect on the differentiation of KO cells. Even though WT
Figure 11. BMP4 reduces smooth muscle gene expression and induce adipogenic genes in WT SVCs

(A) Differentiation protocol used for stromal vascular cells. BMP4 was supplemented to the media from day -3 to day 0.

(B) WT SVCs were treated with vehicle or 6.3 nM BMP4 for 3 days and underwent adipogenesis. Protein samples were harvested day -2, -1, 0 and 6 hours after adipogenic induction. Protein samples from different stage of adipogenesis were probed for smooth muscle-selective genes and adipogenic genes. Cyclophilin A (CA) served as loading control.

(C) WT and MRTFA−/− SVCs were treated with vehicle or 6.3 nM BMP4 for 3 days and underwent adipogenesis. Protein samples were harvested day -1, 0 and 6 hours after adipogenic induction. Protein samples from different stages of adipogenesis were probed for smooth muscle-selective genes and adipogenic genes. Cyclophilin A served as loading control.
Figure 12. CCG induces beige adipogenesis in SVCs at least in part by inhibiting MRTFA.

(A) Differentiation protocol used for stromal vascular cells. CCG203971 or CCG1423 were supplemented to the media from day -3 to day 0.

(B) WT-mCherry SVCs were treated with Vehicle or 10 µM CCG203971 for 3 days and underwent adipogenesis. Fluorescence imaging of mCherry were taken at 2 days-intervals. At day 6, SV adipocytes were treated with or without forskolin (Lee et al.) for 4 hours prior to isolation of total RNA for analysis. (n = 3, ***p ≤ 0.001)

(C) WT and SVCs were treated with Vehicle or 10 µM CCG1423 for 3 days and underwent adipogenesis. At day 7, cell cells were imaged by phase contrast microscopy. SV adipocytes were treated with or without forskolin (Lee et al.) for 4 hours prior to isolation of total RNA for analysis. (n=3, **p ≤ 0.01) Relative mRNA expression was normalized to WT DMSO without FSK stimulation.
and KO cells differentiated to the same extent, the response to FSK (fold induction of UCP-1) was much greater in the KO cells (Figure 12C). Interestingly, CCG1423 increased UCP-1 expression by FSK to a larger extent in WT compared to KO cells, suggesting that the browning activity of the CCG1423 is primarily through inhibition of MRTFA-SRF transcriptional activity.
Summary

In this Chapter we hypothesized that the deficiency of MRTFA would promote beige adipocyte formation rather than smooth muscle cell differentiation. We showed that MRTFA ING WAT expressed higher beige precursor markers and lower smooth muscle markers and this finding was supported by *in vitro* SVC differentiation studies. In addition, *MRTFA*−/− SVCs from ING WAT expressed higher levels of adipogenic regulators including as C/EBPβ and PPARγ during the expansion phase and after adipogenic induction. It appears that BMP and the CCG molecules can induce brown adipogenesis *in vitro*, at least in part, by inhibiting MRTFA-SRF pathway.
CHAPTER FIVE

DISCUSSION

Obesity is a major risk factor for many human diseases and, consequently, there is an urgent need for novel therapeutics to treat obesity and associated comorbidities. Numerous drugs have been approved for the treatment of obesity. However, most of them have been withdrawn from the market because of their adverse effects (Kang and Park, 2012). Beige adipocytes have begun to draw attention as a potential target for treating obesity. Understanding the biological circuits that regulate the formation of beige adipocytes will help in identifying more targets for anti-obese drugs and therapies. Here, we identified the MRTFA-SRF signaling pathway that, when inhibited in mice, leads to beige adipocyte formation in ING WAT and protects against DIO, insulin resistance and improves whole-body metabolism.

*MRTFA deficiency promotes physiologically functional beige adipocyte formation in ING WAT of mice*

The appearance of UCP-1+ adipocytes in the ING WAT of MRTFA-/- mice (Figure 1A and 1B) supports a suppressive role for MRTFA-SRF in beige adipocyte development. Enhanced mRNA expression of other BAT-selective markers (Fabp3, Cox7a1, Elovl3, Cox8b) (Figure 3) confirmed beige adipocyte development in ING WAT at a molecular level. MRTFA-/- mice were smaller, with lower body weight and smaller
fat pads (Figure 1C, 1D, 1E), suggesting that beige adipocyte formation in ING WAT might inhibit the overall growth of all white fat depots. WAT-specific genes Agt, Chemerin and Pank3 (Massiera et al., 2001; Vernochet et al., 2009) (Figure 3) were down-regulated in the ING WAT of MRTFA−/− mice. Activation of the browning of white fat has been shown to improve glucose homeostasis and adipokine secretion in vivo (Cederberg et al., 2001; Seale et al., 2011). Our data also showed that these metabolic parameters were improved in MRTFA+/− mice (Figure 3), suggesting that beige adipocyte development in MRTFA−/− mice might be physiologically functional.

Brown-like, multilocular adipocytes respond to β3-adrenergic receptor stimulation by inducing expression of thermogenic genes most notably UCP-1 (Barbatelli et al., 2010; Cousin et al., 1992; Wu et al., 2012). Upon 1 week of β3-adrenergic agonist treatment, MRTFA−/− mice responded more dramatically to CL316,243 than their WT littermates by producing larger regions of UCP-1 positive multilocular adipocytes in ING WAT depots as well as producing higher amounts of select brown genes Elovl3, Fabp3, Cidea, Cox7a1 and Dio2 (Figure 5B) in addition to UCP-1 mRNA and UCP-1 protein (Fig 5C). Importantly, UCP-1+ adipocytes were also observed in EPI WAT of MRTFA−/− mice but not in their WT littermates after CL316,243 injection, suggesting that MRTFA might also regulate the beige/brite adipocyte formation in EPI WAT. In fact, β3-adrenergic signaling promotes the browning of WAT by de novo recruitment of beige progenitors (Lee et al., 2012; Schulz et al., 2011; Vegiopoulos et al., 2010; Wu et al., 2012) or trans-differentiation of pre-existing white adipocytes (Himms-Hagen et al., 2000; Vitali et al., 2012). By lineage tracing, it was suggested that EPI WAT responds to activation of the
β3-adrenergic pathway by both trans-differentiation of white adipocytes and recruitment of beige precursors (Wang et al., 2013). Since MRTFA is undetectable in mature adipocytes (Figure 10), it appears unlikely to play a role in trans-differentiation. Instead, MRTFA may regulate the recruitment of beige precursors during the browning of EPI WAT.

Beige adipocyte formation can prevent DIO induced weight gain, glucose intolerance, insulin resistance and enhance energy expenditure in mice (Cederberg et al., 2001; Kiefer et al., 2012; Seale et al., 2011). We showed that MRTFA−/− mice had limited weight gain, improved glucose homeostasis, less EPI WAT inflammation, no hepatic steatosis and improved adipokine profile (Figure 6 and Figure 7). Prolonged high-fat feeding induces adipose tissue fibrosis (Sun et al., 2013). Smooth muscle markers e.g. SMA are induced during adipose tissue fibrosis (Michailidou et al., 2012). Interestingly, ING WAT of MRTFA−/− mice expressed less smooth muscle markers (SMA and CRP2) compared to WT littermates (Figure 7E) after high fat feeding, suggesting that the deficiency of MRTFA would prevent prolonged diet-induced adipose tissue fibrosis.

Until recently, it was difficult to establish a role for beige adipocytes in controlling whole-body energy expenditure (Shabalina et al., 2013). BAT was still considered to be the predominant source of thermogenesis, since the thermogenic density (UCP-1-dependent oxygen consumption per gram of tissue) of inguinal white adipose tissue was maximally one-fifth of interscapular brown adipose tissue, and the total quantitative contribution of all inguinal mitochondria was maximally one-third of all interscapular brown-fat mitochondria (Shabalina et al., 2013). As mentioned in Chapter
One, beige adipocytes might contribute to whole-body metabolism (Cederberg et al., 2001; Seale et al., 2011; Kiefer et al., 2012) by enhancing glucose homeostasis and insulin sensitivity. In addition, Cohen et al., have demonstrated that absence of beige adipocyte development in mice leads to DIO and insulin resistance, without any changes in BAT development or activity (Cohen et al., 2014), indicating that beige tissue might contributes to the whole-body energy balance. Consistent with this notion, $MRTFA^{-/-}$ mice enhance whole-body energy expenditure without any changes in BAT development, leading to enhanced heat and CO$_2$ generation as well as O$_2$ consumption (Figure 7). Our study reinforced the importance of beige adipocytes in whole-body energy expenditure.

**MRTFA controls the balance between smooth muscle-like cells and beige precursors**

Searching for beige and white adipose precursors has been difficult due to the complexity of the stromal vascular compartment and the lack of biological functional markers for beige adipocytes in WAT. Understanding the signaling circuit that potentially controls beige precursor determination would provide relevant functional markers for future studies investigating the origin of beige precursors. Here, we identified MRTFA as a negative regulator for beige precursor determination, possibly by enhancing the specification of smooth muscle–like cells.

Beige adipocytes can be detected in subcutaneous WAT of mice maintained at thermo-neutrality (Figure 1) (Wu et al., 2012). Following β3-adrenergic stimulation, beige adipocytes can develop either by trans-differentiation of mature white adipocytes (Himms-Hagen et al, 2000; Vitali et al., 2012) or by the de novo differentiation of
adipose precursor cells (Lee et al., 2013; Wang et al., 2013). For MRTFA/SRF activity to regulate beige adipocyte formation during trans-differentiation would require the suppression of MRTFA in mature adipocytes. This is unlikely to occur because MRTFA abundance is already at undetectable levels (Figure 10), suggesting that the conversion of white to beige adipocytes requires activation or attenuation of other pathways. Most likely, MRTFA’s regulation of beige adipocyte is a developmental process. Consistently, we showed that whole ING WAT or SVCs from ING WAT of MRTFA−/− mice expressed higher developmental beige precursor markers Tbx1, CD137 and Tmem26 (Wu et al., 2012) at mRNA level (Figure 7).

Several lines of evidence suggest a similar origin of beige precursor and smooth muscle precursor (Lapid et al., 2014; Long et al., 2014). We showed that SVCs isolated from MRTFA−/− mice expressed lower smooth muscle markers SMA and CRP2 compared to WT SVCs, this can be observed before or after adipogenic induction in vitro (Figure 10 and Figure 11). In addition, compared to WT SVCs, MRTFA−/− SVCs efficiently differentiated into beige adipocytes that express BAT-selective genes. These genes could be stimulated to maximum level by forskolin, a cAMP activator (Figure 4B and 4C). These results implied that MRTFA deficiency might preferentially promote the determination of beige precursors rather than smooth muscle-like cells.

**BMP4, CCG1423 and CCG203971 as negative regulators of MRTFA**

Several studies have identified BMP as an inducer of brown adipogenesis in vitro (Tseng et al., 2008) and beige adipocyte formation in vivo (Qian et al., 2013). Our lab has shown that BMP7 can disrupt the actin cytoskeleton and inhibit Rho kinase that would
hypothetically deactivate MRTF-SRF transcriptional activity (Mcdonald et al., 2015). Conversely, BMP’s effect in other systems is mainly to promote MRTF-SRF transcriptional activity (Lagna et al., 2007; Wang et al., 2012). Here, we showed that while BMP4 enhanced the expression of C/EBPβ (a regulator of brown and beige adipocyte formation) (Karamanlidis et al., 2007; Tanaka et al., 1997) in WT SVCs, it had no additional effect on expression C/EBPβ in KO SVCs (Figure 11C), suggesting that the induction of C/EBPβ by BMP4 is at least in part through inhibition of MRTFA activity.

CCG molecules were designed to inhibit Rho-GTPase-A signaling (Evelyn et al., 2010; Evelyn et al., 2007). However, the inhibitory effect of CCGs on MRTFA-SRF is still unclear: it was suggested recently that CCG-1423 could target to the RPEL domain of MRTFA and compete with G-actin’s binding (Hayashi et al., 2014), which in turn inhibits MRTFA-SRF transcriptional activity. Our results are consistent with this notion by showing that CCG-1423 and CCG-203971 can enhance brown adipogenesis in WT SVCs but not in MRTFA−/− SVCs (Figure 12C). Taken together, these findings suggest that the browning activity of the CCG1423 is dependent on MRTFA.

**Identification of C/EBPβ as an MRTFA-SRF target gene that is responsible for enhanced beige adipocyte formation.**

Although we concluded that MRTFA-SRF negatively regulates beige adipocyte formation *in vivo*, possibly due to an enhanced beige precursor specification, understanding the mechanism of MRTFA-SRF’s action requires the identification of MRTFA-SRF target genes. C/EBPβ, an essential regulator of brown and beige adipocyte formation *in vivo* (Jimenez-Preitner et al., 2011; Tanaka et al., 1997), was recently
identified as a gene whose mRNA is repressed by MRTFA-SRF (Esnault et al., 2014). We showed that C/EBPβ is more abundant in $MRTFA^{+/−}$ SVCs than WT SVCs during progenitor expansion and after adipogenic induction (Figure 11C), indicating that MRTFA-SRF might inhibits the expression of C/EBPβ in WT SVCs. In $MRTFA^{-/-}$ SVCs however, C/EBPβ might not be repressed and thus available to promote beige adipogenesis.

Identification of MRTFA/SRF target genes by microarray would be of great importance for future studies to understand the mechanism of MRTFA-SRF in negatively regulating beige adipocyte determination and formation.

**Summary**

Taken together, we have identified a novel role for MRTFA in the negative regulation of physiologically functional beige adipocytes formation within ING WAT of mice, which protects mice from DIO-induced obesity and insulin resistance. SVCs of ING WAT from $MRTFA^{-/-}$ mice express higher levels of beige precursor markers and lower levels of smooth muscle markers, implicating a negative role for MRTFA/SRF in promoting beige precursors. In addition, we provide evidence that BMP4 and CCG might inhibit MRTFA-SRF transcriptional activity and promote brown adipogenesis *in vitro*. The identification by others that C/EBPβ is a target gene repressed by MRTFA-SRF provides a potential mechanism for MRTFA-SRF’s negative regulation of beige adipocyte formation (Scheme 4).
Scheme 4. MRTFA negatively regulates beige adipocyte formation, possibly by inhibiting smooth muscle-like cell differentiation. MRTFA plays a negative role in beige adipocyte lineage determination and formation. BMP4 and CCG might inhibit MRTFA-SRF transcriptional activity and promote brown adipogenesis in vitro. C/EBPβ might be a target gene that is repressed by MRTFA-SRF and promote brown adipogenesis.


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