# Depot-specific mechanisms determining human fat distribution

A thesis submitted to the University of Oxford in candidature for the degree of Doctor of Philosophy




**By**

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## Memorandum

The work in this thesis is the original work of the author. Experiments were undertaken at the Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, under the supervision of Professor Fredrik Karpe and Dr Katherine Pinnick. Funding was provided by the Radcliffe Department of Medicine.

I hereby state that no part of this thesis has been submitted for any other degree at this or any other university. This thesis is approximately 45,000 words (excluding references).

# Depot-specific mechanisms determining human fat distribution

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Body fat distribution is a strong determinant of human metabolic health but the mechanisms underpinning regional deposition of white adipose tissue (WAT) remain poorly understood. WAT also exhibits striking depot-specific functional properties. The aim of this project was to investigate the potential role of specific candidate genes implicated in regulating WAT development and/or function in a depot-specific manner. Cartilage oligomeric matrix protein (COMP) is an extracellular matrix protein that is highly differentially expressed between subcutaneous abdominal and gluteal WAT but has primarily been studied in the context of bone biology. WAT *COMP* expression was found to be significantly up-regulated in obesity; *COMP* expression in preadipocytes was increased by glucocorticoids; and COMP promoted adipogenesis in (immortalised) subcutaneous abdominal and gluteal preadipocytes.

Building on a finding during the COMP study, bone morphogenetic protein (BMP)-2 was identified as another candidate. BMP2 exerts positive adipogenic effects in murine models and a recent genome-wide association study meta-analysis identified a significant association between *BMP2* and body fat distribution. BMP2 was found to exert a pro-adipogenic effect specifically in subcutaneous abdominal preadipocytes, with this effect requiring activation of SMAD1/5/8 signalling via type 1 BMP receptors. These data identify BMP2 as a novel depot-specific regulator of human adipogenesis.

Particularly lipid-laden cells were formed when conventional adipogenic medium was supplemented with fatty acids; these cells were captured, de-differentiated (DFAT) and expanded to generate immortalised abdominal and gluteal DFAT cells. These DFAT cells exhibit a greatly enhanced adipogenic potential compared to the mixed stromovascular (SVF) population from which they derive and retained an intrinsic memory of their anatomical origin. The use of DFAT cells is likely to represent a valid and enhanced model system to study various depot-specific aspects of WAT biology such as adipogenesis. Overall, the data from this thesis emphasise the striking depot-specific biology exhibited by WAT and provide novel insights into the mechanisms governing the regional distribution of WAT in humans.

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## Contents

**Chapter 1 Introduction** ..... 12
1.1 Adipose tissue biology ..... 13
1.1.1 The types of adipose tissue and their anatomy ..... 13
1.1.2 Composition of WAT ..... 16
1.1.3 Fatty acid metabolism in WAT ..... 17
1.1.4 WAT as an endocrine organ: bioactive peptide secretion ..... 19
1.1.5 WAT as an endocrine organ: steroid hormone metabolism ..... 20
1.2 Development of WAT ..... 21
1.2.1 Embryonic origin of WAT ..... 21
1.2.2 Commitment to the white adipose lineage ..... 22
1.2.3 Transcriptional regulation of white adipogenesis ..... 25
1.2.4 Epigenetic regulation of white adipogenesis ..... 28
1.2.5 Mitotic clonal expansion in white adipogenesis ..... 31
1.3 Body composition, body fat distribution and metabolic disease risk ..... 32
1.3.1 Body Mass Index ..... 32
1.3.2 Waist-to-Hip Ratio ..... 32
1.3.3 Dual-energy X-ray absorptiometry ..... 33
1.3.4 Computed tomography ..... 33
1.3.5 Magnetic resonance imaging ..... 34
1.3.6 Limitations of imaging modalities ..... 34
1.3.7 Relationship of body composition to health ..... 34
1.3.8 Relationship between regional WAT distribution and metabolic health ..... 36
1.4 Depot-specific WAT biology ..... 39
1.4.1 Regional variation in fatty acid handling ..... 39
1.4.2 Depot-specific cell biology of WAT ..... 41
1.4.3 Depot-specific transcriptional and epigenetic profiles in subcutaneous WAT ..... 43
1.4.4 Relationship of adipocyte size to metabolic health ..... 44
1.4.5 AT inflammation in obesity and metabolic disease ..... 47
1.4.6 The extracellular matrix in WAT ..... 48
1.5 Determinants of regional AT distribution ..... 50

1.5.1 Genetics of body fat distribution....50
1.5.2 Pharmacological determinants of body fat distribution....52
1.5.3 Surgical manipulation of body fat distribution....53
1.5.4 Sex steroid regulation of body fat distribution....54
1.5.5 Glucocorticoid regulation of body fat distribution....54
1.6 Approaches to understanding regional AT biology....57
1.6.1 Aims....59
**Chapter 2 Methods**....69
2.1 The Oxford Biobank....61
2.2 Cell culture of human preadipocyte cell lines....63
2.2.1 Immortalised human subcutaneous abdominal and gluteal preadipocytes....63
2.2.2 Isolation and culture of human primary white preadipocytes....63
2.2.3 Generation of human preadipocyte cell lines....64
2.2.4 Adipogenic differentiation of primary and immortalised human preadipocytes....65
2.2.5 Proliferation analysis using CyQuant assay....65
2.3 Gene expression analysis....66
2.3.1 RNA extraction....66
2.3.2 Complementary DNA synthesis....66
2.3.3 Quantitative real-time PCR....67
2.4 Measurement of intracellular TAG content....68
2.5 Western blotting....69
2.5.1 Cellular protein extraction....69
2.5.2 Protein extraction from AT biopsies....69
2.5.3 Gel electrophoresis....69
2.5.4 Enhanced chemiluminescence....70
2.5.5 Densitometric analysis of Western blot images....71
2.6 Enzyme-linked immunosorbency assay....71
2.7 Statistical analysis....72
2.8 Data contribution....75
**Chapter 3 Cartilage oligomeric matrix protein is a differentially expressed regulator of human adipogenesis**....77
3.1 Introduction....78
3.1.1 Aims....80

3.2 Methods....81
3.2.1 Adipose tissue sample collection and analysis....81
3.2.2 Immunohistochemistry....81
3.2.3 Preadipocyte culture and adipogenic differentiation....82
3.2.4 Gene expression analysis....83
3.2.5 Measurement of intracellular TAG content....83
3.2.6 Western Blotting....83
3.2.7 COMP ELISA....83
3.2.8 Data analysis and statistics....84
3.3 Results....85
3.3.1 *COMP* is highly differentially expressed between abdominal and gluteal AT....85
3.3.2 *COMP* expression in AT is enhanced in obesity....87
3.3.3 Glucocorticoids regulate *COMP* expression in preadipocytes....89
3.3.4 COMP may represent a novel adipokine....91
3.3.5 COMP is a positive adipogenic regulator....94
3.3.6 COMP may interact with ECM components to modulate adipogenesis....96
3.4 Discussion....99
3.4.1 COMP expression in AT....99
3.4.2 COMP as a novel adipokine....100
3.4.3 COMP as a pro-adipogenic matricellular protein residing in AT ECM....102
3.4.4 Glucocorticoid regulation of AT COMP expression....109
3.4.5 Concluding statements....111
**Chapter 4 Bone morphogenetic protein 2 is a depot-specific regulator of human adipogenesis**....113
4.1 Introduction....114
4.1.1 Aims....117
4.2 Methods....118
4.2.1 Adipose tissue sample collection and analysis....118
4.2.2 AT histology....118
4.2.3 Preadipocyte culture and adipogenic differentiation....118
4.2.4 Analysis of preadipocyte proliferation....119
4.2.5 Gene expression analysis....119
4.2.6 Measurement of intracellular TAG content....119

4.2.7 Western Blotting ..... 119
4.2.8 BMP2 ELISA ..... 120
4.2.9 Data analysis and statistics ..... 120
4.3 Results ..... 121
4.3.1 BMP2 regulates adipogenesis in a depot-specific manner ..... 121
4.3.2 BMP2 is a paracrine factor in WAT ..... 122
4.3.3 BMP2 activates SMAD1/5/8 signalling in preadipocytes via type 1 BMP receptors ..... 124
4.3.4 BMP2's pro-adipogenic effects are mediated via type 1 BMP receptor kinase activity ..... 126
4.3.5 Differential expression of SMAD1/5/8 signalling components and its regulators may underlie the depot-specific action of BMP2 ..... 128
4.3.6 The rs979012/rs2145270 genetic locus is an eQTL for BMP2 expression in AT ..... 129
4.3.7 The rs979012/rs2145270 genotype is associated with increased abdominal adipocyte size in males ..... 132
4.3.8 Erythroferrone is a depot-specific regulator of BMP2 action in preadipocytes ..... 134
4.4 Discussion ..... 136
4.4.1 BMP2 as a paracrine factor ..... 136
4.4.2 BMP2-SMAD1/5/8 signalling and adipogenesis ..... 137
4.4.3 Ligand structure as a determinant of depot-specific BMP action ..... 138
4.4.4 BMP antagonists as a determinant of depot-specific BMP action ..... 140
4.4.5 BMP receptor subtypes as a determinant of depot-specific BMP action ..... 142
4.4.6 BMP2-SMAD1/5/8 target genes may determine BMP2's depot-specific action ..... 144
4.4.7 Epigenetic modifications as a determinant of depot-specific BMP action ..... 145
4.4.8 *BMP2* and *SMAD6* genetics ..... 149
4.4.9 Concluding statements ..... 152
**Chapter 5 De-differentiated adipocytes exhibit an enhanced adipogenic potential and retain depot-specific properties** ..... 153
5.1 Introduction ..... 154
5.1.1 Aims ..... 158
5.2 Methods ..... 159
5.2.1 Generation of DFAT cells via ceiling culture ..... 159

5.2.2 Culture and adipogenic differentiation of immortalised SVF and DFAT cells .....160
5.2.3 Measurement of intracellular TAG content .....160
5.2.4 Measurement of lipolysis in differentiated preadipocytes *in vitro* .....160
5.2.5 Osteogenic differentiation .....161
5.2.6 Alizarin red staining and quantification .....162
5.2.7 DNA extraction from cell lysates .....163
5.2.8 Gene expression analysis .....163
5.2.9 Data analysis and statistics .....164
5.3 Results .....165
5.3.1 DFAT cells exhibit a greatly enhanced adipogenic potential .....165
5.3.2 Differentiated SVF and DFAT cells exhibit lipolytic activity .....167
5.3.3 DFAT cells retain their depot-specific gene expression pattern .....168
5.3.4 Cell expansion during differentiation is positively correlated with adipogenic endpoints .....170
5.3.5 DFAT cells exhibit a greater osteogenic differentiation capacity compared to SVF cells .....172
5.4 Discussion .....175
5.4.1 Adipogenesis and lipogenesis in immortalised SVF and DFAT cells .....175
5.4.2 Depot-specific gene expression in SVF and DFAT cells .....178
5.4.3 Lipolysis in differentiated SVF and DFAT cells .....180
5.4.4 Studying mitotic clonal expansion and adipogenesis in SVF and DFAT cells .....181
5.4.5 Potential practical uses of SVF and DFAT cells .....184
5.4.6 Concluding statements .....187
**Chapter 6 Discussion** .....188
6.1 The importance of depot-specific AT biology .....189
6.2 Identifying appropriate candidates .....191
6.3 Studying AT under the optimal conditions and time-scale .....195
6.4 Using the most appropriate model systems .....198
6.5 Concluding statements .....200
**Bibliography** .....202

## Figures

### Chapter 1 Introduction

Figure 1.1 WAT anatomy in humans .....13
Figure 1.2 Overview of adipocyte fatty acid handling .....18
Figure 1.3 Overview of commitment and differentiation in white adipogenesis .....27
Figure 1.4 Overview of WAT expandability .....44

### Chapter 3 Cartilage oligomeric matrix protein is a differentially expressed regulator of human adipogenesis

Figure 3.1 *COMP* is differentially expressed between abdominal and gluteal AT. ....85
Figure 3.2 *COMP* is differentially expressed between abdominal and gluteal cells ..86
Figure 3.3 *COMP* expression in AT is enhanced in obesity.....87
Figure 3.4 Glucocorticoids regulate *COMP* expression in preadipocytes .....90
Figure 3.5 COMP may represent a novel adipokine .....92
Figure 3.6 COMP is a positive adipogenic regulator .....95
Figure 3.7 BMP2 is a depot-specific regulator of adipogenesis .....97

### Chapter 4 Bone morphogenetic protein 2 is a depot-specific regulator of human adipogenesis

Figure 4.1 BMP2 regulates adipogenesis in a depot-specific manner .....121
Figure 4.2 BMP2 is a paracrine factor in WAT .....123
Figure 4.3 BMP2 activates SMAD1/5/8 signalling in preadipocytes via type 1 BMP receptors .....124
Figure 4.4 BMP2's pro-adipogenic effects are mediated via type 1 BMP receptor kinase activity .....126
Figure 4.5 Differential expression of SMAD1/5/8 signalling components and its regulators may underlie the depot-specific action of BMP2 .....128
Figure 4.6 Expression quantitative trait locus analysis of *BMP2* and *SMAD6* expression in subcutaneous abdominal and gluteal AT.....131
Figure 4.7 Adipocyte size analysis according to rs979012/rs2145270 genotype in male subcutaneous abdominal AT.....133
Figure 4.8 - Erythroferrone is a depot-specific regulator of BMP2-SMAD1/5/8 signalling in preadipocytes .....135
Figure 4.9 Potential determinants of BMP2's depot-specific pro-adipogenic action 149

### Chapter 5 De-differentiated adipocytes exhibit an enhanced adipogenic potential and retain depot-specific properties

Figure 5.1 Representative image of lipid-laden SVF cells used for ceiling culture ..159

Figure 5.2 Differentiated immortalised abdominal and gluteal SVF and DFAT cells .....165
Figure 5.3 DFAT cells exhibit a greatly enhanced adipogenic potential .....166
Figure 5.4 Differentiated SVF and DFAT cells exhibit lipolytic activity .....167
Figure 5.5 DFAT cells retain their depot-specific gene expression pattern .....169
Figure 5.6 Cell expansion during differentiation is positively correlated with adipogenic endpoints .....171
Figure 5.7 DFAT cells exhibit a greater osteogenic differentiation capacity compared to SVF cells .....172

## Tables

**Chapter 2 Methods**

Table 2.1 Reverse-transcription reaction volumes for cDNA synthesis.....66

Table 2.2 Data contribution .....75

**Chapter 3 Cartilage oligomeric matrix protein is a differentially expressed regulator of human adipogenesis**

Table 3.1 Anthropometric characteristics of plasma COMP study participants.....93

**Chapter 4 Bone morphogenetic protein 2 is a depot-specific regulator of human adipogenesis**

Table 4.1 Adipocyte size analysis according to rs979012/rs2145270 genotype in male subcutaneous abdominal AT.....134

**Chapter 6 Discussion**

Table 6.1 Results of PubMed search for specific adipose depots.....190

# Chapter 1

# Introduction

## 1.1 Adipose tissue biology

### 1.1.1 The types of adipose tissue and their anatomy

There are two main functionally and morphologically distinct types of adipose tissue (AT) in mammals, white and brown, that together form the 'adipose organ' (Cinti, 2001). White adipose tissue (WAT) stores energy as triacylglycerol (TAG) and secretes hormones/cytokines which modulate whole body metabolism, appetite and insulin sensitivity (Luo & Liu, 2016). WAT surrounding soft organs also confers protection against mechanical forces and provides thermal insulation (Trayhurn, 2007).

WAT is located throughout the body: under the skin (subcutaneous); around internal organs (visceral); in bone marrow (yellow bone marrow); and in breast tissue. Approximately 80 - 90% of total body AT is stored in the subcutaneous areas surrounding the abdominal (waist), sub-scapular (upper back), gluteal (buttock), and femoral (thigh) areas. Visceral AT surrounds digestive organs in the omental and mesenteric depots and accounts for 6 - 20% of total body AT (Gesta, Tseng, & Kahn, 2007). The anatomy of subcutanous and visceral WAT depots is outlined in Figure 1.1.

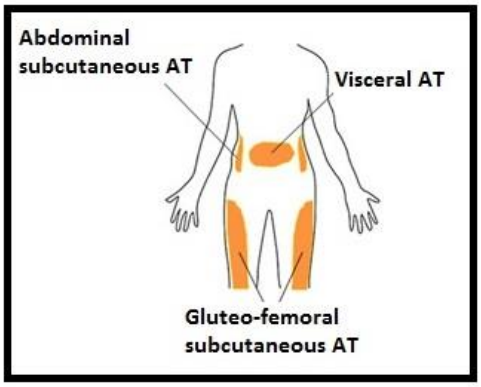



**Figure 1.1** WAT anatomy in humans. Diagram adapted from (Kwok, Lam, & Xu, 2016).

While clinically relevant data are ideally generated from studies of human AT, a great deal of our understanding of AT biology derives from experimental investigations of

mice. Murine AT is a multi-depot organ; there are two main subcutaneous fat pads, one located between the scapulae (anterior) and another located between the lumbar vertebrae that descends to the gluteal region. The latter is known as the inguinal pad and is anatomically similar to gluteo-femoral AT in humans. In addition to the subcutaneous depots there is visceral AT located in the perigonadal region (epididymal AT in males and periovarian AT in females). Murine AT shares many features in its functional and regulatory properties with human AT; regional variation in the expression of various developmental genes (including members of the *HOX* family) is broadly conserved across species (Gesta et al. 2006) as is the re-distribution of AT association with aging, which comprises expansion of visceral AT with a concomitant reduction in subcutaneous AT mass (Tchkonia et al. 2010).

It is important to consider some of the important differences between murine and human AT though (Chusyd, Wang, Huffman and Nagy 2016). Perigonadal AT is the most frequently studied in the literature and is thought to be analogous to human visceral AT. However, as this murine depot lacks portal vein drainage (unlike omental and mesenteric AT in humans) it has been suggested that this AT should be treated as 'peri-visceral'. Other species differences pertain to the depot-specific mechanisms by which AT expands in mice upon overfeeding (section 1.4.2). In addition, it has been shown that AT-specific knockout of collagen VI in mice permits almost limitless expansion upon high fat diet feeding and protects against the associated metabolic complications (Khan et al., 2009); however, this biology does not seem to translate directly to humans (McCulloch et al., 2015). Evidently differences at the molecular, tissue and anatomical level exist that can make extrapolation from one system to another difficult in the absence of direct comparison studies.

There are various other types of AT that each play a distinct role in human physiology. Over recent years there has been great interest in brown adipose tissue (BAT) a type of AT which shares a developmental lineage with muscle and is specialised for heat production via non-shivering thermogenesis mediated via uncoupling protein 1 (Bargut, Aguila, & Mandarim-de-Lacerda, 2016). BAT is primarily found in the suprascapular region in new-borns but has been detected in the supraclavicular region in adults (Marlatt et al. 2017). A related but distinct strand of research has focussed on beige AT which concerns certain (subcutaneous) WAT depots that can acquire the thermogenic properties of BAT under certain conditions, specifically cold-exposure and exercise (Aldiss et al. 2017). This process has also been detected in mice but species differences have been detected (Zuriaga, Fuster, Gokce and Walsh 2017). Following observations that BAT mass is reduced in obese humans (Pfannenberg et al. 2010), it has been proposed that BAT and beige AT may regulate overall metabolic health and play a causal role in the development of obesity (Trayhurn 2017).

Interest in the role of AT located within bone marrow (BMAT) has also grown in recent years following reports that BMAT may be a significant source of circulating adiponectin whose contribution increases during periods of calorie restriction (Scheller et al. 2016). BMAT has a distinct molecular lineage to WAT and BAT and has been reported to influence osteogenesis and haematopoiesis (Ambrosi and Schulz 2017). The role of BMAT in energy balance, (regional) adiposity and overall metabolic health currently remains unclear but certainly represents a fertile ground for future research.

Epicardial AT resides between the myocardium and the visceral pericardium and reportedly shares a developmental origin with visceral AT (Nagy et al 2017). This type of AT has been found to secrete a variety of adipokines and cytokines that modulate vascular health and function (Patel, V. B. et al. 2017). Observations that epicardial AT

volume is reportedly increased in patients with type 2 diabetes and is positively correlated with BMI and waist circumference have prompted suggestions that this AT depot is implicated in the development of diabetic coronary atherosclerosis (Wang, C.P. et al. 2009).

### 1.1.2 Composition of WAT

WAT is a type of loose connective tissue comprising lipid-laden adipocytes held in a network of collagen fibres. Several non-adipocyte cell types also reside within WAT which collectively form the stromovascular fraction (SVF). Adipocytes are the primary cellular constituent of (subcutaneous abdominal) AT with the SVF accounting for ~3% of total AT in a normal weight human; this proportion decreases with obesity (Fraser et al. 2008). Cells within the SVF include (mesenchymal) stem cells, endothelial cells, immune cells, smooth muscle cells, pericytes and preadipocytes (Nguyen et al., 2016); note that the relative proportion of each cell type and their function varies between different AT depots and can be affected by age, sex and/or adiposity (Yang, H. J. et al. 2014). The preadipocyte population comprises cells that are committed to the adipose lineage but have not yet been recruited to undergo adipogenesis and accumulate lipids. Many different cell types residing within AT secrete bioactive peptides and contribute to its endocrine role (section 1.1.4; (Galic, Oakhill, & Steinberg, 2010); (Fasshauer & Bluher, 2015)).

An important component of WAT is the extracellular matrix (ECM; section 1.4.6). This is a collagenous network that provides mechanical strength to AT by acting as an external scaffold which protects the lipid droplet from disruption by physical forces (Mariman & Wang, 2010). The structure and composition of the ECM at any given moment reflects a complex interplay between gene expression and the enzymatic degradation of its

structural components (Lin, Chun, & Kang, 2016). Despite being viewed as a passive, inert structure, the ECM is actually highly dynamic and co-ordinates many important processes within AT such as (hypertrophic and/or hyperplastic) expansion, retraction and vascularisation (Divoux & Clement, 2011).

### 1.1.3 Fatty acid metabolism in WAT

The major metabolic role of WAT is the tightly controlled storage and release of lipids, which is stored in adipocytes as triacylglycerol (TAG) and released into the systemic circulation as non-esterified fatty acids (NEFA). Metabolic flux can range from fatty acid storage in the post-prandial state to fatty acid mobilisation during fasting. It is generally thought that subcutaneous (abdominal) WAT acts as a buffer against the daily influx of dietary lipids to protect extra-adipose tissues from lipid overflow and the deleterious effects of 'lipotoxicity' (K. Frayn, 2002); this function is analogous to the role of the liver in glucose homeostasis. An overview of adipocyte fatty acid handling is provided in Figure 1.2.

Hydrophobic TAGs are transported in lipoprotein particles such as chylomicrons from the gut and very low density lipoprotein from the liver (Luo & Liu, 2016). These particles are too large to escape from the capillaries so adipocytes rely on lipoprotein lipase (LPL) to hydrolyse circulating lipoprotein-TAG (Kersten, 2014); locally-produced NEFA generate a concentration gradient in the interstitial fluid which favours their entry into adipocytes (K. N. Frayn et al., 1994). However, not all liberated fatty acids are taken up; some NEFA enter the systemic circulation and 'spill-over' (K. N. Frayn, Coppack, Fielding, & Humphreys, 1995). The physiological role of this process remains unclear but as spill-over varies between AT depots (Almandoz et al., 2013) it may be involved in AT re-distribution (section 1.4.1). Absorbed NEFA are converted to an acyl-CoA

derivative and re-esterified with glycerol-3-phosphate to be stored as TAG (K. N. Frayn, Karpe, Fielding, Macdonald, & Coppack, 2003). Stored fatty acids are mobilised from TAG through the sequential action of several lipases (Nielsen, Jessen, Jorgensen, Moller, & Lund, 2014); liberated NEFA are carried to tissues (e.g. muscle and liver) bound to albumin in the plasma.

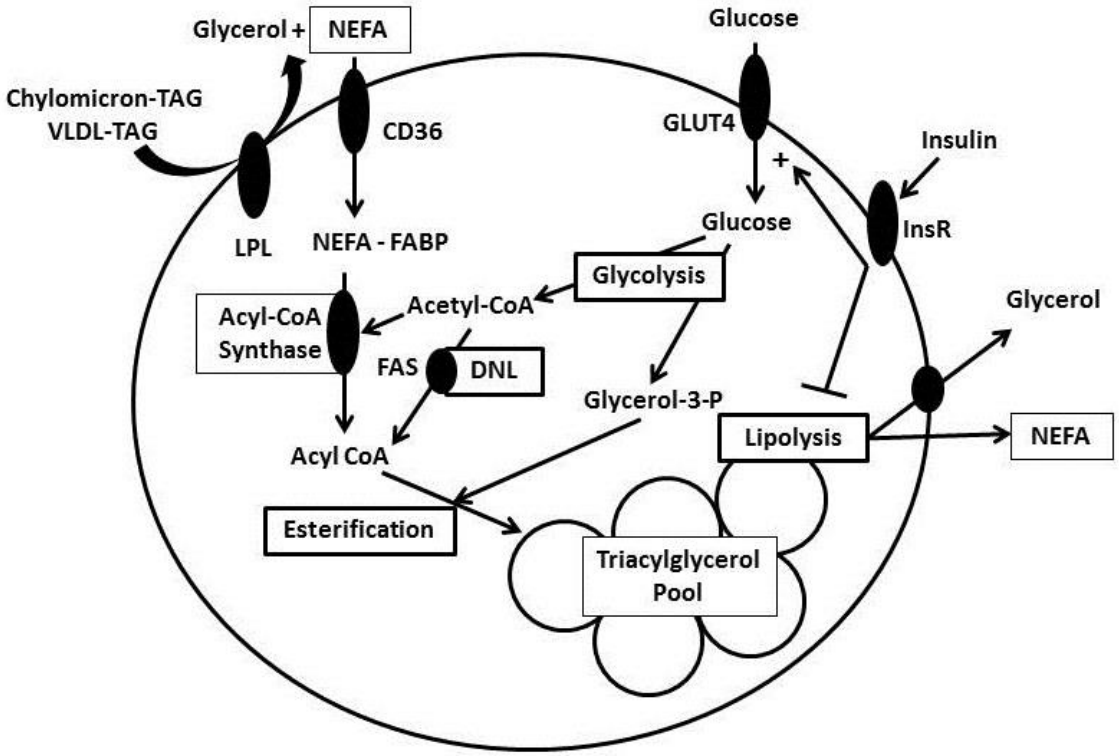



**Figure 1.2 Overview of adipocyte fatty acid handling.** Triacylglycerol (TAG) carried in chylomicrons or very low density lipoproteins (VLDL) are hydrolysed by lipoprotein lipase (LPL) to yield glycerol and non-esterified fatty acids (NEFA). NEFA are transported into adipocytes via CD36 and bind to fatty acid binding protein (FABP). An acetyl-CoA group is added to the NEFA via acyl-CoA synthase. Glycerol-3-phosphate generated via glycolysis of glucose (absorbed in an insulin-dependent manner via GLUT4 upon insulin-mediated activation of the insulin receptor; InsR) is then used as a backbone for NEFA esterification to form TAG that joins the TAG pool. Fatty acid synthase (FAS) uses acetyl-CoA and malonyl CoA to generate new acyl-CoA molecules via *de novo* lipogenesis. NEFA are liberated from the TAG pool via lipolysis (through the sequential action of adipose triacylglycerol lipase; hormone sensitive lipase; and monoacylglycerol lipase) and are transported to target tissues such as skeletal muscle and liver for β-oxidation bound to albumin. Liberated glycerol exits via aquaporin 7 and travels in the plasma to the liver where it is used for gluconeogenesis.

AT can also perform *de novo* lipogenesis (DNL; the synthesis of fatty acids from non-lipid precursors). Studies using stable isotopes suggest that newly stored TAG contributed by AT DNL ranges from 20% of total (Strawford, Antelo, Christiansen, & Hellerstein, 2004) to almost negligible (Diraison et al., 2003). AT DNL may be important for the synthesis of lipokines like palmitoleate (Pinnick et al., 2012) which are fatty acid species that modulate systemic metabolic health (Yilmaz, Claiborn, & Hotamisligil, 2016).

### 1.1.4 WAT as an endocrine organ: bioactive peptide secretion

Cells residing within WAT secrete many different peptide hormones (adipokines) that regulate appetite, insulin sensitivity, glucose and lipid metabolism as well as inflammation and immunity (Fasshauer & Bluher, 2015). Leptin is the archetypal adipokine. It is a 16kDa peptide secreted by adipocytes (Y. Zhang et al., 1994) that circulates in the plasma at levels directly proportionate to the degree of adiposity (Schwartz et al., 1997). Leptin crosses the blood-brain barrier to exert an appetite-suppressing effect by modulating neuronal activity in the arcuate nucleus of the hypothalamus (Dieguez, Vazquez, Romero, Lopez, & Nogueiras, 2011).

Adiponectin is another well-established adipokine secreted by adipocytes (Maeda et al., 1996) that exerts insulin-sensitising effects (Pajvani et al., 2004). Other adipokines include resistin, interleukin-6 and plasminogen activator inhibitor 1 (Fasshauer & Bluher, 2015). Thrombospondin (TSP)-1 has also been identified as an adipokine (Kong, Cavalera, & Frangogiannis, 2014) whose expression in AT reportedly correlates positively with BMI but negatively with insulin sensitivity (Varma et al., 2008). In addition to adipocyte-derived factors, other molecules have been identified that are released by non-adipocyte cells residing within the SVF, potentially in a depot-specific manner. For example, omentin is an adipokine that is primarily expressed and secreted

by SVF cells residing within omental (visceral) AT but is essentially undetectable in subcutaneous AT (Yang, R. Z. 2006). Consistent with omentin's insulin sensitising effects in human adipocytes, circulating omentin levels are reportedly reduced in obesity and type 2 diabetes (de Souza Batista, 2007).

### 1.1.5 WAT as an endocrine organ: steroid hormone metabolism

WAT also possesses the full complement of enzymes required for the activation, interconversion and inactivation of sex steroid hormones (J. Li, Papadopoulos, & Vihma, 2015). Knowledge of this aspect of AT biology significantly pre-dates the identification of any adipose-derived bioactive peptides (Siiteri, P. K. 1987). Both adipocytes and non-adipocyte SVF cells express distinct but overlapping arrays of steroid hormone-metabolising enzymes (Tchernof 2015). The expression of these enzymes can vary between different AT depots (Blanchette 2005) and according to obesity (Corbould 2002) and regional adiposity (Wake 2007).

Together this means that many cell types within AT contribute to the highly complex, bi-directional interplay between (regional) adiposity, sex steroid metabolism and overall metabolic health. The regulation of body fat distribution by sex steroids is discussed in more detail in section 1.5.4. WAT is also a key site for glucocorticoid metabolism. The regulation of regional adiposity by glucocorticoids is discussed in more detail in section 1.5.5.

## 1.2 Development of WAT

### 1.2.1 Embryonic origin of WAT

With respect to human embryogenesis, the first lobules of WAT start to develop between weeks 14 to 16 of gestation; WAT is detectable at all predicted subcutaneous and visceral depots by week 28 (Poissonnet, Burdi, & Garn, 1984). White adipocytes are principally mesodermal in origin (Gesta, Tseng, & Kahn, 2007) although non-mesodermal AT has been detected in mice; craniofacial AT in mice reportedly derives from neuroectoderm (Billon et al. 2007); (Takashima et al. 2007). It has also been reported that AT progenitor cells reside in the vasculature and may be endothelial in nature (section 1.2.2; (Nishimura et al., 2007); (W. Tang et al., 2008). It is clear that much work remains to be done in order to confirm these complex findings in humans.

A mounting body of evidence suggests that anatomically distinct AT depots have unique developmental origins. In support of this, a lineage tracing study in mice identified that the developmental regulator Wilm's tumour 1 (*Wt1*) is expressed by preadipocyts residing in visceral but not subcutaneous WAT (Chau et al. 2014). Such data build upon the observation that many differentially expressed genes between anatomically distinct subcutaneous and visceral AT depots in humans and mice are developmental regulators (Gesta et al., 2006); (Pinnick et al., 2014). Identification of differentially expressed developmental regulators between WAT depots is consistent with the burgeoning data indicating that AT and its constituent cells exhibit distinct depot-specific functional properties and gene expression profiles in humans and mice alike (Tchkonia et al., 2013).

In addition to complexity arising from inter-depot differences, however, recent data suggest that the population of progenitor cells within a given AT depot is not homogenous. A recent lineage tracing study in mice highlighted that within a given AT

depot, white adipocytes develop from a population of progenitor cells comprising multiple molecularly-distinct lineages which can change dynamically in response to external and internal factors (Sanchez-Gurmaches & Guertin, 2014). This observation might provide a reason explaining how functionally distinct sub-populations of SVF cells can be isolated from a single AT depot (Tchkonia et al., 2005) and suggests that the functional properties of an AT depot emerge from the aggregate behaviour of the depot's constituent cells.

Based on these data, it would be more correct and precise to identify a preadipocyte as 'subcutaneous' or 'visceral' based on its anatomical origin; however, such a label may still under-represent the complexity of its associated biology. It follows that each anatomically and functionally distinct AT depot likely derives from a unique development process involving a distinct population of progenitor cells (Hepler, Vishvanath and Gupta 2017). Although isolating cells from AT that can perform adipogenesis is a routine procedure, it is apparent that our understanding of the factors and processes involved in committing MSCs to the adipose lineage remains poor.

### 1.2.2 Commitment to the white adipose lineage

Various signalling pathways have been implicated in the commitment of MSCs to the adipose lineage including the bone morphogenetic protein (BMP) family. BMP-Smad1/5/8 signalling is required for commitment of murine pluripotent MSCs to the adipose lineage; BMP2 pre-treatment enhances lipid accumulation and expression of adipogenic markers upon differentiation compared to untreated cells (Wang, Israel, Kelly, & Luxenberg, 1993); (Huang et al., 2009). This effect is achieved via up-regulation of *PPARG2* expression by Schnurri-2-mediated binding of Smad1/4 and C/EBPα heterodimers to its promoter (Jin et al., 2006). Pre-treatment of murine MSCs with BMP4

also promotes commitment to the adipose lineage (Q. Q. Tang, Otto, & Lane, 2004). The type of BMP receptor (BMPRs) expressed by MSCs may also influence how BMPs affect lineage commitment; BMPR-1A is reportedly pro-adipogenic whereas BMPR-1B conveys a pro-osteogenic signal in murine (D. Chen et al., 1998) and human (Zielins et al., 2016) SVF cells.

The Wnts are also involved in commitment to the adipose lineage; many Wnt genes are expressed upstream of several key transcriptional regulators of adipogenesis in murine pluripotent MSCs (Bowers & Lane, 2008). In murine 3T3-L1 preadipocytes, Wnt10b negatively regulates adipogenesis by inhibiting *PPARG2* expression (Ross et al., 2000) while mutations within *Wnt10b* are associated with early onset obesity in humans (Christodoulides et al., 2006). Other factors regulating adipose commitment include the hedgehog ligands (Zehentner, Leser, & Burtscher, 2000) and retinoblastoma protein (Fajas et al., 2002).

Lineage tracing studies have provided important insights into AT development. While the exact molecular characteristics of each depot-specific preadipocyte population remains elusive, mounting data suggest this population may reside in AT vasculature and be endothelial in nature (Nishimura et al., 2007); (W. Tang et al., 2008). Such findings build upon the proposal that AT vascularisation and expansion (and development) are closely linked via one of two models. One proposed model suggests that during positive energy balance, AT would expand via hypertrophy and/or hyperplasia until a point is reached where the oxygen and energy demands of the expanded tissue are not met by the pre-existing vasculature. This scenario would precipitate a hypoxia signal (e.g. activation of hypoxia inducible factor-1 and the release of pro-angiogenic signals such as vascular endothelial growth factor) that stimulates angiogenesis which acts to mitigate the local hypoxia (Corvera and Gealekman 2014). The role of hypoxia in AT is somewhat

controversial, however; while hypoxic AT has been documented in obese rodents and potentially contributes to AT dysfunction (Rausch et al. 2008), this scenario may not directly translate to humans (Hodson 2014).

An alternative model states that the growth of the vasculature precedes the generation of adipocytes (Corvera and Gealekman 2014). In this scenario, a positive energy balance would result in the release of trophic factors (e.g. insulin) that stimulate angiogenesis in AT; adipogenesis would consequently occur in response to exposure to both the trophic factors and excess nutrients, thus resulting in hyperplastic AT expansion. Experimental data suggest that the latter scenario occurs during development and in expanding adult tissue with expression of *Zfp423*, an adipogenic transcription factor, providing a mechanism that couples AT development and expansion to vascularisation.

*Zfp423* is a zinc finger transcription factor that regulates the expression of *Pparg* and therefore adipogenesis in murine NIH 3T3 cells (Gupta et al., 2010). The link between angiogenesis and adipogenesis derives from the observation that cells expressing this gene reportedly reside in the vasculature (Gupta et al., 2012). While the *Zfp423*-expressing cells exhibit an endothelial phenotype, treatment with PPARG agonists prompts them to undergo adipogenesis and develop into fully functional, mature adipocytes (Tran et al., 2012); this phenomenon has been documented in both murine and human AT.

As the *Zfp423* gene is highly evolutionarily conserved (*ZNF423* is the human homologue), it seems likely that it represents a key component of the mechanism linking angiogenesis and adipogenesis. This is supported by recent human data in which *ZNF423* expression was found to be reduced in obese subcutaneous (abdominal) AT accompanied by adipocyte hypertrophy (Longo et al. 2017). It follows that the relationship between

angiogenesis and adipogenesis might vary between AT depots and will likely influence the mechanism by which a given AT depot expands (i.e. hypertrophy vs. hyperplasia). The extent of coupling between adipogenesis and angiogenesis will therefore likely determine a depot's 'expandability' and influence an individual's 'personal fat threshold' and therefore their overall metabolic health (section 1.4.4; (R. Taylor & Holman, 2015)).

### 1.2.3 Transcriptional regulation of white adipogenesis

Preadipocytes develop into mature adipocytes via adipogenesis, a tightly-regulated, temporally co-ordinated, multi-step process involving the sequential activation of several transcription factors. Peroxisome proliferator-activated receptor gamma (*PPARG*) is a member of the nuclear receptor superfamily and is regarded as the 'master regulator' of adipogenesis *in vivo* and *in vitro* in mice (Rosen, E.D. et al 1999) and humans (Fajas, Landsberg, Huss-Garcia, Sardet, Lees and Auwerx 2002). Ectopic over-expression of *Pparg* in murine fibroblasts is sufficient to enable them to undergo adipogenesis (Tontonoz, Hu, & Spiegelman, 1994) whereas genetic deficiency of *Pparg* completely abrogates adipogenesis *in vivo* and generates lipodystrophic mice (E. D. Rosen et al., 1999).

Persistent *Pparg* expression is required to maintain the differentiated state; introduction of a dominant negative *Pparg* isoform into differentiated murine 3T3-L1 cells precipitates loss of accumulated lipid and reduced expression of adipogenic markers (Tamori, Masugi, Nishino, & Kasuga, 2002). Clinical observations have confirmed that the *PPARG2* isoform plays a crucial role in human AT biology; *PPARG2* mutations cause specific forms of congenital partial lipodystrophy defined by the localised absence of WAT (Fiorenza, Chou, & Mantzoros, 2011). *PPARG2* is the target of the thiazolidinediones (TZD); these drugs are synthetic PPARG agonists which mimic

endogenous ligands to improve insulin sensitivity and glucose control by promoting metabolically-favourable (subcutaneous) adipogenesis (Carey et al., 2002).

Experiments utilising murine 3T3-L1 preadipocytes have been instrumental in the characterisation of various members of the CCAAT/enhancer bind protein (C/EBP) transcription factor family (specifically C/EBPα, C/EBPβ, and C/EBPδ) which also regulate adipogenesis (E. D. Rosen & Spiegelman, 2000). In 3T3-L1 cells, the expression of the different *C/ebps* proceeds in a temporally co-ordinated fashion and culminates in the *C/ebp*-dependent up-regulation of *Pparg* expression to drive adipogenesis and maintain the terminally differentiated state.

*C/ebpβ* and *C/ebpδ* expression is induced early during adipogenesis (Q. Q. Tang & Lane, 1999); these proteins proceed to activate the expression of *C/ebpα* (Q. Q. Tang, Zhang, & Daniel Lane, 2004) and *Pparg* (Zuo, Qiang, & Farmer, 2006). After induction, *C/ebpα* maintains its own expression via an auto-regulatory feedback loop (Timchenko et al., 1995) and promotes *Pparg* expression (Wu et al., 1999). This transcriptional activity generates a positive feedback loop that drives adipogenesis forward to terminal differentiation (E. D. Rosen et al., 2002) and then maintains the mature adipocyte phenotype (B. O. Park, Ahrends, & Teruel, 2012).

Several other proteins are involved in the transcriptional regulation of adipogenesis. For example, the sterol regulatory element-binding proteins (SREBPs) are basic helix-loop-helix, leucine zipper transcription factors that regulate the expression of lipogenic genes (Horton, 2002). The expression of *ADD1* (the mouse homologue of *SREBP1c*) increases early during adipogenesis in 3T3-L1 cells (Tontonoz, Kim, Graves, & Spiegelman, 1993) and has been found to link the acquisition of a terminally differentiated adipocyte phenotype with lipid accumulation (J. B. Kim & Spiegelman, 1996).

While the molecular regulation of adipogenesis has not been as extensively characterised in human preadipocytes as in murine cells, mounting data suggest that there are many similarities conserved between species (Moreno-Navarrete et al. 2013); (Todoric et al. 2011), especially with respect to key adipogenic regulators such as *PPARG* (Perrini et al. 2008). However, species-specific differences have also been detected meaning that extrapolation between systems in the absence of direct comparative evidence should be minimised (Gagnon, Foster, Landry and Sorisky 2013). An overview of the commitment and differentiation processes in white adipogenesis is provided in Figure 1.3.

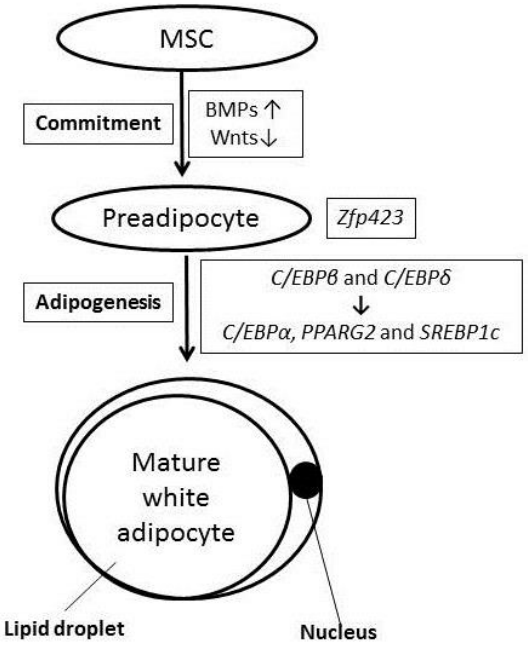



**Figure 1.3 Overview of commitment and differentiation in white adipogenesis.** Mesenchymal stem cells (MSC) become committed to the white adipose lineage by integrating pro-adipogenic (e.g. BMPs) and anti-adipogenic (e.g. Wnts) signals; this culminates in the expression of commitment factors such as *Zpf423* (or *ZNF423*, the human homologue). Upon exposure to the appropriate (and currently unknown) stimuli, preadipocytes (i.e. cells committed to the white adipose lineage) perform adipogenesis. This involves a tightly regulated, temporally co-ordinated transcriptional cascade under the control of various CCAAT/enhancer binding proteins (C/EBPs), peroxisome proliferator-activated receptor gamma (PPARG) 2 and sterol regulatory element binding protein 1c (SREBP1c) and results in the acquisition of a mature adipocyte phenotype (e.g. lipid accumulation).

### 1.2.4 Epigenetic regulation of white adipogenesis

Mounting evidence indicates that epigenetic mechanisms play a crucial role in co-ordinating the highly complex process of (white) adipogenesis; the well-documented transcriptional changes exhibited by differentiating preadipocytes appear to be accompanied by a parallel remodelling of the epigenetic landscape (Sarantopoulos, Banyard, Ziegler, Sun, Shaterian, and Widgerow 2017).

DNA methylation is classically associated with transcriptional silencing (Jones, 2012); the genome-wide DNA methylation profile of human preadipocytes has been reported to change substantially during adipogenesis (Broholm et al., 2016); (van den Dungen, Murk, Kok, & Steegenga, 2016). It follows that DNA methylation has also has been implicated in the regulation of *PPARG* expression; the *Pparg* promoter is hypermethylated and transcriptionally silent in undifferentiated 3T3-L1 preadipocytes but upon induction of adipogenesis there is a progressive increase in *Pparg* expression and parallel demethylation of this genomic region (Fujiki, Kano, Shiota and Murata 2009). DNA hypomethylation has also been observed in conjunction with increased expression of other markers of terminal adipogenic differentiation such as leptin (Yokomori, Tawata, and Onaya 2002).

Genome-wide remodelling of chromatin has also been detected during the process of adipogenesis, with such changes underpinning the shift in transcriptional profile that accompanies preadipocyte differentiation (Siersbæk, Nielsen and Mandrup 2012). Detailed characterisation of murine 3T3-L1 preadipocytes has highlighted that upon induction of adipogenesis, transcription factors (particularly the C/EBPs) bind to the genome in a co-operative manner at distinct sites, thereby forming ‘hotspots’ (Siersbæk et al. 2011). These hotspots manifest in a temporally-controlled manner and appear to

precede chromatin remodelling (e.g. DNA demethylation) which facilitates altered gene transcription.

It has been observed that the transcription factor hotspots (~400 base pairs long) implicated in regulating gene expression often cluster together in the genome within several kilobases of one another and thereby form 'super-enhancers' (Siersbæk and Baek et al. 2014). Dynamic crosstalk in a temporally-co-ordinated manner between hotspots and super-enhancers plays a crucial role in regulating the transcriptional reprogramming underpinning adipogenesis; these sites also serve as regulatory hubs that can integrate external stimuli, such as exposure to glucocorticoids, into altered gene expression (Siersbæk and Rabiee et al. 2014).

It appears that the nature of the primary epigenetic mechanism regulating gene expression (at least in 3T3-L1 cells) appears to change dynamically as adipogenesis progresses. Recent data suggest that the retinoid X receptor plays a key role co-ordinating gene expression early in differentiation whereas terminal differentiation seems to be defined by histone methylation-mediated repression of gene transcription which may serve to stabilise the expression of a principally adipogenic transcriptional network (Sarusi Portuguez et al. 2017).

More focused characterisation of the epigenetic regulation of adipogenesis in murine NIH 3T3 cells has yielded data suggesting C/ebpβ reportedly displaces histone deacetylase 1 from the *C/ebpα* promoter in a glucocorticoid-dependent manner to enhance *C/ebpα* expression and promote adipogenesis (Abdou, Atlas, & Hache, 2013). Other histone modifications can impede adipogenesis; when fed a high-fat diet, transgenic mice over-expressing *high-mobility-group-A1* - a regulator of chromatin structure and gene expression - under the control of an AT-specific promoter displayed less weight gain and

reduced AT mass as a result of impaired adipogenesis (Arce-Cerezo et al. 2015). Such a phenotype is entirely consistent with the growing body of experimental data indicating that the expression of *PPARG* in human and rodent systems alike is subject to extensive epigenetic regulation (Lee and Ge 2014).

The expression of microRNAs (miRNA), a class of small RNA molecules that regulate entire networks of genes by directing the degradation of messenger RNA (mRNA) molecules, has also been implicated in the co-ordination of adipogenesis (McGregor & Choi, 2011). Indeed, the expression of many miRNAs is significantly different between undifferentiated human preadipocytes and mature adipocytes generated *in vitro* (Ortega et al. 2010). Moreover, the various miRNAs whose expression changes during differentiation are not just passive bystanders but instead play an instrumental role in co-ordinating adipogenesis. For example, miR-103 expression increases during differentiation of murine 3T3-L1 preadipocytes; inhibiting miR-103 action via anti-sense oligonucleotide prevents adipogenesis from occurring (Esau et al. 2004).

While our understanding of the epigenetic regulation of adipogenesis in human and murine systems is growing Sarantopoulos, Banyard, Ziegler, Sun, Shaterian, and Widgerow 2017), it is clear that a tremendous amount remains to be learnt. It should also be noted that a parallel line of research into the clinical relevance of epigenetics has provided new insights into the role of DNA methylation in obesity and metabolic disease. It has already been shown that the DNA profile of AT varies between lean and obese individuals and that such changes are associated with differential gene expression (Arner et al. 2015); these associations can vary between different AT depots (Keller et al. 2015).

Epigenetics evidently represents a fertile ground for future research into the molecular regulation of adipogenesis and AT function and may provide opportunities for novel

therapeutic approaches for the treatment of obesity and its associated complications. For instance, the results of a recent genome-wide investigation involving transcriptional and epigenetic profiling of murine 3T3-L1 preadipocytes implicated aberrant glucocorticoid receptor-and vitamin D receptor-mediated signalling in the pathogenesis and/or maintenance of insulin resistance in adipocytes (Kang et al. 2015).

### 1.2.5 Mitotic clonal expansion in white adipogenesis

Mitotic clonal expansion (MCE) is a key process required for successful adipogenesis. MCE involves growth-arrested preadipocytes re-entering the cell cycle to undergo approximately two rounds of division before proceeding to a terminally differentiated state (Q. Q. Tang, Otto, & Lane, 2003). Studies in 3T3-L1 cells indicate that this process occurs under the control of C/EBPs within the first four days post-induction of adipogenesis (J. W. Zhang, Tang, Vinson, & Lane, 2004); (Q. Q. Tang & Lane, 1999). The importance of MCE is highlighted by data indicating adipogenesis is abrogated by inhibition of DNA synthesis (Jacobsen, Mazloumi Gavgani, Mellgren, & Lewis, 2016) or blockade of cell cycle progression (Patel & Lane, 2000). MCE has been linked to the production of endogenous PPARG ligands which promote adipogenesis (Hallenborg et al., 2014). Most data on MCE have been generated in murine 3T3-L1 cells; it is unclear whether MCE is equally important to human adipogenesis and/or displays similar characteristics.

## 1.3 Body composition, body fat distribution and metabolic disease risk

### 1.3.1 Body Mass Index

Body mass index (BMI) is calculated as weight (kg) / [height (m)]$^2$ and is a useful, albeit rough, index of adiposity used in population studies. Several categories of adiposity have been devised that are specific to different ethnic groups ("Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies," 2004). For individuals of European ancestry, the following cut-offs are used: <18.5 underweight; 18.5 - 24.9 normal weight; 25 - 29.9 overweight; and >30 obese. Due to its method of calculation, BMI has numerous limitations (R. S. Taylor, 2010) and can give misleading estimates of adiposity in very tall or short individuals as well as those with high/low amounts of lean mass. The most notable limitation, however, is that BMI provides no information on AT distribution.

### 1.3.2 Waist-to-Hip Ratio

Waist to hip ratio (WHR) is calculated as waist circumference (cm) / hip circumference (cm); WHR provides an index of abdominal obesity. Due to the sexual dimorphism of body shape, WHR categories are sex-specific; males with a WHR >0.9 are defined as centrally obesity whereas for females the cut-off is a WHR >0.85. As for BMI, ethnicity-specific cut-offs for WHR have been proposed (Qiao & Nyamdorj, 2010). It is important to note, however, that a given body circumference reflects not only regional AT but also underlying bone structures and lean mass (i.e. muscle). Several powerful imaging methods that can precisely measure body composition and capture the spatial distribution of adipose, lean and bone tissue are currently available (Seabolt, Welch, & Silver, 2015).

### 1.3.3 Dual-energy X-ray absorptiometry

Dual-energy X-ray absorptiometry (DEXA) was originally designed to measure bone mineral density but has proven highly useful in the measurement of regional AT mass (Toombs, Ducher, Shepherd, & De Souza, 2012). By measuring the absorption of X-ray photons at two different energies, DEXA can distinguish between fat and fat-free soft tissue. DEXA can conveniently and accurately determine body AT percentage across a range of body shapes and sizes in a reproducible manner (Silver et al., 2013) and is scalable, thereby making large studies examining body composition and WAT distribution feasible (Stults-Kolehmainen, Stanforth, & Bartholomew, 2012; Stults-Kolehmainen et al., 2013). Although DEXA cannot directly discriminate different types of WAT, algorithms that can predict visceral AT mass using regional AT DEXA data have been developed and validated against computed tomography data (Kaul et al., 2012); (Bosch et al., 2015).

### 1.3.4 Computed tomography

Computed tomography (CT) is the gold-standard technique used to determine body composition. Utilising a similar principal as DEXA but with much higher doses of radiation, CT scans are generated by making X-ray measurements from different projection angles at specific levels on an individual's body. Software then processes the high resolution 2D images ('slices') to distinguish AT from non-adipose tissue with exquisite sensitivity before calculating 3D tissue volumes from contiguous or non-contiguous 2D slices (Silver, Welch, Avison, & Niswender, 2010). CT has been used to study ectopic lipid accumulation in metabolic disease (Lim & Meigs, 2013).

### 1.3.5 Magnetic resonance imaging

Magnetic resonance imaging (MRI) distinguishes AT from non-adipose tissues based on their unique magnetic resonance properties. In contrast to CT and DEXA, MRI does not involve exposure to ionising radiation so it is suitable for use in vulnerable populations and longitudinal studies involving serial measurements. MRI is being increasingly used to investigate ectopic lipid accumulation in the liver, pancreas and heart (Thomas, Fitzpatrick, Malik, Taylor-Robinson, & Bell, 2013) as well as body composition and regional AT distribution.

### 1.3.6 Limitations of imaging modalities

MRI, CT and DEXA are powerful tools that can accurately measure body composition and regional AT mass. However, these techniques are not interchangeable meaning it is crucial to choose the most appropriate imaging modality for a study. As AT distribution varies considerably between individuals (Thomas & Bell, 2003); (Greenfield, Samaras, Chisholm, & Campbell, 2002), the results of a study can change substantially depending on where an image is taken (So et al., 2012). Multi-slice protocols are better able to detect changes in AT mass and so are preferred for the construction of large, robust datasets like the UK Biobank (Sudlow et al., 2015).

### 1.3.7 Relationship of body composition to health

Excessive adiposity (defined as overweight or obesity) is implicated in the pathogenesis of many chronic diseases and conditions such as type 2 diabetes, cardiovascular disease, several different types of cancer and osteoarthritis (Meldrum, Morris, & Gambone, 2017). As the illnesses exacerbated by and/or caused by obesity are associated with significant reductions to the length and quality of life, the massive global prevalence of obesity

(Kelly, Yang, Chen, Reynolds, & He, 2008) presents not just a public health problem but also an economic one (Finkelstein et al., 2012). The obesity pandemic reflects a confluence of many factors that overall encourage the consumption of calorie-rich food and a sedentary lifestyle (Hruby & Hu, 2015). New ways of thinking about obesity need to be developed so effective interventions can be devised and implemented (B. Y. Lee et al., 2017).

BMI has proven to be highly useful as an index of adiposity in epidemiological studies and has strong predictive value for various health outcomes; a meta-analysis involving 10 million participants in 239 studies across four different continents clearly identified that overweight (BMI 25 - 30 $kg/m^2$) and obesity (BMI >30 $kg/m^2$) are strongly associated with increased all-cause mortality (Global et al., 2016). The link between high BMI and the development of type 2 diabetes is robust; data from Mendelian randomisation (Corbin et al., 2016) and large scale observational studies (Abdullah, Peeters, de Courten, & Stoelwinder, 2010) support a causal relationship. Increasing BMI also predicts the development of cardiovascular and metabolic disease (Kivimaki et al., 2017). However, BMI is only a crude anthropometric index of adiposity; more detailed studies have highlighted the importance of body composition to metabolic health.

'Normal weight obesity' is a state where an individual has a normal BMI but excessive adiposity and as such is at increased risk of developing cardiovascular and metabolic disease (De Lorenzo, Martinoli, Vaia, & Di Renzo, 2006). While the global prevalence of normal weight obesity is currently unknown, it is estimated to be high (Franco, Morais, & Cominetti, 2016). Adiposity is only one half of the equation though; the relative proportion of AT mass to non-AT (i.e. muscle) mass is also important. 'Sarcopenic obesity' refers to individuals characterised by high AT mass in conjunction with low non-AT mass (T. N. Kim & Choi, 2015) who are at increased risk of cardiovascular and

metabolic disease (Srikanthan, Horwich, & Tseng, 2016); (Thomas et al., 2012). The counterpoint to normal weight or sarcopenic obese people are 'metabolically healthy obese' individuals who exhibit increased (subcutaneous) adiposity in the absence of metabolic complications such as dyslipidaemia and hypertension (Karelis, 2008); (Stefan et al., 2008); (Kloting et al., 2010). Evidently the current definition of obesity needs to be updated to emphasise the importance of excessive adiposity relative to overall body weight. Despite this compelling evidence, it should be noted that regional distribution of AT is an even stronger determinant of cardiovascular and metabolic disease risk compared to overall adiposity.

### 1.3.8 Relationship between regional WAT distribution and metabolic health

The link between regional AT distribution and cardiovascular/metabolic disease was originally made during the 1940s and 1950s in a pair of seminal studies which identified that upper body/central obesity was associated with increased incidence of type 2 diabetes and atherosclerosis, whereas these diseases were significantly less common in individuals exhibiting lower body AT accumulation (Vague 1947, 1956). These initial observations have been conclusively confirmed in numerous subsequent studies; a recent meta-analysis comprising over 650,000 individuals highlighted that increased waist circumference is associated with higher rates of all-cause mortality in males and females across the BMI spectrum (Cerhan et al., 2014). Indeed, AT distribution, rather than overall adiposity, is a major determinant of metabolic health (Manolopoulos, Karpe, & Frayn, 2010). The relationship between regional adiposity and metabolic health is likely underpinned by depot-specific AT biology (section 1.4).

Increased waist circumference is a strong independent predictor of increased diabetes risk (Langenberg et al., 2012) and is associated with hypertension and raised levels of

circulating TAGs (Canoy et al., 2004). More detailed analyses have identified that visceral AT accumulation is particularly associated with insulin resistance (Hayashi et al., 2008), especially when this depot expands at the expense of subcutaneous AT (Taksali et al., 2008). Increasing data suggest, however, that visceral AT expansion (and central obesity) may simply be a surrogate marker of (subcutaneous) AT dysfunction (Bays, 2014). In contrast to upper body AT obesity, lower body AT accumulation is associated with protection against the metabolic and cardiovascular disease in men and women; the INTERHEART study of 27,000 individuals reported the risk of myocardial infarction decreased with increasing hip circumference, independent of BMI (Yusuf et al., 2005).

Many other large-scale studies have replicated and confirmed this finding; increased hip circumference was associated with a reduced risk of developing coronary heart disease in the EPIC study (Canoy et al., 2007) while increased hip and thigh circumference were independently associated with lower incidence of diabetes in the Hoorn study (Snijder et al., 2003). Increased hip circumference has also been associated with lower fasting insulin and plasma TAG levels (Seidell, Perusse, Despres, & Bouchard, 2001). Furthermore, increased leg AT mass is independently associated with decreased risk of developing cardiovascular disease (Han et al., 2017) whereas reduced gluteo-femoral AT mass was independently associated with a less favourable glucose and lipid profile in the Health ABC study (Snijder et al., 2005). As for the relationship between BMI and cardio-metabolic disease risk, the results from a recent Mendelian randomisation study provide evidence that abdominal obesity plays a causal role in increasing the risk of type 2 diabetes and cardiovascular disease (Emdin et al. 2017).

Given these associations, it should be noted that body composition and regional adiposity exhibit striking sexual dimorphism. For instance, women have ~10% absolute higher body AT mass compared to BMI-matched men (A. S. Jackson et al., 2002). Regional AT

distribution also varies by sex; the typical male/android body shape is characterised by upper body AT accumulation whereas the female/gynoid shape is defined by lower body AT accumulation, particularly on the hips and thighs (Karastergiou, Smith, Greenberg, & Fried, 2012). At a comparable level of adiposity, women have more subcutaneous AT in the abdominal (Schreiner et al., 1996) and gluteo-femoral depots (Yim, Heshka, Albu, Heymsfield, & Gallagher, 2008) than men. Women also tend to have a smaller visceral AT mass than men (Schreiner et al., 1996).

Body composition and regional AT distribution also vary by ethnicity; for example, Asian Indians tend to exhibit greater overall and abdominal adiposity and have smaller amounts of skeletal muscle compared to Europeans and Polynesians (Rush, Freitas, & Plank, 2009). Such differences are likely underpinned by a strong genetic component that has been influenced by historical exposure to nutritional, economic and social pressures (Heymsfield et al. 2016). Such data further confirm that universal BMI cut-offs are probably not appropriate when attempting to compare the prevalence of obesity between ethnic groups.

Based on the differences in body composition and regional adiposity between men and women, it follows that cardiovascular and metabolic disease risk are sexually dimorphic (Regensteiner et al., 2015). For instance, while cardiovascular disease is the leading cause of death in women, the incidence of coronary heart disease lags 10 years behind men and 20 years for myocardial infarction and sudden death (Go et al., 2014). The prevalence of metabolic disease is lower in women at all ages but the gap between men and women closes with increasing age (Pannier et al., 2006). The loss of protection against metabolic disease may be due to menopause (section 1.5.4), a phenomenon which is associated with a transition from a gynoid to android pattern of AT distribution (Abdulnour et al., 2012).

## 1.4 Depot-specific WAT biology

### 1.4.1 Regional variation in fatty acid handling

Numerous *in vivo* studies of humans indicate that fatty acid handling varies between different AT depots; abdominal AT tends to be more active and dynamic compared to the relatively quiescent gluteo-femoral AT (Karpe & Pinnick, 2015). AT blood flow also varies in a similar depot-specific manner (K. N. Frayn & Karpe, 2014) with blood flow to gluteo-femoral AT in the fasted state being significantly lower compared to subcutaneous abdominal AT (Tan, Goossens, Humphreys, Vidal, & Karpe, 2004).

Measurement of fatty acid release via direct measurement and isotope dilution methods has shown that abdominal subcutaneous AT is more lipolytically active and contributes the majority of NEFAs in the circulation during the fasted state (Martin & Jensen, 1991); (Jensen & Johnson, 1996); (Z. Guo, Hensrud, Johnson, & Jensen, 1999). These data are consistent with the regional variation in the rate of fatty acid turnover; the half-life of subcutaneous abdominal AT TAG was calculated to be 12 months but was 18 months in femoral AT (Marin, Rebuffe-Scrive, Smith, & Bjorntorp, 1987).

The response of AT to various lipolytic stimuli also varies by depot; adrenaline specifically activates lipolysis in upper body AT but has no effect in lower body AT (Jensen, Cryer, Johnson, & Murray, 1996); this effect is probably driven by higher expression of inhibitory α2-adrenoceptors in gluteal adipocytes (Wahrenberg, Lonnqvist, & Arner, 1989). Gluteo-femoral AT also exhibits marked resistance to other lipolytic stimuli such as exercise (Arner, Kriegholm, Engfeldt, & Bolinder, 1990) and fasting (Gjedsted et al., 2007).

Fatty acid uptake in the post-prandial state also exhibits regional variation; dietary fatty acids carried in chylomicrons are stored more efficiently in subcutaneous abdominal AT compared to the gluteo-femoral AT (Marin, Rebuffe-Scrive, & Bjorntorp, 1990); (Romanski, Nelson, & Jensen, 2000); (Koutsari, Snozek, & Jensen, 2008). During the post-prandial period, insulin fully suppresses lipolysis in gluteo-femoral AT but only partially in subcutaneous abdominal AT (Meek, Nair, & Jensen, 1999); (Z. Guo et al., 1999). AT blood flow increases to abdominal and gluteo-femoral AT in the post-prandial state but might increase more in the latter depot in women; this could promote and/or maintain lower body AT accumulation in women.

Although there is preferential uptake of dietary fatty acids by upper body AT, this is not necessarily the final destination of these molecules. Indeed, fatty acids undergo substantial re-distribution over time (Marin et al., 1990) and the short-term depot-specific variation in fatty acid uptake has been found to not predict long-term regional AT expansion (Votruba & Jensen, 2012). Fatty acid re-distribution may involve depot-specific differences in the direct uptake of circulating NEFA; the direct uptake of free fatty acids has been reported to be greater in abdominal AT in men whereas in women there is either no depot difference (Shadid, Koutsari, & Jensen, 2007) or greater uptake in gluteal AT (Koutsari, Ali, Mundi, & Jensen, 2011). Gluteo-femoral AT also displays a preference for fatty acids liberated from very low density lipoprotein particles (McQuaid, Humphreys, et al., 2010). These observations have prompted the suggestion that gluteo-femoral AT acts as a ‘metabolic sink’ which accumulates fatty acids recycled into the circulation and captures them for long-term storage.

The fatty acid composition of TAG varies between abdominal and gluteo-femoral AT; palmitoleate is more abundant in and released at a higher rate by gluteo-femoral AT

(Pinnick et al., 2012). Circulating palmitoleate levels have been reported to be significantly positively associated with insulin sensitivity in humans (Stefan et al., 2010), consistent with their insulin-sensitising effect in mice (Cao et al., 2008). However, other data suggest that circulating palmitoleate actually reflects increased hepatic DNL as it is elevated in individuals with fatty liver (J. J. Lee et al., 2015) who are at increased risk of developing cardiovascular/metabolic disease (Merino et al., 2016).

### 1.4.2 Depot-specific cell biology of WAT

A substantial body of evidence indicates that the depot-specific properties observed at the tissue level are retained at the cellular level *in vitro* (Karpe & Pinnick, 2015); (Tchkonia et al., 2013). Many studies have compared preadipocytes from subcutaneous abdominal and visceral AT in humans; visceral preadipocytes proliferate more slowly (Tchkonia et al., 2005) and are more susceptible to apoptosis than their subcutaneous counterparts (Niesler, Siddle, & Prins, 1998) whereas subcutaneous (abdominal) preadipocytes tend to exhibit a greater ability to undergo adipogenesis *in vitro* (Tchkonia et al., 2002); (Adams et al., 1997); (Hauner, Wabitsch, & Pfeiffer, 1988). Many studies have highlighted that murine preadipocytes share many of the depot-specific functional properties displayed by human cells (Macotela et al., 2012); (Lee, Wu and Fried 2013).

The cellular mechanisms invoked upon overfeeding reportedly vary in a depot-specific manner. In humans, visceral AT seems to preferentially expand via hypertrophy (in which pre-existing cells increase in size but not number), a process associated with deleterious cardiovascular and metabolic outcomes (Rydén, Andersson, Bergström and Arner 2014); (Drolet et al. 2008). On the contrary, subcutaneous AT expands via hyperplasia (i.e. increased adipocyte number via preadipocyte recruitment).

There is further regional variation, however, in the mechanism by which subcutaneous AT expands in humans; subcutaneous abdominal AT reportedly expands via hypertrophy whereas gluteo-femoral AT expands via hyperplasia (Tchoukalova, Votruba, et al., 2010). This is supported by data indicating that the formation rate of new preadipocytes and adipocytes is significantly higher in subcutaneous gluteal AT compared to subcutaneous abdominal AT (White, Fitch, Beyl, Hellerstein, & Ravussin, 2016). Taken together, it seems that in humans, subcutaneous gluteo-femoral AT expands via hyperplasia, subcutaneous abdominal AT expands through both hypertrophy and hyperplasia (the balance likely determined by various factors such as sex and genetic predisposition) while visceral AT expands via hypertrophy. Given these data, it should be noted that the mechanisms of AT expansion invoked in mice appear to be at odds with the human data.

The 'Adipochaser' mouse is a transgenic experimental system which enables the permanent labelling of adipocytes in an inducible manner. Using this powerful tool it has been found that upon high fat diet feeding, subcutaneous WAT in mice expands via hypertrophy whereas the epididymal (deemed analogous to human visceral AT) depot expands via hyperplasia (Wang, Tao, Gupta and Scherer 2013). This pattern is opposite to that observed in humans and so indicates that there is significant species-specific divergence in the regulation of AT cellularity. However, much remains to be learnt from this powerful, highly flexible model system that will undoubtedly yield novel insights into mammalian AT biology.

Returning to human data, it has also been determined that approximately 10% of adipocytes are renewed in subcutaneous abdominal AT every year and that this occurs at a fairly stable rate throughout adulthood in lean and obese individuals (Spalding et al., 2008); it is unclear whether adipocyte turnover in gluteo-femoral AT is affected by obesity. Indeed, extensive functional comparative studies of human subcutaneous

abdominal and gluteal preadipocytes are lacking, but it is known that the fatty acid composition of TAG generated via DNL in differentiated subcutaneous abdominal and gluteo-femoral preadipocytes exhibits the same depot-specific differences observed in whole AT (Pinnick et al., 2012). Overall, these data further support that AT exhibits distinct depot-specific biology and that the resident preadipocytes have an 'intrinsic memory' of their anatomical origin which likely determines their functional properties.

### 1.4.3 Depot-specific transcriptional and epigenetic profiles in subcutaneous WAT

Subcutaneous abdominal and gluteo-femoral AT exhibit depot-specific gene expression profiles (Karastergiou et al., 2013); (Rehrer et al., 2012); (Pinnick et al., 2014); (Passaro et al., 2017). A substantial proportion of the most differentially expressed genes are developmental regulators, including members of the *HOX* and *TBX* families of transcription factors; it follows that each anatomically distinct AT depot likely has unique developmental origins (section 1.2.1; (Hepler, Vishvanath and Gupta 2017)). Expression of specific differentially expressed genes, such as *HOXA5*, also correlates with measures of overall and regional adiposity (Gesta et al., 2006). Differential gene expression does not simply reflect anatomy but is also functional; knockdown of *TBX5* in subcutaneous abdominal preadipocytes (where it is exclusively expressed) completely abrogates adipogenesis (Pinnick et al., 2014).

Depot-specific epigenetic profiles have also been identified in subcutaneous abdominal and gluteo-femoral AT; DNA methylation profiles vary significantly between these AT depots (Gehrke et al., 2013). DNA methylation (section 1.2.4) has been implicated in the regulation of depot-specific gene expression. For example, *HOTAIR* is a long non-coding RNA exclusively expressed in gluteal AT; its promoter region is hyper-methylated and transcriptionally silent in abdominal preadipocytes (Pinnick et al., 2014). MicroRNA

(section 1.2.4) expression also varies significantly between subcutaneous abdominal and gluteo-femoral AT (Rantalainen et al., 2011). It follows that differentially expressed developmental regulators may modulate AT function and/or adipogenesis via epigenetic mechanisms. For instance, TBX5 has been shown to interact with TAZ, a protein that interacts with histone acetyltransferases to modify the transcription of TBX5-dependent genes (Murakami, Nakagawa, Olson, & Nakagawa, 2005).

### 1.4.4 Relationship of adipocyte size to metabolic health

Adipocyte size has been proposed to represent an important index of AT function and metabolic health (Laforest, Labrecque, Michaud, Cianflone, & Tchernof, 2015); regional variation in the mechanism by which AT expands may provide the crucial link between AT distribution and metabolic health (section 1.4.2; (Tchoukalova, Votruba, et al., 2010)). Indeed, adipocyte size fits neatly into the conceptual framework of 'adipose expandability' (Virtue & Vidal-Puig, 2010).

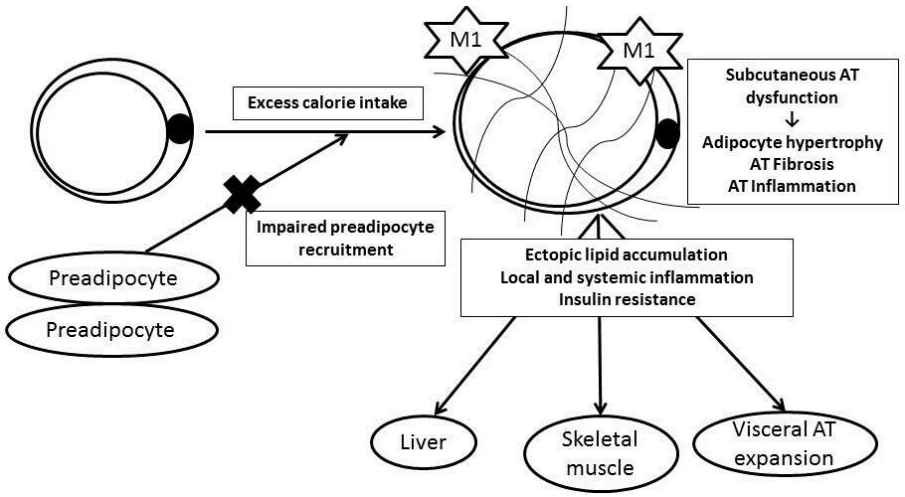



**Figure 1.4 Overview of WAT expandability.** Under extended periods of excess calorie intake, WAT expands through recruitment of preadipocytes. However, if this process is impaired or the preadipocyte pool gets exhausted, adipose tissue (AT) expands via adipocyte hypertrophy. This is associated with subcutaneous AT dysfunction and often results in fibrotic remodelling of AT ECM and inflammation (via M1 polarised macrophages). Continued positive energy balance results in ectopic lipid accumulation in visceral AT, skeletal muscle and liver and is associated with systemic and local inflammation as well as insulin resistance. The ability to expand WAT without deleterious metabolic consequences varies between individuals as outlined in the 'personal fat threshold' hypothesis (R. Taylor & Holman, 2015).

This AT expandability hypothesis states that a given individual has a finite capacity to store excess calories as TAG in AT with no adverse metabolic consequences; the capacity to expand is determined by the lipid buffering ability of subcutaneous AT. If this capacity is exceeded, a continued state of positive energy balance will result in ectopic lipid deposition, precipitating 'lipotoxicity' and metabolic dysfunction. An overview of this concept is provided in Figure 1.4.

As smaller adipocyte size is associated with increased insulin sensitivity and better lipid handling (Bjorntorp et al., 1972) it has been proposed that hyperplasia (i.e. increased cell number) underpins metabolically favourable AT expansion. AT with a reduced hyperplastic capacity will preferentially expand via adipocyte hypertrophy (i.e. increased cell size) and may have a smaller maximal carrying capacity. As adipocyte function and insulin sensitivity decrease with increasing adipocyte size (Laforest et al. 2015), hyperplastic expansion is the optimal method for the safe storage of excess calories as it preserves the functionality of the expanding AT depot through the formation of new, normal-sized cells, thereby protecting overall metabolic health. Conversely, adipocyte hypertrophy is associated with impaired adipocyte function, increased insulin resistance and various adverse cardiovascular/metabolic outcomes; adipocyte hypertrophy probably reflects AT filled beyond its carrying capacity and may indicate that ectopic lipid deposition which is negatively impacting overall metabolic health has occurred.

As the ability of AT to expand varies significantly between individuals (Cuthbertson et al., 2017), the concept of the 'personal fat threshold' has been developed (R. Taylor & Holman, 2015). Individuals with a reduced capacity for (subcutaneous) WAT expansion (i.e. low personal fat threshold, as in lipodystrophy; section 1.5.1) may be predisposed to developing metabolic disease, even at a normal weight, due to ectopic lipid deposition. Conversely, individuals whose subcutaneous AT has a greater capacity to expand (i.e.

high personal fat threshold) can accrue substantial amounts of AT without experiencing any metabolic disturbance (Cuthbertson et al., 2017).

Hypertrophy of subcutaneous (abdominal) adipocytes is a key phenotype displayed by normal weight individuals predisposed to developing metabolic disease (Henninger, Eliasson, Jenndahl, & Hammarstedt, 2014) or those who have type 2 diabetes (Acosta et al., 2016); this relationship is independent of overall adiposity (Weyer, Foley, Bogardus, Tataranni, & Pratley, 2000). Indeed, (subcutaneous abdominal) adipocyte size is inversely associated with systemic insulin sensitivity and circulating adiponectin levels (Hammarstedt, Graham, & Kahn, 2012). Subcutaneous abdominal adipocyte size also predicts the improvement in insulin sensitivity upon weight loss in overweight/obese individuals (Eriksson-Hogling et al., 2015). A similar positive association between adipocyte hypertrophy and worse metabolic outcomes has been detected in relation to visceral AT (Rydén et al. 2014); (Drolet et al. 2008).

As discussed in section 1.2.2, adipogenesis and angiogenesis are closely related processes in AT. Furthermore, the observation from human studies that lower body subcutaneous AT expands via hyperplasia whereas subcutaneous abdominal (and visceral) AT primarily expand via hypertrophy (section 1.4.2; (Laforest et al. 2015)) suggests that the extent to which these processes are coupled varies between different AT depots. It follows that the extent of coupling in each AT depot may determine its 'expandability' and thereby influence an individual's person fat threshold. Given this, it has been proposed that perturbed angiogenesis may be involved in the development of obesity and its associated metabolic sequelae, a notion that is supported by the observation that many mouse models of obesity exhibit inadequately perfused AT which also tends to be inflamed and hypoxic (Crewe, An, and Scherer 2017). AT blood flow is dysregulated in obese humans (Frayn and Karpe 2014) but there is no compelling evidence that human

(subcutaneous) AT is hypoxic, at least at the whole tissue level (Hodson 2014). Evidently the (depot-specific) relationship between angiogenesis, adipogenesis and hypoxia in the context of human AT function and metabolic health in normal weight and obese states remains to be clarified.

It has also been observed that subcutaneous abdominal adipocyte size is inversely related to the expression of lipogenic genes as well as insulin sensitivity (R. Roberts et al., 2009). As lipogenic gene expression is reportedly down-regulated in obese subcutaneous abdominal AT (Diraison, Dusserre, Vidal, Sothier, & Beylot, 2002); (Guiu-Jurado et al., 2015), down-regulated AT DNL may represent a link between adipocyte enlargement and metabolic dysfunction, potentially via perturbed lipokine synthesis (Yilmaz et al., 2016). Overall, the evidence strongly suggests that adipocyte size is an important index of AT function and metabolic health.

### 1.4.5 AT inflammation in obesity and metabolic disease

Increasing amounts of data suggest that obesity and insulin resistance/metabolic disease are associated with a state of chronic AT inflammation (Pirola & Ferraz, 2017). Whether inflammation of AT is a cause or consequence of metabolic dysfunction remains unclear (Bluher, 2016), especially considering that inflammation has been shown to be absolutely required for metabolically favourable AT remodelling and expansion (Wernstedt Asterholm et al., 2014) yet blockade of inflammatory signalling in humans can (partially) rescue insulin sensitivity (Esser, Paquot, & Scheen, 2015). AT inflammation is evidently a double-edged sword whose activity level and type must be tightly regulated for the maintenance of metabolic homeostasis.

While obesity/metabolic dysfunction is associated with upper body AT inflammation (Michaud, Drolet, Noel, Paris, & Tchernof, 2012), data pertaining to gluteo-femoral AT

are inconsistent. Depot-specific differences in the expression and release of pro-inflammatory cytokines between subcutaneous abdominal and gluteo-femoral AT have been reported (Evans et al., 2011); (Pinnick et al., 2014) whereas others have found no difference (Malisova et al., 2014); (You et al., 2014). Further studies are evidently required to dissect the interplay between depot-specific AT inflammation and metabolic health (Dam, Sikder, & Santosa, 2016).

### 1.4.6 The extracellular matrix in WAT

The extracellular matrix (ECM) within AT can mechanistically link adipose expandability to depot-specific AT biology, adipocyte size and metabolic health (section 1.4.4; (Carobbio, Pellegrinelli, & Vidal-Puig, 2017)). AT ECM remodelling co-ordinates cell morphology, number and function with nutritional cues (Alligier et al., 2012) and involves altering the expression and activity of structural components, degradative enzymes (Lin et al., 2016) and the immune system (Choe, Huh, Hwang, Kim, & Kim, 2016). AT ECM structure and composition are perturbed in obesity and metabolic disease (Henegar et al., 2008); AT from obese and/or insulin resistant individuals is fibrotic (i.e. exhibits excess deposition of ECM components like collagens) compared to AT from healthy, lean individuals (Spencer et al., 2011). As fibrotic AT is physically stiffer (Lackey et al., 2014), it has been proposed that it may have a reduced ability to expand, thereby predisposing individuals to ectopic lipid accumulation and metabolic dysfunction (section 1.4.4). Consistent with this, the subcutaneous (abdominal) AT of individuals genetically predisposed to developing metabolic disease was found to exhibit increased fibrosis and adipocyte hypertrophy compared to healthy, lean controls (Henninger et al., 2014).

The interplay between ECM structure and AT expandability is exemplified by the phenotype of collagen VI-knockout mice; collagen VI-deficient AT was able to undergo almost limitless expansion (via adipocyte hypertrophy) upon high fat diet feeding yet these animals were completely protected from the metabolic disturbances typically associated with such extreme adiposity (Khan et al., 2009). While this phenotype highlights how the ECM influences AT expandability and metabolic health it has not translated directly to the human condition (Pasarica et al., 2009); (McCulloch et al., 2015). A further consideration is that degradation products generated by ECM remodelling may act as signals themselves. For instance, endotrophin is a cleavage product of collagen VI α3 which promotes AT fibrosis and exacerbates the metabolic derangements of high fat diet feeding in mice (Sun et al., 2014). Endotrophin can also enter the circulation from AT and promote mammary tumour formation (J. Park & Scherer, 2012).

Overall, the ECM plays a crucial, yet underappreciated role in AT biology and is involved in the pathogenesis of metabolic disease (Divoux & Clement, 2011). The structure and composition of the ECM co-ordinates the cellular processes employed during expansion and likely determines the maximal expansion capacity of a given AT depot. Dissection of the depot-specific properties of AT ECM in the context of AT expansion and metabolic disease represents fertile ground for future study.

## 1.5 Determinants of regional AT distribution

### 1.5.1 Genetics of body fat distribution

Overall (Stunkard, Foch, & Hrubec, 1986) and regional adiposity (Malis et al., 2005); (Selby et al., 1989) are strongly heritable traits; heritability is a measure of the proportion of phenotypic variation that can be attributed to genetic variation i.e. single nucleotide polymorphisms. Indeed, studies of monozygotic twins have provided estimates that genetic inheritance may explain ~75% of the variance for BMI and waist circumference (Wardle et al. 2008). Studies of adopted children also support a strong genetic component of obesity, as highlighted by the finding that the BMI of adopted children correlates more to that of their biological rather than adopted parents (Stunkard, A. J., Sørensen, T.I., et al. 1986).

Genome-wide association studies (GWAS) have identified numerous genomic loci that are significantly associated with obesity (Locke et al., 2015) and body fat distribution independent of overall adiposity (Shungin et al., 2015), some of which exhibit sexual dimorphism (Sung et al., 2016). Despite the indication that obesity and regional adiposity are strongly heritable, the combined contribution of the currently identified genomic loci explain only 2.7% of the variance for BMI (Locke et al. 2015) and 1.4% of WHR (Shungin et al., 2015). This somewhat paradoxical situation has led to the proposal that the 'missing heritability' may be explained by other factors, such as genetic susceptibility and epigenetics (Pigeyre, Yazdi, Kaur, & Meyre, 2016). It has also prompted the analogy that "genetics loads the gun but the environment pulls the trigger" (Olden & White, 2005). It has been proposed that copy number variants (CNVs) contribute to the 'missing' heritability of obesity and/or regional adiposity but are not usually picked up in GWAS;

CNVs comprise chromosomal segments encompassing gene replications or deletions (Manolio et al. 2009).

The true power of genetics to influence regional adiposity is exemplified by the phenotype of congenital lipodystrophy patients. Congenital lipodystrophy disorders arise from mutations in genes involved in adipogenesis, adipocyte survival, lipid droplet formation and lipid synthesis (Vigouroux et al. 2011). If the loss of AT is total/generalised, severe metabolic complications such as insulin resistance, hypertriglyceridaemia and non-alcoholic fatty liver disease typically occur (Lightbourne and Brown 2017). Conversely, selective loss and compensatory expansion of specific AT depots precipitates metabolic complications consistent with the relationship between regional AT distribution and metabolic health (section 1.3.8). For instance, the familial partial lipodystrophy of the Dunnigan variety (FPLD) is defined by loss of AT in the lower body coupled with expansion of upper body AT and is associated with severe metabolic consequences (Garg 2004). When upper body AT is lost, however, as in the Barraquer-Simons syndrome, the accompanying metabolic sequelae are usually mild and can even be absent (Misra et al. 2004).

Documentation of the different types of congenital lipodystrophy (partial and generalised) and identification of the causative mutations have provided intriguing insights into AT biology and how certain aspects may vary in a depot-specific manner. For instance, congenital generalised lipodystrophy type 1 is associated with mutations in the 1-acylglycerol-3-phosphate O-acyltransferase 2 (*AGPAT2*) gene (Agarwal et al. 2002). AGPAT2 catalyses the formation of phosphatidic acid, an intracellular signalling molecule required for normal adipocyte function and TAG synthesis. More detailed study of these patients has highlighted that *AGPAT2* mutations primarily affect subcutaneous and visceral WAT involved in metabolic homeostasis but leaves mechanically important

WAT depots unaffected (Simha and Garg 2003). Such data suggest that even within a generalised lipodystrophy disorder there can be some depot-specific phenomena, an observation which further supports the regional heterogeneity of AT biology.

More convincing evidence which supports the notion that AT biology varies in a highly depot-specific manner comes from investigations into congenital partial lipodystrophy patients. Various mutations within the *PPARG2* gene have been associated with partial lipodystrophy disorders characterised by the selective loss of specific AT depots. For example, the F388L mutation disrupts the transactivation activity of PPARG2, thereby greatly impairing its ability to support *in vitro* adipogenesis in murine 3T3-L1 cells (Hegele et al. 2004). While the F388L mutation greatly impairs the functionality of the 'master regulator of adipogenesis' (section 1.2.3) and so might be assumed to affect adipogenesis in AT in general, it is fascinating to note this mutation only precipitates a partial lipodystrophy phenotype characterised by preferential loss of lower body subcutaneous AT (Hegele et al. 2004).

Taken together with the observations of congenital generalised lipodystrophy patients, it seems likely that there may be a number of core pathways crucially involved in AT development, survival and/or function but that these are modulated in a highly depot-specific manner. Such regional heterogeneity may derive from the properties of the resident preadipocytes and involve depot-specific variation in gene expression profiles, epigenetic profiles, and/or local hormone concentrations, for example.

### 1.5.2 Pharmacological determinants of body fat distribution

The environment harbours a diverse array of factors that can influence regional AT distribution (Pramyothin & Karastergiou, 2016). Amongst those factors, various pharmacological agents can affect AT distribution and therefore metabolic health in both

beneficial and deleterious ways. For example, PPARG agonists/TZDs such as pioglitazone are used as insulin-sensitising agents by promoting expansion of subcutaneous AT (Carey et al., 2002), sometimes at the expense of visceral AT (Y. Miyazaki et al., 2002). These agents can be used to successfully treat certain forms of lipodystrophy (sections 1.2.3 and 1.5.1) by promoting AT accumulation which can somewhat restore metabolic homeostasis (Iwanishi et al., 2009). On the other hand, highly active anti-retroviral therapy (HAART) used to treat human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) can precipitate HAART-associated lipodystrophy syndrome where gluteo-femoral AT wastes away while subcutaneous abdominal and visceral AT expand (Shlay, Sharma, Peng, Gibert, & Grunfeld, 2009).

### 1.5.3 Surgical manipulation of body fat distribution

Regional adiposity can be changed directly through surgical removal of AT; such interventions represent a unique opportunity to investigate the role of specific AT depots in humans. Liposuction is the most commonly performed AT removal procedure; this technique is predominantly undertaken by women and is typically used for the removal of subcutaneous abdominal AT (Pramyothin & Karastergiou, 2016). While substantial reductions in overall AT mass can be achieved with this procedure, this reportedly does not affect cardiovascular or metabolic disease risk (Klein et al., 2004); (Mohammed, Cohen, Reeds, Young, & Klein, 2008); (Martinez-Abundis, Molina-Villa, Gonzalez-Ortiz, Robles-Cervantes, & Saucedo-Ortiz, 2007).

The protective role of lower body AT is further highlighted by the report that femoral lipectomy is associated with impaired post-prandial lipid handling, specifically resulting in hypertriglyceridemia (Hernandez et al., 2015). On the other hand, the role of visceral

AT as a causative factor in the pathogenesis of metabolic disease has been questioned after data indicating individuals exhibited no improvement in insulin sensitivity after omental lipectomy (Fabbrini et al., 2010). Overall these data indicate that metabolic health cannot be improved by simply excising AT.

### 1.5.4 Sex steroid regulation of body fat distribution

The striking sexual dimorphism in regional AT distribution is strongly influenced by sex steroid action (White & Tchoukalova, 2014). Differences in body shape and composition emerge as body mass increases during puberty, with this increase driven mainly by lean mass in males while AT makes a larger contribution in females (Maynard et al., 2001). Sex steroids also maintain regional AT distribution patterns (Palmer & Clegg, 2015) as highlighted by the re-distribution of AT from a gynoid towards an android pattern, which is associated with cessation of oestrogen and progesterone production in menopause (Abdulnour et al., 2012). The power of sex steroids to modulate regional AT distribution is exemplified by their effect in transsexual individuals; testosterone administered to female-to-male transsexuals reduces subcutaneous AT mass and increases visceral adipose mass (Elbers, Asscheman, Seidell, & Gooren, 1999) whereas oestradiol treatment in male-to-female transsexuals causes expansion of subcutaneous gluteo-femoral AT (Elbers et al., 2003).

### 1.5.5 Glucocorticoid regulation of body fat distribution

Glucocorticoids are potent regulators of regional AT distribution. These hormones are principally catabolic and act to quickly mobilise energy stores during times of stress. However, chronic exposure to high levels of these hormones results in central obesity characterised by excessive expansion of visceral AT coupled with wasting of gluteo-femoral AT (Geer et al., 2010); (Rockall et al., 2003). This re-distribution of AT is

associated with an insulin-resistant phenotype associated with an increased risk of developing cardiovascular and metabolic disease (Ferrau & Korbonits, 2015). Glucocorticoid action in AT is complex owing to the interplay between several dynamic factors such as: circulating glucocorticoid levels; local pre-receptor hormone metabolism; glucocorticoid receptor (GR) expression and activity (influenced via post-translational modifications); and epigenetic regulatory mechanisms (influencing GR binding plus post-transcriptional regulation of GR-mediated gene expression). These factors can vary between depots and over time (Peckett, Wright, & Riddell, 2011).

The transcriptional responses to glucocorticoid treatment can vary in magnitude, sensitivity and/or direction between different AT depots (Pickering, Lee, Karastergiou, Gower, & Fried, 2016). Distinct gene networks within the same AT depot are often differentially regulated; glucocorticoid treatment can suppress the expression of inflammation- and apoptosis-related genes but simultaneously increase the expression of genes involved in adipogenesis and lipid/glucose metabolism (M. J. Lee, Gong, Burkey, & Fried, 2011). Glucocorticoids also modulate fatty acid handling in a depot-specific manner; they potentially promote central obesity by preferentially increasing the expression and activity of LPL (Fried, Russell, Grauso, & Brolin, 1993) and other TAG storage genes (M. J. Lee et al., 2011) in visceral compared to subcutaneous (abdominal) AT.

Pathologically enhanced local glucocorticoid action mediated via 11β-HSD type 1 in WAT represents a major phenomenon underpinning Cushing's syndrome (Morgan, Hassan-Smith, & Lavery, 2016) and central obesity (Bujalska, Kumar, & Stewart, 1997). Expression of 11β-HSD type 1 is increased in obese subcutaneous (abdominal) and visceral AT and correlates positively with adiposity, waist circumference and insulin resistance as well as adipocyte size (Engeli et al., 2004); (Kannisto et al., 2004); (Paulsen,

Pedersen, Fisker, & Richelsen, 2007). Based on these data, 11β-HSD type 1 inhibitors have been developed; while they promote weight loss, their small glucose-lowering effect has precluded further commercial development (Stomby, Andrew, Walker, & Olsson, 2014).

## 1.6 Approaches to understanding regional AT biology

As the world is facing a health crisis of an unprecedented scale driven principally by central obesity, the need to understand the determinants of regional AT distribution has never been greater. The management, treatment and prevention of metabolic and cardiovascular disease represent tasks of increasingly overwhelming proportions. Identifying what determines regional AT distribution and how this relates to metabolic health may provide some opportunity to contain or even reverse the current health crisis. Such information could facilitate optimising methods that can accurately predict an individual's risk of developing central obesity (and therefore cardiovascular and metabolic disease) as well as inform the development of novel ways to enhance or protect AT function and alleviate metabolic disease.

The scientific realm is going through a state of rapid evolution in which extraordinary amounts of data are being generated. This newfound wealth of data is a double-edged sword as it has become apparent that older methods of analysis are generally inadequate for new data and developing new ways of thinking has slowed progress. However, effective use of these new data may provide answers to fundamental questions and potentially shift the balance in favour of containing the global obesity crisis. It follows that the more effective and efficient approach to enhance our understanding will involve assimilating different forms of currently available data so the most attractive hypotheses and candidates can be identified to be followed up in the most appropriate manner; that is the approach underpinning this DPhil thesis.

The identification of the candidates chosen in this project involved integrating various types of data believed to be most relevant to the determination of regional AT distribution. It is well-established that subcutaneous abdominal and gluteo-femoral AT exhibit distinct

gene expression profiles and are functionally distinct (section 1.4). Given the under-appreciated importance of the extracellular matrix in AT function and metabolic health (section 1.4.6), cartilage oligomeric matrix protein (*COMP*) was identified as a candidate suitable for further study as microarray data indicated that it is an ECM component which is highly differentially expressed between subcutaneous abdominal and gluteo-femoral AT (Pinnick et al., 2014).

*BMP2* was identified as another suitable candidate to study in the context of depot-specific adipogenesis as a substantial body of compelling data implicated BMP2-Smad1/5/8 signalling as an important regulator of murine adipogenesis and a recent GWAS meta-analysis identified genomic loci encompassing components of this pathway as being significantly associated with regional AT distribution independent of overall adiposity (Shungin et al., 2015). In addition, an appreciation of the processes underpinning disease states defined by regional AT distribution phenotypes, such as Cushing's syndrome, helped inform experimental design and ensure that biologically meaningful questions were asked. It should be noted, however, that the currently available models of (human) adipogenesis are useful but also limited, as exemplified by their relatively poor lipogenic capacity and heterogeneous pattern of differentiation.

### 1.6.1 Aims

1) Investigate the potential role of COMP as a novel extracellular matrix protein expressed by AT in the context of regional AT distribution in humans.

2) Investigate the potential role of BMP2-SMAD1/5/8 signalling in the context of regional AT distribution in humans.

3) Develop and optimise new cellular models of (depot-specific) human adipogenesis.

# Chapter 2

# Methods

## 2.1 The Oxford Biobank

The Oxford Biobank (OBB; http://www.oxfordbiobank.org.uk/) is composed of 30 - 50 year old healthy Caucasian men and women living in the Oxfordshire area who have undergone detailed examination at a screening visit and given informed consent to be re-approached. Extensive phenotypic information is available on participants within the OBB. Numerous biochemical parameters are measured on the fasting blood sample provided by each individual (e.g. fasting glucose, TAG and cholesterol are measured using an iLAB clinical analyser whereas fasting insulin is measured via radioimmunoassay). Additional plasma samples are frozen and can be obtained for further analysis upon request.

The regional AT distribution of each participant is accurately determined via DEXA scan (section 1.3.3) in addition to basic anthropometric measurements used to calculate BMI and WHR. Each individual also provides a blood sample for genotyping; LGC Genomics extract DNA from the blood samples in preparation for high-throughput genotyping using the Illumina Human Core Exome and Affymetrix UK biobank chips. Custom genotyping using standard TaqMan chemistry can also be performed. This detailed genetic information is then incorporated into the OBB database to facilitate genotype-phenotype association analyses.

Individuals within the OBB give consent to be re-approached to participate in studies; the detailed information within the OBB allows individuals to be recruited on the basis of a specific genotype and/or phenotype of interest. Studies vary in scale and complexity but typically involve the collection of plasma samples and subcutaneous abdominal and/or gluteal AT biopsies under various conditions (e.g. in the fasted or fed state); the Oxfordshire Clinical Research Ethics Committee approved the taking of human plasma

and AT samples. Depot-specific AT biology can be studied *in vivo* as the Clinical Research Unit (CRU) has the expertise to collect arterio-venous plasma samples across subcutaneous abdominal and gluteal AT. AT biopsies can be processed for cell culture, histology, qPCR, Western blotting and/or biochemical analysis of lipid species.

The OBB has been used to generate various useful tools that facilitate the study of AT biology. For instance, a complementary (c) DNA panel for quantitative real-time PCR analysis was prepared from paired abdominal and gluteal AT samples taken from 200 males and females (median age 45 years; range 33 - 53 years) within the OBB across a range of BMI (median 25.6 kg/m$^2$; range 18.8 - 46.2 kg/m$^2$). AT samples were taken by needle biopsy under local anaesthetic (1% lignocaine) from the periumbilical (subcutaneous abdominal AT) and upper buttock (subcutaneous gluteal AT) areas. RNA was extracted from AT biopsies as outlined in section 2.3.1; cDNA synthesis was performed as described in section 2.3.2; qPCR analysis was performed as per section 2.3.3. This AT panel was used as part of the investigations into the role of COMP (sections 3.3.1 and 3.3.2) and BMP2 (section 4.3.6) in human AT.

## 2.2 Cell culture of human preadipocyte cell lines

### 2.2.1 Immortalised human subcutaneous abdominal and gluteal preadipocytes

The majority of the cell work presented in this thesis was performed using immortalised preadipocyte cell lines derived from the stromovascular fraction (SVF) of paired subcutaneous abdominal and gluteal AT biopsies taken from a healthy 50 year old male (BMI 24.4 kg/m$^2$). These cell lines represent a useful tool to study the mechanisms involved in determining regional AT distribution in humans as they are derived from human tissue (unlike murine 3T3-L1 cells) and retain the depot-specific properties of their anatomical origin in a manner similar to primary SVF cells (Todorčević et al., 2017). These cells have been used to successfully characterise various depot-specific adipogenic processes (Pinnick et al., 2014); (Loh et al., 2015).

### 2.2.2 Isolation and culture of human primary white preadipocytes

Preadipocytes were isolated from subcutaneous AT using a previously described protocol (Hauner, Skurk, & Wabitsch, 2001); (Collins, Neville, Hoppa, & Frayn, 2010). AT was broken up using scissors and washed twice with Hanks' buffered salt solution (HBSS) before enzymatic digestion in 1mg/ml collagenase (Roche) in HBSS in a shaking water bath (90 rpm) at 37°C for 45 min. Digested AT was centrifuged at 1000 rpm for 5 min at 4°C before the supernatant (containing buoyant mature adipocytes) was removed.

The pellet containing the SVF cells was re-suspended in HBSS before being filtered twice through nylon mesh (SEFAR), first through 250μm mesh and then 100μm mesh to remove undigested tissue debris. The filtrate was spun (1000 rpm for 5 min at 4°C) and the supernatant was removed. The remaining pellet was then re-suspended in growth medium (Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham's (v/v, 1:1;

Sigma)) supplemented with 10% foetal bovine serum (Labtech); 0.25ng/ml fibroblast growth factor (Sigma); 2mM L-glutamine (Invitrogen); and 100 units/ml penicillin and 100µg/ml streptomycin (Invitrogen). Cells were then seeded in tissue culture flasks (Sarstedt) and maintained in a humidified atmosphere at 37°C in a 5% $CO_2$ incubator. After 1 day, residual contaminating red blood cells were washed off with phosphate-buffered saline (PBS), and growth medium was replaced.

### 2.2.3 Generation of human preadipocyte cell lines

The isolated SVF of a paired ASAT and GSAT biopsy taken from a healthy 50 year old male (BMI 24.4 $kg/m^2$) was immortalised according to a previously described protocol (Darimont & Mace, 2003); (Todorčević et al., 2017). Immortalisation was achieved by co-expression of the type 16 human papilloma virus E7 oncoprotein (HPV16-E7) and human telomerase reverse transcriptase (hTERT) which were sub-cloned into the pLenti6.3/V5-DEST lentiviral expression vector (Invitrogen) from the pBABE-neo-h-hTERT and pGEX2T E7 plasmids (Addgene) respectively. Transgene expression is controlled by the human cytomegalovirus (CMV) immediate-early promoter within the pLenti6.3/V5-DEST vector. Lentiviral particles were generated from HEK 293T cells using the ViraPower HiPerform Lentiviral expression System (Invitrogen).

Primary abdominal and gluteal cells were pre-treated with 8µg/ml hexadimethrine bromide (Sigma) before transduction with hTERT lentiviral particles. Cells were cultured in the presence of 2µg/ml blasticidin to select preadipocytes constitutively expressing hTERT. Blasticidin-resistant cells were then transduced with HPV16-E7 lentiviral particles. Immortalised preadipocytes were maintained under the same conditions as primary cells (section 2.2.1).

### 2.2.4 Adipogenic differentiation of primary and immortalised human preadipocytes

Preadipocytes were seeded onto 6 well plates (2 x $10^5$ cells/well) and grown to confluence for 48 hr before stimulation for 14 days with adipogenic medium comprising Dulbecco's modified Eagle's medium/Nutrient F-12 Ham (v/v, 1:1; Sigma) supplemented with 2mM L-glutamine (Invitrogen); 100 units/ml penicillin and 100µg/ml streptomycin (Invitrogen); 17µM pantothenate (Sigma); 100nM human insulin (Invitrogen); 1nM tri-iodo-L-thyronine (Sigma); 33µM biotin (Sigma); 10µg/ml transferrin (Sigma); and 1µM dexamethasone (Sigma). The adipogenic medium was supplemented with 2µM troglitazone (Sigma) and 250µM 3-isobutyl-1-methylxanthine (Sigma) for the first 4 days and was changed on days 2, 4, 7, 10 and 12.

### 2.2.5 Proliferation analysis using CyQuant assay

Preadipocyte proliferation was measured using the CyQuant direct cell proliferation assay (Invitrogen). Cells were seeded on 96 well plates (8 x $10^3$ cells/well) and cultured overnight. Growth medium was then changed and the cells were incubated with a biological molecule of interest (e.g. BMP2) for 48 hr. After this time had elapsed, growth medium was changed and cells were incubated with the assay's nucleic acid stain and background suppressor for 1 hr at 37˚C. The fluorescence of each well was then measured on a fluorimeter (excitation at 485nm; emission at 530nm). The fold change in live cell number relative to control cells within the same depot was then determined.

## 2.3 Gene expression analysis

### 2.3.1 RNA extraction

RNA was isolated from cells according to a previously described protocol (Collins et al., 2010). In brief, cells were homogenised in Tri-reagent (Thermo Scientific) and transferred to a 2ml PhaseLock gel tube (Eppendorf) before 200µl chloroform were added. The samples were mixed and spun (12000 × *g* for 20 min at 4°C). The RNA-containing aqueous supernatant was transferred to a clean tube and an equal volume of isopropanol plus 2µl glycoblue (Invitrogen) were added to each sample. Samples were mixed thoroughly and incubated at -30°C overnight to precipitate.

To collect the RNA, the samples were spun (12000 × g for 20 min at 4°C) and the isopropanol was aspirated. The RNA pellets were washed twice in 300µl 80% ethanol and collected by centrifugation (12000 × *g* for 3 min at 4°C). The ethanol was then aspirated and the pellets were air-dried at room temperature before each RNA pellet was re-suspended in nuclease-free $H_2O$. The concentration of RNA was determined using a NanoDrop spectrophotometer (Thermo Scientific). The OBB AT cDNA panel (section 2.1) was generated from total RNA which had previously been extracted from subcutaneous abdominal and gluteal AT biopsies using the same extraction method detailed above (Neville, Collins, Gloyn, McCarthy, & Karpe, 2011).

### 2.3.2 Complementary DNA synthesis

Double-stranded complementary (c) DNA was synthesised from total RNA (500ng from biopsies; 1000ng from cells) using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). The composition of the reverse transcription reaction mix is outlined in table 2.1.

**Table 2.1 Reverse-transcription reaction volumes for cDNA synthesis**

| Reagent | Volume per reaction (µl) |
|---|---|
| 10x RT Buffer | 2.0 |
| 25x dNTP mix (100mM) | 0.8 |
| 10x RT Random primers | 2.0 |
| Multiscribe™ Reverse transcriptase (50U/µl) | 1.0 |
| Nuclease-free $dH_2O$ | 4.2 |
| Total volume | 10.0 |

A no template control (NTC) was also included in which RNA was substituted for nuclease-free $H_2O$ for quality control. The cycling parameters for cDNA synthesis were: 25°C for 10 min; 37°C for 120 min; 85°C for 5 min; and 4°C for 10 min.

### 2.3.3 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed on cDNA diluted in 10mM Tris-HCl (pH 8.0) using Kapa Probe Fast Mastermix (Kapa Biosystems) and TaqMan Assays-on-Demand (Applied Biosystems). A cDNA pool made from experimental samples was serially diluted to generate a standard curve (1/10; 1/20; 1/40; 1/80; 1/160; and 1/320). Experimental samples and standards plus an NTC and Tris-HCl blank (pH 8.0) were analysed in triplicate (3.6µl/well) in a final 8µl/well reaction on an ABI Prism 7900HT; data were collected on SDS 2.3 software (Applied Biosystems). The cycle parameters were: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Relative transcript expression was calculated using the ΔΔCt relative quantification method (Pfaffl, 2001). The reaction efficiency for each qPCR assay was determined by the slope of the standard curve (i.e. serially diluted cDNA); this value was used to calculate the ΔCt for each sample according to the following formula:

$$\Delta Ct = \text{reaction efficiency}^{(\text{min Ct} - \text{Sample Ct})}$$

The ΔCt values of target genes were normalised to the corresponding ΔCt of the reference transcript *PPIA* in a given sample. *PPIA* expression has been shown to be relatively stable in AT and preadipocytes (Neville et al., 2011). As *PPIA* exhibited uniformity and stability across all experimental conditions used in this thesis, it represented an appropriate internal reference gene that could be used to identify pipetting errors and/or experimental variation during the calculation of ΔΔCt.

### 2.4 Measurement of intracellular TAG content

On adipogenic differentiation day 14, whole cell lysates were harvested in lysis buffer (1% IGEPAL CA-630; 150mM NaCl; 50mM Tris-HCl, pH 8.0). Cell lysates were sonicated before the concentration of protein was determined using the DC protein assay kit (Bio-Rad). The remaining lysate was heated at 95˚C for 30 min before being mixed and spun (14000 x *g* for 10 min at 4˚C) to clear cell debris. The concentration of TAG in the clear supernatant of the experimental samples (plus quality controls and a lysis buffer-only blank) was determined using the enzymatic TAG assay kit (Instrumentation Laboratories) on an ILAB 650 Clinical Chemistry System (Instrumentation Laboratories). Normalised TAG content was calculated by dividing TAG by the corresponding cellular protein concentration and reported as nanomoles TAG/milligram protein.

## 2.5 Western blotting

### 2.5.1 Cellular protein extraction

Whole cell lysates were harvested in lysis buffer (1% IGEPAL CA-630; 150mM NaCl; 50mM Tris-HCl, pH 8.0) supplemented with phosphatase inhibitors (10mM sodium fluoride and 1mM sodium orthovanadate) and an EDTA-free protease inhibitor cocktail (Roche). Samples were homogenised via sonication and the concentration of protein was determined using the DC protein assay kit against BSA standards (Bio-Rad).

### 2.5.2 Protein extraction from AT biopsies

Whole AT biopsies (taken via needle aspiration) were homogenised using an IKA homogeniser in ice-cold tissue lysis buffer (8M Urea; 1% SDS; 5% glycerol; 10mM Tris-HCl; pH 6.8) supplemented with EDTA-free protease inhibitor cocktail (Roche). After homogenisation, the processed biopsy was spun (9000 rpm for 5 min at 4°C). The protein-containing aqueous phase was collected using a 22-gauge needle and transferred to a clean 1.5ml tube. The concentration of protein for each sample was determined using the DC protein assay kit (Bio-Rad).

### 2.5.3 Gel electrophoresis

To prepare samples for gel electrophoresis, equal amounts of protein (cell or tissue lysate) were mixed with 4x Laemmli sample buffer (Bio-Rad) containing 10% β-mercaptoethanol as a reducing agent. Samples were heated (85°C for 3 min) and then loaded onto a 4 - 20% Criterion TGX gel submerged in running buffer (25mM Tris; 190mM glycine; 0.1% SDS; pH 8.3); a protein standard (All Blue protein ladder; Bio-Rad) was also loaded. Proteins were resolved by passing electrical current through the gel (150V for 1 hr) and then transferred onto 0.2µm PVDF membranes (Bio-Rad) via semi-

dry transfer on a TransBlot turbo (Bio-Rad). After transfer, the blot was incubated on a shaker (90 rpm) in blocking buffer comprising 5% skimmed milk powder or BSA dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hr at room temperature. After blocking, the blot was incubated overnight at 4˚C with a primary antibody diluted in blocking buffer directed against the target protein of interest (see chapters 3 and 4 for details).

### 2.5.4 Enhanced chemiluminescence

The blot was recovered the following day and washed with TBST (five times for 5 min each) before incubation with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody dissolved in the blocking buffer for 1 hr at room temperature. The blot was then washed with TBST (five times for 5 min each). To visualise the protein of interest, the blot was incubated in a 1:1 mixture of Clarity Western enhanced chemiluminescence (ECL) substrate mixture (Bio-Rad) for 5 min at room temperature. Excess ECL reagent was then drained off and the blot was placed on the stage of the Chemi-Doc XRS+ (Bio-Rad). The chemiluminescent signal was captured and analysed using Image Lab software (Bio-Rad). After a satisfactory image had been obtained, the protein standards were visualised using the 'stain-free' image option.

After imaging the target protein, blots were washed in TBST (five times for 5 min each). To visualise an appropriate reference protein (e.g. β-actin), blots were blocked and re-probed if the bands were of sufficiently different size that there would be no overlap. If two proteins of interest were of a similar molecular weight, previously-bound antibodies were removed by incubation for 80 min with stripping buffer (0.1M glycine; 20mM magnesium acetate; 50mM KCl; pH 2.2). After blocking the stripped blot and checking

for the absence of any previous ECL signal, these blots were ready to be re-blocked and re-probed (as described in sections 2.5.3 and 2.5.4).

### 2.5.5 Densitometric analysis of Western blot images

Images of the ECL signal were exported from Image Lab software (Bio-Rad) as .tiff files and opened in ImageJ (National Institute of Health, USA). The contrast was adjusted accordingly to make the signal clearer. The average density of each band (including a background 'blank') was determined using the 'Measure' function with the rectangular selection tool. After importing the densitometry data into Microsoft Excel, each experimental data point was 'blanked' by subtracting the corresponding background measurement and then normalised by division with that of the corresponding reference protein.

## 2.6 Enzyme-linked immunosorbency assay

The solid-phase, quantitative sandwich enzyme-linked immunosorbency assay (ELISA) technique was used to determine the concentration of COMP (R&D Systems) or BMP2 (R&D Systems) protein present in cell culture medium and/or human plasma; both kits involve the same workflow. All samples and standards were analysed in duplicate.

The immobilised primary antibody was first reconstituted by adding 100µl/well assay diluent to the microplate. Standards and samples (50µl/well) diluted in calibrator diluent (where appropriate) were then added to the plate. The microplate was then covered and incubated for 2 hr on a horizontal orbital shaker set to 500 rpm at room temperature. The microplate's contents were then aspirated and discarded before each well was rinsed with wash buffer; this process was repeated 3 more times for a total of 4 washes. After washing, the microplate was inverted and dried thoroughly using paper towels.

For the second stage, 200µl of an enzyme-linked antibody were added to each well. The microplate was then covered and incubated at room temperature for another 2 hr on a horizontal orbital shaker set to 500 rpm. The microplate was then washed 4 more times as described in the previous paragraph. A substrate mixture was then added to each well before the microplate was incubated at room temperature for 30 min covered in tin-foil (to protect it from light). After this time had elapsed, an acid stop solution (50µl/well) was used to terminate the colour development reaction.

The absorbance for each well was then measured on a spectrophotometric VersaMax tuneable plate reader (Molecular Devices) at 450nm and 540nm. Absorbance at 540nm was subtracted from the absorbance at 450nm (to correct for optical imperfections in the microplate) for each well before the average absorbance for each standard or sample analysed in duplicate was calculated. A standard curve generated using a 4-parameter logistic (4-PL) curve-fit in the Softmax Pro program (Molecular Devices) was then used to determine the concentration of target protein in each sample.

### 2.7 Statistical analysis

Data are presented as mean $\pm$ standard error of mean (SEM). Statistical analyses were performed in SPSS Statistics version 22 (IBM). Differences were considered statistically significant at $P<0.05$.

Paired T-tests were used to compare a continuous variable (e.g. mRNA or protein levels) between groups with paired samples. Paired samples comprised abdominal and gluteal AT/cells derived from the same individual (Figure 3.1A) or plasma samples obtained from individuals matched for sex, age, BMI and total fat mass %. A T-test was used as it was assumed that the samples were obtained from a normally distributed population of

cells or people. This statistical test has been used to analyse gene expression in paired AT samples (Todorčević et al., 2017).

The Mann-Whitney U-test was used to compare a continuous variable (i.e. plasma COMP protein concentration) in independent samples that were taken from a non-normally distributed population. This non-parametric statistical test was used to analyse plasma COMP data (Figures 3.5C, 3.5D and 3.5E) as re-sorting the data according to Android/Gynoid fat mass ratio rendered the population non-normally distributed (according to the Shapiro-Wilk test). This test has been used previously in the analysis of human AT blood flow (Karpe et al., 2002).

Analysis of variance (ANOVA) was used to test whether there was a significant difference between the means of three or more independent groups, each of which were assumed to be normally distributed. This test was primarily used to determine whether a continuous variable (e.g. mRNA or protein levels) varied significantly under different experimental conditions (e.g. different doses of exogenous BMP2 as in Figure 4.3D). If a significant difference was detected, post-hoc unpaired T-tests were used to determine which specific groups were significantly different from each other. This approach has been used previously to test whether COMP co-administration enhances BMP2-induced Smad signalling (Kazunari Ishida et al., 2013).

Repeated-measures ANOVA was used to determine whether a continuous variable (e.g. mRNA levels) that was measured multiple times in the same sample varied over time and between groups (i.e. different AT depots), and to assess whether there were any time x depot interactions. This test was principally used to analyse mRNA levels measured in abdominal and gluteal preadipocytes measured at multiple time-points during a differentiation time-course (see Figure 4.1D). It was assumed that the samples derive

from cell populations which are normally distributed. This test was used as an alternative to a multivariate general linear regression model and has been used to test similar outcomes (Todorčević et al., 2017).

Analysis of covariance (ANCOVA) was used to test whether there was a significant difference between the means of three or more independent groups whilst statistically removing the effect of one or more continuous variables (covariates) that might be related to the variable of interest but are not of primary interest (e.g. age or sex). This test was used when all variables were assumed to be normally distributed. This test was primarily used to investigate whether rs979012 or rs1440372 were expression quantitative trait loci for *BMP2* or *SMAD6* mRNA levels in abdominal and gluteal AT, respectively, whilst removing the possible effects of age, sex and/or BMI. This test was used as an alternative to the multivariate general linear regression models which have been used to perform similar analyses to examine whether various genotypes for specific *BMPR1A* SNPs were associated with altered levels of *BMPR1A* mRNA expression (Böttcher et al., 2009).

To test the strength of a linear association between two continuous, normally-distributed variables, the Pearson's correlation analysis was used; the Spearman's rank correlation analysis was used where data were non-normally distributed. The Pearson's test was primarily used to investigate whether different adipogenic endpoints were correlated (e.g. cellular protein and TAG accumulation as in Figure 5.6C). This test has also been used to determine whether AT *TSP-1* mRNA levels are correlated to obesity and insulin sensitivity (Varma et al., 2008).

The Pearson's partial correlation test was used to measure the strength of a linear association between two independent, normally distributed variables whilst removing the effect of one or more other variables that may be related to either variable of interest. This

analysis was mainly used to test the strength of association between *COMP* mRNA levels in abdominal/gluteal AT and waist/hip circumference whilst controlling for the effect of overall obesity as in Figure 3.3; note that *COMP* mRNA level was log-transformed to approximate normality before correlation analysis was performed. This type of analysis has been used previously to analyse AT *BMP2* expression in relation to various anthropometric and metabolic parameters (Guiu-Jurado et al., 2016).

### 2.8 Data contribution

This thesis draws upon data generated from a variety of different resources and people within the OXLIP laboratory so credit must be given to all contributors. An overview of contributions is provided in Table 2.2. Contributions are also outlined in each figure legend where appropriate. All other experiments were performed by myself.

**Table 2.2 Data contribution**

| Figure | Data | Contributor |
|---|---|---|
| 3.1 | AT biopsies and sections | CRU, OBB and Dr Anne Clark |
| 3.3 | AT cDNA panel and phenotype data | OBB and Dr Matt Neville |
| 3.5 | Fasting plasma samples and phenotype data | OBB |
| 4.1 | Primary preadipocyte cDNA for qPCR | CRU and Dr Marijana Todorčević |
| 4.2 | Growth medium Western blotting data | Dr Nellie Loh |
| 4.3 | Cell culture and Western blotting data | Mohamed Eghleilib |
| 4.5 | Preadipocyte qPCR data | Sama Al-Sharifi |
| 4.6 | rs2145270 (proxy for rs979012) genotyping and *BMP2* qPCR analysis | Mohamed Eghlielib |
| 4.7 | AT histological analysis | Dr Katherine Pinnick and Dr Marijana Todorčević |

# Results

# Chapter 3

# Cartilage oligomeric matrix protein is a differentially expressed regulator of human adipogenesis

## 3.1 Introduction

The three-dimensional arrangement of the extracellular matrix (ECM) is co-ordinated by matricellular proteins which physically interact with matrix components, such as collagens and the basement membrane, but do not directly contribute to the ECM's structural integrity (Murphy-Ullrich & Sage, 2014). Matricellular proteins are modular, secreted molecules implicated in numerous processes ranging from cell proliferation, migration and differentiation to angiogenesis and wound healing (Bornstein & Sage, 2002).

The thrombospondins (TSPs) are a prominent matricellular family comprising five calcium-binding matricellular proteins that have been found to regulate AT biology in humans and mice. TSP-1 has been identified as an adipokine implicated in the development of obesity and metabolic disease in a mouse model (Kong et al., 2014). In humans, *TSP-1* mRNA levels are higher in visceral compared to subcutaneous abdominal AT (Ramis et al., 2002); mRNA levels in the latter have been reported to positively correlate with BMI but negatively correlate with insulin sensitivity (Varma et al., 2008). Further, the concentration of circulating TSP-1 protein was found to correlate positively with hypertension, hyperglycaemia and central obesity in a Japanese cohort (Matsuo et al., 2015).

Three independent mouse studies have highlighted that genetic deficiency of *TSP-1* largely protects against the deleterious metabolic consequences of diet-induced obesity. Upon high-fat diet feeding, *TSP-1* knock-out mice retained insulin sensitivity (Y. Li, Tong, Rumala, Clemons, & Wang, 2011), exhibited markedly attenuated AT inflammation (Kong et al., 2013) and were protected from skeletal muscle fibrosis (Inoue et al., 2013). In addition to TSP-1, it has been reported that TSP-2 may exert anti-

adipogenic effects in mice (Shitaye, Terkhorn, Combs, & Hankenson, 2010) although this finding has not been replicated (Van Hul, Frederix, & Lijnen, 2012).

Building on the data implicating TSPs in AT biology, *TSP-5* (or cartilage oligomeric matrix protein; *COMP*) was found to exhibit a striking depot-specific expression pattern between subcutaneous abdominal and gluteal AT ((Pinnick et al., 2014), supplementary data). Given that most differentially-expressed genes between WAT depots tend to be development-related transcriptional regulators such as *HOX* genes (Karastergiou et al., 2013); (Gesta et al., 2007), regional variation in the expression of ECM components may be important in the context of AT biology and certainly warrants further study.

*COMP* has primarily been studied in the context of bone biology as genetic mutations cause severe skeletal malformations, namely pseudoachondroplasia (PSACH; (Posey, Alcorn, & Hecht, 2014)). COMP protein co-ordinates collagen fibrillogenesis (Halasz, Kassner, Morgelin, & Heinegard, 2007) but PSACH-causing mutations disrupt this process (Hansen et al., 2011) typically by causing intracellular retention of mutant COMP protein which results in chondrocyte death (Hecht et al., 2004). Serum COMP protein levels have also been investigated as a potential biomarker of osteoarthritis progression (Hoch, Mattacola, Medina McKeon, Howard, & Lattermann, 2011).

It is important to note that bone, cartilage and WAT share a common mesodermal lineage when considering the growing body of literature highlighting the relationship between body fat distribution, metabolic health and osteoarthritis. Central obesity not only increases the risk of developing metabolic and cardiovascular disease but also predicts poorer outcomes in osteoarthritis (Duclos, 2016). Indeed, there is a growing appreciation of the extensive cross-talk between bone and AT via circulating factors in both health and disease (Thijssen, van Caam, & van der Kraan, 2014).

### 3.1.1 Aims

- Validate prior data indicating that *COMP* exhibited a depot-specific expression pattern by measuring *COMP* mRNA and protein levels in subcutaneous abdominal and gluteal AT biopsies and preadipocytes via qPCR and Western blotting respectively.
- Investigate whether *COMP* mRNA levels in AT (measured via qPCR) are related to adiposity and/or regional AT distribution according to detailed phenotype data from the OBB.
- Identify and characterise biologically relevant regulators of *COMP* expression (measured via qPCR and Western blotting) using immortalised human subcutaneous abdominal and gluteal preadipocytes as a model cellular system.
- Investigate the potential (depot-specific) role of *COMP* in the regulation of adipogenesis by culturing immortalised subcutaneous abdominal and gluteal preadipocytes in differentiation medium supplemented with exogenous COMP protein and measuring the effect on TAG accumulation and adipogenic gene expression.

## 3.2 Methods

### 3.2.1 Adipose tissue sample collection and analysis

As described in section 2.1, a previously generated panel of AT cDNA was used for gene expression analysis (section 3.2.4). Previously collected AT biopsies were also used for Western blotting (section 3.2.6).

### 3.2.2 Immunohistochemistry

AT biopsies were collected via gun biopsy under local anaesthetic (1% lignocaine) to obtain intact samples that were suitable for immunohistochemistry. AT biopsies were fixed in 10% formaldehyde, embedded in paraffin wax and cut into 5µm sections. For staining, tissue sections were dewaxed in xylene and rehydrated in absolute and then 70% ethanol. Antigen retrieval was performed on rehydrated sections (heated in 1mM sodium citrate) followed by blockade of endogenous peroxidase activity (incubated in 0.3% $H_2O_2$ and methanol for 30 min). Sections were incubated in 1.5% glycine for 15 min in the dark to reduce auto-fluorescence, washed in TBS and then incubated for 20 min in 1/20 swine serum (Dako). Sections were incubated with goat anti-COMP (1/100; AF3134; R&D Systems) and rabbit anti-collagen 1 (1/300; ab34710; Abcam) primary antibodies diluted in 0.2M Tris buffer overnight at 4˚C.

Sections were retrieved the following day, washed in TBS and incubated with the first secondary antibody (donkey anti-goat HRP; 1/50; sc2020; Santa Cruz Biotechnology) to label COMP staining. Sections were washed and subjected to tyramide amplification (incubation in the dark with 0.3% $H_2O_2$ and 5% tyramide 488 for 30 min) to enhance the COMP signal. Sections were washed in TBS in the dark and then incubated in 1/20 goat serum (Dako) in the dark; this was to reduce cross-reactivity between secondary

antibodies. The sections were then incubated with the second secondary antibody (goat anti-rabbit 568; 1/250; A11036; Life Technologies) for 30 min in the dark to co-label collagen 1 staining. Sections were washed in TBS and stained with DAPI-containing mounting solution before coverslips were mounted. Sections were visualised using a Radiance 2100 laser scanning system confocal microscope (Bio-Rad) and images were captured using Laser sharp software (Bio-Rad).

### 3.2.3 Preadipocyte culture and adipogenic differentiation

Immortalised preadipocytes were cultured and differentiated as outlined in section 2.2.4. Where indicated, adipogenic medium was supplemented with recombinant COMP protein (ACRO Biosystems) reconstituted in 10mM Tris-buffered saline containing 0.1% BSA and 2mM $CaCl_2$ (H. Chen, Deere, Hecht, & Lawler, 2000), recombinant human BMP2 (Sigma) reconstituted in sterile PBS containing 0.1% BSA and/or recombinant human TGF-β1 (Life Technologies) reconstituted in sterile 4mM HCl solution containing 0.1% BSA. All experiments were performed using standard CellBIND tissue culture plates (Corning) except where collagen 1-coated tissue culture plates (Corning) were used.

For drug treatment experiments, cells were plated ($1.5 \times 10^5$ cells/well) and cultured overnight. Growth medium was then changed and cells were incubated for 48 hr in growth medium containing human insulin (Invitrogen), cortisol (Sigma), dexamethasone (Sigma) and/or mifepristone (Sigma) before cells and/or conditioned medium were harvested.

### 3.2.4 Gene expression analysis

Extraction of total RNA from AT biopsies and preadipocytes was performed as described in section 2.3.1; cDNA synthesis and qPCR were performed as outlined in sections 2.3.2 and 2.3.3 respectively. The following TaqMan Assays-on-Demand were used: *COMP* (Hs00164359_m1); *PPARG2* (Hs01115510_m1); and *PPIA* (Hs99999904_m1).

### 3.2.5 Measurement of intracellular TAG content

Determination of cellular TAG content was performed as outlined in section 2.4.

### 3.2.6 Western Blotting

Western blotting was performed as outlined in section 2.5. Equal amounts of protein were loaded (50µg/biopsy; 100µg/cell lysate) and resolved by SDS-PAGE, transferred onto PVDF membranes (Bio-Rad) via semi-dry transfer using a Transblot Turbo (Bio-Rad) and immunoblotted with the following antibodies: COMP (0.1µg/ml; AF3134; R&D Systems); phospho-ERK1/2 (0.5µg/ml; MAB1825; R&D Systems); β-actin (1:2000; sc1616; Santa Cruz); and α-tubulin (1:2000; ab15246; Abcam) followed by an HRP-conjugated secondary antibody; goat anti-rabbit IgG (1:5000; 31460; ThermoFisher Scientific).

### 3.2.7 COMP ELISA

The concentration of COMP in preadipocyte-conditioned media and plasma was determined using ELISA (R&D Systems) as outlined in section 2.6. Plasma samples were taken from age-, sex-, BMI- and total fat mass %-matched subjects (80 males; 72 females) recruited from the OBB. Anthropometric characteristics for the study cohorts are presented in Table 3.1. Depot-specific release of COMP protein was measured in arterio-venous plasma samples (taken from 9 healthy, lean females in the fasted state collected

during previous studies) taken across subcutaneous abdominal and femoral AT using a previously described method; a superficial epigastric vein draining subcutaneous abdominal AT (K. N. Frayn, Coppack, Humphreys, & Whyte, 1989), a branch of the great saphenous vein draining femoral AT (McQuaid, Manolopoulos, et al., 2010), and an arterialized dorsal hand vein (with the hand kept in a warming box at 60°C) were cannulated. Cannulae were kept patent with 0.9% saline. Plasma creatinine concentrations were used to indicate contamination by muscle venous blood (McQuaid, Manolopoulos, et al., 2010).

### 3.2.8 Data analysis and statistics

Data are presented as means $\pm$ SEM. Statistical analyses were performed in SPSS Statistics version 22 (IBM). Statistical tests used are as described in section 2.7. Differences were considered statistically significant where $P<0.05$.

## 3.3 Results

### 3.3.1 *COMP* is highly differentially expressed between abdominal and gluteal AT

Previous microarray data indicated that *COMP* mRNA levels were significantly higher in gluteal subcutaneous AT compared to abdominal AT ((Pinnick et al., 2014); supplementary data). Gene expression analysis of unselected paired subcutaneous abdominal and gluteal AT biopsies (n=187) across a range of BMIs (20.7 - 45.5 kg/m$^2$) validated the microarray data and showed that *COMP* mRNA levels were about 3-fold higher in gluteal AT ($p=1.7x10^{-31}$; paired T-test).

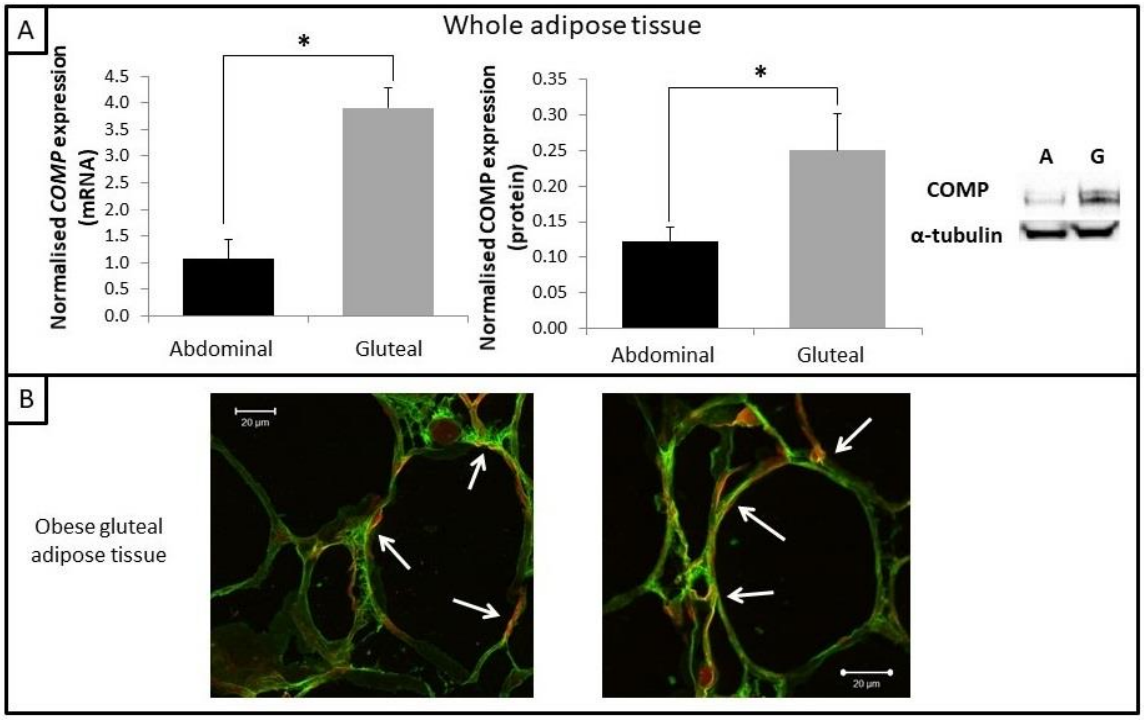



**Figure 3.1 *COMP* is differentially expressed between abdominal and gluteal AT.** A - Left-hand graph depicts *COMP* mRNA levels (normalised to *PPIA*) in abdominal and gluteal AT biopsies (corrected for age, sex and BMI) taken across a range of BMIs (20.7 - 45.5 kg/m$^2$; n=187). Right-hand graph outlines COMP protein levels (normalised to α-tubulin) measured by Western blot in paired AT biopsies (n=6 healthy females); image of a representative blot. Data presented as mean ± SEM; data analysed via paired T-test; *P<0.05. B - Representative immunohistochemistry image from staining (obese gluteal) AT for COMP (green) and collagen 1 (red); white arrows highlight co-localisation (yellow). AT biopsies and sections courtesy of the OBB. Immunohistochemistry data courtesy of Dr Anne Clark.

Consistent with the qPCR data, Western blot analysis of paired AT biopsies indicated COMP protein levels exhibited the same depot-specific expression pattern (Figure 3.1A). Immunohistochemical analysis of (obese gluteal) AT suggested that COMP protein resides in a perivascular location within the extracellular space and may co-localise with collagen 1, an abundant ECM protein (Figure 3.1B).

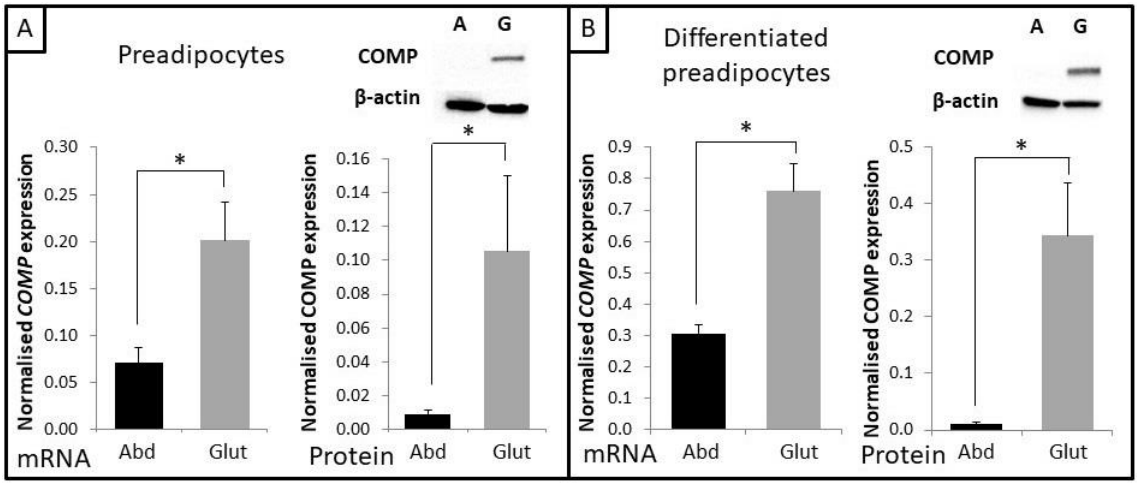



**Figure 3.2 *COMP* is differentially expressed between abdominal and gluteal cells.** A - Left-hand graph shows *COMP* mRNA levels (normalised to *PPIA*) in immortalised abdominal and gluteal preadipocytes (n=6); right-hand graph outlines COMP protein levels (normalised to β-actin) in the same cells measured by Western blot (n=3). B - Left-hand graph presents *COMP* mRNA levels (normalised to *PPIA*) in immortalised abdominal and gluteal preadipocytes after 14 day treatment with adipogenic medium (n=3) whereas the right-hand graph outlines COMP protein levels in the same cells measured by Western blot (n=3). Data presented as mean ± SEM; data analysed via paired T-test; *P<0.05.

Analysis of *COMP* expression in (immortalised) preadipocytes showed that the depot-specific expression pattern was retained *ex vivo* at the level of transcription and translation. Using a paired T-test it was found that *COMP* mRNA and protein levels were significantly higher in proliferating gluteal preadipocytes compared to abdominal cells (Figure 3.2A); this depot-specific expression pattern was recapitulated in differentiated preadipocytes (Figure 3.2B). Collectively these data confirm that *COMP* is highly differentially expressed between subcutaneous abdominal and gluteal AT and that this expression pattern is retained *ex vivo.*

### 3.3.2 *COMP* expression in AT is enhanced in obesity

To investigate whether *COMP* expression in AT is correlated with adiposity and/or regional AT distribution, anthropometric phenotype data on individuals within the AT expression panel data were examined.

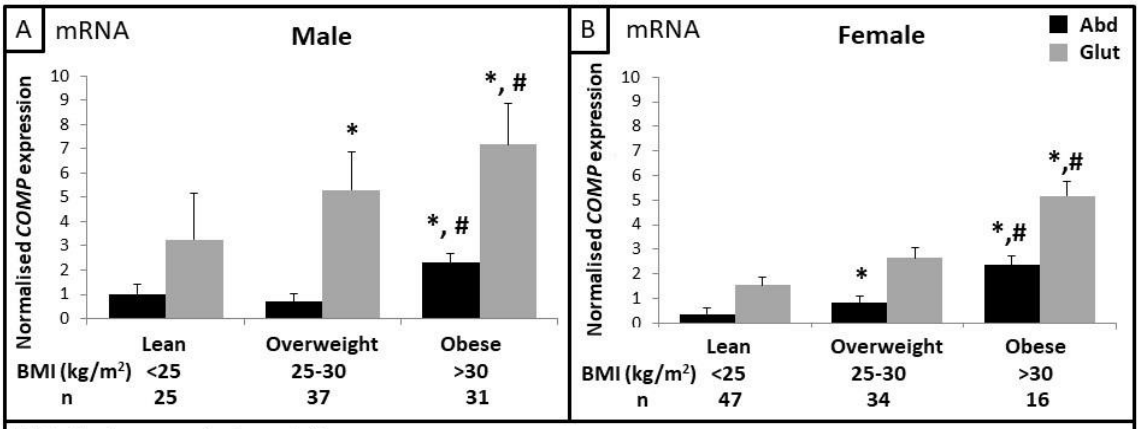



**Table 1 – Age-corrected correlations**

| | | BMI | | Waist circumference | | Hip circumference | | Fasting Insulin | |
|---|---|---|---|---|---|---|---|---|---|
| | | Pearson | p | Pearson | p | Pearson | p | Pearson | p |
| Male | Log Abd *COMP* | 0.400 | $7.0x10^{-5}$* | 0.350 | 0.001* | 0.338 | 0.001* | 0.484 | $2.0x10^{-6}$* |
| | Log Glut *COMP* | 0.329 | 0.001* | 0.291 | 0.006* | 0.294 | 0.005* | 0.194 | 0.07 |
| Female | Log Abd *COMP* | 0.529 | $3.0x10^{-8}$* | 0.463 | $2.0x10^{-6}$* | 0.370 | $2.1x10^{-4}$* | 0.398 | $6.0x10^{-5}$* |
| | Log Glut *COMP* | 0.424 | $1.6x10^{-5}$* | 0.390 | $8.6x10^{-5}$* | 0.361 | 0.003* | 0.358 | $4.0x10^{-4}$* |

**Table 2 – Age and BMI-corrected correlations** **Note – BMI correlations were age- and insulin-corrected**

| | | Waist circumference | | Hip circumference | | Fasting Insulin | | BMI | |
|---|---|---|---|---|---|---|---|---|---|
| | | Pearson | p | Pearson | p | Pearson | p | Pearson | p |
| Male | Log Abd *COMP* | -0.068 | 0.53 | -0.045 | 0.67 | 0.320 | 0.002* | 0.247 | 0.02* |
| | Log Glut *COMP* | -0.052 | 0.63 | -0.008 | 0.94 | 0.002 | 0.99 | 0.299 | 0.005* |
| Female | Log Abd *COMP* | -0.022 | 0.84 | -0.214 | 0.037* | 0.117 | 0.26 | 0.445 | $6.0x10^{-6}$* |
| | Log Glut *COMP* | 0.029 | 0.78 | -0.017 | 0.87 | 0.146 | 0.16 | 0.310 | 0.02* |

**Figure 3.3 *COMP* expression in AT is enhanced in obesity.** A - *COMP* mRNA levels (normalised to PPIA) in abdominal (Abd) and gluteal (Glut) AT in males stratified by BMI (n=93). B - *COMP* mRNA levels in abdominal and gluteal AT in females stratified by BMI (n=97). Data presented as age-adjusted mean ± SEM; data analysed via ANOVA; *P<0.05 compared to lean expression in same depot; #P<0.05 compared to overweight expression in same depot. Table 1 - Partial (Pearson's) correlation analysis (corrected for age) for AT *COMP* mRNA levels with anthropometric and biochemical parameters. Table 2 - Partial (Pearson's) correlation analysis (corrected for age and BMI) for AT *COMP* mRNA levels with anthropometric and biochemical parameters. Right-hand column is correlation analysis between *COMP* mRNA level and BMI (corrected for age and fasting insulin); *P<0.05. AT cDNA courtesy of the OBB.

Using the conventional BMI ranges for Western Europeans, subcutaneous abdominal and gluteal AT *COMP* mRNA levels were found to be significantly higher in obese (BMI >30 kg/m$^2$) individuals compared to their lean (BMI <25 kg/m$^2$) counterparts upon analysis via ANOVA; this expression pattern was observed in males (Figure 3.3A) and females (Figure 3.3B). Furthermore, the ratio of abdominal/gluteal AT *COMP* expression was not significantly different between BMI groups indicating that its depot-specific expression pattern was maintained between the different groups (data not shown).

More detailed analyses using the partial Pearson's correlation test indicated that subcutaneous abdominal and gluteal AT *COMP* mRNA levels were significantly positively correlated with BMI in both men and women (Figure 3.3, Table 1) and that this correlation was not affected by correcting for age, a parameter which showed only a nominally significant association with *COMP* mRNA levels in both depots (data not shown). In addition to the strong positive association between abdominal and gluteal AT *COMP* mRNA levels with BMI, similar positive correlations were detected for waist and hip circumference as well as fasting insulin levels in men and women (Figure 3.3, Table 1). However, these relationships were principally driven by BMI as most of them became non-significant after including a correction for BMI (except hip circumference in women and fasting insulin in men which remained nominally significant; Figure 3.3, Table 2).

As BMI and insulin exhibited a strong positive correlation ($r^2 = 0.537$, $p = 1.2x10^{-15}$), further analysis was performed to investigate whether insulin mediated the correlation between BMI and *COMP* mRNA levels. As the positive correlation between subcutaneous abdominal and gluteal AT *COMP* mRNA levels and BMI remained significant after correcting for fasting insulin (and age), this suggested that insulin probably does not mediate the correlation between *COMP* mRNA levels and BMI. This is consistent with the observation that 48 hr insulin treatment (100nM) did not increase

*COMP* mRNA levels in abdominal or gluteal preadipocytes (data not shown). Overall, subcutaneous abdominal and gluteal AT *COMP* mRNA expression was found to be strongly positively correlated with BMI in both men and women, suggesting it may reflect general adiposity/obesity.

### 3.3.3 Glucocorticoids regulate *COMP* expression in preadipocytes

Glucocorticoids are potent regulators of regional AT distribution classically linked to the development of central obesity (M. J. Lee, Pramyothin, Karastergiou, & Fried, 2014); section 1.5.6). As *COMP* expression exhibits such a striking depot-specific pattern and local glucocorticoid action has been reported to vary between different AT depots (M. J. Lee et al., 2008), a series of experiments was performed to investigate whether these hormones contribute to the depot-specific regulation of *COMP* expression.

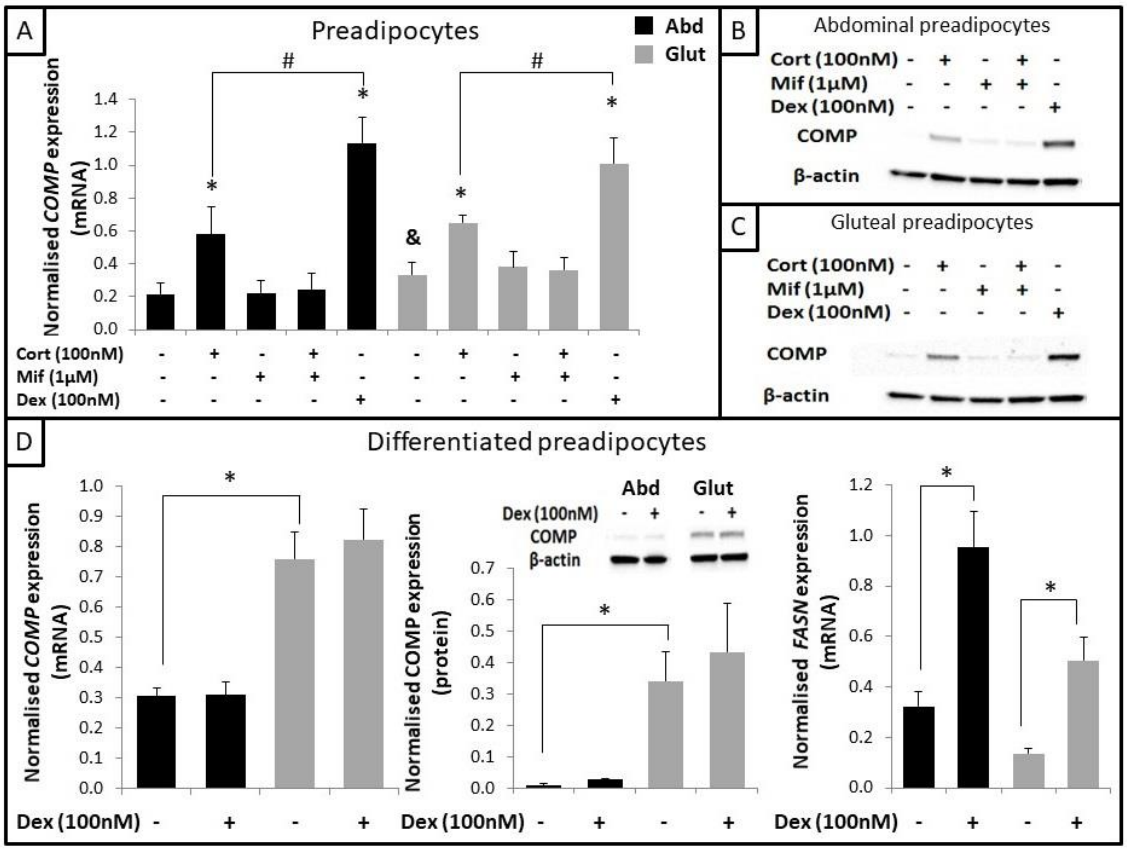



**Figure 3.4 Glucocorticoids regulate *COMP* expression in preadipocytes**. A - *COMP* mRNA levels (normalised to *PPIA*) in abdominal (Abd) and gluteal (Glut) preadipocytes (n=6) treated with cortisol (Cort), mifepristone (Mif) and/or dexamethasone (Dex); *P<0.05 compared to untreated in same depot; &P<0.05 compared to untreated in other depot. B - Representative image from Western blot analysis of COMP protein (normalised to β-actin) in abdominal preadipocytes (n=3) and C - gluteal preadipocytes (n=3). D - Left-hand graph outlines *COMP* mRNA levels (normalised to *PPIA*) in differentiated preadipocytes treated with dexamethasone (n=3). Middle section shows a representative image from Western blot analysis of COMP protein levels in dexamethasone-treated differentiated preadipocytes (n=3). Right-hand graph depicts fatty acid synthase (*FASN*) mRNA levels (normalised to *PPIA*) in dexamethasone-treated differentiated preadipocytes (n=3). All data analysed via ANOVA; *P<0.05.

The levels of *COMP* mRNA and protein levels in proliferating immortalised preadipocytes were robustly increased by cortisol (but more potently by the synthetic glucocorticoid dexamethasone) in both abdominal and gluteal preadipocytes (Figures 3.4A, 3.4B and 3.4C). This effect was mediated via the glucocorticoid receptor (GR) as it was blocked by co-administration of mifepristone, a GR antagonist. Previous data indicate that GR is expressed in abdominal and gluteal AT at approximately similar levels

((Pinnick et al., 2014); supplementary data). It should be noted that the depot-specific pattern of *COMP* expression was lost in glucocorticoid-treated preadipocytes.

In contrast to proliferating cells, glucocorticoid treatment did not modulate *COMP* mRNA or protein expression in differentiated preadipocytes (Figure 3.4D). However, dexamethasone up-regulated fatty acid synthase expression in differentiated preadipocytes (Gathercole et al., 2011) thereby indicating it was bioactive. Together these data highlight that *COMP* expression (mRNA and protein) in subcutaneous abdominal and gluteal preadipocytes - but not differentiated cells - is regulated by glucocorticoids, a class of hormone known to exert strong effects on regional AT distribution and adiposity. Furthermore, *COMP* transcription and translation appear to be tightly linked.

### 3.3.4 *COMP* may represent a novel adipokine

Other members of the TSP family, particularly TSP-1, have been proposed to represent a novel class of adipokine (Varma et al., 2008). To investigate whether *COMP* fulfils the necessary criteria to be labelled an adipokine, conditioned medium taken from preadipocytes treated with dexamethasone for 48 hr was analysed via ELISA. Although undetectable in conditioned medium taken from untreated preadipocytes, COMP protein was readily detectable in medium obtained from glucocorticoid-treated cells (Figure 3.5A); consistent with previous data, the level of COMP protein in the conditioned medium obtained from glucocorticoid-treated cells did not vary by depot. The detection of COMP protein in conditioned medium therefore raised the possibility that adipose-derived COMP protein might enter the circulation and act as an adipokine.

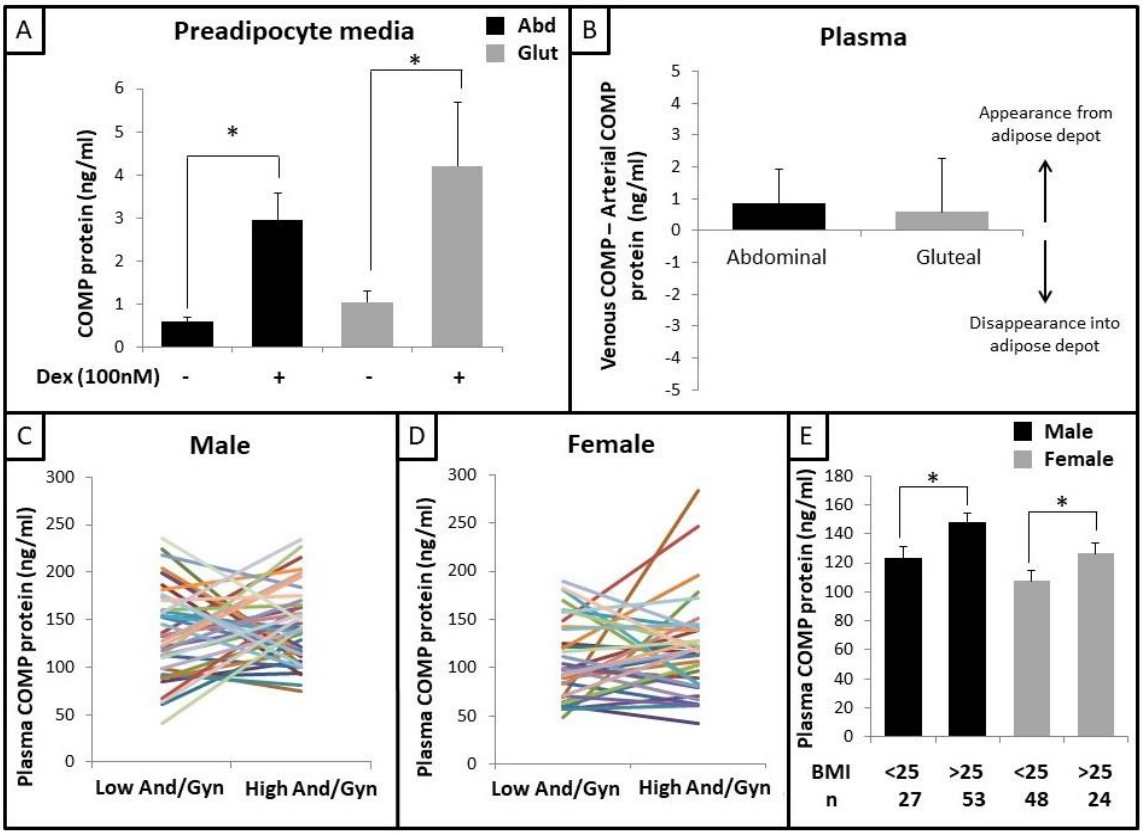



**Figure 3.5 COMP may represent a novel adipokine.** A - Concentration of COMP protein in conditioned medium obtained from dexamethasone (Dex)-treated abdominal (Abd) and gluteal (Glut) preadipocytes measured via ELISA (n=5). Data analysed via ANOVA. B - ELISA used to measure concentration of COMP protein in arterio-venous plasma samples taken across abdominal and gluteal AT from 9 healthy, lean females in the fasted state. Data presented as mean ± SEM; data analysed via paired T-test. C - ELISA used to measure plasma COMP in males (n=40) matched to an individual with the same total AT mass % but the opposite body fat distribution phenotype according to android/gynoid AT mass ratio (And/Gyn). Each line connects the raw plasma COMP concentration for each matched pair. D - Same analysis as for D in females (n=36). E - Analysis of plasma COMP protein concentration measured via ELISA in individuals stratified by BMI. Data presented as mean ± SEM; data in panels C, D and E analysed via Mann-Whitney U-test; *P<0.05. Plasma samples courtesy of the OBB.

As *COMP* is differentially expressed between subcutaneous abdominal and gluteal AT plus its transcription and translation appear to be tightly linked, plasma samples taken across abdominal and gluteal subcutaneous AT (from 9 healthy, lean females in the fasted state) were analysed via ELISA to test whether COMP is released by AT in a depot-specific manner. Consistent with previous data, COMP protein was readily detectable in plasma (Verma & Dalal, 2013). However, no arterio-venous difference was observed

across either abdominal or gluteal subcutaneous AT (Figure 3.5B) and there was no significant difference between depots (paired T-test). Together these data indicate there was no detectable release or sequestration of COMP protein across either AT depot in this experimental set-up.

As the turnover of COMP protein in AT may be too slow for the rapid sampling involved in arterio-venous difference studies to permit detection (as for adiponectin; (Karpe & Pinnick, 2015)), it was proposed that the concentration of systemic circulating COMP protein may better represent an individual's COMP status over a longer term (although the source of COMP protein may not be determined). Therefore, to investigate whether the concentration of circulating COMP protein was associated with a regional or overall adiposity phenotype, COMP protein levels were measured in plasma samples from pairs of individuals matched for total fat mass percentage but exhibiting extreme opposite upper/lower body AT distribution phenotypes across a range of BMIs.

The selection process involved sorting the OBB database according to android/gynoid fat mass ratio with individuals selected from the first tertile (i.e. centrally-distributed adiposity) matched to a corresponding individual from the third tertile (i.e. peripherally-distributed adiposity) with the same total fat mass %. The anthropometric characteristics of the study participants are detailed in Table 3.1.

**Table 3.1 Anthropometric characteristics of plasma COMP study participants**

| | Males (n=80) | | | Females (n=72) | | |
|---|---|---|---|---|---|---|
| **Parameter** | **Low And/Gyn** | **High And/Gyn** | **P** | **Low And/Gyn** | **High And/Gyn** | **P** |
| n | 40 | 40 | | 36 | 36 | |
| Age (years) | 42.3 (0.9) | 43.3 (0.7) | 0.59 | 40.2 (0.8) | 41.1 (0.9) | 0.43 |
| BMI ($kg/m^2$) | 26.4 (0.5) | 26.4 (0.5) | 0.98 | 24.2 (0.6) | 24.2 (0.6) | 0.98 |
| Total fat (%) | 26.8 (0.6) | 26.9 (0.6) | 0.30 | 32.4 (1.0) | 32.5 (1.0) | 0.14 |
| Waist (cm) | 91.3 (1.2) | 94.4 (1.3) | 0.002* | 74.7 (1.1) | 82.6 (1.6) | $1.0x10^{-6}$* |
| Hip (cm) | 103.5 (0.9) | 100.3 (1.0) | 0.0009* | 99.1 (1.3) | 96.6 (1.1) | 0.01* |
| Waist-to-hip ratio | 0.88 (0.01) | 0.94 (0.01) | $7.7x10^{-9}$* | 0.75 (0.01) | 0.85 (0.01) | $8.1x10^{-8}$* |
| Android fat (g) | 1713.4 (100.3) | 2455.8 (125.5) | | 977.0 (89.3) | 1711.0 (127.4) | |
| Gynoid fat (g) | 3837.6 (127.0) | 2832.3 (87.1) | | 4297.4 (191.2) | 3405.1 (142.4) | |
| Android fat/Gynoid fat | 0.44 (0.1) | 0.85 (0.1) | | 0.21 (0.1) | 0.48 (0.1) | |

The data were separated by sex for further analyses as plasma COMP protein levels were significantly higher in men than women (Mann-Whitney U-test, P=0.0002). Pairwise analysis of the 40 male (Figure 3.5C) and 36 female (Figure 3.5D) pairs highlighted that there was no significant difference in the concentration of circulating COMP protein between individuals with extreme opposite regional AT distribution phenotypes. While plasma COMP protein concentration did not vary with respect to body fat distribution, non-parametric analysis (Mann-Whitney U-test) indicated that the concentration of circulating COMP protein was significantly higher in overweight/obese (BMI >25 kg/m$^2$) males (P=0.007) and females (P=0.019) compared to their lean (BMI <25 kg/m$^2$) counterparts (Figure 3.5E).

Additionally, plasma COMP was positively correlated with total fat mass % in males (Spearman's rho=0.352, P=0.001) and females (Spearman's rho=0.315, P=0.007). Collectively these data provide further support for the idea that COMP protein is a general obesity/adiposity factor whose circulating levels reflect overall adiposity. However, it was not possible to confirm whether COMP protein represents a *bona fide* adipokine.

### 3.3.5 COMP is a positive adipogenic regulator

Having demonstrated that *COMP* is expressed in a depot-specific fashion in subcutaneous AT as well as proliferating and differentiated preadipocytes, and that its expression positively correlates with adiposity, the focus shifted to investigate whether it regulated adipogenesis. An initial study involved measuring *COMP* mRNA levels during a standard 14-day adipogenic time-course in immortalised abdominal and gluteal preadipocytes (Figure 3.6A). *COMP* mRNA levels changed dynamically throughout the time-course, specifically declining between days 2 - 10 before returning to their respective depot-

specific baselines by day 14. Despite *COMP* mRNA levels fluctuating over the duration, the depot-specific expression pattern was always maintained.

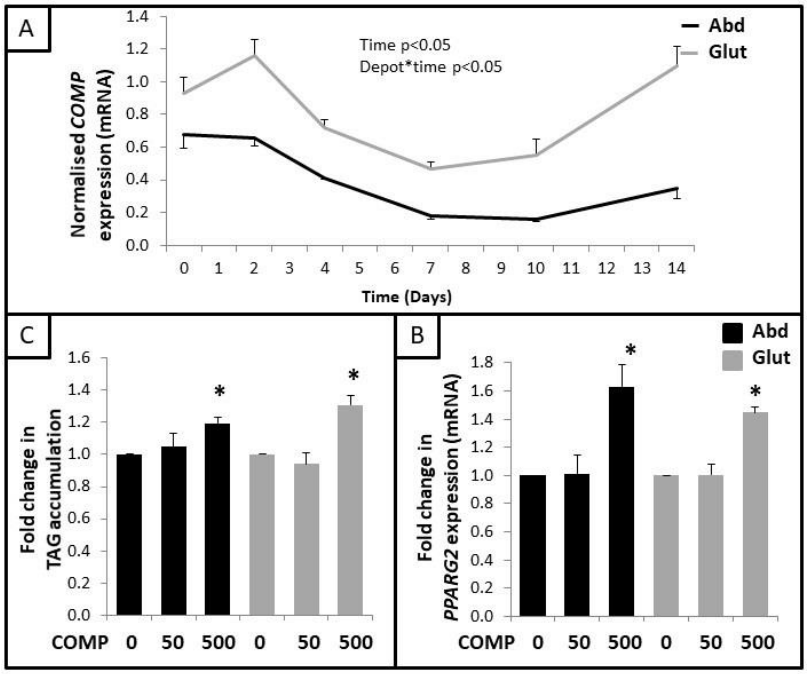



**Figure 3.6 COMP is a positive adipogenic regulator.** A - The expression profile of *COMP* mRNA levels (normalised to *PPIA*) in (immortalised) abdominal (Abd) and gluteal (Glut) preadipocytes (n=6) during a 14 day adipogenic time-course. Data analysed using repeated measures ANOVA. B - Triacylglycerol (TAG) accumulation in preadipocytes differentiated for 14 days in adipogenic medium supplemented with increasing amounts of exogenous COMP protein (n=3). C - *PPARG2* mRNA levels in differentiated preadipocytes cultured for 14 days in adipogenic medium supplemented with increasing amounts of exogenous COMP protein (n=3). Data presented as mean ± SEM; data analysed via ANOVA; *P<0.05 compared to untreated cells from the same depot.

To further explore the potential role of *COMP* in adipogenesis, abdominal and gluteal preadipocytes were differentiated in adipogenic medium supplemented with exogenous COMP protein for the 14 day duration. While no effect was observed with the 50ng/ml treatment, 500ng/ml COMP protein robustly increased day 14 *PPARG2* expression 1.6 fold (Figure 3.6B) and TAG accumulation 1.3 fold (Figure 3.6C). This positive effect on adipogenesis is consistent with the positive correlation between BMI/adiposity and AT *COMP* mRNA levels. Building on this, a series of experiments was performed that aimed to identify mechanism of action of COMP protein.

### 3.3.6 COMP may interact with ECM components to modulate adipogenesis

As a matricellular protein, COMP has been reported to interact with many different components of the ECM (Acharya et al., 2014), some of which have been implicated in the regulation of adipogenesis. It was therefore postulated that COMP may bind to various ECM components and modify their bioactivity to affect preadipocyte differentiation.

Collagen 1 is commonly used as a substratum in tissue culture; it is expressed by human preadipocytes (Romanov, Darevskaya, Merzlikina, & Buravkova, 2005). Collagen 1 reportedly enhances adipogenesis in murine 3T3-L1 cells (Gusinjac, Gagnon, & Sorisky, 2011) although its effect on human cells is unclear (O'Connor, Song, Rosenzweig, & Jansen, 2003). Immunohistochemistry data presented in Figure 3.1B showed that collagen 1 is expressed in human AT and co-localises with COMP. To investigate whether collagen 1 mediates COMP action, preadipocytes were seeded on standard or collagen 1-coated tissue culture plates before differentiation for 14 days with adipogenic medium supplemented with 500ng/ml COMP or vehicle. Collagen 1-coated plates did not affect TAG accumulation or *PPARG2* mRNA levels in abdominal or gluteal cells and this was not modulated by the administration of exogenous COMP protein (data not shown).

BMP2 has been shown to physically interact with COMP (K. Ishida et al., 2013), with such binding enhancing BMP2's bioactivity as highlighted by increased BMP2-induced Smad signalling and greater osteogenesis in murine systems *in vitro* and *in vivo* (P. Guo et al., 2014); (K. Ishida et al., 2013); (Refaat et al., 2016). BMP2 is pro-adipogenic in 3T3-L1 cells (Sottile & Seuwen, 2000) but its effect on human adipogenesis remains unclear. To investigate whether *COMP* regulated adipogenesis by modifying BMP2

bioactivity, preadipocytes were differentiated in adipogenic medium supplemented with 50ng/ml BMP2 and/or 500ng/ml COMP protein.

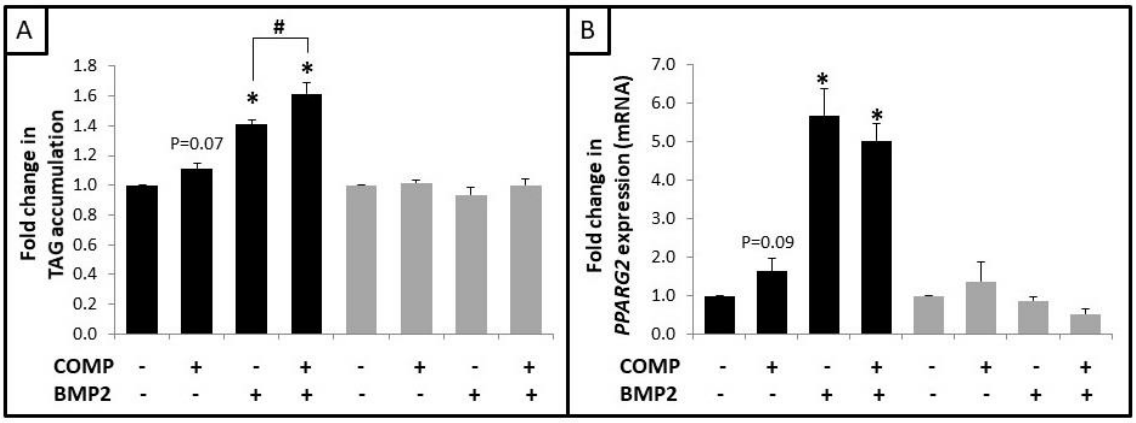



**Figure 3.7 BMP2 is a depot-specific regulator of adipogenesis.** A - Triacylglycerol (TAG) accumulation in (immortalised) abdominal (Abd) and gluteal (Glut) preadipocytes differentiated for 14 days in adipogenic medium supplemented with exogenous COMP (500ng/ml) and/or BMP2 (50ng/ml) protein (n=3). C - *PPARG2* mRNA levels (normalised to *PPIA*) in immortalised preadipocytes differentiated for 14 days in adipogenic medium supplemented with exogenous COMP (500ng/ml) and/or BMP2 (50ng/ml) protein (n=3). Data presented as mean ± SEM; data analysed via ANOVA; *P<0.05 compared to untreated cells from the same depot.

Consistent with previous data, exogenous COMP protein exerted a modest positive effect on TAG accumulation and *PPARG2* mRNA levels. BMP2 exerted profound depot-specific effects on adipogenesis in which it significantly increased TAG accumulation (Figure 3.7A) and *PPARG2* expression (Figure 3.7B) in abdominal preadipocytes but had no effect in gluteal cells. COMP and BMP2 co-administration had an additive, pro-adipogenic effect on abdominal TAG accumulation but this was not observed for *PPARG2* mRNA levels. These data therefore suggest that COMP and BMP2 acted independently in this experimental system to modulate adipogenesis. These data also identify BMP2 as a novel, depot-specific regulator of human adipogenesis. A more detailed characterisation of BMP2's depot-specific, pro-adipogenic effects is provided in chapter 4.

COMP protein has also been reported to bind and enhance the bioactivity of TGF-β1 according to a transcriptional reporter assay (Haudenschild et al., 2011). While classically associated with fibrosis and wound healing (Verrecchia & Mauviel, 2002), TGF-β1 expression (Alessi et al., 2000); (Porreca et al., 2002); (Spencer et al., 2010) and secretion (Fain, Tichansky, & Madan, 2005) by subcutaneous abdominal AT is increased in obesity. Similar to BMP2, the effect of TGF-β1 on (depot-specific) human adipogenesis remains unclear. To investigate whether COMP modified TGF-β1 action, preadipocytes were differentiated in adipogenic media supplemented with 1 - 5ng/ml TGF-β1 and/or 500ng/ml COMP protein.

TGF-β1 was potently anti-adipogenic in both abdominal and gluteal cells as 5ng/ml inhibited TAG accumulation by 60% and *PPARG2* expression by 70% (data not shown). Similar to BMP2, co-administration of COMP protein did not modulate the effect of TGF-β1 suggesting there was no interaction between these molecules in this experimental system. Collectively these data identify *COMP* as a novel positive regulator of human adipogenesis localised within the ECM whose mechanism of action currently remains elusive.

## 3.4 Discussion

COMP is a matricellular protein that plays a key role in bone and cartilage biology (Posey et al., 2014) as well as processes such as inflammation and angiogenesis (Acharya et al., 2014). The data presented in this study highlight that: *COMP* expression (mRNA and protein) are detectable in human AT and vary in a depot-specific manner; *COMP* mRNA levels in AT are positively correlated with BMI/adiposity; and exogenous COMP protein enhanced adipogenesis. These results identify COMP as a novel regulator of AT biology and thereby highlight a previously unknown role for this multi-functional protein.

### 3.4.1 COMP expression in AT

Data from qPCR and Western blotting analysis of AT confirmed that *COMP* is expressed at a significantly higher level in gluteal compared to abdominal subcutaneous AT at both the mRNA and protein level respectively, in agreement with previous microarray data ((Pinnick et al., 2014), supplementary data). This depot-specific expression pattern was retained in (immortalised) preadipocytes during and after differentiation. As most differentially-expressed genes between WAT depots tend to be development-related transcriptional regulators such as *HOX* genes (Karastergiou et al., 2013); (Gesta et al., 2007), the identification of an ECM component exhibiting depot-specific expression in AT is somewhat novel and may have relevance to regional AT distribution, a strong determinant of metabolic health (section 1.3.8; (Karpe & Pinnick, 2015)).

Despite the striking differential expression pattern, however, *COMP* mRNA levels in subcutaneous abdominal or gluteal AT were not associated with a regional AT accumulation phenotype (independent of overall adiposity). Although subcutaneous abdominal and gluteal AT *COMP* mRNA levels were significantly positively correlated with waist and hip circumference, correcting for BMI rendered these correlations non-

significant. Rather than regional AT accumulation, *COMP* mRNA levels in subcutaneous abdominal and gluteal AT were significantly higher in overweight/obese compared to lean individuals, suggesting that *COMP* mRNA levels more likely represent a novel biomarker of overall adiposity.

As measuring mRNA levels was the focus in this aspect of the study, it would be appropriate to measure COMP protein in abdominal and gluteal AT taken from individuals across a range of adiposity as well as characterise the relationship between gene transcription and translation *in vivo*. However, it should also be considered that COMP protein might modulate AT function and/or structure (e.g. cellularity) in the absence of changes to overall depot size; this is discussed in more detail in section 3.4.3.

### 3.4.2 COMP as a novel adipokine

Consistent with the data pertaining to *COMP* mRNA levels in AT, circulating COMP protein levels were also positively correlated with BMI and total fat mass % in males and females. This relationship is consistent with reports that the concentration of plasma COMP protein correlates positively with BMI (El Defrawy, Gheita, Raslan, El Ansary, & El Awar, 2016) and declines upon weight loss (King, Henneicke, Seibel, March, & Anandacoomarasmy, 2015). Although relationships between regional adiposity and the concentration of circulating adipose-derived molecules have been reported previously (e.g. circulating adiponectin levels correlate negatively with central obesity; (Cnop et al., 2003)), plasma COMP protein levels were not significantly different between individuals matched for total fat mass % but exhibiting opposite AT distribution phenotypes.

While fulfilling similar criteria as TSP-1 (Varma et al., 2008) to be granted the 'adipokine' label (i.e. COMP protein was readily detectable in preadipocyte-conditioned medium and plasma plus there was a positive correlation between COMP protein levels

and BMI/adiposity), release of COMP protein was not detectable across either abdominal or gluteal AT upon analysis of arterio-venous plasma samples. When considering other adipokines, it should be noted that leptin release from AT is detectable using the same arterio-venous sampling technique (Pinnick et al., 2014) but adiponectin release is not, principally due to its slow turnover (Karpe & Pinnick, 2015). Given this, not being able to detect COMP protein release from AT does not mean that COMP is not an adipokine.

It is possible that the arterio-venous plasma samples used were not collected under optimal conditions for detecting the release of COMP protein, especially considering that the analysed samples were taken from healthy, normal weight females in which *COMP* mRNA levels would be predicted to be relatively low and transcription and translation appear to be tightly linked. This experiment evidently needs to be repeated but perhaps using individuals with different characteristics; the release of COMP protein from abdominal and/or gluteal AT may be detectable in obese men or from individuals being treated with glucocorticoids. It is also unclear whether the expression and/or release of COMP protein by AT follows a circadian pattern or is affected by feeding status and/or exercise (H. M. Roberts, Moore, Griffith-McGeever, Fortes, & Thom, 2016).

Additionally, it is important to consider that the circulating pool of COMP protein may reflect its turnover not only in AT but also in cartilage (Acharya et al., 2014), bone (Posey et al., 2014), the vasculature (Riessen et al., 2001) and potentially the liver (Magdaleno et al., 2016) and immune system (Fu, Gao, et al., 2016). Impact exercise like running has also been found to cause acute increases in circulating COMP protein levels (H.M. Roberts et al. 2016) by facilitating migration of COMP protein from the synovium into the plasma (Hyldahl et al., 2016). As several studies have suggested that COMP protein may represent a useful biomarker of osteoarthritis progression (El Defrawy et al., 2016); (Verma & Dalal, 2013) and (central) obesity is a risk factor for osteoarthritis (Duclos,

2016), it might be appropriate to investigate how AT *COMP* expression (mRNA and circulating protein) relates to adiposity and bone/cartilage health.

Based on the current data, however, it was not possible to confirm whether COMP represents a novel adipokine. Evidently there are multiple factors that need to be carefully considered so a future study might provide the optimal conditions that would facilitate COMP protein release by AT meaning the question of whether it is an adipokine can be conclusively resolved.

### 3.4.3 COMP as a pro-adipogenic matricellular protein residing in AT ECM

The ECM plays a critical role in regional AT biology yet remains relatively under-studied and poorly understood (Divoux & Clement, 2011). AT ECM structure and composition have been shown to vary between AT depots (Mori, Kiuchi, Ouchi, Hase, & Murase, 2014). Furthermore, perturbations to AT ECM structure and composition have been implicated in adipocyte dysfunction (Baker et al., 2017), the development of obesity (M. J. Lee, Wu, & Fried, 2010); (Catalan, Gomez-Ambrosi, Rodriguez, & Fruhbeck, 2012) and the pathogenesis of insulin resistance/metabolic disease (Williams, Kang, & Wasserman, 2015).

Remodelling of the ECM is crucial for adipogenesis (Nakajima, Muroya, Tanabe, & Chikuni, 2002); (Nakajima, Yamaguchi, Ozutsumi, & Aso, 1998) and involves changes to its structure and composition (Chun, 2012) through co-ordinated alterations in gene transcription and translation as well as post-translational processing (e.g. enzymatic degradation of ECM components via matrix metalloproteases). *COMP* mRNA levels were found to change dynamically throughout adipogenesis, during which time they followed the same pattern in both abdominal and gluteal preadipocytes whilst remaining differentially expressed.

Although data from experiments investigating glucocorticoid regulation of *COMP* expression suggest its transcription and translation are tightly linked, this relationship was not studied extensively during adipogenesis; evidently COMP protein and mRNA levels should be measured in parallel over a differentiation time-course. COMP protein is degraded by various matrix metalloproteases, specifically members of the 'a disintegrin and metalloprotease with thrombospondin motifs' (ADAMTS) family, namely ADAMTS-7 (Liu, Kong, Ilalov, et al., 2006) and ADAMTS-12 (Liu, Kong, Xu, et al., 2006), whose activity is regulated by inhibitory molecules such as granulin-epithelin precursor (F. Guo et al., 2010).

Many potential experiments remain to be conducted to determine whether these post-translational regulators of COMP protein contribute to the depot-specific expression of *COMP*, modulate its expression during adipogenesis and/or facilitate increased *COMP* expression (mRNA and protein) in obesity. This could involve measuring the expression of the post-translational regulators of COMP protein during adipogenesis and in AT obtained from individuals across a range of adiposity as well as investigating whether their over-expression and/or knockdown affects adipogenesis.

Supplementation of the adipogenic medium with exogenous COMP protein enhanced differentiation in both abdominal and gluteal preadipocytes. This pro-adipogenic effect is consistent with the positive correlations between AT *COMP* expression/plasma COMP levels and BMI/adiposity. However, the mechanism by which COMP protein enhanced adipogenesis remains unclear but probably involved physical interaction with components of the ECM such as collagens, fibronectin, growth factors or integrins (Acharya et al., 2014). As COMP protein has also been found to interact with various dietary fatty acids in a way that can alter its structure and potentially bioactivity (McFarlane et al., 2012), this property may be of particular significance to its potential

role in AT. It would be pertinent to investigate whether the effect of COMP on adipogenesis is altered by the presence of fatty acids such as palmitate.

The importance of the ECM as a 3D structure cannot be emphasised enough. Any future experiments proposed below aimed at investigating the role of COMP protein in AT should recognise this crucial property and endeavour to recapitulate the native environment as closely as possible so biologically meaningful data can be generated. For instance, the 3D nature of the ECM can be incorporated into experiments by utilising preadipocytes seeded in matrigel or de-cellularised AT ECM (section 6.4; (Baker et al., 2017)).

In the current study, COMP did not modulate the bioactivity of several adipogenic regulators. Such negative findings may be due to several reasons, one of which may pertain to the highly context-specific nature of COMP action. Murine data have highlighted that COMP protein physically binds BMP2 and enhances BMP2-induced Smad1/5/8 signalling and osteogenesis *in vitro* and *in vivo* (P. Guo et al., 2014); (K. Ishida et al., 2013); (Refaat et al., 2016). However, COMP protein has also been shown to inhibit BMP2 action in vascular smooth muscle cells to prevent vascular calcification (Du et al., 2011). As COMP has been shown to exert opposing effects on the same molecule in two different tissue types, it is possible that this interaction may not occur in AT due to the expression of some unknown factor(s) in AT. Alternatively, the absence of a synergistic interaction between COMP and BMP2 (or TGF-β1) may be due to the method of co-administration and/or concentrations used.

COMP-mediated enhancement of BMP2 bioactivity has been observed in experiments where both proteins were delivered exogenously (K. Ishida et al., 2013) as well as through overexpression of the *COMP* gene (P. Guo et al., 2014). Enhancement of TGF-β1 activity

was also achieved by overexpression of the *COMP* gene (Haudenschild et al., 2011). Given this, it is not clear whether COMP-mediated modulation of BMP2 or TGF-β1 signalling can occur in AT or whether suboptimal conditions were used in the current experimental set-up (i.e. *COMP* gene overexpression was actually required). It is also possible that the concentrations used in previous co-administration studies examining BMP2-mediated osteogenesis (K. Ishida et al., 2013) were not appropriate in the context of adipogenesis. It follows that the effect of COMP (as well as co-administration with BMP2 and TGF-β1, for example) on adipogenesis should be re-examined in preadipocytes over-expressing the COMP gene. Although no synergistic interaction with COMP protein was detected in the current experiments, the striking depot-specific actions of BMP2 clearly identify it as a novel regulator of adipogenesis.

Based on this finding, BMP2 was taken forward for further study (chapter 4). It is also possible that the pro-adipogenic effect of COMP protein did involve modification of the bioactivity of ECM component(s) but the candidate molecules chosen for further investigation were not relevant in this particular cellular model of adipogenesis. A more valid, targeted approach to identify potential binding partners would involve pulling down COMP protein from AT via co-immunoprecipitation before determining the identity of any bound molecules via mass spectrometry. The identity of bound molecules could then inform future experiments involving COMP co-administration.

The expression of matricellular proteins such as COMP typically increases upon tissue injury to co-ordinate wound healing response processes such as ECM remodelling and angiogenesis (Bornstein & Sage, 2002). It follows that increased *COMP* mRNA and/or circulating protein levels have been linked to various pathological states underpinned by perturbed ECM remodelling in a number of tissue types. Circulating COMP protein has recently been identified as a sensitive, non-invasive biomarker of liver fibrosis in patients

with chronic viral hepatitis (Zachou et al., 2017); this is consistent with data highlighting that COMP protein promotes collagen deposition in liver cells (Magdaleno et al., 2016). It is therefore possible that COMP protein mediates collagen deposition in AT to promote fibrosis, particularly in obesity; AT fibrosis has been associated with obesity, insulin resistance and metabolic disease (Divoux et al., 2010); (Williams et al., 2015). To investigate whether *COMP* is involved in the development of AT fibrosis in obesity, it would be appropriate to measure the mRNA and protein levels of *COMP* and other ECM components (such as collagen) in AT obtained from individuals across a spectrum of adiposity.

Based on previous data generated in chondrocytes, it is unclear whether COMP will just alter the spatial arrangement of ECM proteins in AT or modulate their expression in AT given that COMP treatment does not change the expression of ECM-related genes in human cartilage (Ruthard et al., 2014). COMP protein might instead regulate the spatial arrangement of these ECM molecules in a manner which facilitates AT fibrosis and dysfunction, particularly in obesity when *COMP* mRNA and protein levels are increased. Indeed, COMP protein has been shown to co-ordinate collagen fibrillogenesis in cartilage (Halasz et al., 2007). As previous data highlighted that COMP protein co-localises with collagen 1 in AT (Figure 3.1B), immunohistochemical studies are warranted to examine whether COMP co-ordinates the spatial arrangement of EC components whose expression is up-regulated in fibrosis, and whether such interactions facilitate AT dysfunction.

Increasing amounts of data highlight that AT fibrosis occurs in obesity and metabolic disease (section 1.4.6) and that this can predict the response to clinical interventions (Divoux et al., 2010); (Abdennour et al., 2014). Given that AT *COMP* mRNA levels are significantly up-regulated in obesity, it might be appropriate to examine it in the context

of AT fibrosis and dysfunction. Evidently protein analysis techniques such as immunohistochemistry, Western blotting and co-immunoprecipitation would be most suitable for studying its expression and localisation in conjunction with other ECM components whose expression is known to be altered in AT fibrosis. Ideally samples would be taken across a spectrum of adiposity with additional analyses possibly including comparisons between healthy controls and individuals with metabolic disease who would be predicted to have some level of AT fibrosis (e.g. patients with type 2 diabetes).

As the ECM plays a key role in determining the capacity for AT to expand as well as the mechanism employed (section 1.4.6; (Khan et al., 2009)), it would be useful to investigate whether *COMP* expression (mRNA and protein) in subcutaneous abdominal and/or gluteal AT is associated with adipocyte size. Additionally, it should be noted that the immunohistochemistry data presented in Figure 3.1B suggest that COMP protein resides in perivascular clusters within AT; this is notable as other data indicate there is a positive association between circulating COMP protein levels and vascular calcification (F. F. Wang et al., 2017). As AT blood flow has been shown to decrease proportionally with increasing adiposity (K. N. Frayn & Karpe, 2014), there would be merit in investigating whether COMP protein is involved in the development of the perturbed AT blood flow observed in obesity and metabolic disease. This could be achieved by measuring COMP expression in AT and/or circulating COMP levels in individuals across a spectrum of adiposity and relating this to their AT vascular function.

It is important to remember that *COMP* is primarily involved in bone and cartilage biology; it has been implicated in chondrogenesis (Haleem-Smith, Calderon, Song, Tuan, & Chen, 2012) and osteogenesis (K. Ishida et al., 2013). It follows that unique insights into the role of *COMP* in AT may be provided by looking to individuals specifically affected by *COMP* pathology. Indeed, mutations in the *COMP* gene are the leading cause

of PSACH (Posey et al., 2014) yet there is a paucity of data pertaining to the overall metabolic health of these individuals, let alone their AT function, structure and/or distribution.

While it might be unethical to involve PSACH patients in overfeeding studies involving repeated invasive interventions such as blood sampling and AT biopsy harvesting, it is well-established that the PSACH phenotype can be readily recapitulated in cell and transgenic mice expressing mutant *COMP* mRNA and protein (Hansen et al., 2011); (Posey et al., 2009). These same models could be easily re-purposed to examine whether the expression of mutant COMP protein affects AT expansion upon overfeeding *in vivo* and/or adipogenesis *in vitro* respectively.

It is notable that the *COMP* knockout mouse reportedly does not exhibit any discernible phenotype under normal conditions (Posey et al., 2008). This particular mouse model would be useful to determine the timescale over which *COMP* mRNA and protein are up-regulated during the development of obesity, as well as whether COMP protein contributes to AT fibrosis and/or dysfunction as well as the development of metabolic complications. The *COMP* knockout mouse could also be used to investigate whether glucocorticoids act through COMP to promote (regional) AT expansion upon overfeeding and whether the presence or absence of COMP protects or exacerbates the metabolic sequelae associated with exposure to chronic high levels of glucocorticoids (section 3.4.4).

COMP protein may also act as a ligand that activates signalling pathways which modulate adipogenesis. For instance, it has been reported that COMP protein stimulates ERK1/2 signalling in rat hepatic stellate cells via activation of CD36 receptors (Magdaleno et al., 2016); ERK1/2 signalling is crucial for adipogenesis (Bost, Aouadi, Caron, & Binetruy,

2005). As the mRNA levels of *CD36* increase in preadipocytes during the latter part of adipogenesis (Todorčević et al., 2017), Western blotting could be used to determine whether COMP's pro-adipogenic action involves activation of ERK1/2 signalling during this phase of differentiation.

Overall, the prevailing literature indicates that *COMP* employs a range of mechanisms whilst participating in various biological processes in a highly context-specific manner; such biological flexibility makes *COMP* an intriguing candidate that has proven difficult to study. While *COMP* is interesting due to its pro-adipogenic effects and the strong positive correlation between AT *COMP* mRNA levels and adiposity, it also serves to highlight the crucial role that the ECM plays in (regional) AT biology and dysfunction. The AT ECM represents a fertile ground that currently remains relatively under-explored. Sitting at the interface between AT structure and function, the ECM certainly warrants greater representation in AT research more broadly, especially given that such work could yield novel approaches to the treatment and/or management of various obesity-related diseases.

### 3.4.4 Glucocorticoid regulation of AT COMP expression

Glucocorticoid treatment robustly enhanced *COMP* mRNA and protein levels in abdominal and gluteal preadipocytes (but not differentiated cells), meaning it is possible that *COMP* represents a molecular link between glucocorticoid action and adiposity. A novel indicator of long-term cortisol metabolism suggests that circulating cortisol levels are elevated in obesity (S. E. Jackson, Kirschbaum, & Steptoe, 2017). As *COMP* mRNA and protein levels in preadipocytes were up-regulated by glucocorticoids and AT *COMP* expression was positively correlated with overall adiposity, it is possible that *COMP* mediates the pro-adipogenic effect of glucocorticoids in obesity. This could be tested *in*

*vitro* by overexpressing or knocking down *COMP* expression in preadipocytes and measuring whether adipogenesis is affected upon differentiation with different concentrations of glucocorticoid in the adipogenic medium. Relevant *in vivo* studies would involve measuring (regional) AT expansion in conjunction with *COMP* mRNA and protein levels upon overfeeding individuals treated with either exogenous glucocorticoids or with mifepristone, an inhibitor of GC signalling. More detailed characterisation could be performed in mice, with additional studies involving *COMP* knockout mice to test whether *COMP* deficiency protects against the weight gain and metabolic disturbances associated with glucocorticoid treatment (M. J. Lee et al., 2014).

It is well-established that central obesity is associated with insulin resistance/metabolic disease (see section 1.3.8) and that many aspects of this metabolic phenotype are shared with Cushing's syndrome patients (M. J. Lee et al., 2014). Such similarities have prompted the suggestion that altered glucocorticoid signalling, particularly in AT, may underlie non-Cushingoid central adiposity and its associated metabolic sequelae (Bujalska et al., 1997). It is becoming increasingly clear that pathologically enhanced local glucocorticoid action mediated via 11β-HSD type 1 - particularly in AT - probably represents a major process underpinning central obesity and Cushing's syndrome (Morgan et al., 2016).

While *COMP* mRNA levels were not associated with a regional AT accumulation phenotype in this particular cohort, depot-specific differences in local glucocorticoid signalling within AT have been previously reported (M. J. Lee et al., 2008). Although *COMP* has the potential to link local re-activation of cortisol from cortisone to regional AT expansion with the development of central obesity, it is not clear whether this actually occurs *in vivo*. This could be tested directly through analysis of *COMP* mRNA and protein levels in abdominal and gluteal AT taken from individuals with Cushing's syndrome. In

addition, the development of 11β-HSD type 1 inhibitors present a unique opportunity for the investigation of *COMP* biology in AT. As these molecules have been shown to exert a beneficial effect on metabolic health and confer weight loss (Stomby et al., 2014), it might be appropriate to measure if/how (depot-specific) AT *COMP* expression changes upon treatment with 11β-HSD type 1 inhibitors and how this may be related to regional weight loss.

As a component of the ECM, *COMP* may influence the mechanism by which AT expands (i.e. hypertrophy vs. hyperplasia). This may be important to consider given that adipocyte size is an important predictor of metabolic disease risk (Laforest et al., 2015) and abdominal adipocyte hypertrophy in Cushing's patients has been observed (Rebuffe-Scrive, Krotkiewski, Elfverson, & Bjorntorp, 1988); (Roerink et al., 2017). Histological studies examining abdominal and gluteal AT from individuals with Cushing's syndrome and non-Cushing's central obesity in comparison to healthy, lean individuals would provide insight into the potential relationship between *COMP* mRNA and protein levels, (regional) adiposity, metabolic health and adipocyte size.

### 3.4.5 Concluding statements

*COMP* is a matricellular protein that exhibits a striking depot-specific expression pattern in AT. *COMP* mRNA levels in abdominal and gluteal AT as well as circulating COMP protein levels were positively correlated with BMI/adiposity but not with a regional AT distribution phenotype. Exogenous COMP protein exerted pro-adipogenic effects in abdominal and gluteal preadipocytes although the mechanism(s) employed remain unclear. *COMP* mRNA and protein levels in preadipocytes (but not differentiated cells) were robustly increased by glucocorticoids, thereby suggesting that *COMP* may represent a molecular link between (local) glucocorticoid action and adiposity. The data indicate

that *COMP* represents an intriguing novel regulator of AT biology whilst also highlighting the important yet poorly understood role of the ECM in AT biology.

# Chapter 4

# Bone morphogenetic protein 2 is a depot-specific regulator of human adipogenesis

## 4.1 Introduction

During the investigation into the role of *COMP* in human AT it was observed that BMP2 exerted striking depot-specific effects on adipogenesis (section 3.3.6). The primary objective of this chapter was to build on this intriguing observation and perform a detailed characterisation of BMP2's potential depot-specific role in human AT biology. Various BMPs have previously been identified as regulators of adipogenesis; BMP4 is a pro-adipogenic factor in human preadipocytes (B. Gustafson & Smith, 2012) (Suenaga et al., 2013) whereas BMP7 (Elsen et al., 2014) and BMP9 (Kuo et al., 2014) promote brown/beige adipogenesis in humans and mice respectively. BMP2 was originally identified as a potent inducer of bone and cartilage formation (Wozney et al., 1988). Since this observation, however, several reports have implicated BMP2 in the regulation of adipogenesis in murine cells.

BMP2 was found to enhance adipogenesis in 3T3-L1 cells when co-administered with rosiglitazone, a PPARG agonist (Sottile & Seuwen, 2000). Pre-treatment of proliferating murine C3H10T1/2 pluripotent stem cells with BMP2 promoted their commitment to the adipose lineage, resulting in greater lipid accumulation and expression of adipogenic markers upon differentiation (Huang et al., 2009). Treatment with exogenous BMP2 reportedly enhanced adipogenesis in murine preadipocytes through activation of Smad1/5/8 and p38 kinase signalling which thereby increased the expression of *Pparg* (K. Hata et al., 2003) and *C/ebpα* (Jin et al., 2006).

BMP4-SMAD1/5/8 signalling may play an important role in promoting metabolically beneficial, hyperplastic AT expansion during weight gain in humans; the apparent importance of BMP4 in AT biology may also be true for BMP2 given that these proteins share ~83% amino acid sequence homology (Shore et al., 1998). Preadipocytes express

and secrete more GREM-1, an endogenous BMP antagonist, in individuals with hypertrophic obesity, which is characterised by enlarged adipocytes (Birgit Gustafson et al., 2015) and may indicate AT dysfunction or impaired AT expandability (section 1.4.4). It follows that GREM-1-mediated inhibition of BMP4-SMAD1/5/8 signalling may prevent the recruitment of new preadipocytes during AT expansion, thereby promoting hypertrophy of pre-existing adipocytes and contributing to AT dysfunction (Laforest et al., 2015). BMP4 has also been implicated in the co-ordination of adipogenesis and angiogenesis during the expansion of white AT (Y. Tang et al., 2016).

Given the importance of regional AT distribution to metabolic disease risk, it is pertinent to note that BMP2 has been shown to exert depot-specific effects in murine preadipocytes; primary visceral preadipocytes were more sensitive to the pro-adipogenic effects of exogenous BMP2 compared to their subcutaneous counterparts according to TAG accumulation and expression of adipogenic genes such as *Pparg* and *C/ebpα* (Macotela et al., 2012). It is possible that such regional differences in BMP2-mediated Smad1/5/8 signalling underlie this phenomenon in murine cells, which may be driven by depot-specific expression of endogenous regulators such as *Grem-1* or *Smad6* (A. Hata, Lagna, Massague, & Hemmati-Brivanlou, 1998) or transcriptional targets of Smad signalling that regulate adipogenesis such as *Id1* (Patil, Sharma, & Satyanarayana, 2014). Regardless, it is currently unclear whether BMP2 regulates human adipogenesis in a depot-specific manner and thus contributes to the determination of body fat distribution.

Emerging genetic data suggests that developmental regulators likely play an important role in the determination of regional AT distribution. Twin studies have demonstrated that body fat distribution, independent of overall adiposity, is heritable (section 1.5.1; (Malis et al., 2005)). Further, a recent genome-wide association study (GWAS) meta-analysis (in which associations were analysed via linear regression using an additive

model) identified numerous genetic loci significantly associated with waist-hip ratio adjusted for BMI (WHRadjBMI) linked to genes involved in developmental signalling (Shungin et al., 2015). While the list of genetic loci associated with regional AT distribution mainly included developmental regulatory transcription factors, such as members of the *HOX*, *TBX* and *KLF* families, it is notable that a SNP located near to the *BMP2* gene (rs979012) was significantly associated with WHRadjBMI in males and females, specifically involving increased waist circumference ((Shungin et al., 2015); supplementary table 4).

While *BMP2* has been implicated in the regulation of bone biology and growth ((Shungin et al., 2015); supplementary table 22), no significant association between rs979012 and height has been reported. However, a significant positive association between rs979012 and BMI has been observed (Guiu-Jurado et al., 2016), thus suggesting that BMP2 might be involved in regulating AT biology. In addition, a different SNP that was significantly positively associated with WHRadjBMI was linked with *SMAD6* (rs1440372), another component of the BMP-SMAD signalling pathway. Based on the growing body of cellular data implicating BMPs in the regulation of adipogenesis and the genetic data suggesting that BMP-SMAD signalling is associated with regional adiposity, this study aimed to investigate the potential depot-specific roles of *BMP2* and *SMAD6* in human subcutaneous abdominal and gluteal AT.

### 4.1.1 Aims

- Validate prior data indicating that BMP2 exerts a depot-specific pro-adipogenic effect by culturing immortalised subcutaneous abdominal and gluteal preadipocytes in differentiation medium supplemented with exogenous BMP2 before measuring TAG accumulation and adipogenic gene expression.
- Analyse *BMP2* mRNA and protein levels in human subcutaneous abdominal and gluteal AT via qPCR and Western blotting respectively.
- Characterise BMP2-SMAD1/5/8 signalling in subcutaneous abdominal and gluteal preadipocytes by measuring SMAD1/5/8 phosphorylation (via Western blotting) in the presence or absence of an inhibitor of BMP signalling. Investigate the role of SMAD1/5/8 signalling in the purported depot-specific, pro-adipogenic effect by culturing preadipocytes in differentiation medium supplemented with exogenous BMP2 and/or an inhibitor of BMP signalling before measuring TAG accumulation and adipogenic gene expression.
- Investigate whether the genetic loci linked with *BMP2* (rs979012) and *SMAD6* (rs1440372) which are significantly associated with WHRadjBMI are related to an AT phenotype, particularly expression of the corresponding gene (measured via qPCR) and/or adipocyte size (measured via histology).

## 4.2 Methods

### 4.2.1 Adipose tissue sample collection and analysis

As described in section 2.1, a previously generated panel of AT cDNA was used for gene expression analysis (section 4.2.5). Previously collected AT biopsies were also used for Western blotting (section 4.2.7).

### 4.2.2 AT histology

Cell size analysis was performed on a dataset of previously collected and processed samples. The dataset contains cell size information on 63 individuals (28 males, 35 females) with a median age of 40 years old (range 30 - 50 years) and a median BMI of 24.9 kg/m$^2$ (range 21.1 - 35.7 kg/m$^2$). AT biopsies were collected via gun biopsy under local anaesthetic (1% lignocaine) to obtain intact samples that were suitable for cell sizing. AT biopsies were fixed in 10% formaldehyde, embedded in paraffin wax, cut into 5µm sections and stained with haematoxylin and eosin. Sections were viewed and photographed at x20 magnification and adipocyte cross-sectional area was calculated using Adobe Photoshop 5.0.1 (Adobe Systems) and Image Processing Tool Kit (Reindeer Games) according to a previously described method (Marinou et al., 2014).

### 4.2.3 Preadipocyte culture and adipogenic differentiation

Immortalised preadipocytes were cultured and differentiated as outlined in section 2.2.4. Where indicated, adipogenic medium was supplemented with recombinant human BMP2 (Sigma) reconstituted in sterile PBS containing 0.1% BSA. For the drug treatment experiments, cells were plated (1.5 x $10^5$ cells/well) and cultured for 48 hr. Cells were then incubated in serum-free medium for 24 hr. On the treatment day, cells were pre-treated for 30 min with serum-free medium containing K02288 (Cayman Chemicals)

reconstituted in DMSO or vehicle before treatment with serum-free medium containing BMP2 and/or K02288 for 30 min before harvesting.

### 4.2.4 Analysis of preadipocyte proliferation

Preadipocyte proliferation upon BMP2 treatment was measured as in section 2.2.5.

### 4.2.5 Gene expression analysis

Extraction of total RNA from AT biopsies and preadipocytes was performed as described in section 2.3.1; cDNA synthesis (section 2.3.2) and qPCR (section 2.3.3) were performed as outlined in previous sections. The following TaqMan Assays-on-Demand were used: *ADIPONECTIN* (Hs00605917_m1); *BMP2* (Hs00154192_m1); *BMPR1A* (Hs01034913_g1); *BMPR1B* (Hs01010965_m1); *BMPR2* (Hs00176148_m1); *CEBPA* (Hs00269972_s1); *GREM1* (Hs01879841_s1); *GREM2* (Hs03986140_s1); *ID1* (Hs03676575_s1); *PLIN1* (Hs00160173_m1); *PPARG2* (Hs01115510_m1); *PPIA* (Hs99999904_m1); and *SMAD6* (Hs00178579_m1).

### 4.2.6 Measurement of intracellular TAG content

Determination of cellular TAG content was performed as outlined in section 2.4.

### 4.2.7 Western Blotting

Western blotting was performed according to the protocol outlined in section 2.5. Conditioned medium samples were generated by incubating preadipocytes for 36 hr in serum-free growth medium supplemented with 5µg/ml heparin (Sigma) to protect BMP2 from degradation and extend its half-life (Zhao et al., 2006). Conditioned medium samples were concentrated on a centrifugal evaporator before blotting. Equal amounts of protein were loaded (50µg/biopsy; 100µg/cell lysate) and resolved by SDS-PAGE,

transferred onto PVDF membranes (Bio-Rad) via semi-dry transfer using a Transblot Turbo (Bio-Rad) and immunoblotted with the following antibodies: BMP2 (1:1000; NBP1-19751; Novus Biologics); phosphorylated (p)-SMAD1/5/8 (1:1000; #13820; Cell Signalling Technology); SMAD1/5/8 (1:500; sc-6031-R; Santa Cruz); β-actin (1:2000; sc1616; Santa Cruz); and α-tubulin (1:2000; ab15246; Abcam) followed by an HRP-conjugated goat anti-rabbit secondary antibody (1:5000; 31460; ThermoFisher Scientific).

### 4.2.8 BMP2 ELISA

The concentration of BMP2 in plasma was determined using ELISA (R&D Systems) as outlined in section 2.6. Depot-specific release of BMP2 protein was measured in arterio-venous plasma samples (taken from 9 healthy, lean females in the fasted state collected during previous studies) taken across subcutaneous abdominal and femoral AT; a superficial epigastric vein draining subcutaneous abdominal AT (K. N. Frayn et al., 1989), a branch of the great saphenous vein draining femoral AT (McQuaid, Manolopoulos, et al., 2010) and an arterialized dorsal hand vein (with the hand kept in a warming box at 60°C) were cannulated. Cannulae were kept patent with 0.9% saline. Plasma creatinine concentrations were used to indicate contamination by muscle venous blood (McQuaid, Manolopoulos, et al., 2010). Samples were analysed in duplicate.

### 4.2.9 Data analysis and statistics

Data are presented as means $\pm$ SEM. Statistical analyses were performed in SPSS Statistics version 22 (IBM). Statistical tests used were as described in the section 2.7. Differences were considered statistically significant at $P<0.05$.

## 4.3 Results

### 4.3.1 BMP2 regulates adipogenesis in a depot-specific manner

To confirm and expand on the previous finding that *BMP2* exerted depot-specific effects on adipogenesis (section 3.3.6), a dose-response experiment was performed in which preadipocytes were differentiated in adipogenic medium supplemented with exogenous BMP2 throughout the time-course.

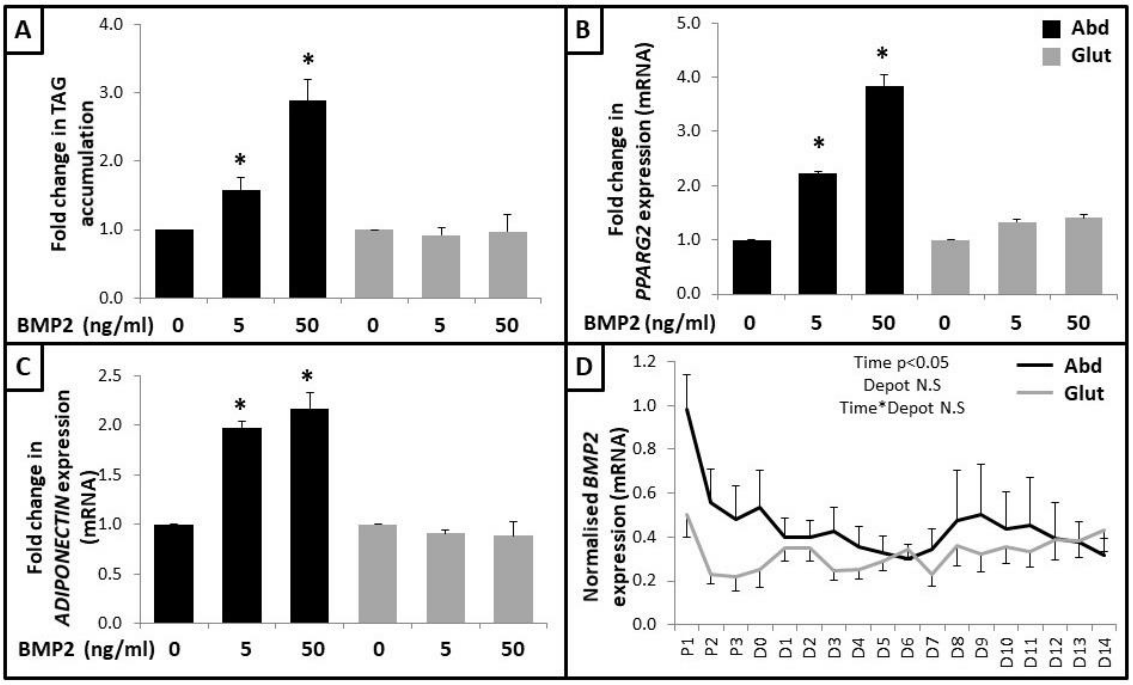



**Figure 4.1 BMP2 regulates adipogenesis in a depot-specific manner.** A - Triacylglycerol (TAG) accumulation; B - *PPARG2* mRNA levels; C - *ADIPONECTIN* mRNA levels (normalised to *PPIA*) in immortalised preadipocytes cultured for 14 days in adipogenic medium supplemented with increasing doses of exogenous BMP2 protein (n=3). Data presented as mean ± SEM; data analysed via ANOVA; *P<0.05 compared to untreated in same depot. D - *BMP2* expression in primary abdominal (Abd) and gluteal (Glut) preadipocytes during 3 days of proliferation (P) followed by 14 days of differentiation (D) in adipogenic medium (n=8; 4 males, 4 females). Data analysed via repeated measures ANOVA. Data in panel D courtesy of Marijana Todorčević.

In agreement with previous data (section 3.3.6), *BMP2* dose-dependently enhanced adipogenesis specifically in abdominal preadipocytes. This effect manifested as increased TAG accumulation (Figure 4.1A) as well as *PPARG2* (Figure 4.1B) and *ADIPONECTIN*

(Figure 4.1C) expression specifically in abdominal preadipocytes; BMP2 treatment had no effect in gluteal cells.

A further experiment was conducted in which *BMP2* expression was measured over a 14 day adipogenic time-course in previously collected cDNA from primary abdominal and gluteal preadipocytes from 4 males and 4 females via qPCR (Figure 4.1D). While considerable inter-individual variation was observed, it was found that *BMP2* expression was not significantly different between depots but changed dynamically over time (repeated measures ANOVA; $P<0.05$). More specifically, the data suggest that *BMP2* expression may be down-regulated in abdominal and gluteal cells during the transition from proliferation to differentiation. Overall, these data confirm previous results and identify BMP2 as a novel regulator of adipogenesis in human preadipocytes that exerts depot-specific effects.

### 4.3.2 BMP2 is a paracrine factor in WAT

Having confirmed that BMP2 exerts depot-specific, pro-adipogenic effects, the focus then shifted to investigate the cell biology of BMP2 in AT. An initial study was performed to measure the expression of BMP2 protein in AT and (immortalised) preadipocytes. It was found that BMP2 protein was readily detectable in the lysates of whole AT (Figure 4.2A), preadipocytes (Figure 4.2B) and differentiated preadipocytes (Figure 4.2C). Consistent with the AT expression panel data, BMP2 expression did not vary by depot. A 44kDa band was detected in lysates which corresponds to the inactive 397 amino acid-long pre-pro-protein (Wozney et al., 1988).

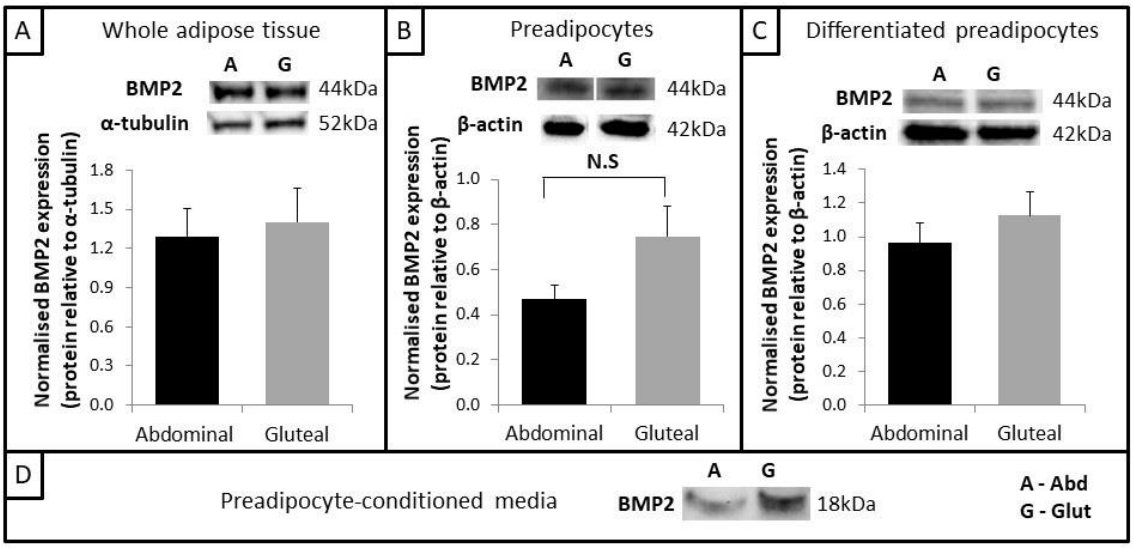



**Figure 4.2 BMP2 is a paracrine factor in WAT.** A - Representative image from Western blot analysis and summary graph of BMP2 protein levels in abdominal (A/Abd) and gluteal (G/Glut) AT homogenates (n=5). B - Representative image from Western blot analysis and summary graph of BMP2 protein levels in proliferating immortalised preadipocyte lysates (n=3). C - Representative image from Western blot analysis and summary graph of BMP2 protein levels in lysates of immortalised preadipocyte cultured in adipogenic medium for 14 days (n=3). D - Representative image from Western blot analysis of BMP2 protein in (heparin-containing) conditioned medium obtained from proliferating (immortalised) preadipocytes (n=3). Data presented as mean ± SEM; data analysed via paired T-test; *P<0.05. AT biopsies courtesy of the OBB. Data in panel D courtesy of Nellie Loh.

BMP2 protein was also detectable in (concentrated) conditioned medium obtained from abdominal and gluteal preadipocytes (Figure 4.2D). Under reducing conditions, an 18kDa single band was detected that corresponds to the cleaved, bioactive, glycosylated 144 amino acid-long monomer (amino acids 283-396; (Wang et al., 1990)). Given this, ELISA was used to measure BMP2 protein in arterio-venous samples taken across subcutaneous abdominal and gluteal AT to investigate whether BMP2 represents a novel adipokine. BMP2 was not detectable in (heparinised) plasma samples indicating it probably circulates at levels lower than 60pg/ml, at least in this group of healthy individuals. These data indicate that abdominal and gluteal preadipocytes express and secrete bioactive BMP2 which probably acts as a paracrine factor.

### 4.3.3 BMP2 activates SMAD1/5/8 signalling in preadipocytes via type 1 BMP receptors

Having detected *BMP2* mRNA and protein expression within AT, a series of experiments was performed to investigate whether an extracellular BMP2 stimulus activates SMAD1/5/8 signalling in abdominal and/or gluteal preadipocytes.

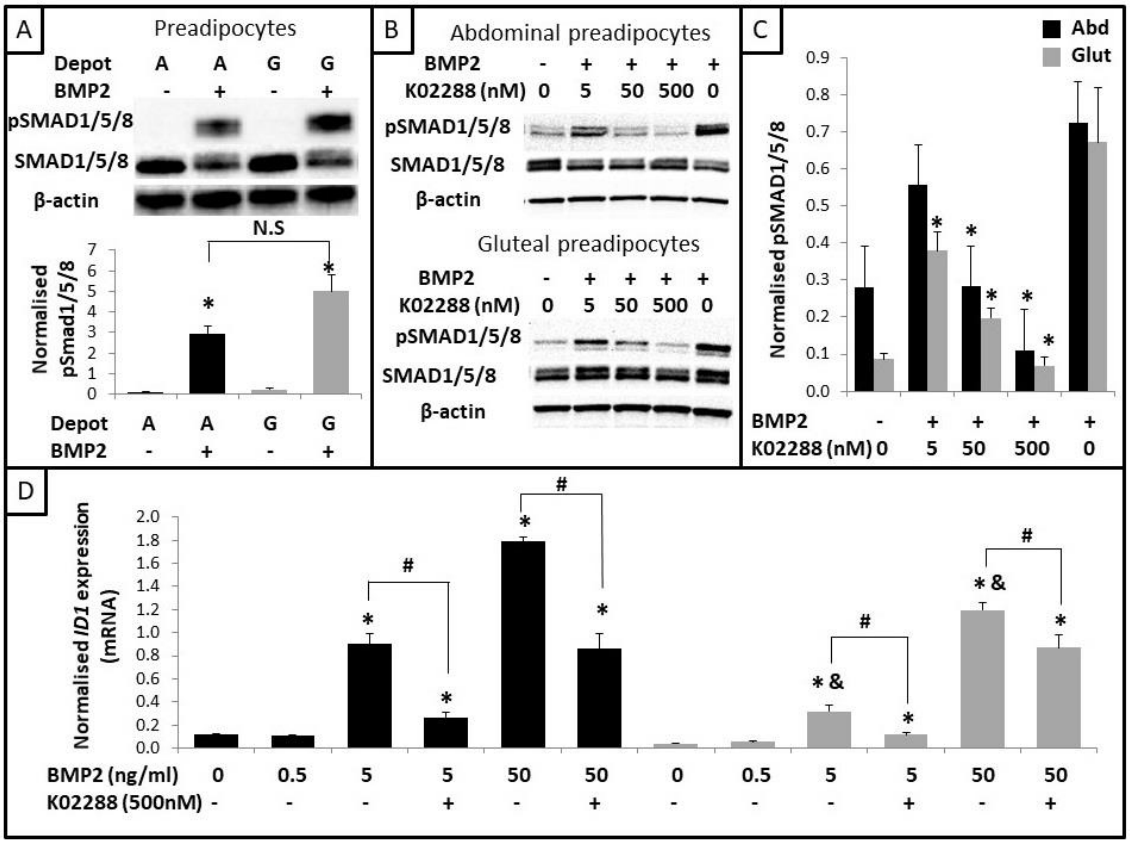



**Figure 4.3 BMP2 activates SMAD1/5/8 signalling in preadipocytes via type 1 BMP receptors.** A - Representative image from Western blotting and summary graph of SMAD1/5/8 phosphorylation in immortalised abdominal (A/Abd) and gluteal (G/Glut) preadipocytes treated with 50ng/ml BMP2 for 20 min (n=3). B - Representative image from Western blotting of immortalised preadipocytes treated with 50ng/ml BMP2 and/or various doses of K02288 for 20 min. C - Summary graph of Western blot data presented in panel B (n=3). D - *ID1* mRNA levels (normalised to *PPIA*) in preadipocytes treated with increasing doses of BMP2 and/or 500nM K02288 for 6 hr (n=3). Data presented as mean ± SEM; *P<0.05 compared to untreated in same depot; $^{\#}$P<0.05 between conditions in same depot; $^{\&}$P<0.05 compared to same condition in abdominal cells. Data in panel B and C courtesy of Mohamed Eghleilib.

Treatment with 50ng/ml BMP2 induced SMAD1/5/8 phosphorylation in proliferating abdominal and gluteal preadipocytes within 20 min of administration (Figure 4.3A). To investigate whether this effect was mediated via type 1 BMP receptors, a novel small molecule inhibitor of type 1 BMP receptor kinase activity (Sanvitale et al., 2013) was used. As anticipated, pre-treatment and co-administration of K02288 dose-dependently inhibited BMP2-induced SMAD1/5/8 phosphorylation (Figure 4.3B). Inhibition of SMAD1/5/8 phosphorylation occurred to approximately the same extent in abdominal and gluteal cells, with near-complete inhibition of SMAD1/5/8 phosphorylation achieved with 500nM K02288 (Figure 4.3C).

To investigate whether the SMAD1/5/8 signalling pathway was functional, qPCR was used to measure the expression of *ID1*, a well-established transcriptional target of BMP2 regulated by SMAD1 and SMAD4 activity (Hollnagel, Oehlmann, Heymer, Ruther, & Nordheim, 1999); (Lopez-Rovira, Chalaux, Massague, Rosa, & Ventura, 2002). Treatment with BMP2 for 6 hr dose-dependently induced *ID1* expression in preadipocytes from both depots, although this occurred to a significantly greater extent in abdominal compared to gluteal cells (Figure 4.3D).

Consistent with its effect on SMAD1/5/8 phosphorylation, pre-treatment followed by co-administration of 500nM K02288 inhibited BMP2-stimulated induction of *ID1* expression in both depots (80% inhibition for 5ng/ml BMP2; 30 - 50% inhibition for 50ng/ml BMP2). These data indicate that both abdominal and gluteal preadipocytes possess the cellular machinery (i.e. functional type 1 BMP receptors and SMAD1/5/8 proteins) required to successfully transduce an extracellular BMP2 signal into altered gene expression. Also, abdominal preadipocytes seemed to display higher sensitivity to BMP2-induced *ID1* expression; this may be relevant to the depot-specific action of BMP2 in human AT.

### 4.3.4 BMP2's pro-adipogenic effects are mediated via type 1 BMP receptor kinase activity

Having previously shown that BMP2 activates SMAD1/5/8 signalling via activation of type 1 BMP receptors, the next objective was to investigate whether the depot-specific, pro-adipogenic effects of BMP2 are mediated through this mechanism.

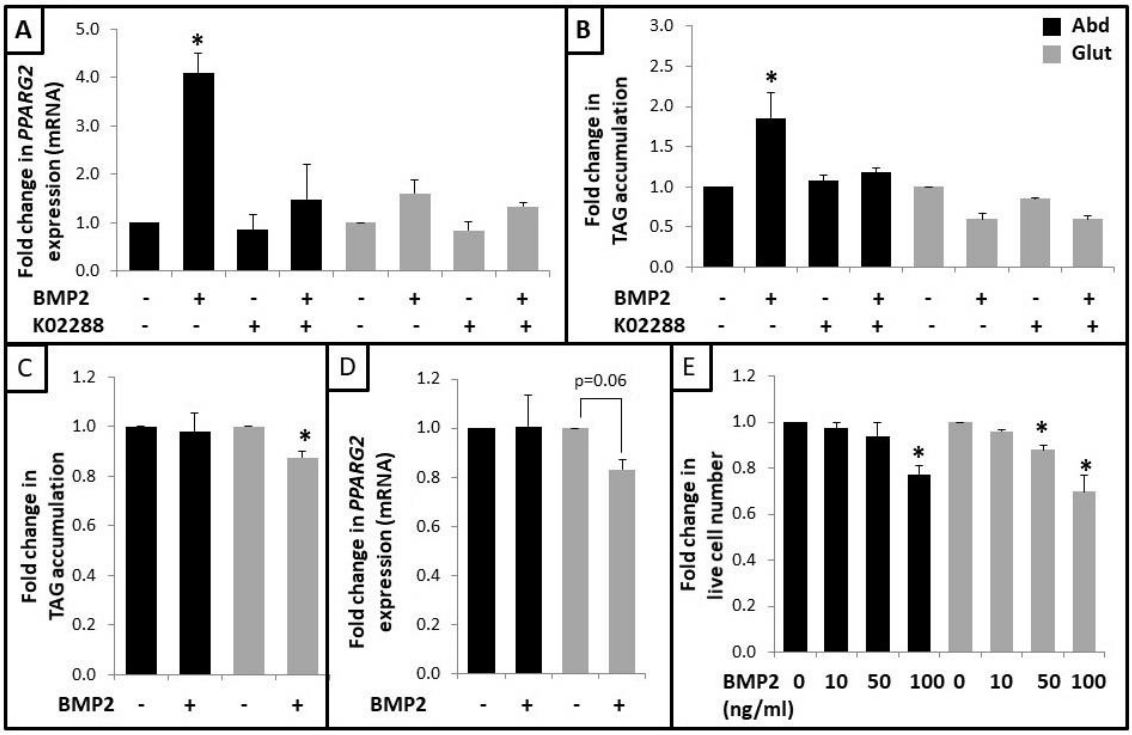



**Figure 4.4 BMP2's pro-adipogenic effects are mediated via type 1 BMP receptor kinase activity.** A - *PPARG2* expression; B - triacylglycerol (TAG) accumulation in immortalised abdominal (Abd) and gluteal (Glut) preadipocytes cultured for 14 days in adipogenic medium supplemented with 50ng/ml BMP2 and/or 500nM K02288 (n=3). C - TAG accumulation; D - *PPARG2* expression in immortalised preadipocytes differentiated for 14 days in adipogenic medium after pre-treatment for 48 hr with 50ng/ml BMP2 or vehicle (n=3). E - Analysis of proliferation in preadipocytes cultured for 48 hr in growth medium supplemented with increasing doses of BMP2 (n=6). All data presented as mean ± SEM; all data analysed via ANOVA; *P<0.05 compared to untreated in same depot.

An experiment was performed in which abdominal and gluteal preadipocytes were differentiated in adipogenic medium supplemented with 50ng/ml BMP2 and/or 500nM K02288. As anticipated, co-administration of 500nM K02288 completely blocked the depot-specific, pro-adipogenic effect of 50ng/ml BMP2 as highlighted by the complete aabrogation TAG accumulation (Figure 4.4A) and *PPARG2* expression (Figure 4.4B) in

abdominal cells. Treatment with K02288 alone did not affect preadipocyte differentiation in either depot.

It has been reported that acute *BMP2* pre-treatment commits pluripotent mesenchymal stem cells to the adipogenic lineage and enhances their subsequent adipogenic differentiation according to experiments in murine cells (Huang et al., 2009). To test whether this phenomenon translated to human cells, abdominal and gluteal preadipocytes were incubated with 50ng/ml BMP2 (or vehicle) for 48 hr before a standard 14-day adipogenic protocol. Pre-treatment of preadipocytes with 50ng/ml BMP2 did not recapitulate the pro-adipogenic effects in abdominal cells according to TAG accumulation (Figure 4.4C) or *PPARG2* expression (Figure 4.4D). However, the acute pre-treatment did modestly inhibit adipogenesis in gluteal cells. Taken together with the previous data, this experimental outcome indicates that a persistent BMP2 stimulus is required for its depot-specific, pro-adipogenic effects to fully manifest.

A further study was performed to analyse the effect of exogenous BMP2 on preadipocyte proliferation. Abdominal and gluteal preadipocytes were cultured in the presence of varying doses of BMP2 for 48 hr before the final cell number was measured using a fluorescent dye-based assay. BMP2 was found to exert a modest anti-proliferative effect (Figure 4.4E). The data suggest that gluteal cells might be more sensitive to the anti-proliferative effects of BMP2 at lower doses as 50ng/ml significantly reduced cell number in this depot but this was not observed in abdominal cells. However, 100ng/ml inhibited cell proliferation by ~15% in both depots.

### 4.3.5 Differential expression of SMAD1/5/8 signalling components and its regulators may underlie the depot-specific action of BMP2

In a bid to understand some of the mechanism(s) potentially underlying the striking depot-specific effects of BMP2 on adipogenesis, the expression of various regulators and components of the SMAD1/5/8 signalling pathway was analysed.

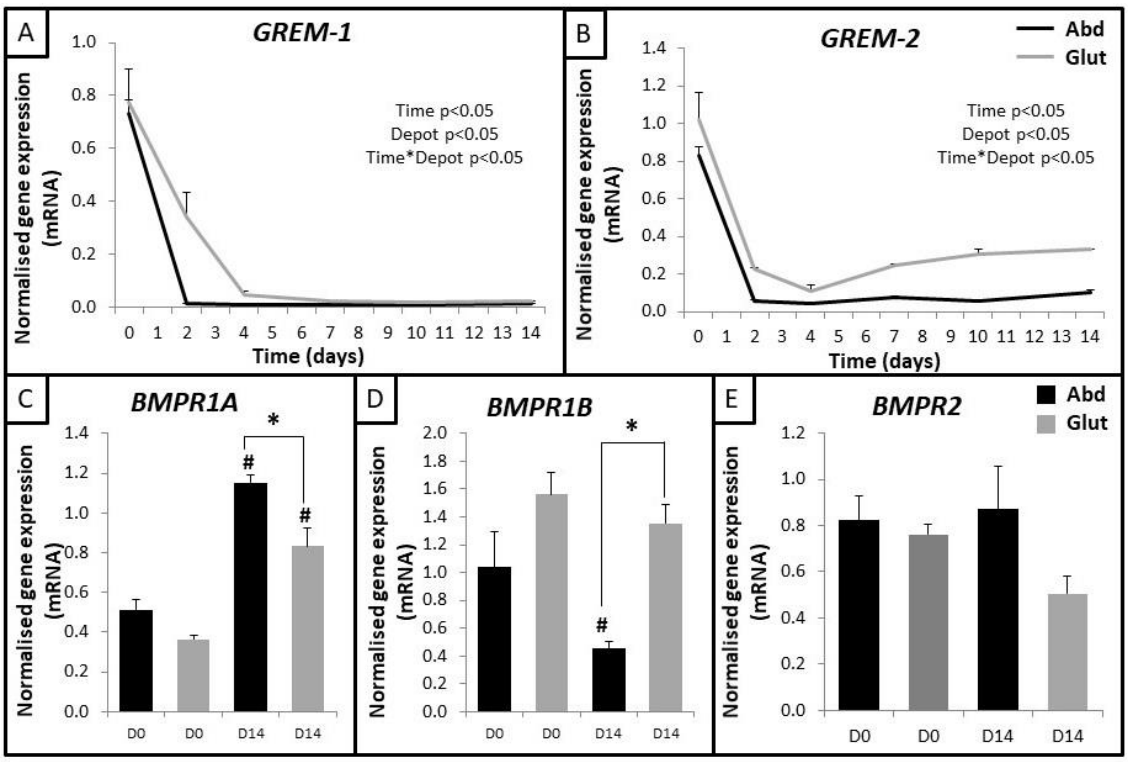



**Figure 4.5 Differential expression of SMAD1/5/8 signalling components and its regulators may underlie the depot-specific action of BMP2.** A - *GREM-1* expression; B - *GREM-2* expression in immortalised abdominal (Abd) and gluteal (Glut) preadipocytes during culture for 14 days in adipogenic medium (n=3). Data analysed via repeated measures ANOVA. C - *BMPR1A* expression; D - *BMPR1B* expression; E - *BMPR2* expression in immortalised abdominal and gluteal preadipocytes on day (D) 0 and 14 days (D) of a standard adipogenic differentiation protocol (n=3); data analysed via ANOVA. All data presented as mean ± SEM; *P<0.05 between depots at the same time-point; $^{\#}$P<0.05 compared to D0 in same depot. Data in panels C, D and E courtesy of Sama Al-Sharifi.

During a 14 day-adipogenic time-course it was found that expression of the endogenous BMP antagonists *GREM-1* (Figure 4.5A) and *GREM-2* (Figure 4.5B) was dramatically down-regulated in both depots within the first 4 days of differentiation (repeated measures

ANOVA, post-hoc T-test). However, this down-regulation was delayed and even partially reversed for *GREM-2* from day 4 onwards in gluteal cells.

With respect to BMP receptor subtypes, it was found that *BMPR1A* expression did not exhibit any depot-specific pattern on day 0 but was significantly higher in abdominal cells on day 14 (Figure 4.5C). *BMPR1B* expression did not vary between different depots on day 0 either but was significantly lower in abdominal cells on day 14 (Figure 4.5D). Other signalling components such as *BMPR2* (Figure 4.5E) and *SMAD6* (data not shown) did not display any distinct depot-specific expression patterns that varied over time. These data indicate that the expression of various components of the BMP-SMAD1/5/8 signalling cascade changes dynamically in a depot-specific manner during differentiation; such variation may contribute to the striking differential effects of BMP2 on adipogenesis in abdominal and gluteal cells.

### 4.3.6 The rs979012/rs2145270 genetic locus is an eQTL for BMP2 expression in AT

Having identified BMP2 as a novel depot-specific regulator of adipogenesis in human preadipocytes and explored some of its cell biology, the focus shifted to explore whether either of the genomic loci associated with body fat distribution also represent expression quantitative trait loci (eQTL) in human AT for the genes located in the associated region that encode components of the BMP2-SMAD signalling pathway. To investigate this, *BMP2* and *SMAD6* mRNA levels were measured in subcutaneous abdominal and gluteal AT biopsies taken from males and females (n ~190) across a range of BMIs (18.8 - 46.2 $kg/m^2$).

Due to the type of arrays used to genotype participants in the OBB (section 2.1), genotype data for rs979012 (T/C) were not available so LDproxy (NIHR; https://analysistools.nci.nih.gov/LDlink/) was used to identify rs2145270 (C/T) as a

suitable proxy. LDlink was used to determine the linkage disequilibrium between rs979012 and rs2145270 (the proposed proxy). The D' value, which is an indicator of allelic segregation, was determined to be 1.000, thus indicating tight linkage of these alleles. In addition, the $r^2$ value, which is a measure of correlation between the two alleles, was determined to be 0.9551. As both D' and $r^2$ values were sufficiently high enough to indicate that these alleles co-segregated so rs2145270 could serve as a valid proxy for rs979012.

The recent genome-wide association study (GWAS) meta-analysis identified a significant positive association between rs979012/rs2145270 and WHRadjBMI upon analysis of data using linear regression under an additive model (Shungin et al., 2015). An initial analysis was performed using a similar additive model (albeit via ANCOVA with corrections for age, sex and BMI), but as *BMP2* expression in the CT and TT groups were not significantly different (data not shown), the analysis was repeated (ANCOVA including corrections for age, sex and BMI) using a recessive model to strengthen the analysis. It was found that *BMP2* expression in subcutaneous abdominal (Figure 4.6A) and gluteal (Figure 4.6B) AT was significantly lower (15%) in rare CC individuals compared to their CT/TT counterparts. *BMP2* mRNA levels did not exhibit a depot-specific expression pattern (data not shown).

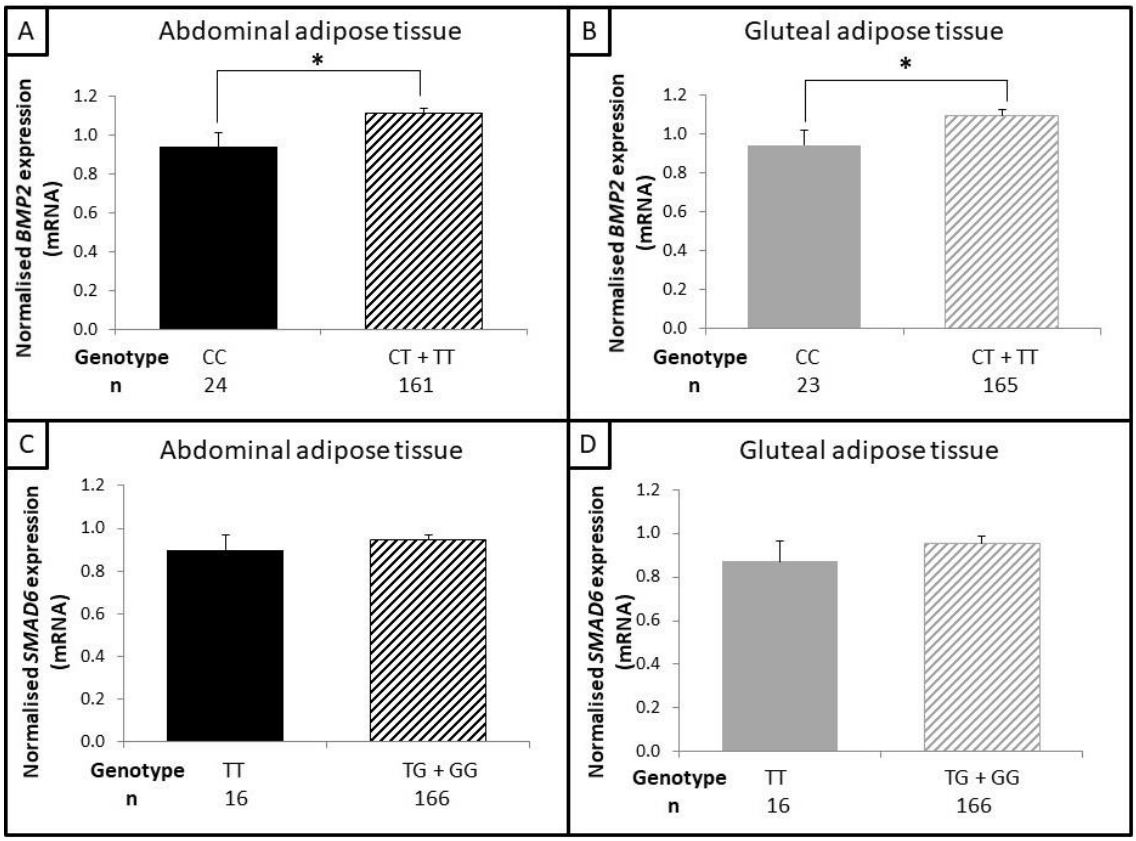



**Figure 4.6 Expression quantitative trait locus analysis of *BMP2* and *SMAD6* expression in subcutaneous abdominal and gluteal AT.** *BMP2* expression was measured via qPCR in abdominal (panel A) and gluteal (panel B) AT biopsies taken from 90 males and 98 females in the OBB. Data were sorted according to rs2145270 (proxy for rs979012) genotype and analysed via ANCOVA (including age, sex and BMI as co-variates). *SMAD6* expression was also measured via qPCR in abdominal (panel C) and gluteal (panel D) AT biopsies taken from 94 males and 97 females in the OBB. Data were sorted according to rs2439385 (proxy for rs1440372) genotype and analysed via ANCOVA (including age, sex and BMI as co-variates). Data are presented as estimated marginal means ± SEM. *P<0.05. *BMP2* qPCR for rs2145270/rs979012 eQTL analysis performed by Mohamed Eghleilib.

A similar approach was taken to investigate whether the SNP linked to *SMAD6* was also an eQTL in AT. As for the other SNP, the OBB did not contain genotype data for rs1440372 (T/C) so LDproxy was used to identify rs2439385 (T/G) as a suitable proxy ($r^2$=0.9744, D'=1.000). The recent genome-wide association study (GWAS) meta-analysis identified a significant positive association between rs979012/rs2145270 and WHRadjBMI upon analysis of data using linear regression under an additive model (Shungin et al., 2015).

An initial analysis was performed using a similar additive model (albeit via ANCOVA with corrections for age, sex and BMI), but as *SMAD6* mRNA levels in the CT and TT groups were not significantly different (data not shown), the analysis was repeated (ANCOVA with corrections for age, sex and BMI) using a recessive model. It was found that *SMAD6* expression in abdominal (Figure 4.6C) and gluteal (Figure 4.6D) AT was not significantly different between TT individuals compared to their TG/GG counterparts. As for *BMP2*, *SMAD6* did not exhibit a depot-specific expression pattern (data not shown). Together these data suggest that rs1440372/rs2145270 does not represent an eQTL for *SMAD6* expression in AT whereas rs979012/rs2145270 genotype was associated with altered AT *BMP2* expression.

### 4.3.7 The rs979012/rs2145270 genotype is associated with increased abdominal adipocyte size in men

Based on the eQTL data, the rs979012/rs2145270 (*BMP2*) genetic locus was taken forward for further analysis. To examine whether rs979012/rs2145270 was associated with a regional AT accumulation phenotype, the OBB database was interrogated. While much smaller in size compared to the number of participants involved in the GWAS meta-analysis (Shungin et al., 2015), the OBB contains DEXA data which provides a more detailed and accurate measure of regional adiposity. The data were separated by sex and grouped according to a recessive model before analysis via ANCOVA (including corrections for age and BMI or fat mass %); no significant associations between rs2145270 genotype and DEXA-derived regional AT accumulation phenotype were detected.

As DEXA (section 1.3.3) does not provide information on important features of AT structure such as adipocyte size (section 1.4.4), a histological database containing cell

size information from previously processed samples was interrogated to investigate whether the rs979012/rs2145270 locus was associated with an adipocyte size phenotype. The data were split by sex and analysed using a recessive model as CT and TT groups were not significantly different (data not shown).

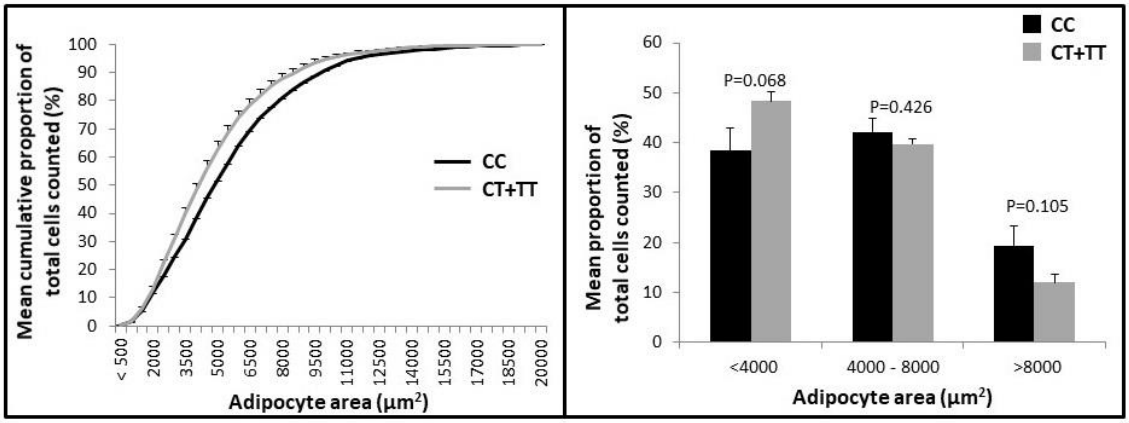



**Figure 4.7 Adipocyte size analysis according to rs979012/rs2145270 genotype in male subcutaneous abdominal AT.** A - Plot of mean cumulative proportion of total cells counted according to size in male subcutaneous abdominal AT; data were separated by genotype. B - Histogram outlining mean cumulative proportion of cells counted according to different cell size bins. Data in panel B presented as estimated marginal means ± SEM; Data analysed via ANCOVA (including age and BMI as co-variates); *P<0.05. Histological analysis performed by Dr Marijana Todorčević and Dr Katherine Pinnick.

When considering the data presented as mean cumulative proportion of total cells counted (Figure 4.7A), a clear trend was observed in male abdominal AT that favoured larger adipocytes at the expense of smaller cells; this trend was mirrored when the proportional frequency data were binned according to cell size and presented as a histogram (Figure 4.7B). Statistical analysis of other indices of cell size distribution via ANCOVA (including corrections for age and BMI) also support this trend. Details are provided in Table 4.1 in which data are presented as mean (SEM).

**Table 4.1 Adipocyte size analysis according to rs979012/rs2145270 genotype in male subcutaneous abdominal AT**

| | CC | CT | P value |
|---|---|---|---|
| Mean | 5988 (360) | 4711 (150) | 0.073 |
| Median | 4913 (330) | 4216 (140) | 0.077 |
| Lower Quartile | 3030 (180) | 2710 (73) | 0.119 |
| Upper quartile | 7339 (570) | 6126 (240) | 0.070 |
| n | 5 | 23 | |

No significant differences were observed in male gluteal AT. Furthermore, there were no significant differences in adipocyte size between genotypes for female abdominal or gluteal AT. Together these data suggest that the WHRadjBMI-increasing rs979012/rs2145270 allele is associated with a shift in adipocyte size that favours larger cells at the expense of smaller cells in subcutaneous abdominal AT in males.

### 4.3.8 Erythroferrone is a depot-specific regulator of BMP2 action in preadipocytes

It is well established that the biology of various BMPs can be modulated by other ligands, potentially other members of the TGF-β superfamily of growth factors (Katagiri, Boorla, Frendo, Hogan, & Karsenty, 1998), or molecules residing in the ECM (Sedlmeier & Sleeman, 2017). Interactions with other molecules that modify the activity of the same signalling pathway can also bring seemingly disparate biological processes together. Amongst the many potential novel regulators of BMP-induced SMAD signalling, erythroferrone (ERFE) is a potentially interesting candidate.

ERFE is an erythroid protein implicated in iron homeostasis; ERFE reportedly acts as a negative regulator of hepcidin to enhance iron availability and absorption (Gozzelino & Arosio, 2016). Recent data indicate that ERFE may achieve this effect by inhibiting hepatic BMP4-SMAD1/5/8 signalling (Drakesmith et al. *unpublished*); (C. Y. Wang et al., 2017). Other data suggest that ERFE is a myokine that modulates fatty acid

partitioning (Seldin, Peterson, Byerly, Wei, & Wong, 2012). Given the striking degree of structural similarity between BMP2 and BMP4 (Shore et al., 1998), an investigation was performed to explore whether ERFE modifies BMP2-SMAD1/5/8 signalling in (abdominal and gluteal) preadipocytes.

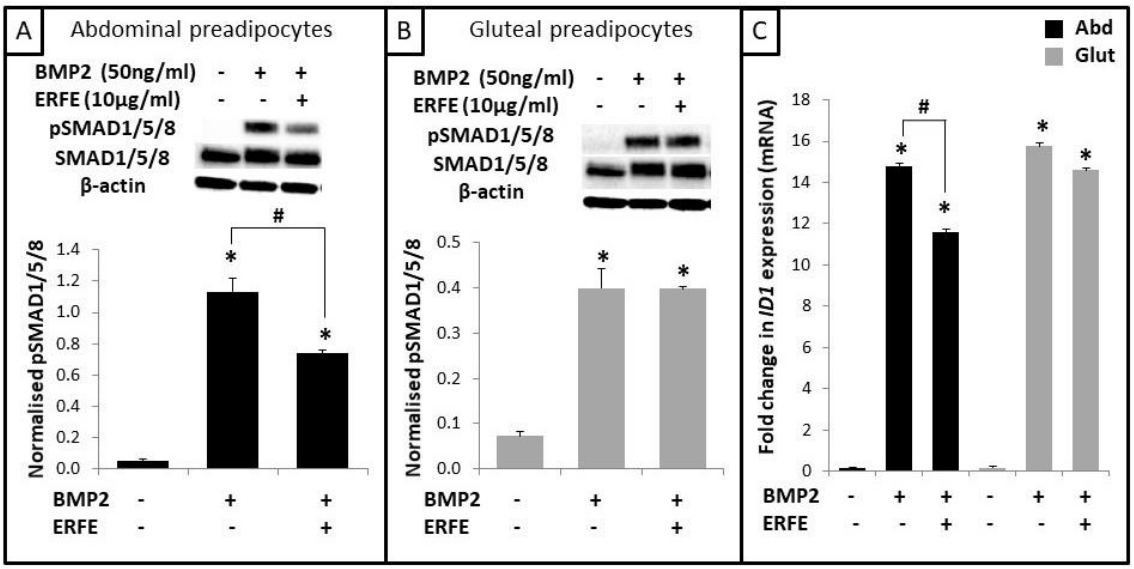



**Figure 4.8 Erythroferrone is a depot-specific regulator of BMP2-SMAD1/5/8 signalling in preadipocytes.** A - Representative image and summary graph of Western blot analysis of SMAD1/5/8 signalling in immortalised abdominal (Abd) preadipocytes treated with 50ng/ml BMP2 and/or 10µg/ml erythroferrone (ERFE) for 30 min (n=3). B - Representative image and summary graph of Western blot analysis of immortalised gluteal (Glut) preadipocytes (n=3). C - *ID1* expression in preadipocytes treated with BMP2 and/or ERFE for 6 hr (n=5). All data presented as mean ± SEM; all data analysed via ANOVA with post-hoc T-tests; *P<0.05 compared to untreated in same depot; #P<0.05 between conditions in same depot. ERFE protein courtesy of Prof. Hal Drakesmith.

It was found that co-administration of 10µg/ml ERFE inhibited BMP2-induced pSMAD1/5/8 levels by ~30% specifically in abdominal preadipocytes (Figure 4.8A) but had no effect in gluteal cells (Figure 4.8B). Consistent with this effect, ERFE significantly reduced *BMP2*-mediated activation of *ID1* expression by ~20% in abdominal preadipocytes whereas no effect was detected in gluteal cells (Figure 4.8C). It is unclear why the regional variation in BMP2-induced *ID1* expression (Figure 4.3) was absent. Regardless, these data identify ERFE as a potential mediator of regional AT expansion that signals from energy sensing organs such as bone marrow and skeletal muscle.

## 4.4 Discussion

BMP2 is a member of the transforming growth factor-β superfamily of growth factors and is classically associated with bone/cartilage biology (V. Rosen, 2009) although more recent data suggest it plays a key role in the regulation of adipogenesis. The data presented in this study highlight that: *BMP2* is expressed in AT and secreted by preadipocytes; and BMP2 modulates adipogenesis in a depot-specific manner despite activating SMAD1/5/8 signalling in both abdominal and gluteal preadipocytes via type I BMP receptors; and a GWAS hit for body fat distribution represents an eQTL for *BMP2* expression in AT. These results identify BMP2 as a novel regulator of human adipogenesis that may be involved in the determination of regional AT distribution.

### 4.4.1 BMP2 as a paracrine factor

Western blotting for BMP2 protein in AT biopsies, cell lysates and conditioned medium generated bands of different heights which indicated that bioactive BMP2 was processed (i.e. expressed, cleaved and glycosylated) and secreted by abdominal and gluteal preadipocytes. *BMP2* expression (mRNA and protein) did not exhibit any depot-specific expression pattern in whole AT or preadipocytes.

BMP2 protein was undetectable in arterio-venous plasma samples taken across subcutaneous abdominal and gluteal AT. As the plasma samples analysed contained heparin, which acts to protect BMP2 (Zhao et al., 2006), the inability to detect BMP2 via ELISA likely reflects low circulating concentrations rather than sample degradation. Other studies using the same ELISA kit in the context of atherosclerosis (M. Zhang et al., 2015) or non-small-cell lung cancer (Choi et al., 2012) have reported circulating concentrations of BMP2 near or below the limit of detection (60pg/ml). Another study also reported relatively low circulating BMP2 levels (<100pg/ml) that were not

significantly different between normal weight and obese individuals (Guiu-Jurado et al., 2016). Taken together, the data presented in this study suggest that BMP2 primarily acts as a paracrine factor secreted by preadipocytes in AT that probably only plays a limited endocrine role.

### 4.4.2 BMP2-SMAD1/5/8 signalling and adipogenesis

Exogenous BMP2 (50ng/ml) activated SMAD1/5/8 phosphorylation in both abdominal and gluteal preadipocytes within 30 min of administration. Activation of SMAD1/5/8 culminated in activation of *ID1* expression, indicating these cells possess the cellular machinery to successfully transduce an extracellular BMP2 signal. This effect was mediated via type 1 BMP receptor kinase activity as both SMAD1/5/8 phosphorylation and *ID1* expression could be blocked in a dose-dependent manner by co-administration of K02288. There was a tendency for abdominal preadipocytes to exhibit a greater sensitivity to BMP2 than their gluteal counterparts, as a given concentration of BMP2 activated *ID1* expression to a greater extent in the former depot; however, this effect was somewhat inconsistent (compare Figure 4.3 to Figure 4.8).

Supplementation of adipogenic medium with BMP2 throughout differentiation selectively enhanced TAG accumulation and *PPARG2* expression in abdominal preadipocytes but had no effect in gluteal cells; this is consistent with initial observations in chapter 3 (section 3.3.6). This depot-specific, pro-adipogenic effect was mediated via activation of type 1 BMP receptors as it was blocked by co-administration of K02288 (Sanvitale et al., 2013). It is notable that treating preadipocytes with BMP2 before differentiation did not recapitulate its pro-adipogenic effects, thus suggesting that a brief exposure to BMP2 did not commit the immortalised SVF cells to the adipose lineage; this

potentially conflicts with data generated in murine cells (Huang et al., 2009); (Wang et al., 1993).

It is possible, however, that BMP2 plays different roles at different times in AT. BMP2 may be involved in AT development by promoting the commitment of pluripotent MSCs to the adipose lineage; if SVF cells obtained from mature AT are already committed, acute exposure to exogenous BMP2 may have no additional effect. Persistent BMP2 exposure to (committed) preadipocytes selectively promoted adipogenesis in abdominal cells but had no effect on gluteal cells, thus suggesting that BMP2 regulates regional AT expansion in adults. As BMP2 activated SMAD1/5/8 signalling in gluteal cells but did not affect adipogenesis, it is possible that BMP2 regulates other biological processes in gluteal cells such as angiogenesis (David, Feige, & Bailly, 2009). Regardless of the underlying reasons, persistent activation of BMP2-SMAD1/5/8 signalling was required for the depot-specific, pro-adipogenic effects to manifest, at least in this *in vitro* experimental system. Evidently such a prerequisite for BMP2's action on adipogenesis would have to be taken into account if these observations were ever to be investigated further *in vivo*, whether that involves administration of exogenous BMP2 or overexpression in a transgenic animal model.

### 4.4.3 Ligand structure as a determinant of depot-specific BMP action

BMP signalling is highly complex and the manifestation of BMP2's pro-adipogenic signal in abdominal preadipocytes may emerge at several different stages. A diagrammatic overview of potential determinants is provided in Figure 4.9. In terms of the active (dimeric) BMP ligand, it is well-established that members of the TGF-β superfamily can form heterodimers which exhibit profoundly different bioactivity compared to the homodimeric forms of their constituents (Israel et al., 1996). Interaction

between different BMPs seems to be essential for normal development, as highlighted by the profound abnormalities observed in animals genetically deficient for more than one type of BMP (Katagiri, Boorla, Frendo, Hogan, & Karsenty, 1998).

BMP2 protein was detected in preadipocyte-conditioned medium as 18kDa monomers on the Western blots. This is because the β-mercaptoethanol present in the sample buffer reduced any disulphide bonds between the seven conserved cysteine residues that hold BMP2 dimers together. Detailed biochemical analysis (e.g. co-immunoprecipitation and proteomic analysis of molecules bound to BMP2) of the structure and composition of BMP2-containing molecules expressed and secreted by subcutaneous abdominal and gluteal preadipocytes and/or AT may provide insight into whether the depot-specific effects of BMP2 emerge at the ligand level.

It is also possible that BMP2 forms non-adipogenic heterodimers in gluteal cells, or that this depot expresses molecules that neutralise the pro-adipogenic action of BMP2. Such molecules may even reside in the extracellular matrix (ECM). For example, heparin-containing molecules can bind to a region within the N-terminus of BMP2, thereby sequestering it and reducing its bioactivity (Ruppert, Hoffmann, & Sebald, 1996). As such interactions are reversible (Nistala et al., 2010) the ECM can act as a reservoir of growth factors which also controls the spatial arrangement of bioactive BMPs over time (Sedlmeier & Sleeman, 2017). Regional variation in AT ECM structure and composition has been reported (Mori et al., 2014) meaning such differences could underlie the depot-specific actions of BMP2. Identifying potential candidate regulators might be best achieved by using minimally-biased techniques involving proteomic screening or microarray analyses.

As BMP2 and BMP4 protein share 83% sequence homology (Shore et al., 1998) and BMP4 has been identified as a pro-adipogenic factor in human preadipocytes (B. Gustafson & Smith, 2012), there would be merit in performing a head-to-head comparison of these two molecules to examine whether the latter exerts the same depot-specific effects on adipogenesis as the former in human subcutaneous abdominal and gluteal preadipocytes. Such an experiment could identify whether the depot-specific pro-adipogenic effects manifest through regional variation in SMAD1/5/8 signalling within preadipocytes or whether there is something unique to BMP2. Additionally, as BMP4 resistance mediated via increased *GREM-1* expression has been implicated in hypertrophic obesity (Birgit Gustafson et al., 2015), investigating whether the expression of BMP2-SMAD1/5/8 signalling components (including *GREM-1* or *GREM-2*) in subcutaneous abdominal and gluteal AT is associated with altered adipocyte size in relation to metabolic health would be appropriate; this analysis would ideally involve samples taken from individuals across a range of adiposity.

### 4.4.4 BMP antagonists as a determinant of depot-specific BMP action

Another potential mechanism underlying the depot-specific, pro-adipogenic action of BMP2 may involve the BMP antagonists GREM-1 and GREM-2. These molecules are secreted glycoproteins which bind BMPs to form heterodimers that prevent ligand-receptor interactions and subsequent downstream signalling (Wordinger, Zode, & Clark, 2008). Gremlin-mediated inhibition of BMP signalling is essential for the proper development of many tissues including the limbs (Capdevila, Tsukui, Rodriquez Esteban, Zappavigna, & Izpisua Belmonte, 1999) and lungs (Lu et al., 2001). GREM-1 has been identified as a potent negative regulator of BMP4-mediated adipogenesis (Birgit Gustafson et al., 2015). In addition to blocking BMP4 action, GREM-1 potently inhibits

BMP2-mediated osteogenesis in human bone marrow-derived mesenchymal stem cells (Hu, Sun, Gui, & Sui, 2017).

*GREM-1* and *GREM-2* expression was down-regulated early in differentiation (between days 0 - 4); this is presumably a permissive act that facilitates adipogenesis. However, the down-regulation of *GREM-1* expression was relatively delayed in gluteal cells. *GREM-2* expression was also greatly reduced early in differentiation but partially rebounded in gluteal cells from day 4 onwards. These expression patterns may inhibit BMP2 action, or at least confer an anti-adipogenic signal, specifically in gluteal cells. Previous data also indicate that *GREM-1* expression is higher in gluteal AT ((Pinnick et al., 2014); supplementary data). Evidently GREM-1 and GREM-2 present themselves as candidate molecules that have the potential to modulate BMP2 activity in a depot-specific manner and therefore warrant further investigation. This could involve examining whether over-expression or knockdown of *GREM-1* and/or *GREM-2* affects adipogenesis of human subcutaneous preadipocytes in a depot-specific manner.

In addition to well-established inhibitors of BMP signalling, the identification of ERFE as a depot-specific regulator of BMP2-SMAD1/5/8 signalling in preadipocytes is potentially fascinating. Not very much is currently known about ERFE except that it is a member of the C1q/TNF-related protein (CTRP) family of cytokines and is involved in iron homeostasis. ERFE is secreted by erythroid cells and enhances iron availability by suppressing the synthesis of hepcidin, a negative regulator of iron availability (Kautz et al., 2014). ERFE (also known as myonectin) has also been described as a myokine secreted by skeletal muscle that affects fatty acid metabolism in liver and AT (Seldin et al., 2012).

Previous data suggest that ERFE is a potent inhibitor of BMP4-SMAD1/5/8 signalling in hepatocytes, specifically HepG2 cells (Drakesmith et al., *unpublished data*). ERFE was found to selectively inhibit BMP2-SMAD1/5/8 signalling in abdominal preadipocytes according to both SMAD1/5/8 phosphorylation and *ID1* expression. While such data identify ERFE as a potential mediator of regional AT expansion that signals from energy sensing organs such as bone marrow and skeletal muscle, it remains uncertain whether the interaction between ERFE and AT actually occurs *in vivo* and under what circumstances.

Initial efforts to better understand ERFE biology have focused on developing a method to measure circulating ERFE protein so that systemic levels can be related to regional AT distribution phenotypes. Based on insights from the literature, it has also been proposed that ERFE may be pro-lipolytic as erythropoietin treatment (i.e. a potential stimulus for ERFE activation) in non-exercised individuals reportedly enhances liver fat content and reduces systemic insulin sensitivity (Christensen et al., 2013). The identification of ERFE serves as an important reminder of the extensive cross-talk between distinct tissues and highlights how supposedly disparate physiological processes may be more closely related than previously thought (e.g. iron metabolism and regional AT expansion). It will be fascinating to see how the role of ERFE in AT biology and body fat distribution develops.

### 4.4.5 BMP receptor subtypes as a determinant of depot-specific BMP action

Regional variation in the expression of type 1 BMP receptor subtypes, most notably in differentiated cells harvested on day 14, may also contribute to the depot-specific actions of BMP2. Previous data indicate that *BMPR1A* conveys a pro-adipogenic signal whereas *BMPR1B* is pro-osteogenic in murine stem cells (D. Chen et al., 1998). This pattern is mirrored in human cells; adipose-derived stem cells enriched for *BMPR1A* expression

exhibit a greatly enhanced adipogenic capacity compared to their *BMPR1A*-null counterparts (Zielins et al., 2016) whereas human adipose-derived stem cells enriched for *BMPR1B* expression display a greatly enhanced osteogenic capacity both *in vitro* and *in vivo* (McArdle et al., 2014). Consistent with the depot-specific, pro-adipogenic action of BMP2, abdominal cells harvested on day 14 expressed higher levels of *BMPR1A* but lower levels of *BMPR1B* compared to their gluteal counterparts. It would be interesting to examine whether preferential expression of *BMPR1A* and *BMPR1B* in AT is associated with adipocyte size and/or the mechanism by which AT expands upon overfeeding (i.e. hypertrophy vs. hyperplasia) in humans and mice.

On this basis, it follows that the profile of BMP receptor subtypes expressed at the cell surface may determine the nature of the signal transmitted within a given preadipocyte and the biological outcome that manifests. This is exemplified by data generated in a spinal cord injury model in which opposing effects on gliosis were driven by deficiency of *BMPR1A* and *BMPR1B* expression (Sahni et al., 2010). Overall, these data support the prevailing view that the wide range of biological outcomes associated with BMP signalling principally arises from complexity within BMP ligands and their cognate receptors (Mueller & Nickel, 2012). Further studies aimed at dissecting the highly complex and dynamic interaction between different BMP receptor subtypes and the associated intracellular signalling (including endogenous antagonists such as *SMAD6*) may provide insight into the depot-specific biology of BMP2 signalling in preadipocytes.

Insight into the BMP2-SMAD1/5/8 signalling may be provided through more detailed characterisation of patients with fibrodysplasia ossificans progressiva (FOP). This condition is caused by mutations within the *ACVR1* gene which encodes type 1 BMP receptors (Kaplan et al., 2009). Mutations causing FOP render type 1 BMP receptors constitutively active; the persistent activation of downstream SMAD1/5/8 signalling

drives progressive ossification (i.e. deposition of mineralised bone) of cartilage and muscle tissue. Having identified BMP2 as a novel depot-specific regulator of adipogenesis, it remains to be seen whether patients with FOP exhibit altered AT function and/or distribution.

It should be noted that K02288 was developed as an inhibitor of type 1 BMP receptor activity (Sanvitale et al., 2013) to be used in the treatment of FOP. While there might be merit in investigating whether K02288 treatment alters regional AT distribution in these patients, this molecule could also be used to investigate whether inhibition of BMP signalling modulates regional AT expansion upon overfeeding in humans and/or murine model systems. Based on the current data, it would be expected that inhibiting BMP2's depot-specific pro-adipogenic effects would promote metabolically-favourable expansion of gluteo-femoral AT upon overfeeding, potentially at the expense of abdominal AT. If this were the case, K02288 may represent the first in a new class of novel drugs that act in a genuinely depot-specific manner to induce metabolically beneficial AT re-distribution that may alleviate the likes of type 2 diabetes and non-alcoholic fatty liver disease.

### 4.4.6 BMP2-SMAD1/5/8 target genes may determine BMP2's depot-specific action

A key outcome for BMP2-mediated signalling is altered gene expression in the target cell, a process which is primarily controlled by phosphorylated SMAD1/5/8. A well-established transcriptional target of BMPs is *ID1* (Hollnagel et al., 1999); (Lopez-Rovira et al., 2002), a protein that modulates the binding of basic helix-loop-helix transcription factors to DNA to regulate gene expression (Benezra, Davis, Lockshon, Turner, & Weintraub, 1990). *Id1* has been shown to regulate the balance between cell proliferation

and differentiation in murine cells (Zebedee & Hara, 2001) but has more recently been implicated in AT biology (Patil et al., 2014).

It has been reported that *Id1* expression in murine 3T3-L1 cells is down-regulated early in differentiation via ubiquitin-mediated proteasomal degradation (Satyanarayana, Klarmann, Gavrilova, & Keller, 2012). Consistent with this, *Id1* knockdown was found to enhance adipogenesis. However, *Id1* over-expression did not impair adipogenesis as expected because excess *Id1* protein was targeted for proteasomal degradation. Evidently the relationship between *Id1* transcription and translation are complex and clearly warrant characterisation in murine and human cells alike.

Although the role of *ID1* in human adipogenesis is currently unclear, the observation that BMP2 increased *ID1* expression to a greater extent in abdominal preadipocytes compared to gluteal cells suggests that *ID1* may represent a potential mediator of the depot-specific, pro-adipogenic effects of BMP2 and thereby warrants further investigation. This could involve assessing adipogenesis after overexpressing or knocking down *ID1* in subcutaneous abdominal and gluteal preadipocytes. To identify other potential candidate effector genes involved in determining the depot-specific action of BMP2, microarray technology could be used to analyse the network of genes modulated by BMP2 treatment in abdominal and gluteal preadipocytes. The role of suitable candidates in modulating BMP2-SMAD1/5/8 signalling could then be investigated *in vitro* through gene overexpression and/or knockdown in subcutaneous abdominal and gluteal preadipocytes.

### 4.4.7 Epigenetic modifications as a determinant of depot-specific BMP action

*ID1* is only one of many genes regulated by BMP2-mediated signalling. As BMP2 activated SMAD1/5/8 phosphorylation in both abdominal and gluteal preadipocytes, it is probable that its depot-specific effects on gene expression and adipogenesis are largely

determined at the epigenetic level. It is well established that subcutaneous abdominal and gluteal AT exhibit striking depot-specific gene expression patterns (Rehrer et al., 2012); (Karastergiou et al., 2013) which are subject to regulation via epigenetic modifications, such as DNA methylation, which also vary in a depot-specific manner (Gehrke et al., 2013); (Pinnick et al., 2014). It is possible that the (depot-specific) epigenetic landscape of the target cell influences what genes BMP2 regulates, thereby determining the nature of BMP2 action. Performing genome-wide epigenetic profiling in conjunction with microarray analysis of human subcutaneous abdominal and gluteal preadipocytes before and after BMP2 treatment could facilitate the identification of transcriptional 'hotspots' (section 1.2.3) and the associated target genes whose altered expression underpins the depot-specific, pro-adipogenic effects of BMP2.

It is striking that activation of SMAD1/5/8 signalling in both abdominal and gluteal preadipocytes to approximately the same extent results in a binary pro-adipogenic signal that is only conveyed in abdominal cells. This divergence in response to the same stimulus suggests there must be a molecule or molecules that direct BMP2-SMAD1/5/8 signalling activity towards the pro-adipogenic machinery in abdominal but not gluteal preadipocytes. A candidate molecular switch determining whether BMP2-induced signalling exerts pro-adipogenic effect (or not) may be *Ahnak*. Murine data from *in vitro* and *in vivo* systems suggest that *Ahnak* represents a key mediator that links BMP2-Smad1 signalling to adipogenesis by positively regulating *Pparg* expression (Shin et al. 2016). It remains to be seen whether the human homologue of *Ahnak* is differentially expressed and/or regulated between abdominal and gluteal preadipocytes; if it is, it may therefore represent the key molecular switch that determines the depot-specific pro-adipogenic effects of BMP2-SMAD1/5/8 signalling in human subcutaneous preadipocytes.

Determining whether the human homologue of *Ahnak* is the key mediator would involve knocking down or over-expressing it in abdominal and gluteal preadipocytes and investigating whether the BMP2's pro-adipogenic effect loses its depot-specificity (i.e. a pro-adipogenic effect is also observed in gluteal cells upon *Ahnak* over-expression) or BMP2 ceases to exert any effect whatsoever (i.e. no pro-adipogenic effect in abdominal cells upon knock-down of *Ahnak* expression). If *Ahnak* proves to be a crucial player, a small molecule inhibitor of *Ahnak* could reduce abdominal adipogenesis to promote metabolically beneficial lower body AT accumulation. This outcome might also be achieved via artificial enhancement of *Ahnak* expression in gluteal preadipocytes, perhaps via retrovirus-mediated overexpression.

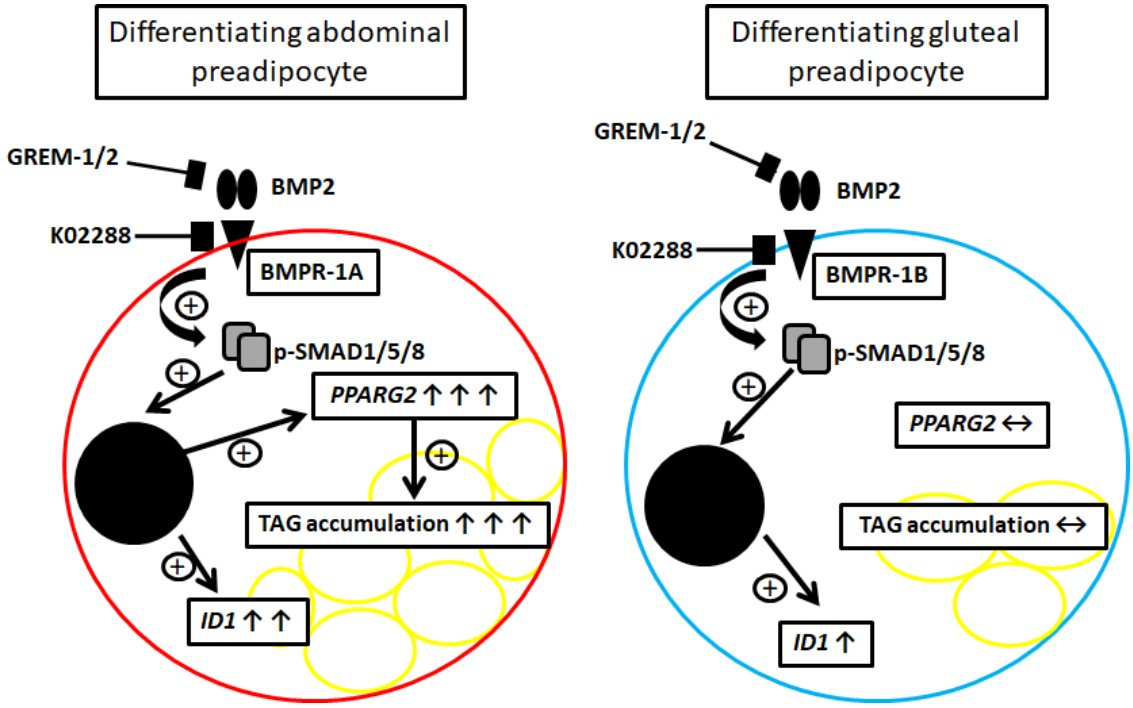
Another candidate determinant of BMP2's depot-specific, pro-adipogenic action could be *ZNF423* (the human homologue of *Zfp423*), the transcription factor expressed by preadipocytes which resides upstream of *PPARG2*. It has been reported that *Zfp423* regulates BMP4's pro-adipogenic effect in murine NIH 3T3 cells by co-ordinating the SMAD-dependent activation of *Pparg* expression (Gupta et al., 2010). Due to the high sequence homology between BMP2 and BMP4 (Shore et al., 1998), it is possible that differential expression of *ZNF423* underpins the striking depot-specific effects of BMP2. Given this possibility, *ZNF423* mRNA and protein levels should be measured in abdominal and gluteal preadipocytes to determine whether *ZNF423* represents the missing depot-specific link between SMAD1/5/8 signalling and adipogenesis in humans.

It should be considered that although the epigenetic profile of a target cell may direct BMP2 action, this relationship is reciprocal as it has been found that BMP2 can modify the epigenetic landscape to regulate gene expression. Indeed, BMP2 has been shown to promote osteogenesis at the expense of adipogenesis in murine C3H10T1/2 cells by down-regulating the expression of the key adipogenic transcription factor *C/ebpα* (Fan,

Tang, Zhang, & Dai, 2009). This effect was achieved by induction of DNA hypermethylation and histone 3 and 4 hypoacetylation within the *C/ebpα* promoter region (Fan et al., 2009). It seems plausible that BMP2 could modulate the expression of *C/EBPα* and other pro-adipogenic genes through similar mechanisms in human adipose-derived SVF cells in a depot-specific manner. Such an investigation could involve chromatin-immunoprecipitation and qPCR analysis of *C/EBPα* expression in subcutaneous abdominal and gluteal preadipocytes incubated with adipogenic medium in the presence or absence of exogenous BMP2 to determine whether BMP2 utilises epigenetic modifications to affect the expression of adipogenic genes.

*PPARG2* potentially represents another useful gene to study given the striking depot-specific effects of BMP2 exerted on its expression coupled with previous observations that this gene is regulated by epigenetic mechanisms such as DNA methylation and histone modification (Zych et al., 2013). A mounting body of evidence already points to the potential importance of epigenetic modifications in AT in both health and disease (Kasinska, Drzewoski, & Sliwinska, 2016). Evidently understanding the epigenetic mechanisms involved in co-ordinating (depot-specific) adipogenic processes and how they integrate stimuli such as BMP2 at the genome-wide level represents a fertile ground for future research (section 1.2.3).

**Figure 4.9 Potential determinants of BMP2's depot-specific pro-adipogenic action**




### 4.4.8 *BMP2* and *SMAD6* genetics

The results of a recent GWAS meta-analysis identified 49 loci associated with WHRadjBMI (Shungin et al., 2015). Amongst the hits were rs979012 (*BMP2*) and rs1440372 (*SMAD6*); within the genomic regions associated with regional AT distribution lay the genes encoding different components of the BMP-SMAD signalling pathway. It is notable that these two hits are linked to components which exert opposing effects on the same signalling pathway yet have the same overall direction of effect on WHRadjBMI. Closer inspection of the data, however, indicates that these loci are associated with differential expansion or retraction of AT; rs979012 (*BMP2*) was associated with increased waist circumference whereas rs1440372 (*SMAD6*) was associated with decreased hip circumference ((Shungin et al., 2015); supplementary table 4). This seemingly paradoxical situation highlights the complexity underlying regional

AT distribution and serves to indicate that genotype is only one factor which may influence this phenotype.

It was found that the rs979012/rs2145270 locus represents an eQTL associated with 15% lower *BMP2* expression in subcutaneous abdominal and gluteal AT. On the contrary, there was no evidence to suggest that the other GWAS locus was an eQTL for *SMAD6* expression in AT. No significant associations between rs979012/rs2145270 and DEXA-derived measures of regional adiposity were detected, although this probably reflects the small size of the OBB dataset. A previous study has also reported that rs979012 (*BMP2*) is significantly associated with increased BMI (Guiu-Jurado et al., 2016). Given these data, *BMP2* was taken forward for further study.

In addition to identifying genetic loci associated with measures of regional AT distribution (however crude), it is important to consider AT biology in a more holistic manner. For instance, adipocyte size is an important biomarker of AT function (section 1.4.4); adipocyte hypertrophy, particularly in visceral AT, seems to reflect an impaired capacity for subcutaneous AT to expand and is associated with deleterious alterations in various metabolic parameters (Laforest et al., 2015). The rs979012/rs2145270 (*BMP2*) genotype was associated with an adipocyte size phenotype; males homozygous for the WHRadjBMI-increasing C allele displayed a shifted cell size distribution favouring larger cells at the expense of smaller cells in abdominal AT. Unfortunately the small size of the dataset precluded any meaningful attempt to investigate whether genotype and/or cell size was associated with a corresponding alteration in metabolic status. Given the borderline-significant trend towards altered adipocyte size in male subcutaneous abdominal AT, recruiting more males with the CC genotype at the rs979012 locus would be sensible to increase the size of the adipocyte sizing dataset in order to repeat the analysis.

Genetic regulation of adipocyte lipolysis has also been investigated; basal and isoprenaline-stimulated lipolysis was measured in dispersed subcutaneous abdominal adipocytes from 789 individuals and related to genotype at 49 genomic loci associated with WHRadjBMI (Strawbridge et al., 2016). Only a handful of nominally significant associations between genotype and lipolysis were reported, none of which included *BMP2* or *SMAD6*. While this suggests that genetic loci associated with WHRadjBMI do not modulate adipocyte lipolysis (at least *in vitro*), it is likely that this dataset was simply too small to generate robust results. With respect to understanding how GWAS hits affect regional AT distribution, perhaps future studies would be more successful if a functional genetics approach were adopted. Based on the current results, it might be appropriate to investigate whether rs979012/rs2145270 (*BMP2*) genotype modulates the mechanism by which subcutaneous abdominal and gluteal AT expands (i.e. hypertrophy vs. hyperplasia) upon overfeeding in humans.

It remains unclear why the association between rs979012 genotype and adipocyte size exhibited sexual dimorphism, especially when the associations with AT *BMP2* expression and WHRadjBMI did not. If the effects of BMP2 are truly sexually dimorphic, then it is fortunate that BMP2 was characterised through *in vitro* experiments conducted on preadipocytes derived from a male participant. Based on the current data, it would be appropriate to repeat the eQTL analysis on a larger number of individuals so a better powered analysis can be performed where the data are separated by sex. While it is possible that BMP2 acts to promote abdominal AT expansion in males and that this effect is absent in females to facilitate lower body AT accumulation, a conclusive answer is required to determine whether BMP2 biology in AT is actually sexually dimorphic. As this investigation has focussed more on the cell biology of BMP2, it should be noted that

detailed genetic characterisation of the causal SNP associated with WHRadjBMI awaits; it is possible that this GWAS locus does not actually act through *BMP2*.

### 4.4.9 Concluding statements

This study confirmed prior observations and identifies BMP2 as a novel, depot-specific regulator of human (subcutaneous white) adipogenesis. *BMP2* is readily expressed in human AT and is secreted in a bioactive form by preadipocytes. However, as BMP2 protein was undetectable in plasma it likely acts as a paracrine factor within AT. Exogenous BMP2 activated SMAD1/5/8 signalling via type 1 BMP receptors in both subcutaneous abdominal and gluteal preadipocytes but only enhanced adipogenesis in the former depot.

The exact molecular mechanism underpinning the striking depot-specific action of BMP2 remains uncertain but could involve regional variation in the expression of BMP receptor subtypes, endogenous BMP antagonists and effector genes as well as the epigenetic landscape of the target cells. The importance of BMP2-SMAD1/5/8 signalling in the regulation of body fat distribution is supported by results of a recent GWAS meta-analysis that identified that the BMP2 gene resides within a genomic region that is significantly associated with central obesity; this locus also represents an eQTL for *BMP2* expression in AT. Overall these data identify BMP2-SMAD1/5/8 signalling as a crucial regulatory system involved in (regional) AT biology in humans.

# Chapter 5

# De-differentiated adipocytes exhibit an enhanced adipogenic potential and retain depot-specific properties

## 5.1 Introduction

During the process of this thesis it was observed that immortalised abdominal and gluteal preadipocytes differentiated in adipogenic medium supplemented with fatty acids (and BMP2) became explosively lipid-laden and buoyant, resulting in many cells detaching from the monolayer. Capitalising on this scenario, the ceiling culture technique was used to isolate this floating cell population (Sugihara, Yonemitsu, Miyabara, & Yun, 1986). After adhering to the tissue culture flask the captured cells de-differentiated (DFAT), lost their lipid content and regained a proliferative fibroblast-like phenotype, thereby becoming immortalised DFAT cells. As these cells had been selected based on a core feature of adipocyte biology (i.e. buoyancy owing to lipid accumulation), it was postulated that the DFAT population might be enriched for some type of progenitor cell residing within the heterogeneous stromovascular fraction (SVF). It has been reported that DFAT cells typically exhibit a greater adipogenic capacity compared to the mixed SVF population (Asada et al., 2011).

It has been acknowledged that the cellular models currently available to study (human) adipogenesis and other aspects of (depot-specific) AT biology *in vitro* are not ideal; primary SVF cells exhibit a limited renewal capacity and high degree of inter-individual variation whereas the adipogenic potential of immortalised SVF cells typically diminishes as the passage number increases (Ruiz-Ojeda, Ruperez, Gomez-Llorente, Gil, & Aguilera, 2016). However, the generation of immortalised abdominal and gluteal DFAT cells from SVF cell-lines which had been previously validated for the study of depot-specific human AT cell biology (Todorčević et al., 2017) provided a unique opportunity to evaluate the characteristics of a new but related cell system against an established model.

With respect to the methods used to isolate adipogenic cells/ASCs residing within the heterogeneous SVF population, it is possible to sort cells based on the expression profile of surface markers. As selecting cells based on surface marker expression relies on a pre-conceived notion of what markers are important it carries the inherent risk that relevant cells might be omitted. While a profile of cell surface markers defining a population of cells that exhibit these multi-potent capabilities has been developed for regenerative medicine (Bourin et al., 2013), there is no such consensus with respect to the molecular identity of the preadipocyte in humans (Cawthorn, Scheller, & MacDougald, 2012).

With mounting evidence suggesting that each anatomically distinct AT depot has a unique developmental origin characterised by a heterogeneous population of progenitor cells that exhibit depot-specific gene expression and epigenetic profiles, it seems increasingly likely that there is no single preadipocyte (section 1.2.1). Furthermore, it is notable that while human ASCs from abdominal subcutaneous and visceral AT exhibit distinct differentiation capacities and gene expression profiles they cannot be distinguished based on their cell surface marker expression (B. Kim et al., 2016).

An alternative approach to the selection process involves distinguishing cells on the basis of their physical properties, such as density/buoyancy. The routine processing of an AT biopsy exploits these properties; when a dispersed AT biopsy is centrifuged, the SVF cells form a dense pellet at the bottom of the tube whereas the lipid-laden adipocytes float (Collins et al., 2010); (Darimont & Mace, 2003). The cells used to generate the DFAT population in this study were also selected based on a physical property (i.e. buoyancy) which manifested from a relevant physiological characteristic (i.e. accumulation and storage of lipid in a unilocular TAG droplet). As a result, it was hoped that the ceiling culture process would yield a cell population that would exhibit most/all of the desired properties expected from a cellular model of AT biology.

An ideal cellular model system of AT should: readily and reproducibly perform adipogenesis (with this ability not diminishing with increasing passage); exhibit a high lipogenic capacity; respond appropriately to lipolytic stimuli; and have an unlimited proliferative capacity. An additional but important criterion pertinent to this thesis mandates that cells should also retain an intrinsic memory of their anatomical origin, whether that manifests as depot-specific functional properties (Tchkonia et al., 2013), regional variation in gene expression (Karastergiou et al., 2013) and/or depot-specific epigenetic profiles (Gehrke et al., 2013); (Pinnick et al., 2014).

Aside from using ASCs to study (depot-specific) human AT biology, interest is growing in these cells owing to their potential to transform regenerative medicine. Mounting data indicate that ASCs exhibit the ability to undergo adipogenesis, osteogenesis, chondrogenesis and myogenesis (Frese, Dijkman, & Hoerstrup, 2016); they can also form neural-like (S. Jang, Cho, Cho, Park, & Jeong, 2010) and hepatocyte-like cells (Fu, Deng, et al., 2016). The multi-potent differentiation capabilities of ASCs reportedly vary in a depot-specific manner; cells from subcutaneous gluteal AT exhibit a greater ability to undergo osteogenesis compared to their abdominal counterparts (Iwen et al., 2014); (Levi et al., 2010).

The yield and differentiation potential of ASCs can be affected by age, sex and BMI (Yang et al., 2014); obesity negatively affects the ability of ASCs to perform *in vitro* adipogenesis (Tchkonia et al., 2013) and osteogenesis (Strong et al., 2016); (Oliva-Olivera et al., 2015). DFAT cells have not been studied as extensively as SVF cells but it has been reported that they can perform adipogenesis, osteogenesis and chondrogenesis (Matsumoto et al., 2008); (Shen, Sugawara, Yamashita, Ogura, & Sato, 2011) and even cardiomyogenesis (Jumabay & Bostrom, 2015).

Having capitalised on a unique opportunity to generate a distinct population of immortalised cells derived from a cell-line model that has been validated for the study of depot-specific human AT biology, a series of experiments was performed to compare the biological characteristics of the immortalised DFAT cells to the immortalised SVF cells from which they derive. The main objective was to determine whether the immortalised DFAT cells represent a valid model system to study depot-specific AT biology. This primarily involved comparing the ability of the immortalised SVF and DFAT cells to perform adipogenesis and lipolysis. Additional experiments aimed to characterise whether the depot-specific properties exhibited by the immortalised abdominal and gluteal SVF cells (particularly gene expression profiles) had been retained during the de-differentiation process and were displayed by the immortalised DFAT cells.

### 5.1.1 Aims

- Compare the ability of immortalised DFAT cells to perform adipogenesis to the immortalised SVF cells from which they derive by measuring TAG accumulation and adipogenic gene expression via qPCR.
- Evaluate whether the differentiated DFAT cells exhibit the functional properties expected of mature adipocytes (principally lipolytic activity) and compare them to their corresponding immortalised SVF counterparts.
- Investigate whether immortalised DFAT cells retain an intrinsic memory of their anatomical origin by measuring gene expression profiles exhibited by the immortalised SVF cells via qPCR.

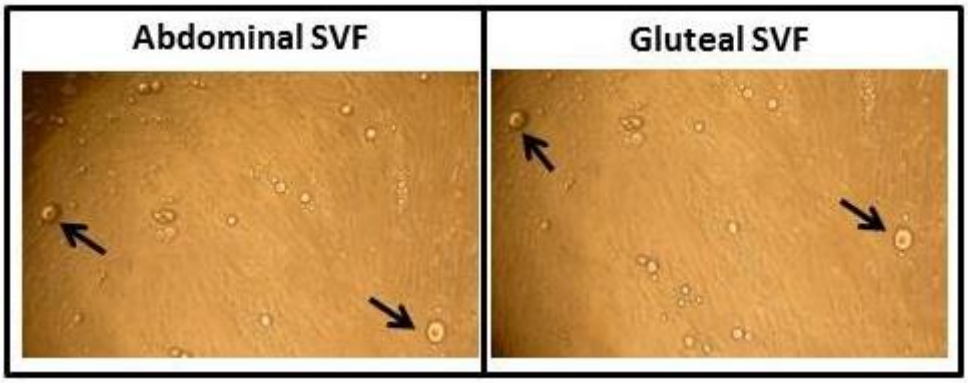
## 5.2 Methods

### 5.2.1 Generation of DFAT cells via ceiling culture

Immortalised SVF cells were differentiated according to a modified protocol described in section 2.2.4. Adipogenic medium was supplemented with 2µM troglitazone (Sigma) throughout the time-course. An 180µM fatty acid mixture comprising 75µM oleate (Sigma); 65µM palmitate (Sigma) and 40µM linoleate (Sigma) was added during days 7 - 14. The ratio of fatty acids in this mixture reflects the proportion of the three major fatty acids present in human AT (Pinnick et al., 2012) and is more physiologically relevant than the administration of palmitate alone, which can cause cell injury (Collins et al., 2010). Fatty acids were reconstituted in Dulbecco's modified Eagle's medium / Nutrient F-12 Ham (v/v, 1:1; Sigma) containing 10% (essentially fatty acid-free) bovine serum albumin (BSA; Sigma).

Medium containing buoyant, lipid-laden cells on day 12/14 of differentiation was transferred to Nunc Lab-tech slide flasks (ThermoFisher Scientific) filled with growth medium. A representative image of the (unilocular) lipid-laden cells (highlighted by arrows) transferred to the slide flasks is provided in Figure 5.1.

**Figure 5.1 – Representative image of lipid-laden SVF cells used for ceiling culture**



After the cells were transferred, each flask was inverted and left for 3 - 5 days to allow the cells to adhere. After 5 - 10 days, adherent cells had de-differentiated/reverted to a fibroblast-like state and started proliferating (Sugihara et al., 1986). Growth medium was changed and each flask was returned to the upright orientation. The DFAT cells were then expanded to make stocks in preparation for experiments. Note that the DFAT cells are also immortalised as they derive from immortalised SVF cells expressing hTERT and HPV16-E7 (section 2.2.1). All subsequent experiments involving DFAT and SVF cells were performed in parallel to allow direct comparison of these populations.

### 5.2.2 Culture and adipogenic differentiation of immortalised SVF and DFAT cells

Immortalised SVF and DFAT cells were cultured as outlined in section 2.2.2. Adipogenic differentiation was performed in immortalised SVF and DFAT cells using glucose-only differentiation medium as outlined in section 2.2.4.

### 5.2.3 Measurement of intracellular TAG content

Determination of cellular TAG content was performed as outlined in section 2.4.

### 5.2.4 Measurement of lipolysis in differentiated preadipocytes *in vitro*

After 14 days culture in adipogenic medium, cells were incubated in serum-free medium comprising Dulbecco's modified Eagle's medium / Nutrient F-12 Ham (v/v, 1:1; Sigma) supplemented with 2mM L-glutamine (Invitrogen) and 100 units/ml penicillin and 100µg/ml streptomycin (Invitrogen) for 48 hr. On the day of the lipolysis experiment, cells were incubated for 1 hr in Krebs-Ringers-HEPES (KRH) solution (1ml/well) comprising: 120mM NaCl; 4.8mM KCl; 2.5mM $CaCl_2.2H_2O$; 1.2mM $KH_2PO_4$; 1.2mM; $MgSO_4.7H_2O$; 15mM $NaHCO_3$; 10mM HEPES (pH 7.0 - 7.6); 6mM Glucose; and 3.5% essentially fatty acid-free BSA (Sigma).

After the initial equilibration, cells were incubated in fresh KRH which was collected after 2 hr (basal sample). The cells were then incubated in KRH containing 10nM isoproterenol (Sigma) and/or 100nM insulin (Invitrogen). After a further 2 hr, the KRH was collected (experimental sample) and cells were harvested for TAG as described in section 2.4. A sample of each KRH solution was taken as a blank for subsequent analysis. All samples were stored immediately at -30°C after collection.

The concentration of free glycerol present in KRH samples and blanks was determined on a 650 ILAB Clinical Chemistry System (Instrumentation laboratories) using an enzymatic glycerol detection assay (Randox) calibrated to standards diluted in deionised water. Free glycerol was used as an index of lipolytic activity as it is not re-used by adipocytes (Steinberg, Vaughan, & Margolis, 1961). Free glycerol was normalised to the concentration of protein in the corresponding sample (determined using the DC protein assay kit against BSA standards; Bio-Rad) and reported as nanomoles glycerol/milligram protein.

### 5.2.5 Osteogenic differentiation

To measure osteogenic differentiation, osteogenic gene expression was analysed via qPCR (section 5.2.8) and mineralisation of the ECM was quantified via Alizarin red staining (ARS; section 5.2.6) normalised to cellular DNA content (section 5.2.7); cells were seeded in separate wells and treated in parallel before being harvested for gene expression, DNA extraction and ARS analysis. Cells were seeded onto 6 well plates (2 x $10^5$ cells/well), grown to confluence and incubated for 14 days in standard osteogenic medium (Jaiswal, Haynesworth, Caplan, & Bruder, 1997) comprising Dulbecco's modified Eagle's medium / Nutrient F-12 Ham (v/v, 1:1; Sigma) supplemented with 10% foetal bovine serum (Labtech); 2mM L-glutamine (Invitrogen); 100 units/ml penicillin

and 100µg/ml streptomycin (Invitrogen); 100nM dexamethasone (Sigma); 50µM L-ascorbic acid-2-phosphate (Sigma); and 10mM β-glycerophosphate (Sigma). Osteogenic medium was changed on days 2, 4, 7, 10 and 12.

### 5.2.6 Alizarin red staining and quantification

After 14 days osteogenic differentiation medium was removed, the cells were washed with PBS and then fixed in 4% paraformaldehyde (PFA) for 15 min. The PFA was then discarded and the cells were washed 3 times with deionised water before ARS solution (Merck-Millipore) was added (1ml/well). Cell culture plates were shaken (90 rpm) at room temperature for 20 min before unincorporated dye was aspirated and washed away with deionised water. Cells were photographed at this stage for the qualitative assessment of ECM mineralisation.

To extract and quantify incorporated dye, 800µl/well 10% (v/v) acetic acid (Sigma) was added and the plates were shaken (90 rpm) for 30 min at room temperature. Cells were scraped and the slurry transferred to a clean tube. Each sample was vortexed for 30 sec, heated (85°C for 10 min) and incubated on ice for 5 min. The cell slurries were spun (20000 x *g* 15 min at 4°C) before 500µl supernatant were transferred to a clean tube and 200µl 10% (v/v) ammonium hydroxide (ThermoFisher Scientific) were added. After mixing, the solution was transferred to a transparent 96-well assay plate (150µl in triplicate). Absorbance at 405nm was measured using a spectrophotometric VersaMax tuneable plate reader (Molecular Devices). ARS was normalised by dividing the colorimetric data (arbitrary units) by the corresponding DNA concentration.

### 5.2.7 DNA extraction from cell lysates

Whole cell lysates were harvested in lysis buffer (1% IGEPAL CA-630; 150mM NaCl; 50mM Tris-HCl, pH 8.0). An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10mM Tris, pH 8.0, 1mM EDTA (Sigma) was added to each lysate before each sample was mixed and spun (12000 x *g* for 1 min at room temperature). The aqueous supernatant was transferred to a clean tube and an equal volume of chloroform was added before the solution was mixed and spun (12000 x *g* for 1 min at room temperature). The aqueous supernatant was then transferred to a clean tube and an equal volume of 5M ammonium acetate was added plus 2.5 times the total volume of 100% ethanol and 2µl glycoblue (to facilitate identification of the nucleic acid pellet; Invitrogen). The solution was mixed thoroughly and incubated on ice for 15 min.

To collect the DNA, the chilled samples were spun (12000 × g for 20 min at 4 °C) and the ethanol was aspirated. The DNA pellets were washed twice in 80% ethanol and collected by centrifugation (12000 × *g* for 3 min at 4 °C). After aspirating the ethanol the pellets were air-dried at room temperature before being re-suspended in nuclease-free $H_2O$. The concentration of DNA was determined using a NanoDrop spectrophotometer (Thermo Scientific).

### 5.2.8 Gene expression analysis

Extraction of total RNA from immortalised SVF and DFAT cells was performed as described in section 2.3.1; cDNA synthesis as in section 2.3.2; and qPCR as in section 2.3.3. The following TaqMan Assays-on-Demand were used: *ADIPONECTIN* (Hs00605917_m1); *ALKALINE PHOSPHATASE* (Hs01029144_m1); *BGLAP/OSTEOCALCIN* (Hs01587814_g1); *FATTY ACID SYNTHASE*/*FASN* (Hs00188012_m1); *HOTAIR*; (Hs03296631_m1); *PERILIPIN-1* (Hs00160173_m1);

*PPARG2* (Hs01115510_m1); *PPIA* (Hs99999904_m1); *SP7/OSTERIX*; (Hs01866874_m1); and *TBX5 (*Hs01052567_m1).

### 5.2.9 Data analysis and statistics

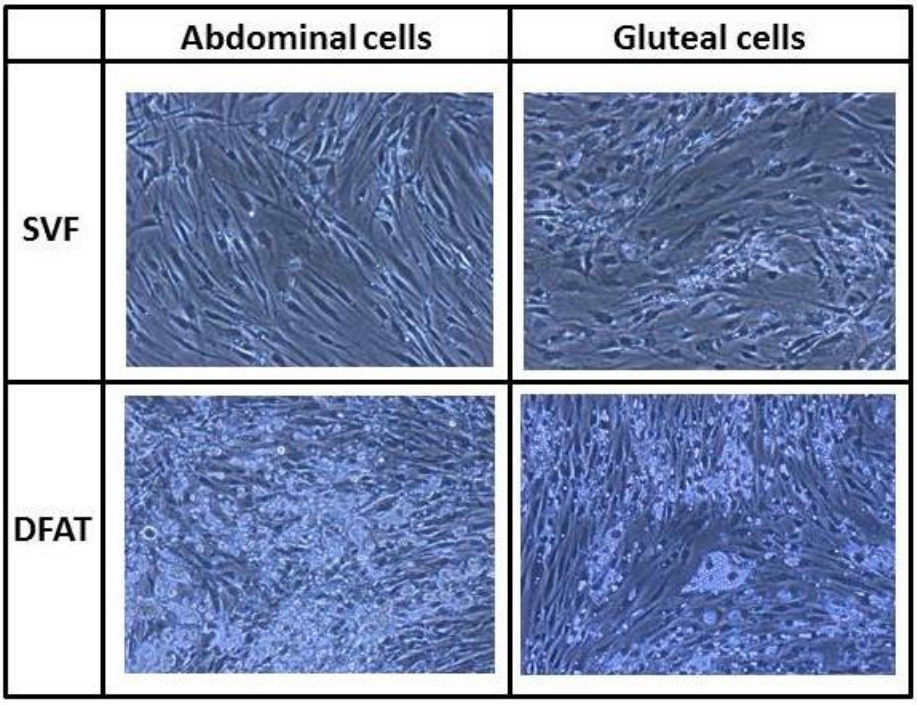
Data are presented as means ± SEM. Statistical analyses were performed in SPSS Statistics version 22 (IBM). Statistical tests used were as described in section 2.7 Differences were considered statistically significant at $P<0.05$.

## 5.3 Results

### 5.3.1 DFAT cells exhibit a greatly enhanced adipogenic potential

To investigate the ability of the immortalised SVF and DFAT cells to perform adipogenesis, both cell populations were cultured in adipogenic medium for 14 days in parallel. As highlighted in Figure 5.2, the DFAT cells accumulated substantial amounts of lipid and displayed a more rounded morphology. Conversely, adipogenesis in the SVF cells occurred in a patchy, non-uniform manner populated by 'islands' of differentiated cells that appeared to contain significantly less TAG compared to their DFAT counterparts.

**Figure 5.2 Differentiated immortalised abdominal and gluteal SVF and DFAT cells**




During the 14 day differentiation time-course, the expression of *PPARG2* followed a distinct cell type-specific pattern (Figure 5.3A). *PPARG2* expression started to increase much earlier in DFAT compared to SVF cells (day 4 vs. day 7) and reached a much higher level by the end of the time-course. Indeed, the expression of *PPARG2* (as well as

*ADIPONECTIN* and *PERILIPIN-1*; data not shown) on day 14 was 3 - 5 fold greater in DFAT compared to SVF cells in both depots (Figure 5.3B).

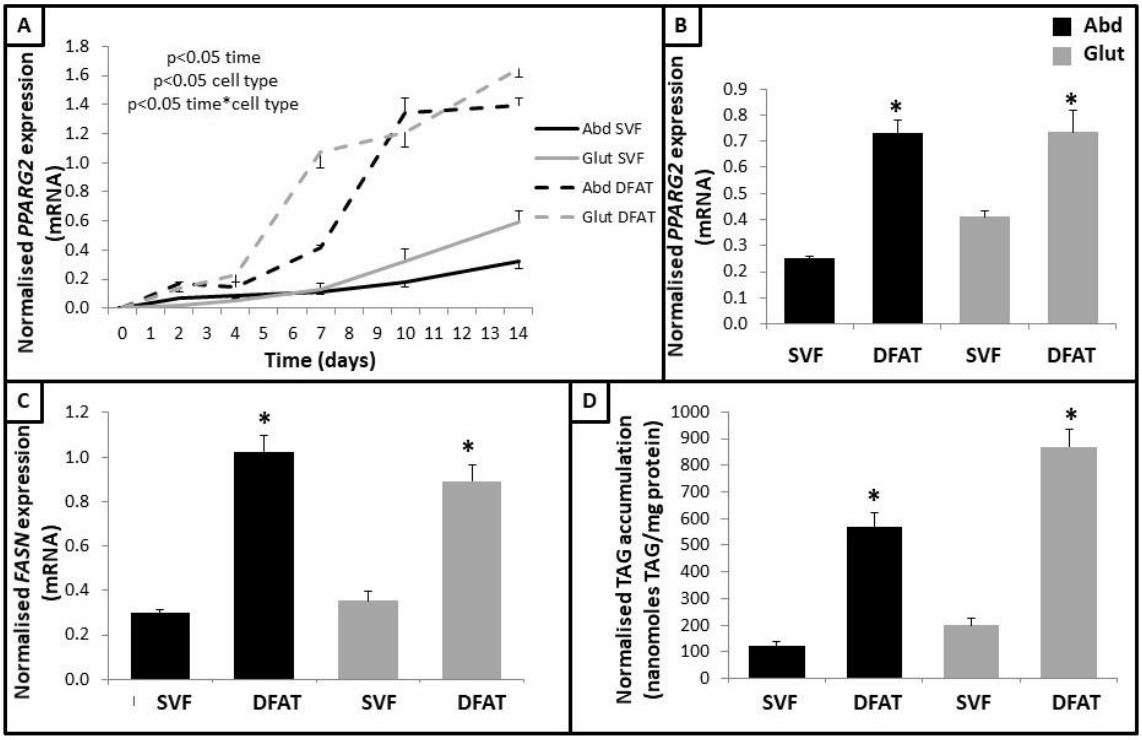



**Figure 5.3 DFAT cells exhibit a greatly enhanced adipogenic potential.** A - *PPARG2* mRNA levels in immortalised abdominal (Abd) and gluteal (Glut) SVF and DFAT cells during culture for 14 days in adipogenic medium (n=3). Data analysed via repeated measures ANOVA. B - *PPARG2* mRNA levels (normalised to *PPIA*); fatty acid synthase (*FASN*) mRNA levels (normalised to *PPIA*); D - Triacylglycerol (TAG) accumulation in SVF and DFAT cells after culture for 14 days in adipogenic medium. Data presented as mean ± SEM. Data in panels B, C and D analysed via ANOVA; *P<0.05 compared to SVF from same depot.

*FASN* expression was also much more highly expressed in DFAT cells (Figure 5.3C), consistent with the much greater TAG accumulation observed in these cells (Figure 5.3D). As all of the cells were differentiated in glucose-only medium, the TAG harvested at day 14 was solely the product of *de novo* lipogenesis (DNL; (Collins et al., 2011)). Together these data suggest that the DFAT cells are a uniform, highly adipogenic population that exhibits an impressive capacity to perform DNL.

### 5.3.2 Differentiated SVF and DFAT cells exhibit lipolytic activity

Having examined the capacity of the immortalised SVF and DFAT cells to perform adipogenesis, the next aim was to characterise the ability of the differentiated cells to perform lipolysis.

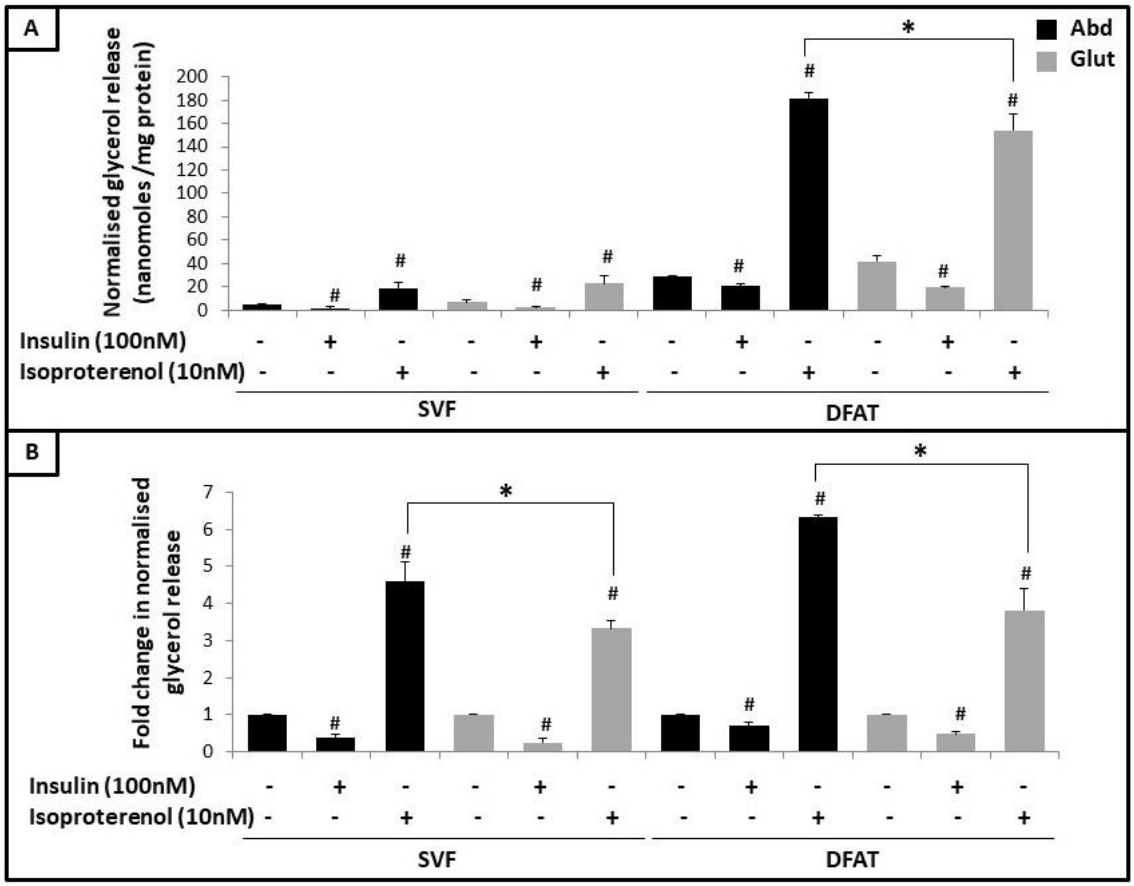



**Figure 5.4 Differentiated SVF and DFAT cells exhibit lipolytic activity.** A - Normalised glycerol release (raw data); B - Fold change in glycerol release by immortalised abdominal (Abd) and gluteal (Glut) SVF and DFAT cells during incubation with KRH supplemented with insulin (100nM), isoproterenol (10nM) or vehicle for 2 hr after culture for 14 days in adipogenic medium (n=3). Data presented as mean ± SEM; data analysed via ANOVA; *P<0.05 compared to abdominal cells of same type; #P<0.05 compared to untreated cells of same type from the same depot.

Both differentiated immortalised SVF and DFAT cells exhibited lipolytic activity that responded to physiological stimuli in the appropriate manner (Figure 5.4). While the absolute level of lipolysis was much greater in DFAT cells for each condition studied (Figure 5.4A), the fold change associated with insulin or isoproterenol (a non-selective β-adrenergic agonist) treatment was similar in both cell types. This probably reflects the

fact that DFAT cells accumulate more TAG during differentiation and so generate a larger output upon stimulation.

Insulin (100nM) suppressed lipolysis in both differentiated SVF and DFAT cells but there was a non-significant trend favouring greater suppression in gluteal SVF and DFAT cells (Figure 5.4B); insulin induced 60% suppression in abdominal SVF cells compared to 75% suppression in gluteal SVF cells and 30% suppression in abdominal DFAT cells compared to 50% in gluteal DFAT cells. Both cell types also responded appropriately to activation of beta-adrenoceptors. Treatment with 10nM isoproterenol robustly stimulated lipolysis (4 - 6 fold compared to basal) in both cell types; this occurred to a significantly greater extent in abdominal SVF and DFAT cells compared to their gluteal counterparts.

### 5.3.3 DFAT cells retain their depot-specific gene expression pattern

It is well-established that AT and its constituent SVF cells exhibit depot-specific gene expression (Karastergiou et al., 2013) and epigenetic profiles (Gehrke et al., 2013); (Pinnick et al., 2014). As the DFAT cells represent a purified population of ASCs, it was not clear whether the depot-specific differences in gene expression, for example, would be retained. Therefore, the expression of several genes known to be differentially expressed between subcutaneous abdominal and gluteal AT was measured in proliferating and differentiated immortalised SVF and DFAT cells.

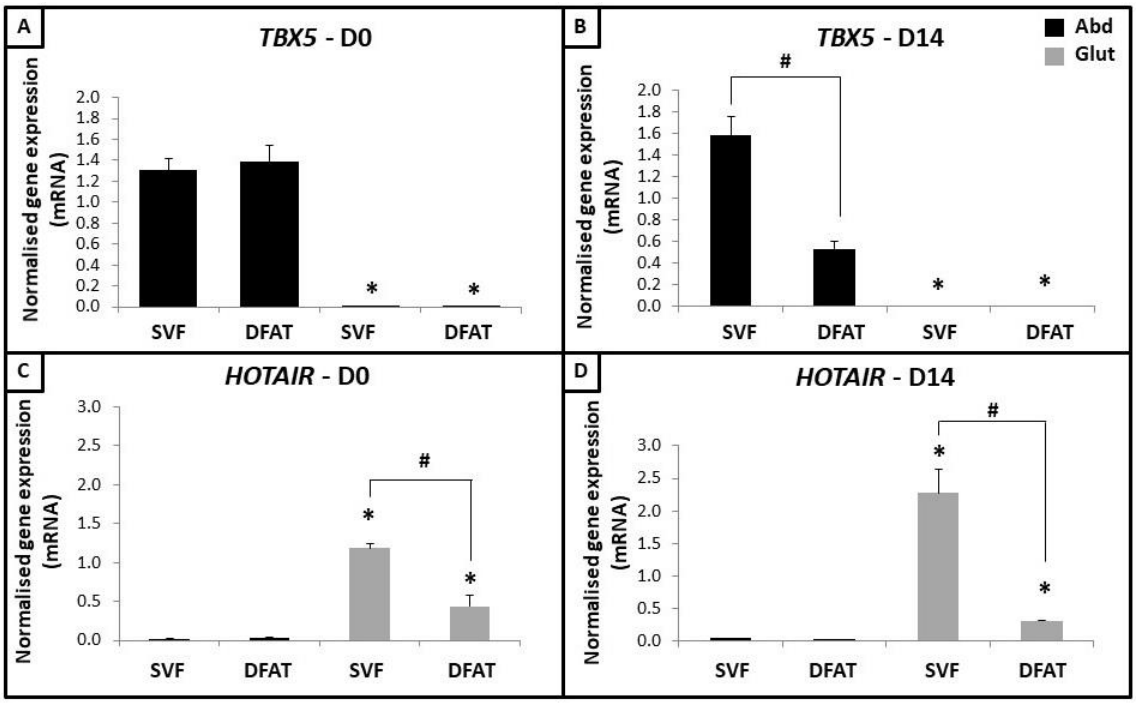



**Figure 5.5 DFAT cells retain their depot-specific gene expression pattern.** A - *TBX5* mRNA levels (normalised to *PPIA*) in proliferating (Day/D 0) immortalised abdominal (Abd) and gluteal (Glut) SVF and DFAT cells. B - *TBX5* mRNA levels in differentiated (D14) immortalised SVF and DFAT cells during culture for 14 days in adipogenic medium. C - *HOTAIR* mRNA levels (normalised to *PPIA*) in proliferating (D0) immortalised SVF and DFAT cells. D - *HOTAIR* mRNA levels in D14 immortalised SVF and DFAT cells (n=3). Data presented as mean ± SEM; data analysed via ANOVA with post-hoc T-tests; *P<0.05 compared to Abd cells of the same type; #P<0.05 compared to the other cell type from the same depot.

It was found that the depot-specific expression pattern of *TBX5*, a differentially expressed transcriptional regulator of adipogenesis (Pinnick et al., 2014), was broadly retained. *TBX5* (Figure 5.5A) was expressed to a similar level in proliferating abdominal DFAT and SVF cells but was undetectable in gluteal cells. Upon differentiation, however, *TBX5* expression was significantly reduced in abdominal DFAT cells compared to the SVF cells from which they derive (Figure 5.5B). *TBX5* expression was undetectable in differentiated gluteal SVF and DFAT cells at both time points.

The depot-specific expression pattern of *HOTAIR*, one of the most differentially expressed genes between subcutaneous abdominal and gluteal AT (Pinnick et al., 2014); (Divoux et al., 2014), was also retained after de-differentiation. *HOTAIR* expression was

only detectable in proliferating (Figure 5.5C) and differentiated abdominal cells (Figure 5.5D) although expression in DFAT cells was significantly lower compared to the corresponding SVF cells at each time point. Overall these data suggest that depot-specific gene expression patterns are broadly maintained after de-differentiation; variation in gene expression between abdominal/gluteal SVF and DFAT cells probably reflects differences in the cellular composition of each population.

### 5.3.4 Cell expansion during differentiation is positively correlated with adipogenic endpoints

During the head-to-head comparisons of SVF and DFAT cells involving their differentiation in parallel, it was consistently observed that despite seeding the same number of cells at the outset of an experiment, DFAT cells not only differentiated better but also yielded significantly greater amounts of cellular material (i.e. TAG, protein and RNA). Many studies in murine 3T3-L1 cells have identified mitotic clonal expansion (MCE) as a necessary process required for successful adipogenesis (section 1.2.5; (Q. Q. Tang et al., 2003); (Q. Q. Tang & Lane, 2012)). However, this process has not been studied extensively in human cells.

An initial study was performed to see how cellular protein content related to cellular DNA content. As no significant depot-specific differences within each cell type were detected (data not shown), data for abdominal and gluteal cells were pooled. A strong positive correlation between cellular protein and DNA was detected in both SVF and DFAT cells (Figure 5.6A) thus indicating that cellular protein content increases proportionally with cell number and so could be used as a valid proxy for cell number. This close relationship also suggests that the increased amount of protein harvested from DFAT cells after differentiation was not due to cellular hypertrophy. Given this, an experiment was

performed to investigate whether cell number changed in a cell type-dependent manner during differentiation.

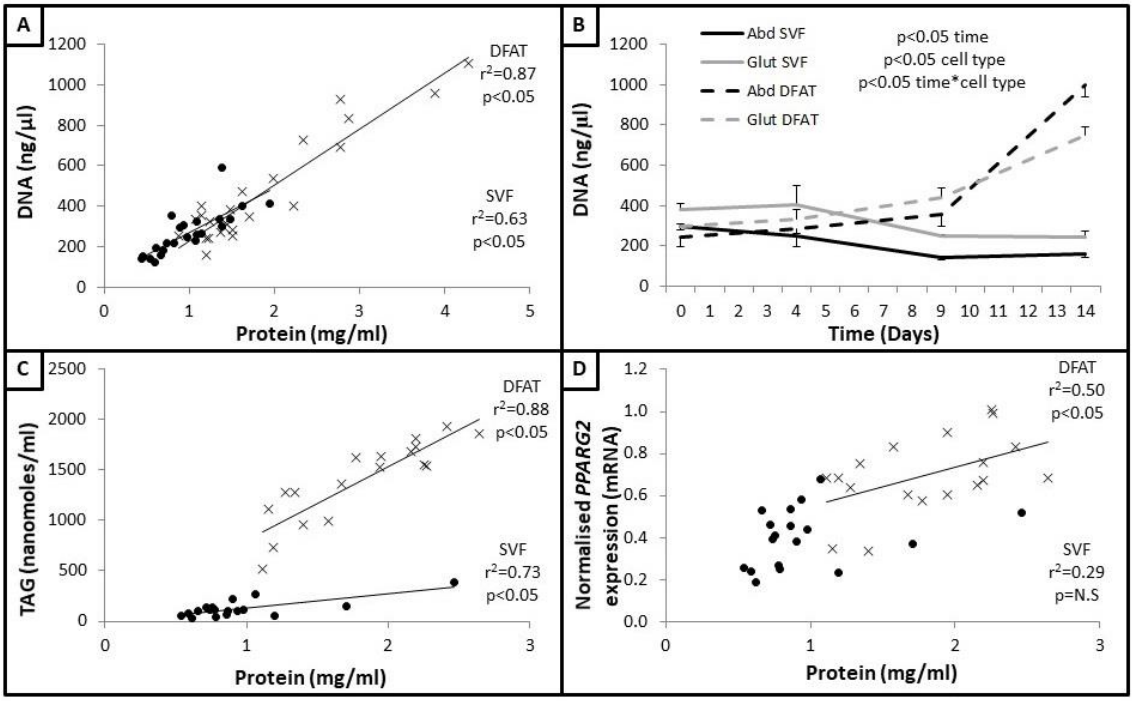



**Figure 5.6 Cell expansion during differentiation is positively correlated with adipogenic endpoints.** A - Pearson's correlation analysis of the concentration of cellular protein and DNA in abdominal and gluteal SVF (n=24) and DFAT cells (n=24). B - DNA concentration of SVF and DFAT cells during culture for 14 days (D) in adipogenic medium. Data analysed via repeated measures ANOVA. C - Pearson's correlation analysis of the concentration of triacylglycerol (TAG) and cellular protein harvested from SVF and DFAT cells after culture for 14 days in adipogenic medium (n=36; 18 SVF, 18 DFAT). D - Pearson's correlation analysis of *PPARG2* mRNA levels (normalised to *PPIA*) and cellular protein harvested from SVF and DFAT cells 14 day culture in adipogenic medium (n=36; 18 SVF, 18 DFAT).

The same number of SVF and DFAT cells were seeded and differentiated in parallel before being harvested at various points during the adipogenic time-course (Figure 5.6B). Cellular DNA content (and protein; data not shown) was found to change dynamically over the 14 day adipogenic time-course in a cell type-specific (but not depot-specific) fashion according to analysis via repeated measures ANOVA. The level of SVF cellular protein started to decline after day 4 with the yield at day 14 being approximately half that at the outset of the experiment. Conversely, the number of DFAT cells remained

relatively stable until day 9 when a robust increase occurred to yield 2 - 3 times the amount of cells at day 14 compared to day 0.

Consistent with earlier observations, the DFAT cells differentiated well and yielded significantly larger amounts of DNA, protein and TAG. To investigate whether these parameters were related, correlation analysis was performed between various adipogenic endpoints and indices of cell number at day 14. A significant positive correlation was detected between D14 protein (and DNA; data not shown) and TAG content in both cell types (Figure 5.6C). However, the slope of the line was far steeper in DFAT compared to SVF cells, indicating that DFAT cells accumulate more TAG than SVF cells for a given increase in cell number. In addition, a significant positive relationship was also detected between D14 protein levels (and DNA; data not shown) and *PPARG2* expression in DFAT (but not SVF) cells (Figure 5.6D).

Together these data suggest that human ASCs perform MCE during adipogenesis; the extent to which this process occurs is probably determined by the proportion of ASCs in the cell population; and that the extent of MCE predicts the quality of differentiation. More detailed investigations of cell proliferation during differentiation, potentially involving Ki67 staining (Scholzen & Gerdes, 2000) and/or analysis of cell cycle regulator expression/activity (Barnum & O'Connell, 2014) are certainly warranted.

### 5.3.5 DFAT cells exhibit a greater osteogenic differentiation capacity compared to SVF cells

ASCs represent an increasingly attractive cell population for a number of tissue engineering applications due to their multi-potent differentiation capabilities (Frese et al., 2016). Given this, an experiment was performed to characterise the ability of SVF and DFAT cells to perform osteogenesis using an established protocol (Jaiswal et al., 1997).

Osteogenesis was primarily evaluated via ARS, which stains calcium-containing ECM. The expression of specific osteogenic marker genes was also measured via qPCR.

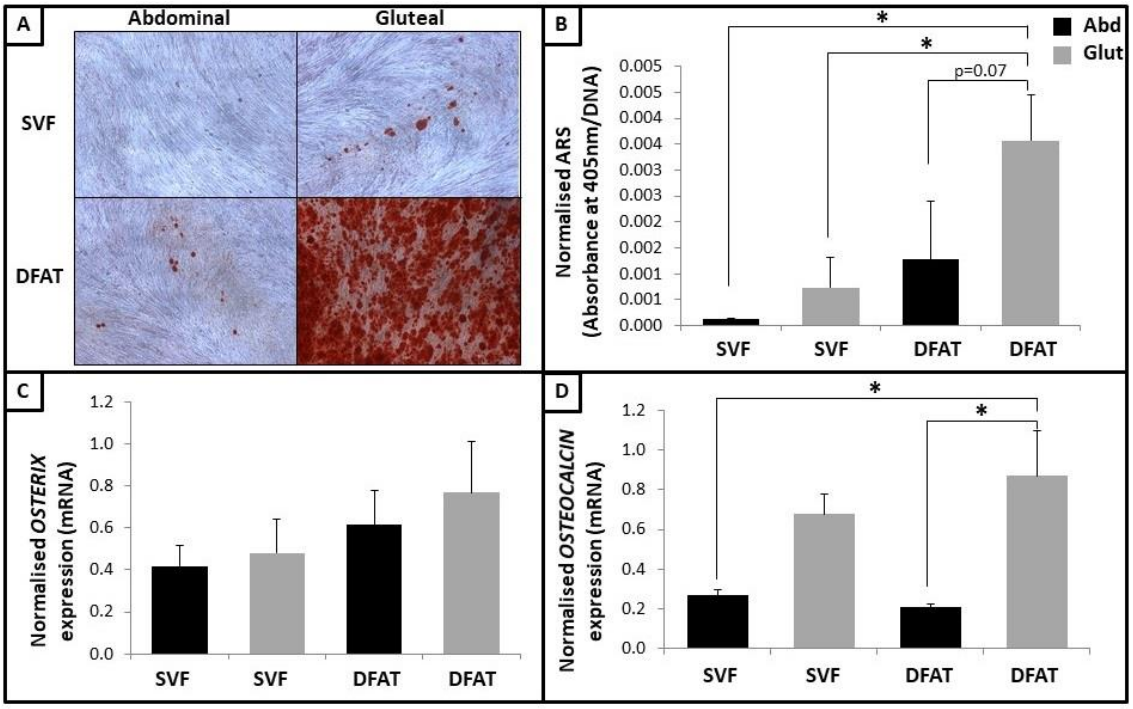



**Figure 5.7 DFAT cells exhibit a greater osteogenic differentiation capacity compared to SVF cells.** A - Representative image from Alizarin red staining (ARS) of immortalised abdominal (Abd) and gluteal (Glut) SVF and DFAT cells cultured for 14 days in osteogenic medium. B - Quantification of extracted ARS normalised to the corresponding cellular DNA content in SVF and DFAT cells. C - *OSTERIX* mRNA levels (normalised to *PPIA*); D - *OSTEOCALCIN* mRNA levels (normalised to *PPIA*) in SVF and DFAT cells cultured for 14 days in osteogenic medium (n=3). Data presented as mean ± SEM; data analysed via ANOVA; *P<0.05.

With respect to depot, it was found that gluteal cells displayed a much greater osteogenic capacity compared to abdominal cells of the same type, which is consistent with previous reports (Iwen et al., 2014); (Levi et al., 2010). However, the osteogenic capacity was also greatly influenced by cell type. ARS was detectable but patchy in gluteal SVF cells but was dense and uniform in gluteal DFAT cells (Figure 5.7A); this pattern of ECM mineralisation essentially mirrors that of TAG accumulation during adipogenesis (Figure 5.2). ARS was undetectable in abdominal SVF cells although some patches were observed in abdominal DFAT cells. The pattern of ARS described was confirmed upon extraction and quantitative analysis of incorporated dye (Figure 5.7B).

In addition to ARS, the expression of several osteogenic genes was measured in cells harvested on day 14. Despite not reaching statistical significance, the expression of *OSTERIX*, the master transcription regulator of osteoblastogenesis (Long, 2012), tended to be highest in gluteal DFAT cells and lowest in abdominal SVF cells (Figure 5.7C); this pattern is broadly consistent with the ARS data. Expression of *OSTEOCALCIN* (a glycoprotein secreted by osteoblasts) was higher in gluteal compared to abdominal cells but was not significantly different between cell types (Figure 5.7D). These data indicate that DFAT cells exhibit a greatly enhanced ability to undergo osteogenesis that reflects the intrinsic osteogenic capacity of the AT depot from which they derive.

## 5.4 Discussion

Capitalising on an opportunity that arose during other experiments, the ceiling culture technique was used to isolate lipid-laden ASCs from immortalised SVF cells differentiated in fatty acid-supplemented adipogenic medium. By comparing the DFAT to the SVF population from which they derive it was found that the DFAT cells retain an intrinsic memory of their anatomical location and exhibit a greatly enhanced (multi-potent) differentiation capacity that reflects their depot of origin. These results identify DFAT cells as a novel cellular model system that could be used to study depot-specific human AT biology.

### 5.4.1 Adipogenesis and lipogenesis in immortalised SVF and DFAT cells

There was a concern that fatty acid supplementation might cause non-specific TAG accumulation within the immortalised SVF population and thereby prejudice the ceiling culture selection process. However, the biological characteristics of the immortalised DFAT cells suggest this did not occur. Upon re-differentiation, adipogenesis occurred uniformly in DFAT cells compared to the patchy lipid accumulation pattern exhibited by the SVF cells, thus indicating that the DFAT cells comprise a homogenous population enriched for ASCs (Figure 5.2).

It remains unclear exactly what cell population the de-differentiation process yields. The surface marker expression profile of DFAT cells has been found to overlap considerably with that displayed by sorted ASCs (T. Miyazaki, Kitagawa, Toriyama, Kobori, & Torii, 2005); (Matsumoto et al., 2008), thus suggesting these approaches isolate essentially the same cell population. Indeed, it has been reported that the expression profile of various stem cell marker genes is not sufficiently different between SVF and DFAT cells to reliably distinguish these cell populations (Gao, Zhao, Song, & Yang, 2012).

During differentiation, the expression of adipogenic genes, specifically *PPARG2*, was activated earlier and reached higher maximal levels in DFAT compared to SVF cells. *FASN* expression followed a similar pattern to *PPARG2* and agreed with the significantly greater TAG accumulation detected. DFAT cells exhibited an impressive intrinsic lipogenic capacity, suggesting this property was selected for during ceiling culture despite the buoyant, lipid-laden cells being generated via fatty acid loading.

The importance of DNL to adipogenesis and (regional) AT biology more broadly is currently unclear (section 1.1.3). While ASCs can synthesise the major fatty acids via *de novo* lipogenesis (DNL) as well as fatty acid elongation and desaturation required for full differentiation *in vitro* (Collins et al., 2011), it is currently unclear whether DNL is essential for adipogenesis and/or whether fatty acid supplementation modulates this process. Addressing this issue is important given that most/all *in vitro* models of human and murine adipogenesis are primarily lipogenic (i.e. they involve differentiating ASCs in the absence of exogenous fatty acids). The high lipogenic capacity of DFAT cells means they represent potentially useful tools that could be used to explore the role of DNL in adipogenesis and thereby complement *in vivo* studies investigating this process in human AT.

A general paucity of data coupled with contradictory findings mean that the relationship between AT DNL, nutrition and metabolic health remains essentially unknown (sections 1.1.3 and 1.4.4). Using stable isotopes it has been demonstrated that DNL occurs in AT *in vivo*; however, the relative contribution of AT DNL to the total amount of stored TAG in AT has been reported to range from 20% (Strawford et al., 2004) to almost negligible (Diraison et al., 2003). Such inconclusive data are not helped by reports indicating that dietary consumption of saturated fatty acids and AT palmitate content do not correlate

(R. Roberts et al., 2009) and that excess carbohydrate consumption does not induce changes in total DNL (Minehira, Vega, Vidal, Acheson, & Tappy, 2004).

As excessive consumption of purportedly lipogenic nutrients like fructose has been associated with increased adiposity and the development of metabolic disease (Legeza et al., 2017), exploring whether substrate type and availability modifies the rate of AT DNL and/or the products generated is potentially important; the immortalised DFAT cells represent an ideal model cellular system that could be used to answer such questions. This could involve biochemical characterisation of the fatty acid species in the TAG droplet within differentiated cells cultured in adipogenic medium containing different metabolic substrates (e.g. glucose, fructose and/or essential/non-essential fatty acids). It has also been suggested that AT DNL represents a mechanism linking adipocyte recruitment and smaller adipocyte size to potentially facilitate metabolically favourable weight gain (R. Roberts et al., 2009). Consistent with this, the rate of AT DNL is reportedly reduced in insulin-resistant individuals (Allister et al., 2015), as is the expression of DNL genes in AT (R. Roberts et al., 2009).

As AT DNL may modulate systemic metabolic health via lipokines - i.e. fatty acid species synthesised and released by AT (section 1.1.3; (Yilmaz et al., 2016)) - determining what molecules the immortalised SVF and DFAT cells synthesise and release could be a useful adjunct to *in vivo* studies. Given the lack of clarity over the importance of this process, clarifying the role of AT DNL in metabolic health should also be a priority and could be achieved by measuring AT DNL across a range of adiposity, as well as in healthy and diabetic individuals; such information could inform detailed investigations of specific lipid species aimed at characterising their role in modulating the function/dysfunction of AT and other metabolic tissues such as the liver, muscle and pancreas.

With respect to regional AT biology, it has been shown that the fatty acid composition of TAG within AT varies between depots; gluteal AT generates more desaturated products such as palmitoleate (Pinnick et al., 2012). More importantly, such differences are retained in isolated preadipocytes differentiated *in vitro*; recapitulation of the depot-specific difference in fatty acid composition between AT depots *in vitro* was driven by DNL due to the absence of fatty acids in the culture medium. It remains to be seen whether the previously observed depot-specific differences in fatty acid composition generated via DNL (Pinnick et al., 2012) are exhibited by the DFAT cells; this could be investigated through analysis of the fatty acid composition of TAG in differentiated abdominal and gluteal SVF and DFAT cells.

In summary, it is clear that much remains to be learned about the role of AT DNL in relation to metabolic health and nutrition. The immortalised DFAT cells represent a highly lipogenic cell population that could be used to answer many questions pertaining to the (depot-specific) biology of AT DNL.

### 5.4.2 Depot-specific gene expression in SVF and DFAT cells

In addition to regional variation in DNL (Pinnick et al., 2012), the immortalised abdominal and gluteal SVF cells used throughout this thesis have been shown to retain other depot-specific properties *ex vivo* similar to primary SVF cells, particularly gene expression profiles (Todorčević et al., 2017). Indeed, these cells have been used successfully in the characterisation of various depot-specific adipogenic processes (Pinnick et al., 2014); (Loh et al., 2015). Data generated in this study suggest that these depot-specific properties are largely retained after the process of de-differentiation, thus meaning that the immortalised abdominal and gluteal DFAT and SVF cell populations

represent useful cellular model systems appropriate for studying depot-specific phenomena in human AT.

The differential expression patterns of *TBX5* and *HOTAIR* previously identified in SVF cells were largely retained in the corresponding population of DFAT cells. Previous data indicate that *TBX5* is a positive adipogenic regulator selectively expressed in abdominal cells (Pinnick et al., 2014) whereas *HOTAIR* is a long non-coding RNA implicated in the regulation of adipogenesis which is exclusively expressed in gluteal cells (Divoux et al., 2014).

Although the previously described depot-specific expression patterns of both genes were broadly retained in DFAT cells, some discrepancies were observed in proliferating and differentiated cells. For instance, *HOTAIR* expression was significantly lower in gluteal DFAT cells compared to gluteal SVF cells while *TBX5* expression in differentiated abdominal DFAT cells was significantly lower compared to their SVF counterparts. The functional significance of these discrepancies is not immediately clear but likely reflects the cellular composition of each population. It follows that it might be possible to discern the contribution of non-ASCs residing in the mixed SVF population to various cellular processes by directly comparing the immortalised DFAT and SVF cell populations. For instance, measuring the effect of overexpressing or knocking down the same gene in both SVF and DFAT before differentiating both cell types in parallel could provide insights into the contribution of that gene in the ASC and non-ASC population in the context of adipogenesis.

The immortalised SVF and DFAT cells also represent appropriate cellular systems that could be used to investigate the epigenetic mechanisms underpinning the depot-specific gene expression profiles and functional properties exhibited by AT and its resident ASCs

(section 1.4); it has previously been shown that the immortalised SVF cells exhibit depot-specific DNA methylation profiles that may be involved in regulating the transcription of differentially expressed genes such as *TBX5* and *HOTAIR* (Pinnick et al., 2014). Given the highly adipogenic nature of the DFAT cells and the relative purity of their composition, they represent a highly attractive cell type with which to study the transcriptional and epigenetic regulation of adipogenesis (sections 1.2.3 and 1.2.4). This could involve genome-wide DNA methylation profiling and chromatin-immunoprecipitation in conjunction with microarray analysis of gene expression in cells at different time-points during adipogenesis.

### 5.4.3 Lipolysis in differentiated SVF and DFAT cells

Differentiated SVF and DFAT cells both exhibited lipolytic activity which changed appropriately in response to various physiological stimuli. While the absolute rate of lipolysis displayed by differentiated DFAT cells under all experimental conditions examined was much larger than that measured in differentiated SVF cells, the fold change in lipolysis upon insulin or isoproterenol treatment was comparable in both cell types. This suggests that the de-differentiation process did not modify the behaviour of the resident ASCs present in the mixed SVF population but simply isolated and enriched the ASC population. On a practical note, the greater absolute level of lipolysis exhibited by the DFAT cells compared to their SVF counterparts made it easier to measure lipolytic activity more accurately across a wider dynamic range.

Lipolysis was detectable in the basal state in SVF and DFAT cells but could be robustly suppressed by insulin in both depots. There was a tendency for insulin treatment to induce greater suppression of lipolysis in gluteal SVF and DFAT cells, consistent with *in vivo* data (McQuaid, Manolopoulos, et al., 2010). Conversely, isoproterenol stimulated

lipolysis in both cell types, albeit to a significantly greater extent in abdominal compared to gluteal cells; this agrees with previous *in vitro* data (Berman, Nicklas, Rogus, Dennis, & Goldberg, 1998).

It remains to be seen whether the immortalised differentiated SVF and DFAT cells retain the well-established depot-specific response to adrenaline treatment (Manolopoulos, Karpe, & Frayn, 2012), which only stimulates lipolysis in abdominal cells owing to the higher expression of inhibitory α2-adrenoceptors in gluteal cells (Wahrenberg et al., 1989). If they do, such data would conclusively identify differentiated immortalised SVF and DFAT cells as ideal model systems that could be used for the study of regional fatty acid handling to characterise both the action of endogenous factors as well as novel small molecules in the process of drug discovery and development.

### 5.4.4 Studying mitotic clonal expansion and adipogenesis in SVF and DFAT cells

One aspect of ASC biology that proved tractable to investigation via comparative studies using SVF and DFAT cells was mitotic clonal expansion (MCE) during adipogenesis (section 1.2.5). Such an investigation was prompted by the observation that despite seeding the same number of cells at the start of an experiment, DFAT cells yielded far more cellular material (i.e. RNA, protein and TAG) upon differentiation compared to the SVF cells. MCE has primarily been studied in murine cells and is the process whereby growth-arrested ASCs re-enter the cell cycle and undergo approximately two rounds of division (Q. Q. Tang et al., 2003). This process is necessary for adipogenesis and typically occurs during the first 4 days in murine 3T3-L1 cells under the control of the C/EBP proteins (J. W. Zhang et al., 2004); (Q. Q. Tang & Lane, 2012). Mounting data suggest that MCE represents a common mechanism that specific ligands (Hallenborg et al., 2014),

genes such as *FTO*; (Merkestein et al., 2015) and even epigenetic modifiers (M. K. Jang, Kim, & Jung, 2017) act through to regulate adipogenesis.

To study MCE in the context of human adipogenesis, cell number was measured in SVF and DFAT cells over a 14 day differentiation protocol. Consistent with prior observations, there was a gradual loss of SVF cells whereas DFAT cell number increased. Expansion of the DFAT cells via MCE occurred from day 9 onwards. With many studies in murine 3T3-L1 cells indicating that MCE is crucial for successful adipogenesis (Q. Q. Tang & Lane, 2012), it is possible to explain qualitative differences in differentiation in terms of MCE.

Adipogenesis in the heterogeneous SVF population was consistently patchy, with 'islands' of differentiated cells likely developing through MCE of the relatively scarce resident ASCs (Figure 5.2). However, it should also be noted that the number of immortalised SVF cells declined during differentiation. To reconcile these observations, it is possible that MCE of differentiating ASCs was not sufficient to balance the loss of non-ASCs which could be dying due to prolonged exposure to adipogenic medium, for example. Conversely, adipogenesis occurred in a uniform manner in the DFAT cell population meaning the yield of cells (according to DNA and protein content) at the end of differentiation was substantially larger than that seeded. As this population is enriched for ASCs, it is possible that nearly every cell performed MCE (probably after day 9) and differentiated; this interpretation could explain both the distribution of adipogenesis as well as the relative yield of cells harvested at the end of the differentiation time-course for each cell type.

Given the strong positive correlations between cell number and adipogenic endpoints such as TAG accumulation and *PPARG2* expression, it is clear that further study of MCE

in human adipogenesis is warranted. There would be merit in measuring cell viability during adipogenesis as well as the expression/activity of cell cycle regulators (Barnum & O'Connell, 2014). For example, performing Ki67 staining (Scholzen & Gerdes, 2000) to physically label and identify the specific cells performing MCE during adipogenesis in conjunction with lipid co-staining would make it possible to determine which cell type drives the formation of the adipogenic islands in the SVF population and also presumably constitutes the majority of the DFAT population; MCE might be driven by expansion of ASCs and/or lipid-filled cells budding off.

MCE has been fairly well-characterised in murine 3T3-L1 cells but the paucity of data concerning MCE in human adipogenesis (section 1.2.5) means it is difficult to contextualise the results presented in Figure 5.5. It has been previously reported that MCE does not occur in primary human SVF cells (Tomlinson, Boudreau, Wu, Atlas, & Hache, 2006), but as the authors only studied DNA synthesis during the first 4 days of adipogenesis it is possible that these cells would have performed MCE later during differentiation. It is currently unclear whether the timing of MCE affects adipogenesis or simply highlights that the molecular regulation of adipogenesis varies by species.

It has been reported that the mechanism by which AT expands varies between depots (section 1.4.2); subcutaneous abdominal AT expands via hypertrophy whereas gluteo-femoral AT expands via hyperplasia (Tchoukalova, Votruba, et al., 2010). This is supported by data indicating that the cell formation rate for new preadipocytes and adipocytes was significantly higher in subcutaneous gluteal AT compared to subcutaneous abdominal AT (White et al., 2016). As these studies were unable to identify the source of new cells, it is possible that depot-specific variation in MCE (rather than ASC proliferation alone) may influence the mechanism by which AT expands upon overfeeding. This means that MCE might sit at the interface between adipocyte

size/number, metabolic health and regional AT distribution (section 1.4.4). Evidently further investigation into the role of MCE in (depot-specific) human adipogenesis is justified and the immortalised SVF and DFAT cells represent valid model systems to use in such studies.

### 5.4.5 Potential practical uses of SVF and DFAT cells

The immortalised DFAT cells retained the regional variation in their multi-potent differentiation capacity. Consistent with previous data (Iwen et al., 2014); (Levi et al., 2010), gluteal cells exhibited a greater osteogenic capacity than abdominal cells. Similar to adipogenesis, osteogenesis occurred uniformly in gluteal DFAT cells but was patchy in the gluteal SVF cell population from which they derive, as highlighted by ARS. This indicates that the intrinsic, depot-specific, multi-potent differentiation capacity was retained after the process of de-differentiation and further supports the notion that the DFAT cell population is enriched for ASCs. The multi-potential differentiation capacity of ASCs makes them attractive candidates to pursue in the development of regenerative medicine therapies; a clear understanding of the depot-specific properties of these cells will be essential for optimising their clinical effectiveness.

A crucial process that will influence the success of regenerative medicine is the technique used to isolate cells. Data from pre-clinical studies of regenerative medicine therapies suggest that purified ASCs are significantly more effective in promoting fat graft retention compared to unsorted SVF cells in mice (Zielins et al., 2017). From an academic standpoint, detailed characterisation of the selected and unselected cell populations would provide some useful insights into the cellular composition of AT and processes such as adipogenesis. With respect to cell sorting, it is notable that DFAT cells generated via ceiling culture reportedly exhibit a very similar expression profile of cell surface markers

(Shen et al., 2011); (T. Miyazaki et al., 2005); (Matsumoto et al., 2008) and stem cell marker genes (Gao et al., 2012) to that displayed by conventionally sorted ASCs. This suggests that the cruder ceiling culture technique isolates a very similar/the same sub-population of ASCs from the SVF as that purified via cell sorting.

It is notable that SVF cells from subcutaneous abdominal and visceral AT cannot be distinguished based on their cell surface marker expression (B. Kim et al., 2016); (Shah et al., 2014); this also seems to be the case for subcutaneous abdominal and gluteal SVF cells (Iwen et al., 2014). While the expression profile of cell surface markers may not vary by depot, the immortalised SVF and DFAT cells can be distinguished based on depot-specific gene expression profiles (Figure 5.5) and functional properties such as lipolysis (Figure 5.4) and multi-potent differentiation capacity (Figure 5.7).

It is probable that the (depot-specific) biological properties of ASCs are largely determined at the epigenetic level. Indeed, treatment of SVF cells with 5'-azacytidine (a chemical which reduces DNA methylation) has been found to enhance their ability to undergo osteogenesis, chondrogenesis and myogenesis (Cifarelli et al., 2012) as well as rescue the osteogenic capacity that is typically impaired in ASCs taken from older donors (Yan et al., 2014). Such data suggest that there might be merit in characterising the role of epigenetics in the regulation of depot-specific gene expression in the immortalised SVF and DFAT cells as well as how such phenomena influence the biological properties of the cells. This could involve investigating what role DNA methylation, histone modifications and/or miRNA expression play in determining the depot-specific adipogenic or osteogenic capabilities of human ASCs. Epigenetic regulators in ASCs could be modified pharmacologically at the genome-wide level via treatment with drugs such as 5'-azacytidine, trichostatin A or miRNA-neutralising anti-sense oligonucleotides.

The characteristics exhibited by the DFAT cells make them a highly useful cell type that could be useful in a variety of non-clinical applications. The DFAT cells are immortalised, proliferate relatively quickly in standard culture conditions, retain the characteristics of their anatomical origin and exhibit a high capacity to perform adipogenesis. In addition to these attractive characteristics, the DFAT cells derive from human tissue. Together, these characteristics mean that these cells represent a model system that could be highly useful for dissecting the molecular regulation of (depot-specific) processes relevant to human AT biology. Further, the DFAT cells might be appropriate to use when screening for (depot-specific) anti-obesity drugs that modulate adipogenesis and/or drugs which aim to modulate lipolysis, adipokine secretion or cellular signalling, for example, in preadipocytes or mature adipocytes. The features of DFAT cells surely make them a strong competitor to the murine 3T3-L1 cells in the academic world as the 'go-to' cell type for studying (human) AT biology (Ruiz-Ojeda et al. 2016).

In summary, the capture and de-differentiation of buoyant, lipid-laden cells cultured in adipogenic medium supplemented with fatty acids may represent a cheap, reproducible method that can be used to purify ASCs from a mixed SVF population. Characterisation of the DFAT cells generated via ceiling culture indicated that they largely retained the depot-specific properties of the immortalised SVF cell population from which they derive. The impressive multi-potent differentiation capacity of DFAT cells holds great promise for various purposes in regenerative medicine. Moreover, the immortalised SVF and DFAT populations represent useful, valid tools that can be used to study many aspects of depot-specific AT and ASC biology in humans.

### 5.4.6 Concluding statements

Data from this study indicate that ceiling culture/de-differentiation represents a viable alternative to conventional cell sorting techniques for generating homogenous ASC populations which exhibit a high differentiation capacity. SVF and DFAT cells retain an intrinsic memory of their anatomical origin, as highlighted by their depot-specific gene expression profiles and multi-potent differentiation capacity. A comparative study of these cell types identified MCE as an important yet poorly understood process that is closely associated with adipogenic endpoints. The immortalised SVF and DFAT cells display various features that make them a potentially powerful tool with many academic and clinical applications.

# Chapter 6

# Discussion

## 6.1 The importance of depot-specific AT biology

The data presented in the three results chapters of this thesis support and build upon the prevailing literature to further emphasise the striking depot-specific properties exhibited by subcutaneous abdominal and gluteo-femoral AT and their constituent cells. The importance of the depot-specific properties of AT cannot be overstated enough. Obtaining a clear understanding of the mechanisms that regulate the depot-specific functional properties of AT will be vital for the development of effective novel therapies for obesity and its associated diseases. Such knowledge will also facilitate the identification of individuals most at risk of developing metabolic and cardiovascular disease so appropriate preventative strategies can be implemented.

For instance, developing a biomarker or genetic test that could robustly predict an individual's 'personal fat threshold' (section 1.4.4) or an appropriate proxy of AT function/expandability (e.g. the propensity of their subcutaneous adipocytes to hypertrophy upon weight gain) would help identify individuals most at risk of developing metabolic complications if they gain weight. At-risk individuals could then be targeted for increased monitoring and encouraged to undertake beneficial dietary and behavioural interventions. Looking to data from studies examining BMP signalling in AT, particularly in the context of hypertrophic obesity (chapter 5; (Birgit Gustafson et al., 2015)), might be useful in developing such a predictive tool.

Many aspects of AT biology have received varying degrees of interest over recent decades including: fatty acid handling; adipogenesis; gene expression and epigenetic profiling; *de novo* lipogenesis; fatty acid handling; inflammation; adipocyte size; preadipocyte and adipocyte turnover; and AT ECM composition and structure. Many of these processes have been studied in a single AT depot (typically subcutaneous abdominal AT) but a

growing appreciation that these processes can vary by depot has led to increasing numbers of studies comparing different AT depots. The depots of choice have been subcutaneous abdominal and visceral AT; this choice likely reflects the relationship between central obesity and metabolic/cardiovascular disease (Cerhan et al., 2014) as well as the fact that mouse models have been used extensively to study these depots, even if they are not directly comparable (section 1.2.3; (Gagnon et al. 2013)). However, the focus on these depots has been at the expense of studying gluteo-femoral AT; the results of a simple PubMed search performed during August 2017 presented in Table 6.1 highlight how gluteo-femoral AT remains grossly understudied relative to upper body AT.

**Table 6.1 Results of PubMed search for specific adipose depots**

| Search terms | Number of articles since 1950 |
|---|---|
| 'gluteal adipose' | 394 |
| 'femoral adipose' | 1158 |
| 'subcutaneous abdominal adipose' | 4422 |
| 'visceral adipose' | 7618 |

This bias in the literature is concerning when considering that the emphasis on upper body AT, particularly visceral AT, may be misplaced. Indeed, mounting data suggest that visceral AT expansion simply reflects subcutaneous AT dysfunction (Bays, 2014) and may not play a causal role in the pathogenesis of metabolic disease. Furthermore, it is remarkable that gluteo-femoral AT has not received more attention given its well-established protective effect against the development of metabolic and cardiovascular disease (section 1.3.8). From an academic standpoint, allowing this under-representation to persist means that our understanding of AT can never be truly complete. From a clinical and health economic perspective, understanding and exploiting gluteo-femoral AT

potentially represents our best chance to contain or reverse the burgeoning pandemic of metabolic and cardiovascular disease.

If one considers that the metabolic and cardiovascular complications associated with obesity and lipodystrophy develop when an individual's energy balance exceeds their 'personal fat threshold' (section 1.4.4), artificially enhancing their AT expandability could alleviate or even reverse such conditions. A comprehensive and detailed understanding of AT progenitor cell's depot-specific properties and the isolation process would facilitate taking a regenerative medicine approach to the treatment of type 2 diabetes, for example. The principle of this approach and the importance of the donor cell type has already been demonstrated in mice; transplantation of subcutaneous but not visceral AT into mice subjected to a high-fat diet significantly enhanced glucose tolerance, insulin sensitivity and lipid handling (Tran, Yamamoto, Gesta and Kahn 2008); (Hocking et al 2015). It follows that autologous transplantation of the highly adipogenic subpopulation of SVF/DFAT cells isolated via ceiling culture from gluteo-femoral AT, the depot which exhibits the most metabolically favourable functional properties (section 1.3.8), could represent a novel approach to the treatment of type 2 diabetes or lipodystrophy.

The remainder of this chapter will consider the main themes that emerged from this thesis whilst providing some suggestions for potential future directions.

### 6.2 Identifying appropriate candidates

When conducting candidate-based scientific investigations, it is crucial to identify the most appropriate candidates. The process of choosing a candidate for further study has been made simultaneously easier and more difficult as a result of technological advances. Data can now be collected, generated and analysed on an industrial scale as a result of

globalisation, increasing computer power and ever-decreasing costs for sample collection and analysis. The new era of 'big data' has provided answers to questions that were until only recently insurmountable. For example, GWAS are now performed on a large enough scale to enable the successful identification of genetic loci associated with regional AT distribution independent of overall adiposity (Shungin et al., 2015). Microarray technology permits expression analysis of hundreds/thousands of genes in a single sample (Pinnick et al., 2014). It is now easy and affordable to determine an individual's genotype at hundreds/thousands of SNPs (section 2.1) and it is also possible to measure DNA methylation across the entire genome in any type of tissue (Gehrke et al., 2013). In addition, modern manufacturing methods mean that nearly any protein can be obtained in a form suitable for almost any type of experiment; it is equally possible to modify the expression of nearly any gene in any tissue at any time in transgenic mice (and cells).

The wealth of data and choice of options are overwhelming and make the process of picking a single candidate incredibly difficult. Big data is rapidly establishing itself as a force in modern science, principally through the acquisition of resources and people that together are contributing to the surge in big data-based publications. The arrival of big data does not mean it is the future of science though; it can only be a part of it. Rather than replacing the traditional method, big data is transforming how science is conducted by providing new types of information that can inform how candidates are picked and experiments are conducted.

As data scientists develop ways to analyse, present and rationalise big data, the current generation of scientists is learning how to glean the most important information from this new phenomenon and use it to their advantage. The identification of the candidates studied in this thesis (COMP and BMP2) involved considering big data relevant to depot-specific AT biology (e.g. GWAS, microarray and DNA methylation array data) and

assimilating it with more traditional scientific literature (e.g. cell biology, transgenic mouse models, clinical genetics etc.). While the initial hypotheses proffered for each of the candidates chosen might not have been supported by the consequently generated data (e.g. COMP is not a depot-specific regulator of AT despite being highly differentially expressed), it is clear that the approach taken in this thesis successfully identified two novel regulators of human subcutaneous AT biology.

Being able to integrate and appraise different types of data in an appropriate manner to identify promising candidates worthy of detailed study will be crucial for the advancement of myriad scientific fields along their optimal trajectory as there are simply not enough resources or time to characterise every single 'hit' identified in big data-driven studies. It should also be considered that investigating a single biological candidate in isolation may represent a somewhat limited approach to science that will soon give way to more sophisticated analytical methods which follow many candidates in parallel. Indeed, pathway or network analyses in which multiple candidates can be considered simultaneously is offering increasing amounts of convincing data that many genetic loci associated with overall obesity contribute to appetite regulation (Locke et al., 2015) whereas regional AT distribution is associated with genomic loci involved in the transcriptional regulation of development (Shungin et al., 2015). Such findings suggest that overall obesity and regional AT distribution are distinct phenomena underpinned by different mechanisms meaning they require different approaches in order to be modified in beneficial ways.

In addition to investigations in which big data is being used as a guiding force, technological advances are facilitating the study of various candidates and topic areas in a variety of novel ways. For instance, imaging modalities (sections 1.3.3, 1.3.4 and 1.3.5) are now exquisitely accurate and sufficiently accessible to permit detailed phenotyping

of multiple AT depots during weight gain and weight loss (Cuthbertson et al., 2017); depot-specific changes in AT mass can then be related to genotype, gene expression, epigenetic markers, adipocyte size and adipokine secretion, for example. Such studies will facilitate the identification of factors which determine whether an individual is predisposed to developing central obesity so increased monitoring and/or preventive action can be implemented. These data could also be used to optimise currently available therapies used to treat metabolic and cardiovascular diseases driven by central obesity.

Building on the interest in the factors that determine which AT depots expand upon overfeeding, the identification of BMP2 as a depot-specific regulator of adipogenesis raises the exciting prospect that AT can be modulated pharmacologically in a depot-specific manner. This aim has been buoyed by the detection of numerous factors that distinguish the constituent cells in a given AT depot from another - e.g. differential expression of developmental genes (Karastergiou et al., 2013); (Pinnick et al., 2014). While TZDs have been used somewhat effectively to enhance insulin sensitivity in numerous individuals by increasing (subcutaneous) AT expandability (Carey et al., 2002), they act in a relatively non-specific manner and so are responsible for various harmful side effects (Bugge and Holst 2017).

Identifying alternative methods to bring about metabolically favourable AT re-distribution represents an attractive approach to the treatment of (central) obesity-related diseases. Indeed, modifying BMP2-SMAD1/5/8 signalling in human AT, potentially via K02288 (Sanvitale et al., 2013), may represent the first incarnation of a new line of pharmacological treatment for metabolic disease underpinned by central obesity, for example. Another therapeutic approach to invoke metabolically beneficial AT re-distribution could involve using anti-sense oligonucleotides that modify the action of

adipogenic regulators which are differentially expressed between AT depots - e.g. *TBX5* (Pinnick et al., 2014).

It follows that identifying the factors which predict and/or modify regional AT distribution and expandability might also provide some insights into the mechanisms which link (central) obesity to the development of other diseases. Central obesity is a well-established risk factor for cardiovascular disease (Cerhan et al., 2014) and osteoarthritis (Duclos, 2016), so understanding the factors which determine AT function (or dysfunction) could provide new opportunities for predicting and/or preventing obesity-related diseases.

For instance, a mouse study examining how the composition of the AT ECM changes in response to high-fat diet feeding culminated in the identification of endotrophin, a cleavage product of collagen VI α3, which not only exacerbates AT fibrosis and dysfunction (Sun et al., 2014) but can also enter the circulation and promote mammary tumour formation (J. Park & Scherer, 2012). It is likely that other factors similar to endotrophin await discovery and that these molecules may represent novel biomarkers of central obesity-related diseases which may also be amenable to therapeutic intervention. Such investigations could be extended to examine what role (if any) bone marrow AT (Ambrosi and Schulz 2017) and/or brown/beige AT (Marlatt et al. 2017) play in determining regional AT distribution, AT expandability and the pathogenesis of central obesity-related diseases such as osteoarthritis.

### 6.3 Studying AT under the optimal conditions and time-scale

During the process of this thesis it became abundantly clear to consider all of the possible conditions under which a study is performed or a sample is harvested. This is especially important when attempting to interpret and reconcile different types of data in preparation

for designing the next experiment based on the currently available information. It should be noted that the most important characteristics of a given sample or experimental set-up may also vary depending on the question posed. However, it is also worth noting that no sample or experiment can ever be ideal owing to finite resources and/or time as well as methodological limitations; these problems can be compounded if samples originally harvested for one purpose end up being used for something else. It follows that the constraints imposed on experimental set-ups, collecting and/or analysing samples may result in many potentially important candidates in various scientific disciplines being deemed 'not interesting' simply because they have been studied under irrelevant or sub-optimal conditions.

This issue became most clear during the investigation of *COMP* when it was observed that its expression was found to be greatly increased in obese subcutaneous abdominal and gluteal AT with additional analyses highlighting that circulating COMP levels were also elevated in overweight/obese individuals (section 3.3.2). While these data were encouraging, their cross-sectional nature meant it was impossible to determine whether *COMP* plays a causative or correlative role in the development of obesity. Furthermore, the dataset was not large enough to examine whether up-regulation of *COMP* expression was associated with better or worse metabolic outcomes. There are two important points here. Firstly, measuring AT *COMP* expression over time in a longitudinal experiment would provide more convincing data that it is (or is not) associated with adiposity and/or ECM remodelling. Secondly, it is important to try and collect a sufficient amount of data to minimise the likelihood of a study being underpowered; this is as important in cell culture as it is in human studies. The latter issue can be overcome by designing studies based on power calculations informed by preliminary experiments.

The significant up-regulation of *COMP* expression in obese AT should perhaps be unsurprising when considering the prevailing literature defining COMP as a matricellular protein whose expression increases upon tissue injury to co-ordinate ECM remodelling and angiogenesis (Murphy-Ullrich & Sage, 2014). This means that COMP may only become an important player in AT biology when a potentially injurious stimulus is presented (i.e. chronic positive energy balance driving AT expansion). Compared to the significant up-regulation of *COMP* expression in obesity, the fairly modest pro-adipogenic effect of exogenous COMP seems slightly underwhelming, that is until it is considered that the preadipocytes used in the experiments were actually derived from a healthy, lean male.

Taken together, it is possible that the currently observed effect of COMP on adipogenesis is a gross under-estimate. With hindsight, it would have been more appropriate to compare how exogenous COMP modulates adipogenesis in preadipocytes obtained from lean and obese individuals, for example. As COMP is thought to act by co-ordinating the spatial arrangement of growth factors and structural components within the ECM, it is possible that the expression of other genes is required for COMP to exert a significant effect on adipogenesis and/or AT function; such factors may not be present in (immortalised) cells derived from a lean individual.

With respect to BMP2, it was observed that the rs979012/rs2145270 genotype was an eQTL for *BMP2* expression in subcutaneous abdominal and gluteal AT from males and females (section 4.3.6). It was also observed that homozygous males carrying the WHRadjBMI-increasing allele exhibited a cell size phenotype favouring adipocyte hypertrophy in subcutaneous abdominal AT (section 4.3.7). As discussed in chapter 4, this observation raised the possibility that BMP2 action is sexually dimorphic (section 4.4.8). As the trend was observed in male AT and the immortalised SVF cells used to

characterise BMP2 action were derived from a healthy 50 year old male (section 2.2.1), it is highly fortunate that these phenomena aligned favourably; it is entirely possible that BMP2 might not have been further explored if it had had no effect on a female preadipocyte cell line. Evidently the sexual dimorphism of BMP2 action remains an exciting prospect waiting to be explored but it is concerning that this promising candidate could have been dismissed so easily.

It is also crucial to appreciate that a given molecule's action can be highly context-specific, a feature that highlights the importance of considering a range of relevant biological scenarios to accurately identify a candidate's role. Certain molecules and/or genetic variants not critical for development and/or routine function may have minimal effects on a normal system but only come into play when the system is 'stressed' by any of a number of potential physiological stressors. Having a clear understanding of an experiment or dataset's limitations is vitally important to minimise the inadvertent use of inappropriate or sub-optimal experimental designs in the future. To generate the most meaningful data, future experiments should endeavour to use an appropriate time-scale and include clearly defined individuals, cells or tissue samples relevant to the question being posed.

### 6.4 Using the most appropriate model systems

Optimising an experimental system to recapitulate the native environment as closely as possible is crucial for designing experiments that will yield the most physiologically relevant data. Equally, acknowledging the limitations of an experimental system is important for drawing appropriate conclusions. This issue is particularly relevant to the work utilising the immortalised abdominal and gluteal SVF (and DFAT) cells to investigate depot-specific processes such as adipogenesis. While it has been demonstrated

that the immortalised SVF cells display depot-specific properties akin to primary cells (Todorčević et al., 2017), these cells might behave differently *in vitro* than they would *in vivo* owing to the loss of the native microenvironment containing hormones, growth factors and nutrients (Tchoukalova, Koutsari, et al., 2010). Despite this, the primary limitation of the current experimental set-up used to study adipogenesis is that it is 2D.

Two-dimensional cell systems are convenient and practical but are often criticised for providing their resident cells with an over-simplistic, unrepresentative environment (Y. Li & Kilian, 2015); this can result in the generation of misleading data which do not translate to the native 3D environment (Van Hul, Bauters, & Lijnen, 2013). However, choosing and optimising an appropriate 3D culture system is a difficult undertaking owing to the inherent dynamism of the ECM as well as numerous practical issues regarding ECM composition, cell seeding, oxygenation and perfusion etc. A common approach taken in 3D culture systems involves seeding preadipocytes into a collagenous matrix. Although the cells may then be in a 3D environment, however, the composition of the matrix provided may not reflect the native setting, meaning such an experimental set-up might not provide any more meaningful data than a 2D culture system.

An emerging technique in 3D culture involves seeding preadipocytes into de-cellularised AT. The de-cellularisation process involves taking a biopsy, rupturing the resident cells and washing away the residue to leave an ECM frame derived from the tissue of interest that can be seeded with cells of the investigator's choice. De-cellularised AT ECM has been shown to exert a strongly pro-adipogenic effect on preadipocytes, even in the absence of adipogenic medium (Flynn, 2010). Furthermore, this technique has been used to demonstrate that the AT ECM potently regulates the biology of the resident cells; healthy preadipocytes seeded in de-cellularised AT ECM obtained from diabetic individuals acquire a diabetic phenotype as highlighted by significantly impaired insulin

sensitivity and fatty acid handling (Baker et al., 2017). The use of de-cellularised AT ECM represents a great opportunity to study AT cell biology (e.g. adipogenesis, fatty acid handling and insulin signalling) in a physiologically and pathologically relevant 3D environment in a depot-specific manner. De-cellularised AT ECM could even be used in combination with other experimental interventions involving drug treatment or gene overexpression and/or knockdown.

On a different but related note, the generation of immortalised DFAT cells now provides future investigators with a choice of cell models to use. Whether it is appropriate to use the heterogeneous immortalised SVF population or the homogenous DFAT population enriched for ASCs will obviously depend on the question being posed. As discussed in section 5.4, by comparing the immortalised SVF and DFAT cell populations, it may be possible to discern the roles played by ASCs and non-ASCs (e.g. fibroblasts, immune cells and endothelial cells) in various processes within AT. Chapter 5 presented data from some initial comparative studies examining adipogenesis, lipogenesis and MCE in these cell lines but the potential uses for these cell lines is nearly limitless. While adipogenesis, lipogenesis and MCE could be examined in more detail, an obvious experiment might involve comparing the effect of COMP and/or BMP2 in these cell lines. The impressive adipogenic capacity exhibited by the DFAT cells makes them particularly attractive candidates to use in 3D culture systems, particularly de-cellularised AT ECM.

### 6.5 Concluding statements

AT exhibits depot-specific properties; this likely underpins the well-established observation that regional AT distribution strongly determines overall metabolic health. The identification of BMP2 as a novel depot-specific regulator of adipogenesis is exciting and important for advancing our understanding of the factors that determine regional AT

distribution. The investigation into the role of COMP in AT serves as an important reminder that the ECM is a crucial yet underappreciated element of AT that warrants much greater attention, especially in the context of upper/lower body AT. The serendipitous development of the immortalised DFAT cells provides an opportunity to develop the experimental models used to study depot-specific AT biology. Many lessons were learned during the process of this thesis; it is hoped that lessons can be learned from it too.

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