ALTERNATIVES FOR IMPROVING THE ADIPOGENIC POTENTIAL IN CELLULAR MODELS OF PERIVASCULAR ADIPOSE TISSUE

By

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ABSTRACT

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Adipose tissue is an important multi-depot and multi-function organ. Each depot exhibits a unique phenotype relative to its anatomical location and specific function. Among adipose depots, the perivascular adipose tissue (PVAT) is a major regulator of blood vessel function due to its proximity to the vasculature and its ability to secrete vasoactive molecules. The comprehensive study of PVAT biology requires in vivo, ex vivo, and reductionist approaches. Adipogenic models of PVAT, where preadipocytes from these tissues are induced to differentiate into adipocytes, are an important reductionist model that can be used to study the perivascular adipocyte. The goal of this thesis was to develop alternative methods to improve the efficiency of current models of adipogenesis in PVAT. We first adapted the use of magnetic activate cell sorting (MCS) to isolate adipocyte progenitor cells (APC). These adipocyte precursors must then be cultured to initiate adipogenesis in order to study mature adipocytes that are phenotypically and functionally similar to primary adipocytes of the same depot. Next, we developed a co-culture method that improved the adipogenesis efficiency and reduced the time required to induce differentiation in a cell culture model of PVAT. Our work provides a method to isolate APC from PVATs and a method using co-culture to differentiate PVAT preadipocytes, both of which are efficient in creating a more accurate depot-specific PVAT model of adipogenesis.

To all those who have dedicated or given their lives
to science and the pursuit of knowledge

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KEY TO ABBREVIATIONS

AdipoQ	adiponectin
AKT	protein kinase B
APC	adipocyte progenitor cells
aPVAT	thoracic aortic perivascular adipose tissue
BAT	brown adipose tissue
BMP	bone morphogenetic protein
BMP4	bone morphogenetic protein 4
C/EBP	CCAAT/enhancer-binding protein
C/EBPα	CCAAT/enhancer-binding protein alpha
CREB	cAMP response element binding protein
DEX	dexamethasone
Dio2	deiodinase iodothyronine type II
DMEM/F12	Dulbecco's Modified Eagle Medium/Ham's F12
EDTA	ethylenediaminetetraacetic acid
EGR2	early growth response protein 2
FABP4	fatty acid binding protein 4
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FGF	fibroblast growth factors
GLUT4	glucocorticoid transporter type 4
GON	perigonadal adipose tissue

GR	glucocorticoid receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBMX	3-isobutyl-1-methylxanthine
INS	insulin
KLF4	Kruppel-like factor 4
KLF5	Kruppel-like factor 5
KRBB	Kreb’s Ringer Modified Bicarbonate Buffer
MCS	magnetic activated cell sorting
mPVAT	mesenteric perivascular adipose tissue
MS	MultiSort
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGFRα	platelet derived growth factor receptor alpha
Pgc1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PLIN1	perilipin 1
PPARγ	peroxisome proliferator activated receptor gamma
Prdm16	PR domain containing 16
PVAT	perivascular adipose tissue
Sca1	stem cells antigen-1
SREBP1c	sterol regulatory element binding protein 1c
STAT5	signal transducer and activator of transcription 5
SVF	stromal vascular fraction
Tbx1	T-box protein 1

Tcf21	transcription factor 21
TH	tyrosine hydroxylase
Tmem26	transmembrane protein 26
TR	thyroid hormone receptor
T3	triiodothyronine
UCP1	uncoupling protein 1
Zfp423	zinc finger protein 423
Zic1	zinc finger protein 1

CHAPTER 1

Introduction

Abstract

Although a single organ, adipose tissue has multiple depots that exhibit various phenotypes and functions. In vitro models of adipogenesis have been developed to study adipocyte biology. However, the diversity of adipose tissue makes it especially difficult to develop in vitro adipogenic models for specific depots. One of the most effective ways to study adipose tissue biology is by using cellular models. These models require the induction of adipogenesis in stromal vascular cells or adipocyte progenitor cells and thus are often termed adipogenesis models. The development of perivascular adipose tissue (PVAT) adipogenic models is challenging because standardized pharmacological induction of adipogenesis does not have the same efficiency when used across multiple adipose depots. Adipogenic models that are effective in emulating specific adipocyte phenotypes are especially important when studying PVAT biology, as each PVAT is unique. Due to its proximity to the vasculature and its ability to secrete vasoactive molecules, PVAT impacts blood vessel function. Although adipogenesis protocols have been standardized for other adipocyte depots, they are not equally efficient when used in PVAT. The use of standard pharmacological inductions in conjunction with various cell culture techniques, such as micro-patterned surfaces, polymer structures, matrices, and co-cultures, have been used to improve adipogenesis. Understanding differences in adipogenesis through PVAT depot-specific in vitro modeling can provide further insight to phenotype and function as well as disease prevention and treatment.

Introduction

Adipose tissue as an organ has important autocrine, paracrine, and endocrine secretory functions. Through its secretome, it is a major regulator of energy balance and storage, nutrient transport, glucose homeostasis, lipid metabolism, immune function, angiogenesis, and blood pressure (Trayhurn, 2013). Adipose tissue is made up of multiple cell populations, including adipocytes and stromal vascular fraction that contains immune cells and preadipocytes. It also has multiple phenotypes and functions depending on depot location. White adipose tissue, whose main function is energy storage, is found in visceral depots, such as perigonadal or perirenal, and subcutaneous depots. Brown adipose tissue, whose major function is thermal regulation, is located in the interscapular region in rodents and human infants and in paravertebral regions in adult humans (Sanchez-Gurmaches et al., 2016). Adipose tissues that are positioned around blood vessels are called perivascular adipose tissue (PVAT). The ability of PVAT to secrete vasoactive molecules makes it an important regulator of vessel function. During metabolic disease (e.g. obesity or lipodystrophy), PVAT function is dysregulated, just like in other adipose tissue, which then alters its ability to modulate vasculature function. For example during obesity, the expansion of PVAT leads to a reduction in its anti-contractile effects on vessels (Bussey et al., 2016; Greenstein et al., 2009). To date, there is not a depot-specific adipogenic protocol that is effective in mimicking the phenotype of PVAT adipocytes in vitro. Most of what is known about adipogenesis has been studied using immortalized cell lines that have high adipogenic potential (Rosen and MacDougald, 2006). The characteristics of these cell lines do not represent the phenotype of all adipose depots. The development of a PVAT adipogenic

model must exemplify each depot's uniqueness in phenotype, and functionality. This would allow for further study of PVAT in respect to its ability to take up and secrete molecules as well as its response changes during disease. This review will focus on the mechanisms of adipogenesis, various adipogenic models, and special considerations for PVAT cultures.

Perivascular Adipose Tissue

Perivascular adipose tissue (PVAT) has a major paracrine signaling component that regulates vascular tone (Watts et al., 2013). During disease, PVAT undergoes a remodeling process that includes expansion, in the case of obesity, or reduction in the case of lipoatrophy. Changes during the remodeling process play a role in the development of cardiovascular disease. PVAT and the vasculature it surrounds also impact the remodeling processes each undergo. The secretion of adipokines, such as adiponectin and leptin, not only alters adipose tissue remodeling but also increases aortic diameter during obesity with increased immune cell influx (Verhagen and Visseren, 2011). During obesity associated expansion of PVAT, the secretion of adiponectin is reduced and is a predisposing factor for hypertension. This change during obesity is directly correlated to the occurrence of atherosclerosis, and when increased around visceral arteries, relates to development of metabolic disorders. Similarly, PVAT surrounding the aorta, as well as the mesenteric arteries, reduces the ability of the vessel to contract, but loses its protective function during disease states (Gao et al., 2005; Verlohren et al., 2004). Still, there is much to be discovered of the true inner-workings of PVAT.

PVAT has also been determined to be a reservoir of catecholamines that is separate from that of the sympathetic neural system (Ayala-Lopez et al., 2014), which may affect adipose remodeling processes (Millet et al., 1998; Morimoto et al., 1997). Further studies on PVAT and its contribution to disease development are necessary to understand the role it plays and its biological function in its entirety, especially since it is not yet fully understood how PVAT impacts blood vessel function. Furthermore, each

PVAT depot is unique based on its anatomical location (Lian and Gollasch, 2016). Aortic and mesenteric PVATs also vary in their tyramine-released catecholamines (Ayala-Lopez et al., 2014). These variations between PVAT depots elicit the need for individualized adipogenesis models for each that retain the phenotype, genotype, and function.

The Need for PVAT Adipogenic Models

To improve our understanding of PVAT vasomodulatory functions, it is necessary to understand changes in PVAT adipocyte biology. During obesity, PVAT expands by hypertrophy and hyperplasia. Adipose tissue remodels itself to accommodate this expansion and undergoes changes in immune cell infiltration and extracellular matrix remodeling (Sun et al., 2011). Understanding the role that the PVAT adipocyte plays in these processes requires reductionist approaches that are only possible with adipogenic models. Immune cell infiltration is largely due to the presence of macrophages in the adipose tissue (Sun et al., 2011), and it is thought in some cases for inflammation to be necessary for adipose tissue to expand at all (Asterholm et al., 2014; Cinti et al., 2005). Angiogenesis and extracellular matrix remodeling are also important for remodeling of adipose tissue. As the rate limiting step in expansion, formation of new blood vessels ensure that new and existing adipocytes are provided nutrients as well as maintained in normoxic conditions (Martinez-Santibanez and Lumeng, 2014; Sun et al., 2011). The extracellular matrix must then change its structure to support the growth of the adipocytes and newly formed blood vessels. Accurate modeling of PVAT expansion and its effect on blood vessels would give powerful insight in the treatment and prevention of cardiovascular disease development. However, there are many difficulties in modeling PVATs. Depot specific phenotypes also determine lipid droplet formation within cells (Table 2). Cultured adipocytes must also have a similar secretome as PVAT in order to understand its effects on vasculature anatomy and physiology. Together, these traits need to be represented under normal conditions as well as in response to stimuli mimicking disease states. In vitro modeling of PVAT adipocytes and would give much

insight into PVAT function during healthy states as well as during disease. Although models of adipogenesis itself exist, there is still a need for functional, depot-specific models that represent the individualities embodied by its adipocytes.

Adipogenesis

Although a comprehensive description of adipogenesis is beyond the scope of this review, it is necessary to give a brief overview of the processes to better understand the alternatives described here to improve adipogenesis modeling. Adipogenesis is the development of mature adipocytes from committed APC (Rosen and Spiegelman, 2000). Once induced, a complex series of events ensues (Figure 1), including, cell cycle arrest, changes to morphology, increased hormone sensitivity and expression of lipogenic genes, and lipid accumulation, [for comprehensive reviews on this topic reader is referred to (Mueller et al., 2002; Rosen, 2002; Rosen and MacDougald, 2006; Rosen and Spiegelman, 2000; Rosen et al., 2000; Siersbaek et al., 2012)]. These events are initially regulated by co-expression of the nuclear receptor PPARγ and transcription factors CCAAT/enhancer-binding protein alpha (C/EBPα) and SREBP-1 (Shao and Lazar, 1997). C/EBPα expression induces terminal adipocyte differentiation, while also expression of SREBP-1, signals downstream PPARγ expression (Rosen and Spiegelman, 2000). C/EBPs also bind to the Kruppel-like factor subfamily zinc finger protein 5 (KLF5) promotor, which then upregulates PPARγ expression(Rosen and MacDougald, 2006). PPARγ, a nuclear receptor and master regulator of adipogenesis, then starts the transcriptional cascade of adipogenic and lipogenic genes and enzymes, including perilipin 1 (PLIN1), fatty acid binding protein 4 (FABP4), acetyl-CoA carboxylase, fatty acid synthetase, and ATP-citrate lyase (Grimaldi et al., 1978; Rosen and MacDougald, 2006). PPARγ then signals a decrease in expression of leptin and increased expression of adiponectin (Park et al., 2011; Rosen and MacDougald, 2006).

Modeling Adipogenesis in PVAT

Many culture techniques have been created in attempt to improve adipogenesis rates, which vary across sites. For example, visceral preadipocytes differentiate at a much lower rate compared to those from subcutaneous depots (Wu et al., 2001), while PVATs have a lower adipogenic rate compared to gonadal adipose (Contreras et al., 2016). These cultures are designed to emulate the natural growth of cells by providing an in vitro environment that is more similar to that occurring in vivo. Much of the knowledge of adipogenesis modeling has been discovered using the immortalized Swiss mouse embryo fibroblast 3T3 cell lines (Green and Meuth, 1974). These cells have become a standard in adipogenesis due to their indefinite proliferation and exceptional capability to differentiate into adipocytes, however, it is important to note that differentiation efficiency in this cell line is reduced by senescence (Rosen and MacDougald, 2006). Although these cells, as well as other immortalized cell line models, including Ob17, EN-1078D, C3H10T1/2, OP9, (Armani et al., 2010; Duval et al., 2017; Ruiz-Ojeda et al., 2016), give insight into adipogenesis itself, they do not accurately model the diversity of adipocyte phenotypes. Decades of research have pursued the creation of more specific adipogenic models.

Hormones play a major role in activating the cascade of events in adipogenesis. Insulin binding causes activation of the MAP Kinase pathway that contributes to differentiation (Rosen and Spiegelman, 2000). In standard pharmacological induction practices, glucocorticoids, often dexamethasone, are used to activate the PPAR and C/EBP receptor families to induce cell maturation (Rosen and Spiegelman, 2000; Wu et al., 1996). Adipogenesis can also be triggered by bone morphogenetic proteins (BMP)

(Huang et al., 2009) and fibroblast growth factors (FGF) (Xiao et al., 2010) binding to preadipocyte surface receptor proteins and causing downstream activation of PPARγ and C/EBP (Figure 1) (Lowe et al., 2011). Standardized pharmacological induction of differentiation has been used in cell culture studies since the 1980's (Hauner et al., 1989; Student et al., 1980). The purpose of supplemental hormones in media is to activate the transcriptional cascades of adipogenesis, beginning with PPARγ. Additional hormones, such as insulin, are also added to stimulate lipid accumulation (Hauner et al., 1989). For the first 48 hours in a standardized induction, cells are incubated with 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone (Armani et al., 2010; Student et al., 1980). IBMX is a phosphodiesterase inhibitor that increases cAMP activity, which then stimulates C/EBP and PPARγ expression (Sun et al., 2003). Dexamethasone is a glucocorticoid that stimulates glucocorticoid receptors and commits preadipocytes to becoming mature adipocytes (Pantoja et al., 2008). The differentiation may then be maintained with media supplemented with fetal or calf serum, high glucose concentrations, and insulin (Armani et al., 2010). Beginning in the 1990's and 2000's, a PPARγ agonist, such as rosiglitazone or troglitazone, was included in adipocyte media (Motoshima et al., 2002). More recently, treatment with bone morphogenetic proteins prior to induction have been used to aid differentiation (Ahrens et al., 1993; Bowers and Lane, 2007; Schulz and Tseng, 2009). Beyond 3T3-L1 cells, rat preadipocytes and adipocyte progenitor cells have been successfully differentiated using standardized pharmacological inductions (Berry and Rodeheffer, 2013; Church et al., 2014; Contreras et al., 2016; Tchkonia et al., 2006). Hormones control cell growth and in cell culture aid in proliferative and lipogenic responses. Standard pharmacological inductions are not

universal for the culture of cells from various adipose depots and thus require additional supplements for custom cultures. The induction of differentiation can quickly become complex and expensive requiring frequent inclusion of additional growth factors. Still, theses inductions do not ensure equivalent induction efficiencies or retention of native cell phenotypes.

Improving Pharmacological Induction Efficiency

Preadipocytes and Adipocyte Progenitor Cells

Not all the fibroblast like cells in the stromal vascular fraction of AT will become adipocytes. Adipocyte progenitor cells (APC) vary from adipocyte-derived stem cells (ASC), which are not committed to a specific cell maturation but can differentiate into various cell types of the mesodermal origin (Yoshimura et al., 2009). APC can be identified by their profile of specific cell surface markers. For example, a specific group of APC presenting CD34 and platelet-derived growth factor receptor alpha (PDGFRα) surface markers was independently identified by two research groups (Berry and Rodeheffer, 2013; Lee et al., 2012). Cells co-expressing these proteins have the potential to differentiate to white or brown adipocytes (Lee et al., 2012). Other preadipocyte surface markers of commitment and differentiation have been identified, including Zfp423, Sca1, and CD24 (Gupta et al., 2012; Rodeheffer et al., 2008). Although the rate of proliferation can vary by site, the differentiation rate of the same isolated APC group can be similar (Contreras et al., 2016; Van Harmelen et al., 2004). Although certain groups of surface markers have identified committed APCs, there is not yet a distinct set recognized (Cawthorn et al., 2012). Using only APC in culture is one of the best ways to improve PVAT in vitro modeling.

Depot	Phenotype	Genotype	Impact
Thoracic Aortic PVAT	Brown, multilocular, high mitochondria density, UCP1 positive	*Pparγ, Cebpα, AdipoQ, Plin1, Ucp1, Pgc1α, Prdm16, Dio2, Zic1*	Catecholamine store, increased mass correlated to atherosclerosis development
Abdominal Aortic PVAT	Beige, multilocular, moderate mitochondria density, UCP1 negative	*Pparγ, Cebpα, AdipoQ, Plin1, Tcf21, Tbx1, Tmem26*	Catecholamine store, increased mass correlated to decreased vessel contraction
Coronary PVAT	Beige, multilocular, moderate mitochondria density, UCP1 positive	*Pparγ, Cebpα, AdipoQ, Plin1, Ucp1, Prdm16, Dio2*	Volume associated with the extent and severity of coronary artery diseases such as atherosclerosis
Mesenteric PVAT	White, unilocular, low mitochondria density, UCP1 negative	*Pparγ, Cebpα, AdipoQ, Plin1, Tcf21, Tbx1, Tmem26*	Catecholamine store, increased mass correlated to decreased vessel contraction
Peripheral/ Skeletal PVAT	White, unilocular, low mitochondria density, UCP1 negative	*Pparγ, Cebpα, AdipoQ, Plin1*	Modulates insulin-dependent microvascular function

Table 1: Overview of depot specific phenotype and genotype differences in perivascular adipose tissue. Adipogenic gene markers: Peroxisome proliferator-activated receptor gamma (*Pparγ*), CCAAT/enhancer-binding protein alpha (*Cebpα*), Adiponectin (*AdipoQ*), Perilipin 1 (*Plin1*). Brown adipocyte markers: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1α*), PR domain containing 16 (*Prdm16*), Deiodinase Iodothyronine Type II (*Dio2*), Zinc-finger protein 1 (*Zic1*). White adipocyte markers: Transcription factor 21 (*Tcf21*), T-box protein 1 (*Tbx1*), Transmembrane protein 26 (*Tmem26*). (Contreras et al., 2016; Gil-Ortega el al., 2015; Padilla et al., 2013)

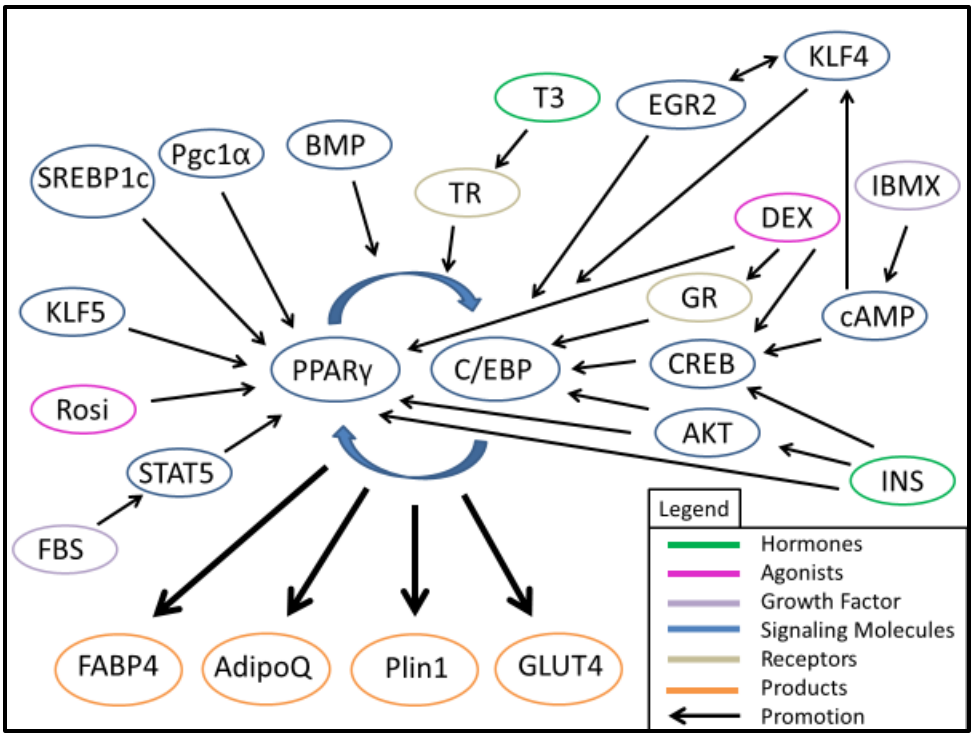

Figure 1: Transcriptional signaling cascade of adipogenesis in brief.

Abbreviations: Adiponectin (AdipoQ), Protein kinase B (AKT), Bone morphogenetic protein (BMP), Cyclic adenosine monophosphate (cAMP), CCAAT/enhancer-binding protein (C/EBP), cAMP response element-binding protein (CREB), Dexamethasone (DEX), Early growth response protein 2 (EGR2), Fatty acid binding protein 4 (FABP4), Fetal bovine serum (FBS), Glucose transporter type 4 (GLUT4), Glucocorticoid receptor (GR), 3-isobutyl-1-methylxanthene (IBMX), Insulin (INS), Kruppel-like factor 4 (KLF4), Kruppel-like factor 5 (KLF5), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc1a), Perilipin 1 (Plin1), Peroxisome proliferator-activated receptor gamma (PPARγ), Rosiglitazone (Rosi), Sterol regulatory element-binding protein 1c (SREBP1c), Signal transducer and activator of transcription 5 (STAT5), Thyroid hormone receptor (TR), Triiodothyronine (T3)

Conditioned Media

Various culture techniques (Table 2) attempt to increase adipogenic differentiation efficiencies by utilizing specific growth factors and culture surfaces in conjunction with pharmacological inductions. To address the challenge of differing induction rates among sites, conditioned media has also been used to differentiate adipocytes. These media contain the secretome or adipocytes of the same site being cultured that can then be introduced to another cell culture to explore relationships and interactions of the proteins. The secretome from primary adipocytes has been collected in conditioned media for use in studies of epidermal cell transformations (Bernard et al., 2014). Conditioned medium can be made; however, this requires simultaneous, independent culture of multiple cell lines. Although these mediums allow the ability to study a single cell type, they don't preserve the contained secretome well and protein degradation occurs rapidly (Khosravi et al., 2007; Yde et al., 2011). Conditioned medias also discount that in vivo, there is more than one cell type present and together influence the proliferation and differentiation of each other (Dowling and Clynes, 2011).

Micro-patterning

Micro-patterning is a form of 2D cell culture that alters the micro-topography of tissue culture plates that gives the cells a larger surface area to adhere to (Chaubey et al., 2008). A study by Chaubey and co-authors (Chaubey et al., 2008) indicated that when differentiating adipocytes, cells cultured micro-patterned surfaces differentiated more quickly than those cultured on non-patterned surfaces and the cells accumulated higher levels of lipid. The study also reported that cells cultured on polystyrene surfaces had a moderate lipid accumulation but a high rate of differentiation when compared to patterned and non-patterned poly-L-lactide surfaces, thus illuminating the importance of culture surfaces (Chaubey et al., 2008). Although these cultures improve proliferation of cells, there is no direct connection to this method in and of itself improving adipogenesis.

Three-dimensional Cultures

One type of 3D culture places cells in or on a polymer matrix. The matrix itself may be made of different material that provides a suitable scaffold for the cells in question to attach. Collagen and reduced growth factor basement membrane matrices work well with sensitive cell types, such as adipocytes. Emont and co-authors (Emont et al., 2015) demonstrated that differentiating visceral preadipocytes in a collagen matrix allows for increased induction rate and functional adipocytes with retained depot characteristics. Due to the support structure provided, this model has also been used in studies of adipose inflammation to understand processes to occur during disease states

(Turner et al., 2015). Isolation of cells from these 3D matrices post growth can be difficult and the matrix may need to be collected along with cells.

Another form of 3D culture uses scaffolds to support cell growth. These scaffolds can be made from biopolymers, such as collagen (Emont et al., 2015), or prefabricated polymers, such polycaprolactone or polystyrene. Scaffolding, especially in adipocyte culture, allow for a more natural space to for cells to grow and provide space for organelles, such as lipid droplets, as opposed to a flat growth area. Prefabricated scaffolds also allow for trypsin use to remove cells from culture environments. Hydrogel cell culture is an in-between of polymer matrices and scaffolds. Hydrogels provide a fluid-like encapsulation while simultaneously supporting cell growth (Emont et al., 2015). The challenge presented when using these hydrogels is removing the cells post growth for analysis. Although there are some obstacles to analysis, these semi-solid structures allow for a controllable stiffness or flexibility of the culture surface and its ability to conform to the growth of the cells, which can affect the migration and differentiation (Davidenko et al., 2010). This stability is a challenge in growth as the hydrogels degrade and may begin to lack support for cells over time.

Co-cultures

The next revolution in differentiation cultures in the last twenty years has been simultaneously culturing multiple cell types together, or co-culture. This type of culture pairs pharmacological induction with 3D and 2D models, using collagen matrices and trans-well inserts respectively. Collagen matrices are used to suspend the two cell types together in an environment similar to that in vivo. Huss et al. (Huss and Kratz, 2001)

have shown use of this method with adipocytes and mammary epithelial cells. Their study reports successful collection of preadipocytes and epithelial cells from a single breast tissue biopsy as well as the culture and differentiation of the adipocytes within a collagen matrix. Although this application was pursued in search of a way to engineer breast tissue post mastectomy due to cancer, this also reveals a viable way to differentiate preadipocytes in an environment similar to that in vivo. Another study by Aoki and colleagues (Aoki et al., 2003) showed that this method may also be used with preadipocytes in conjunction with endothelial cells and that endothelial cells may promote the differentiation of preadipocytes into adipocytes. This finding supports the knowledge of PVAT growth and communication between adipose and blood vessels. Collagen suspensions, even as a suitable growth environment, still present the problem of collecting the cells once they have been differentiated for post culture applications, especially when there is more than one cell type present in the matrix.

Transwell inserts have been used in co-cultures with fibroblasts, adipose tissue explants, and adipocytes. In these cultures, preadipocytes are seeded in plate wells and then a hanging transwell insert with a mesh bottom, with either an explant or adipocytes, is also placed inside the well such that the media is in contact with both groups of cultures. This model of culture may be beneficial to cell growth as it provides an environment similar to *in vivo* and thus allow cells to communicate to one another, while also allowing separation. A study conducted by Subramaniyan et al. (Subramaniyan et al., 2016) demonstrated that the use of transwell insert co-culture between 3T3-L1 adipocytes and bovine fibroblasts improved cell viability under oxidative stress, thus indicating cross-talk of protein secretions in the media improves

cell vigor. Although this model is applicable to combating oxidative stress and the use of 3T3-L1 cell lines may be slightly less expensive than using primary isolated cells, it does not allow for the same in vitro versus in vivo comparisons or accurately model depot variations (Ruiz-Ojeda et al., 2016). Furthermore, the differentiation protocols for these cells takes over two weeks achieve mature adipocytes, which can get expensive to maintain.

In order to study the effect that whole tissues can have on cells, explants have also been used in co-cultures. Challa and co-authors (Challa et al., 2015) showed that using adipose explants in transwell inserts reduces the lipid uptake during adipogenesis of preadipocytes from the same subcutaneous and visceral adipose depots when compared to a standard hormone cocktail induction alone. This result may have been caused by the other cell types, such as macrophages, found within the adipose tissue explant that limited the differentiation process. Due to the buoyancy of mature adipocytes, transwell inserts may be used in conjunction with a collagen matrix to suspend the adipocytes such that they are retained by the transwell inserts (Stacey et al., 2009; Zhao et al., 2015). A study by Zhao et al. (Zhao et al., 2015) demonstrated that using co-culture with primary adipocytes suspended in a collagen matrix in a transwell insert over adipose-derived stem cells can induce lineage commitment through paracrine signaling. This indicates that the secretome of the adipocytes present in the media can aid in the differentiation of adjacent cells. Similarly, a study by Stacey et al. (Stacey et al., 2009) attached human primary adipocytes to the bottom of inserts using a collagen suspension and differentiated isolated preadipocytes establishing that this co-culture technique is more effective when compared to standard hormone cocktail

differentiation and while maintaining the expression of adipogenic markers. Although this form of modeling allows for cell-cell communication, it does require collection of preadipocytes and then primary adipocytes separately.

Protocol	Pro	Con
* Standard Pharmacological Induction	• Standardized since 1980's • Works well with 3T3-L1 cells	• Alterations to increase efficiency get complicated and expensive • May not retain native phenotype
* Isolation of Adipocyte Progenitor Cells	• Mirrors depot specific adipogenesis	• Requires rigorous pharmacological inductions
Conditioned Media	• Contains cell secretome of the same site being cultured	• Requires multiple simultaneous cell cultures • Proteins secreted into media degrade quickly
Micro-patterning	• Provides larger surface area for cell attachment	• Not indicated to significantly improve adipogenesis alone
3D Polymer Matrix	• Allows 3D growth for increased structural support	• Difficult to isolate cells post growth for analyses
Scaffolds/Hydrogels	• Provides a 3D structure for cells to attach to	• Difficult to remove cells from structures for analyses • Hydrogels may degrade too quickly for optimal cell growth
Co-culture: Collagen Matrix	• Allows growth of two cell types in environment similar to in vivo	• Difficult to isolate and separate cells from matrix for post growth analysis
Co-culture: Explant Transwell Inserts	• Provides entire tissue secretome to differentiating cells	• May reduce adipogenesis due to other cell types present
Co-culture: Primary Adipocytes Transwell Inserts	• Primary adipocyte paracrine signaling aids in differentiation while maintaining	• Must have cultured preadipocytes and then isolate primary adipocytes

Table 2: Pros and cons of using various methods of improving adipogenesis.

Although methods of differentiation vary, all utilize some form of pharamacological induction in synchrony with the individualized protocol. * Indicates use in PVAT cultures.

Conclusion

Thus far, multiple studies have revealed the complexity of adipose tissue not only as a location of energy storage but also as a dynamic organ. APC undergo adipogenesis by differentiating into mature adipocytes. The populations of APCs found in PVAT vary by anatomical location and site and may influence adipogenic potential. The multiple phenotypes that are encompassed in various PVATs add to the expansive role that this adipose depot plays in body homeostasis. PVAT expansion impacts blood vessel function and the risk of cardiovascular disease development. Protocols inducing adipogenesis have begun to elucidate the mechanisms and extent that differentiation occurs; however, current methods do not accurately represent each individual PVAT depot. There remains a need for a model that emulates phenotype and function based on anatomical location. Further studies are needed for a full understanding and representation of depot-specific PVAT adipocyte dynamics.

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CHAPTER 2

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Expansion and Adipogenesis Induction of Adipocyte Progenitors from Perivascular Adipose Tissue Isolated by Magnetic Activated Cell Sorting

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Short Abstract

Here we report a method for isolation of adipocyte progenitor cell (APC) populations from perivascular adipose tissue (PVAT) using magnetic activated cell sorting (MCS). This method allows for an increased isolation of APC per gram of adipose tissue when compared to fluorescence activated cell sorting.

Long Abstract

Expansion of perivascular adipose tissue (PVAT), a major regulator of vascular function through paracrine signaling, is directly related to the development of hypertension during obesity. The extent of hypertrophy and hyperplasia depends on depot location, sex, and the type of adipocyte progenitor cell (APC) phenotypes present. Techniques used for APC and preadipocytes isolation in the last 10 years have drastically improved the accuracy at which individual cells can be identified based on specific cell surface markers. However, isolation of APC and adipocytes can be a challenge due to the fragility of the cell, especially if the intact cell must be retained for cell culture applications.

MCS provides a method of isolating greater number of viable APC per weight unit of adipose tissue. APC harvested by MCS can be used for in vitro protocols to expand preadipocytes and differentiate them into adipocytes through use of growth factor cocktails allowing for analysis of the prolific and adipogenic potential retained by

the cells. This experiment focused on the aortic and mesenteric PVAT depots, which play key roles in the development of cardiovascular disease during expansion. These protocols describe methods to isolate, expand, and differentiate a defined population of APC. This MCS protocol allows isolation to be used in any experiment where cell sorting is needed with minimal equipment or training. These techniques can aid further experiments to determine the functionality of specific cell populations based on the presence of cell surface markers.

Introduction

Perivascular adipose tissue (PVAT), due to its close proximity to blood vessels, is a major paracrine signaling component in vasculature function (Watts et al., 2013). Expansion of this adipose tissue is dependent on the phenotype of the adipocyte progenitor cells (APC) present (Dodson et al., 2014; Police et al., 2009). Isolation of cells from adipose tissues is difficult as primary adipocytes are fragile, buoyant, and range in size. Certain isolation techniques can also alter cell phenotype and morphology by increasing inflammatory protein synthesis and reducing adipogenic gene expression (Ruan et al., 2003), emphasizing the importance of a protocol that maintains the integrity of the cells.

Culture of primary cells and specific preadipocyte subpopulations gives a reductionist approach to *in vivo* growth and maintains equivalent cellular genetic makeup (Stacey, 2001), although working time with these cells is limited due to deterioration with aging, or senescence (Swim and Parker, 1957). Preadipocytes from various adipose depots, including subcutaneous and omental depots, also demonstrate differences in proliferation (Van Harmelen et al., 2004), which emphasizes the importance of collecting cells from specific anatomical sites. Precursor cells from non-PVAT white adipose depots have been characterized in previous studies (Church et al., 2014; Roncari et al., 1981; Van Harmelen et al., 2004), but less is known about PVAT APC phenotypes.

The techniques described here allow for the analysis of specific and defined APC populations with minimal impact on their morphology, viability, and potential to proliferate and differentiate. Magnetic activated cell sorting (MCS) is amenable to

downstream applications, such as culture, as the beads dissolve without altering the cell. MCS is also economical, and once the antibody concentrations have been standardized, the need for flow cytometry assays is minimal. *In vitro* studies with PVAT precursors can also give a glimpse of the potential that these primary cells may have.

Protocol

All procedures described in this paper follow guidelines established by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University. All buffers and medias should be protected from light.

Preparation of Buffers, Media, and Instruments

1.1 Prepare Krebs Ringer Bicarbonate Buffered Solution (KRBB): 135 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium sulfate, 0.4 mM potassium phosphate dibasic, 5.5 mM glucose, 1% antibiotic/antimycotic (10,000 units/mL penicillin, 10,000 µg/mL streptomycin, 25 µg/mL amphotericin B), and 10 mM HEPES (pH = 7.4). This solution is stable for 3 weeks when kept sterile and at 4 °C.

1.2 Prepare Collagenase Type 1 Solution: 1 mg/mL in KRBB with 4% bovine serum albumin. This solution should be kept at 37 °C and is stable for 4 h.

1.3 Prepare Erythrocyte Lysis Buffer Solution: 154 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA. Keep at 4 °C for up to one month.

1.4 Prepare MCS Blocking Buffer: DMEM/F12 Media base, 10% fetal bovine serum, 5% normal donkey serum, 40 µL/mL F(ab) Fragment Donkey Anti-Rat IgG. Keep at 4 °C for up to one month.

1.5 Prepare MCS Buffer Solution: PBS (pH 7.5), 0.5% BSA, and 2 mM EDTA. Keep at 4 °C for up to one month. De-gas solution by heating to 37 °C in a glass container and then applying a vacuum for 15 s. Leave unused buffered sealed.

1.6 Prepare Stromal Vascular Fraction (SVF) Basal Media: Dulbecco's Modified

Eagles Medium (DMEM):F12, 15% fetal calf serum, 1% antibiotic/antimycotic (10,000 units/mL penicillin, 10,000 µg/mL streptomycin, 25 µg/mL amphotericin B), 44.05 mM sodium bicarbonate, 100 µM ascorbic acid, 33 µM biotin, 17 µM pantothenate, 2 mM L-glutamine, and 20 mM HEPES. Keep sterile and at 4 °C for up to 2 weeks.

1.7 Prepare Adipocyte Progenitor Cell (APC) Media: Basal Media with additional growth factors including epidermal growth factor (10 ng/mL), leukemia inhibitory factor (10 ng/mL), platelet-derived growth factor BB (10 ng/mL), and basic fibroblast growth factor (5 ng/mL). Keep sterile and at 4 °C for up to 2 weeks.

1.8 Prepare Adipocyte Progenitor Cell (APC) Induction Media: APC Media with 10% fetal bovine serum, 2.5 µg/mL insulin, 0.5 mM 2-isobutyl-1-methylaxanthine (IBMX), 1 µM dexamethasone, and 200 pM T3 (triiodothyronine thyroid hormone). Keep sterile and at 4 °C for up to 2 weeks.

Adipocyte Progenitor Isolation

2.1 Anesthetize rat according to institutional guidelines. Place the rat in dorsal recumbency. Confirm depth of anesthesia via a toe-pinch and the loss of reflex response to this painful stimulus.

Note: This protocol uses 10-week-old Sprague Dawley rats and 70 mg/kg of pentobarbital delivered via an intraperitoneal injection.

2.2 Make a vertical midline incision with scissors along the sternum in the thoracic

area and to the perineal area. Access the abdominal cavity and expose the superior mesenteric artery, the small mesenteric resistance vessels (mPVAT) and the thoracic aorta (aPVAT).

2.3 Sever all connections to the mesentery and aorta and remove vessels from animal. Isolate PVAT by using a dissecting microscope and Petri dish filled with KRBB to view vessels and isolate PVAT.

2.4 In this experiment, collect gonadal (GON) adipose to represent a non-PVAT adipose depot. Place isolated fat pads on ice in KRBB with 10 mM HEPES (pH = 7.4).

2.5 In a biosafety hood, transfer about 50 mg of tissue to a 1.7 mL tube with 1 mL of collagenase type I solution and mince with tissue scissors (1-3 mm pieces).

2.6 Digest samples by incubating at 37 °C in a rotisserie incubator (or incubator with an orbital shaker) for 1 h. In a biosafety hood, sequentially filter digested material through 100 and 40 µm cell strainers into a 50-mL tube. Centrifuge resulting filtrate at 4 °C for 10 min at 300 × g.

Note: All steps in the protocol from here forward are to be performed in a biosafety hood to keep cells sterile.

2.7 Pour off supernatant and resuspend pellets containing the SVF cells in 1 mL of 1X Erythrocyte Lysis Buffer Solution and transfer to a 1.7 mL microfuge tube. Incubate cells for 5 minutes at room temperature protected from light and centrifuge at 4 °C for 5 min at 300 × g.

2.8 Pour off supernatant and resuspend remaining cell pellet in SVF Basal Media. Collect a 20 µL sub-sample to count live cells with Trypan Blue Solution.

Note: The number of SVF cells that can be isolated will vary by site. Average numbers of SVF harvested per mg of tissue are: aPVAT = 5.0 ± 2.0x10^3, mPVAT = 1.04 ± 0.62x10^4, GON = 2.4 ± 1.2x10^5.

Magnetic Activated Cell Sorting

Note: Isolate APC from SVF based on CD34 and PDGFRα cell surface markers by performing all steps at 4 °C.

3.1 Spin cells for 5 min at 300 × g. Pour off supernatant and resuspend the cell pellet in MCS Blocking Buffer at 1x10^6 cells/mL and incubate for 20 min.

Note: Cell suspensions of 1x10^6 to 2x10^8 cells/mL can be separated effectively.

3.2 Incubate cells with 5 µL of FITC-conjugated Mouse anti-CD34 (1 µg/1x10^6 cells) for 30 minutes at 4 °C. Spin cells for 5 min at 300 × g and 4 °C.

3.3 Incubate with 4 µL of anti-FITC microbeads and 96 µL of MCS buffer (total volume of 100 µL) for 5 min at 4 °C in the dark to separate CD34+ and CD34- cells.

3.4 Attach magnetic separator to the stand and place the MultiSort (MS) Column, with the column wings to the front, into the separator. Place a 5-mL collection tube under the MS Column in the upper tube holder.

3.5 Prepare MS Column by rinsing with MCS Buffer Solution. Apply 500 µL of MCS Buffer on top of the column and let the buffer run through. Discard effluent and change collection tube.

3.6 Load antibody labeled cell suspension onto the prepared MS Column. Collect flow-through containing unlabeled cells.

3.7 Wash MS Column with 500 µL of degassed MCS Buffer 3 times. Collect unlabeled cells that pass through and combine with the flow through from previous step.

3.8 Remove MS Column from the magnetic separator and place it on a new collection tube. Pipette 1 mL of MCS Buffer onto the MS Column. Immediately flush out fraction with the magnetically labeled cells by firmly, but slowly, applying the plunger supplied with the column as to not allow excess gas into the column.

Note: Isolated cell numbers will vary by site. Average numbers of CD34+ APC isolated from the SVF population per mg of tissue are: aPVAT = 2.6 ± 0.43x10^2, mPVAT = 9.6 ± 1.4x10^2, GON = 1.3 ± 0.22x10^3.

3.9 Spin cells for 5 min at 300 × g and 4 °C. Incubate the CD34+ fraction collected in 10 µL of a 1:200 solution per 1x10^6 cells Rabbit anti-PDGFRα for 30 min at 4 °C.

3.10 Centrifuge cells again for 5 min at 300 × g and 4 °C. Incubate labeled cells with 4 µL of anti-rabbit IgG microbeads and 96 µL of MCS buffer by repeating steps 3.3 through 3.7 to isolate.

Note: Isolated cell numbers will vary by site. Average numbers of PDGFRα+ APC isolated from the CD34+ population per mg of tissue are: aPVAT = 2.4 ± 0.64, mPVAT = 8.4 ± 2.4, GON = 10.4 ± 1.9, which is 0.5-10% of the previously isolated population.

Cell Culture and Adipogenesis Induction

4.1 Culture the SVF and APC in 6-Well tissue culture plates in basal media with replacement every 2 days. After 3 serial passages, plate in black 96-Well tissue culture plates at $1x10^2$ cells/well for proliferation assays, which are evaluated at 8, 24, 48, and 96 h, and at 50,000 cells/well in 24-well plates or 10,000 cells/well in 48-Well tissue culture plates for adipogenesis assays, which are qualitative and quantitative.

4.2 Supplement APC basal media for 48 h post-confluency and prior to induction with bone morphogenic protein 4 (3.3 nmol/L) as indicated10 for differentiation. Induce cells after 48 h of 100% confluency (day 0) using the APC Induction Media to incubate the cells.

4.3 After 48 h, change media to maintain cells in APC Induction media without IBMX and dexamethasone, for 14 days with media changes every 48 h.

MCS Isolation Validated By FACS

5.1 Take a 50 µL (50,000 cells) sub sample of magnetically separated cells and wash with FACS solution.

5.2 Centrifuge cells and resuspend in 100 µL of a 1:1,000 solution of donkey anti-

rabbit IgG Dylight 405 to label the PDGFRα+ cells. Incubate for 30 min protected from light and at 4 °C.

5.3 Wash cells and resuspend in 200 µL of 2% formaldehyde solution until time for analysis using 488 nm (FITC) and 405 nm (Dylight 405) filters on a flow cytometer.

Note: Lipid accumulation by cultured cells is assessed quantitatively using a lipophilic adipogenesis fluorescence assay in a microplate reader measuring fluorescence and using preadipocytes as calibrators for lipid accumulation. Lipid accumulation is also measured qualitatively by lipid dye staining and imaging performed on an inverted microscope equipped with a camera, making the percentage of total cells that do or do not contain lipid observable. Any plate reader that is able to measure fluorescence with excitation at 485 nm and emission at 572 nm is suitable for analysis as well as any microscope with a camera capable of capturing digital images.

Results

Proliferative capacity of preadipocytes and adipogenic potential of adipocyte precursors are characteristics that are maintained in vitro11. In vitro proliferation of isolated SVF and APC from aPVAT, mPVAT, and GON of male rats was evaluated at 8, 24, 48, and 96 h after plating using a quantitative DNA assay. No site differences in SVF expansion rate were observed at any time point except for the APC from aPVAT, which had less proliferation by 96 h compared to SVF cells from the same site (Figure 2). Confluent APC stimulated with bone morphogenic protein-4 for 48 h prior to standard induction12 exhibited differentiation. This was evident by greater lipid accumulation in droplets as evaluated by both fluorescent lipid uptake assay (Figure 3A) and Oil Red O staining (Figure 3B). When comparing the yield of APC, MCS isolation produced a greater number of cells ready for culture compared to FACS. (Figure 4) Importantly, the distribution and viability of APC populations (CD34+ and PDGFRα+) was similar between MCS and FACS. Cell viability determined by Trypan Blue staining for counting post isolation was similar for both isolation procedures (FACS = 71.57% ±11.09; MCS = 79.25% ± 7.47). These data demonstrate that the MCS isolation of APC yields a higher number of viable APC compared to MCS.

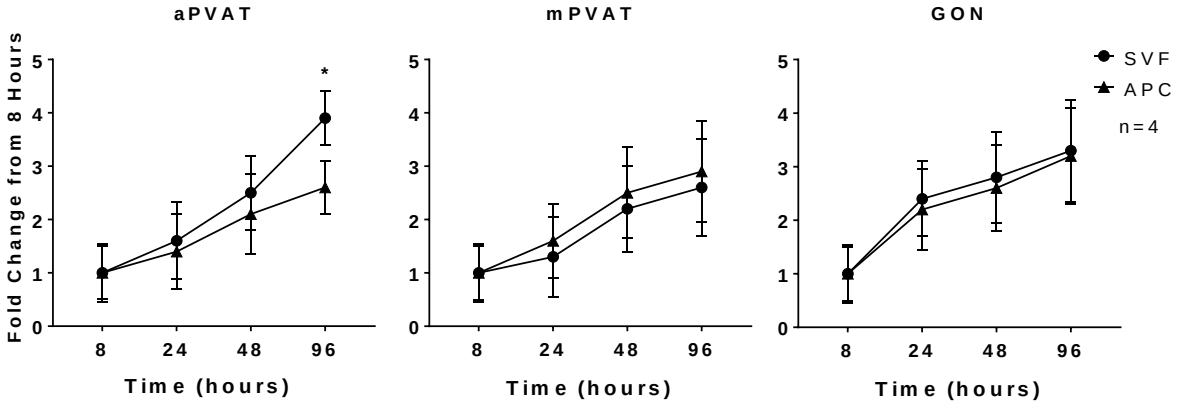

Figure 2: In vitro proliferation of stromal vascular fraction (SVF) is affected by anatomical location. Stromal vascular fraction (SVF) and APC were isolated from aortic and mesenteric perivascular adipose tissue (aPVAT and mPVAT, respectively) and gonadal adipose from 10-week old male rats. Proliferation of cells was measured by a DNA quantification assay at 8, 24, 48, and 96-h time points after seeding. Data are expressed as fold increase over 8 h baseline ± SEM (N=4). Significance is indicated by * ($P<0.05$). Figure modified from Contreras et al. 2016 (Contreras et al, 2016).

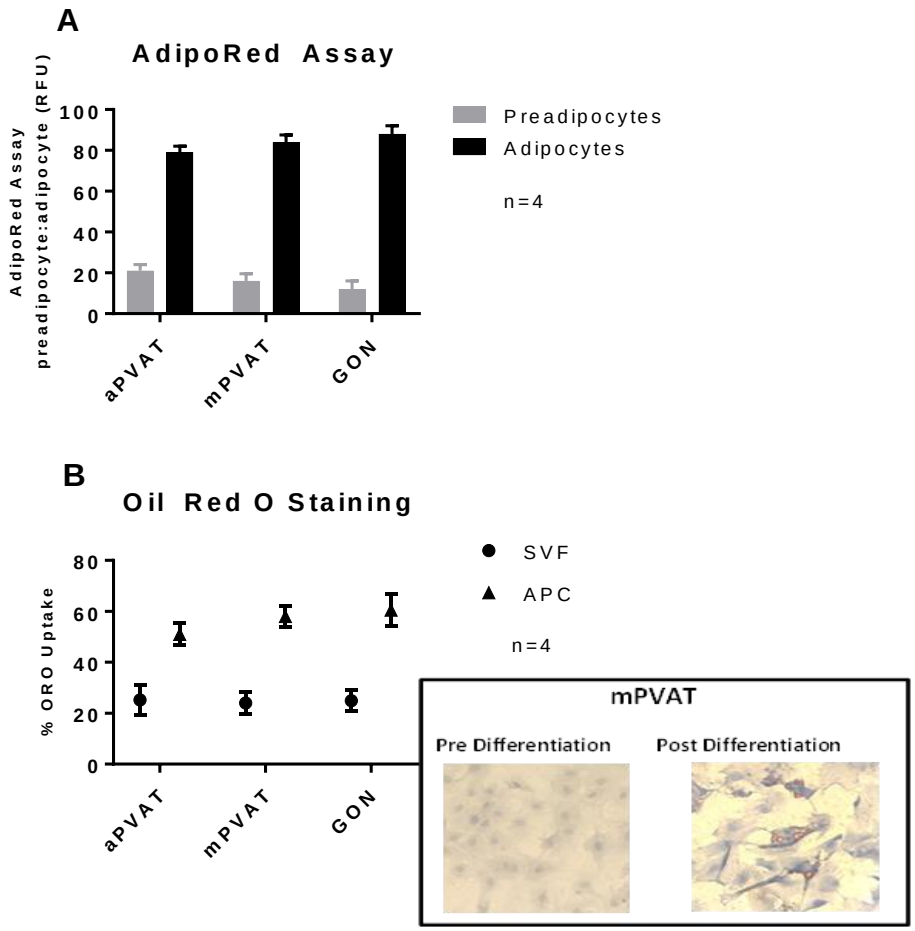

Figure 3: Adipocyte progenitor cells (APC) show no variation in differentiation abilities between depots but greater lipid accumulation compared to stromal vascular fraction (SVF). Cells were induced after 48 h of confluency and exposure to bone morphogenic protein-4 followed by 48 h of exposure to dexamethasone and 3-isobutyl-1-methylxanthine and then sustained in maintenance media 14 days with media changes every 48 h. (A) Lipid uptake assay of differentiated APC with data expressed as ratio of undifferentiated preadipocytes: differentiated adipocytes (preadipocytes: adipocytes) in relative fluorescence units (RFU) ± SEM (N=4). (B) Oil Red O (ORO) staining of APC and SVF with data expressed as percent of cells with

Figure 3 (cont'd)

lipid (ORO Uptake) ± SEM (N=4). Significance is indicated by * ($P<0.05$). Figure modified from Contreras et al. 2016 (Contreras et al, 2016).

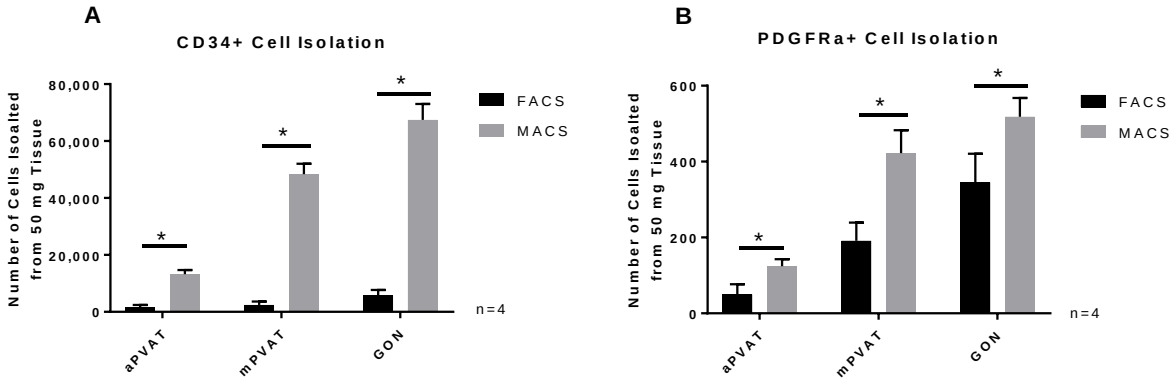

Figure 4: Isolation yield of viable adipocyte progenitor cells (APC) is improved by Magnetic Activated Cell Sorting (MCS). Surface marker expression of CD34+ (A) and PDGFRα+ (B) in SVF isolated from perivascular adipose tissues (aortic=aPVAT; mesenteric=mPVAT) using MCS and FACS. Data are expressed as mean number of cells isolated from 50 mg of tissue ± SEM (N=4). Significance is indicated by * ($P<0.05$).

Discussion

The central focus of the present experiment is the isolation, expansion, and adipogenic induction of APC from PVAT depots. Here we present a protocol for the isolation of APC based on the identification of cells expressing the surface markers CD34 and PDGFRα. These surface proteins were previously identified on APC with high proliferation rates and the potential to differentiate into white or brown adipocytes in various adipose depots (Lee et al., 2013; Lee et al., 2012). By selecting cells based on these specific markers, we were able to isolate similar APC populations from multiple adipose depots that match the adipocyte phenotype that is observed in the PVAT selected (Contreras et al., 2016). In our experiments we improved APC differentiation by supplementing the growth factor BMP4. Previously, Macotela and colleagues demonstrated that specific APC populations from visceral adipose depots had less BMP activity than those from subcutaneous depots when differentiated unless culture media was supplemented with the adipogenic growth factors BMP2 or BMP4(Ahrens et al., 1993; Bowers and Lane, 2007; Macotela et al., 2012; Schulz and Tseng, 2009)' .

Since isolation of individual cells from adipose tissue is difficult due to the fragility of the cells, using MCS provides an efficient method for cell isolation at a smaller cost of consumables, equipment, and training resources. It is important to note that APC yield can be affected by the animal's age or size and by changes in the temperature of media and failure to maintain a sterile isolation environment. Culturing cells in normoxia is also important for proliferation and differentiation (Choi et al., 2015), thus maintenance of incubation air conditions is integral to culture practice. Centrifugation speeds can be increased to 800 x g if sufficient cell pellets do not form during the isolation process.

Checking the expiration of the antibodies may also be necessary as the conjugated FITC fluorochrome can degrade over time. Changing lots of Collagenase Type I may also require alterations to digestion procedures as lots can vary in potency. If using a different species other than rat, specific serum and IgG in the blocking buffer may also be necessary if the host species of the antibodies is different from the ones used here.

Among the advantages of MCS over FACS are that it is more economically feasible than FACS as kits are inexpensive and can be easily purchased. This also avoids the purchase, maintenance, and usage training of a flow cytometer. Using MCS also allows for specific binding without adjustment of gates for selection and background fluorescence involvement.

A limitation to this study is that it relied on two preadipocyte surface markers. Other preadipocyte surface markers of commitment and differentiation, such as Zfp423, Sca1, and CD24, have been identified (Gupta et al., 2012; Rodeheffer et al., 2008). These markers may accurately identify committed adipocyte progenitor cells of specific phenotypes; however, the surface markers used here were selected since cells expressing these markers have the ability of inducing to both brown and white phenotypes (Gupta et al., 2012; Rodeheffer et al., 2008). Another limitation of this study was the selective use of growth factors in APC culture. Other growth factor cocktails have been effective in the induction of adipogenesis (Scott et al., 2011). Cell culture in this study was also limited by the fact that all culture was done in two-dimensional culture plates. Although this is the culture norm, cells *in vivo* do not proliferate in this way. Culturing in three-dimensional environments may allow for further hypertrophy and

hyperplasia as it replicates the natural form of the adipose structure (Edmondson et al., 2014).

Due to the practicality and efficiency of using MCS, this protocol is ideal for the isolation of APC as well as other cell types in PVAT. This procedure also provides a more effective way to induce differentiation in preadipocyte cultures. The minimal cost, equipment, and training required allows this method to be used in any lab wishing to isolate cells based on specific surface markers. Future applications may allow for isolation of other cell populations or use of more specific cell markers. Use of growth factor cocktails in cell commitment may be useful in stem cell activation. Pairing MCS isolation of APC with other methods to improve adipogenic efficiency, such as co-culture, may also aid in the creation of a robust model of adipogenesis in PVAT.

Acknowledgements

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CHAPTER 3

Co-culture of Primary Adipocytes and Preadipocytes Improves Adipogenic Efficiency of Mesenteric Perivascular Adipose

Introduction

Perivascular adipose tissue (PVAT) is an important modulator of vascular function given its anatomical location and its capacity to secrete vasoactive molecules including proteins and lipids (Watts et al., 2013). PVAT's capacity to modulate vascular function is affected in part by the major phenotype held by the adipose depot: white or brown. White adipose is characterized as having a low mitochondrial density, being uncoupling protein 1 (UCP1) negative, having one large lipid droplet, and functions largely as an energy storing unit. In contrast, brown adipose has a high mitochondrial density, is UCP1 positive, has multilocular lipid storage, and its main function is thermogenesis (Sanchez-Gurmaches et al., 2016). Adipocyte phenotypes vary depending on PVAT anatomical location (Rajsheker et al., 2010; Sanchez-Gurmaches et al., 2016). For example, PVAT surrounding the thoracic aorta has a brown phenotype while PVAT surrounding the small mesenteric resistance vessels has a white phenotype (Contreras et al., 2016). PVATs ability to regulate vasculature function becomes impaired during disease states (Lian and Gollasch, 2016). During metabolic diseases resulting from excessive energy intake, such as obesity and metabolic syndrome, adipose tissue is expanded by hyperplasia and hypertrophy (Wang et al., 2013). These processes allow for increased cell number with greater lipid storage capacity; but, when uncontrolled, lead to adipose dysfunction. PVAT dysfunction, leads to alterations in the capacity to modulate vessel function predisposing to cardiovascular disease (Lian and Gollasch, 2016).

To study the mechanisms by which adipocytes adapt to lipid overload, models, including 3T3-L1 cells (Shoham and Gefen, 2011; Student et al., 1980), have been

created to study adipogenesis and adipose expansion. However these models do not fully represent the variations between adipose depots. Furthermore, adipogenic models of visceral adipose tissue, where mesenteric PVAT is located, have low adipocyte differentiation rates compared to subcutaneous depots (Tchkonia et al., 2002; Tchkonia et al., 2006). Despite its importance in modulating blood pressure and vascular function, to date, there is not a depot-specific adipogenic model for PVATs that accurately represents their specific phenotype and functions. This would allow for further study of PVAT in respect to its ability to take up and secrete molecules as well as its response to changes during disease states. The objective of this study was to create a depot-specific adipogenic model for various PVATs. We present a co-culture approach for preadipocyte differentiation by exposing them to homologous primary adipocytes isolated from the same PVAT depots. Our study shows that this method provides a greater adipogenic response when compared to using a pharmacological induction of preadipocytes isolated from PVATs.

Methods

All animal use described here follow guidelines established by the Institutional Animal Care and Use Committee (IACUC) of Michigan State University.

Animals and Tissue Processing

Eight to ten weeks of age male Sprague Dawley rats were euthanized using 70 mg/kg of pentobarbital delivered via an intraperitoneal injection. PVAT from small mesenteric resistance vessels (mPVAT) and the thoracic aorta (aPVAT), as well as gonadal (GON) adipose, were collected in KRBB supplemented with HEPES 10 mM (pH = 7.4) as previously described (Contreras et al., 2016; Thelen et al., 2017). In brief, adipose tissues (50 mg) were digested with 1 mL collagenase type I (Worthington Biochemical) solution (1 mg/mL) and then centrifuged to separate the primary adipocytes from the SVF. Primary adipocytes were washed in 1 mL of KRBB with 4% bovine serum albumin and centrifuged. Final adipocyte population was retained for use in transwell inserts for inductions. The SVF was then sequentially filtered through 100 and 40 µm cell strainers (Falcon). Filtrate was then centrifuged and resulting cell pellet was resuspended and incubated in erythrocyte lysis buffer. After another centrifugation, resultant cells were cultured in preadipocyte media containing 10% fetal bovine serum (Corning), 44.05 mM sodium bicarbonate (Fisher), 100 µm ascorbic acid (Sigma-Aldrich), 33 µm biotin (Sigma-Aldrich), 17 µm pantothenate (Sigma-Aldrich), 1% L-glutamine (Gibco), 1% antibiotic/antimycotic solution (Gibco), and 2% HEPES (Fisher). To eliminate immune cells, preadipocytes populations were expanded for two serial passages in culture flasks (Nest).

Standard Induction

After two serial passages, preadipocytes were seeded in 6 well and 24 well plates (Corning, Corning, New York) at a concentration of 20,000 cells/cm^2 and allowed to proliferate to confluency. Confluent cells were then induced to differentiate using a adipocyte induction media containing 10% fetal bovine serum (Corning, Corning, New York), 5µg/ mL insulin (Sigma-Aldrich, St. Louis, MO), 200 pmol/L T3 (Sigma-Aldrich, St. Louis, MO), and 0.5 µM Rosiglitazone (Caymen Chemical, Ann Arbor, MI). The following supplements from Sigma-Aldrich were used for the first 48 hours of culture: 0.5 mM IBMX and 0.25 µM Dexamethasone. Media was changed every 48 hours.

Co-culture

Expanded preadipocytes populations were seeded in 6 well and 24 well plates (Corning, Corning, New York) at a concentration of 20,000 cells/cm^2 and allowed to proliferate to confluency. Confluent cells were then induced to differentiate using adipocyte induction media used in standard induction cultures and also included a 0.4 µm transwell insert (Greiner Bio-One, Kremsmünster, Austria) containing 900 cells/cm^2 primary adipocytes over the preadipocytes. Media was changed every 48 hours.

Culture Optimization

To determine optimal conditions for co-culture, preadipocytes were cultured in 24 well plates for a total of 7 days (aPVAT, mPVAT, GON) or 10 days (mPVAT). Inserts of primary adipocytes were removed at 96, 120 and 144 hours post induction while preadipocytes were allowed to continue to differentiate. At the end of the of the

differentiation period (d7 for aPVAT, mPVAT and GON, and d10 for mPVAT), triacylglyceride accumulation was analyzed via AdipoRed™ assay (Lonza, Basel, Switzerland). Live cell counts of primary adipocytes were done after 0 and 144 hours of co-culture using Trypan Blue (Sigma-Aldrich, St. Louis, MO). Adipocytes were also cultured using pharmacological induction alone for the same differentiation period as their co-cultured pairs (d7 for aPVAT, mPVAT and GON, and d10 for mPVAT), as well as for 14 days as a control for standard induction protocols.

Efficiency

Expanded preadipocyte populations were seeded in a glass bottom 24 well plate (Corning, Corning, NY) at a concentration of 20,000 cells/cm^2 and allowed to proliferate until confluency. Confluent cells were then induced to differentiate by either standard induction or co-culture methods described above. After differentiation, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and then stained with NucBlue™ and HCS LipidTox™ (Life Technologies, Carlsbad, CA) to identify nuclei and triacylglycerides respectively. Cells were imaged using an Olympus FluoView FV1000 filter-based Confocal Laser Scanning Microscope paired with Olympus FV10-ASW software (Olympus, Waltham, MA). Efficiency was determined by counting the number of differentiated versus non-differentiated cells. ImageJ software was used to measure triacylglyceride (TAG) accumulation.

Functional Assays

Cultured adipocytes differentiated by either coculture of standard pharmacological induction were removed from 6-well plates (Corning, Corning NY) using Trypsin (ThermoFisher, Waltham, MA). Adipocytes were then seeded in triplicate at 100,000 cells/well in black wall 96-well plates (Nunc, Roskilde, Denmark). Functionality of adipocytes was assessed by quantification of lypolytic glycerol release and lipogenic fatty acid uptake using a Glycerol Assay (Sigma-Aldrich, St. Louis, MO) and the QBT Fatty Acid Uptake Assay (Molecular Devices, Sunnyvale, CA) respectively. To measure lipolysis, cells were serum starved for 4 hours and then stimulated to undergo lipolysis by incubating with 1 µM isoproterenol (Sigma-Aldrich, St. Louis, MO), a beta-agonist. After 2 hours of incubation, supernatant was removed and glycerol content was measured. The CyQUANT Assay (Life Technologies, Carlsbad, CA) was used to determine cell number in each well post functional analysis. Fatty acid uptake was measured by first serum starving cells for 16 hours and then stimulating fatty acid uptake by incubating cells with 10 nM insulin (Sigma-Aldrich, St. Louis, MO). Reagent from the QBT Fatty Acid Uptake assay was then added and uptake was measured every 20 seconds for one hour.

Results

To investigate if coculture conditions with primary adipocytes would decrease viability, Trypan Blue staining was used to assess primary adipocyte viability at 0 and 144 hours of cell culture. There was no difference between the viability of cells at either time point (Figure 5). To determine the optimal time of primary adipocyte incubation, preadipocytes were induced to differentiate by incubating in adipocyte induction media and exposing them to primary adipocytes from the same site for 96, 120, and 144 hours. After 7 days of total differentiation, aPVAT and GON revealed an increase in lipid accumulation after 144 hours and 120 hours of primary adipocyte incubation respectively over standard induction, while mPVAT saw no change (Figure 6). The total differentiation time was increased to 10 days in mPVAT, which showed an increase of lipid accumulation after 10 days and at least 120 hours of primary adipocyte incubation over standard induction (Figure 7). Once culture conditions were optimized (Table 3), cells from each depot were cultured to perform functional assays and RNA analyses.

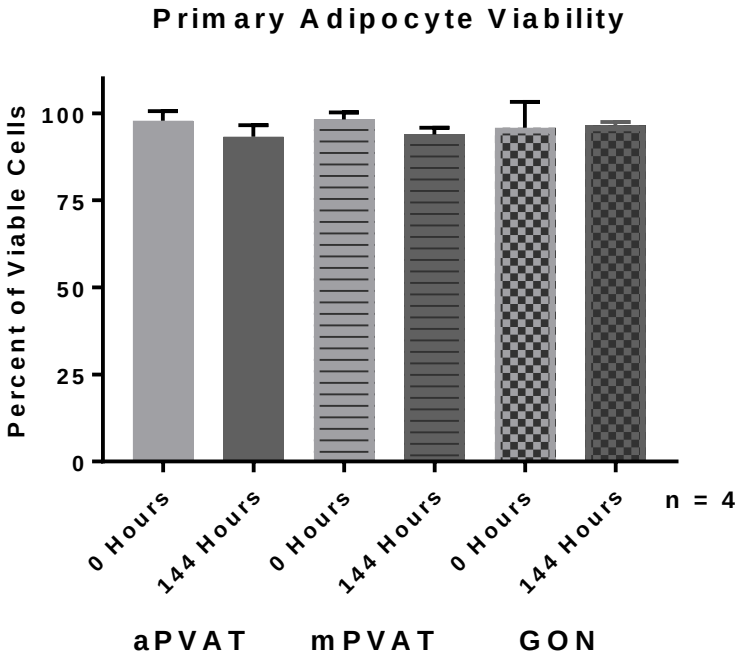

Figure 5: Health of primary adipocytes is not affected by co-culture. Percentage of live cells at 0 hours and 144 hours of co-culture. Data are represented as mean percent viability ± SEM.

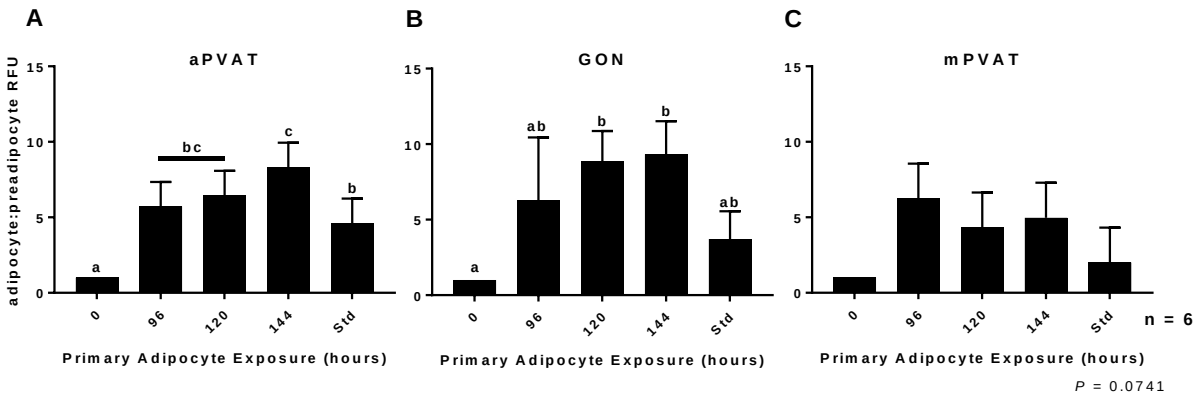

Figure 6: Co-culture of improves aPVAT and GON preadipocyte differentiation compared to pharmacological induction. Co-culture of primary adipocytes and preadipocytes for 0 (preadipocytes), 96, 120, 144 hours and standard pharmacological induction (Std) over 7 days isolated from (A) aortic PVAT (aPVAT), (B) gonadal adipose (GON), and (C) mesenteric PVAT (mPVAT). Analysis of adipogenesis was done by AdipoRed™ assay. Data are represented as the ratio preadipocytes: adipocyte relative fluorescence units (RFU) ± SEM. Significant differences are indicated by letters a, b ($P < 0.05$).

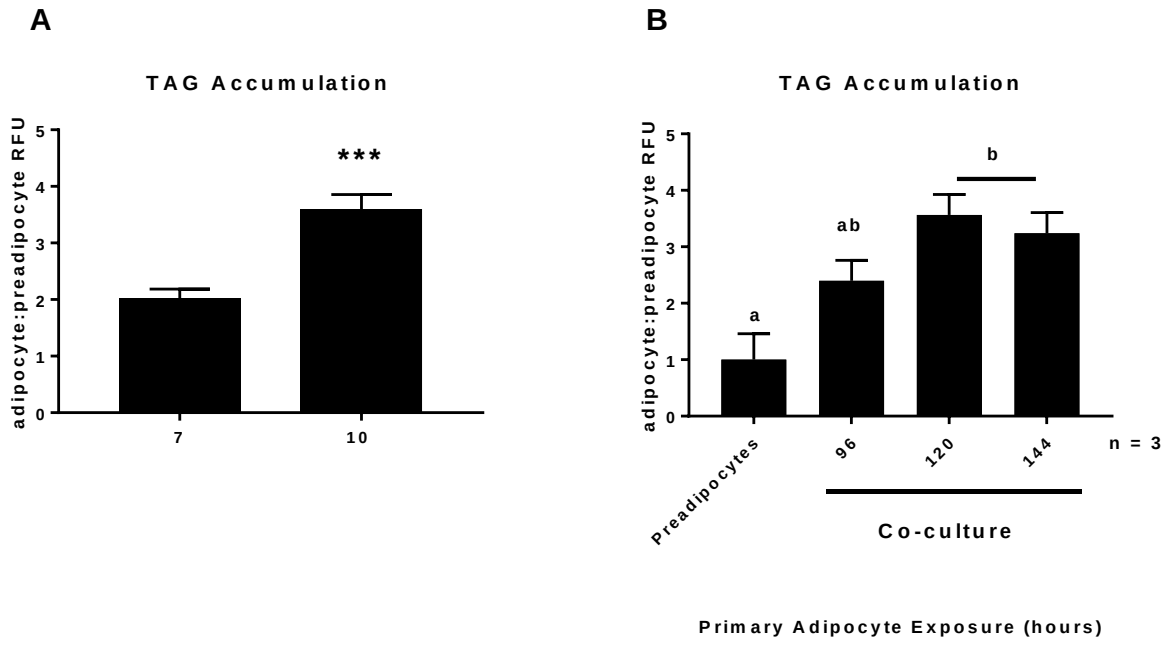

Figure 7: Co-culture improves mesenteric preadipocyte differentiation compared to standard induction methods when cultured for ten days. Co-culture of primary adipocytes and preadipocytes for 7 versus 10 days (A) and culture of preadipocytes and co-culture for 96, 120, and 144 hours (B) isolated from mesenteric PVAT. Analysis of adipogenesis was done by AdipoRed™ assay. Data are represented as the ratio preadipocytes: adipocyte relative fluorescence units (RFU) ± SEM. Significant differences are indicated by * and letters a, b ($P < 0.05$).

Site	Primary Adipocyte Exposure (hours)	Total Differentiation Time (days)
Aortic PVAT	144	7
Mesenteric PVAT	120	10
Gonadal Adipose	120	7

Table 3: Optimal conditions for differentiation of preadipocytes and primary isolated adipocytes. Most efficient exposure and total differentiation times for co-culture of primary adipocytes and preadipocytes as determined by time course trial and assessed using the AdipoRed™ assay.

It was first necessary to determine if co-culture improved the efficiency of pharmacological induction. Co-culture and the 14 day standard induction had similar differentiation efficiencies in aPVAT and GON cultures, with lower efficiency in the 7 day standard induction. Both standard induction protocols in the mPVAT cultures produced moderate efficiencies, while co-cultures differentiated at 76.87 ± 3.36% (Figure 8). All induction protocols yielded similar triacylglyceride (TAG) accumulation when used in the aPVAT and GON depots. However, the most TAG accumulation was seen in mPVAT adipocytes differentiated by co-culture (Figure 9 and Figure 10).

Adipocyte functionality was assessed in both lipolytic and lipogenic facets. Lipolysis was measured by stimulating differentiated adipocytes with the beta-agonist, isoproterenol and then measuring glycerol content in the supernatant after 2 hours. Glycerol release was adjusted to the number of cells per well determined by CyQUANT™ Assay. In both aPVAT and GON depots, co-culture differentiated adipocytes had a higher glycerol release than those differentiated by pharmacological induction alone (Figure 11). Adipocytes in the mPVAT had similar lipolysis rates regardless of differentiation protocol. Those adipocytes differentiated by pharmacological induction did not have a different lipolysis rate from basal (Figure 11).

Fatty acid uptake was first stimulated by insulin and uptake was then measured. Although aPVAT adipocytes cultured using standard induction for 7 days had a lesser uptake than basal, those cultured for 14 days and co-cultured had increased uptake with co-cultured adipocytes have the highest response (Figure 12). Adipocytes from the mPVAT depot had a similar response with an the most increased uptake seen in co-cultured cells, however there were no differences between basal uptake and cells

differentiated by standard inductions. GON adipocytes also had no differences between basal uptake and standard induced cells as well as an increased response from co-cultured adipocytes (Figure 12).

Adipocytes differentiated by co-culture (CC) and 14 day pharmacological induction (Std 14d) and the stromal vascular fraction (SVF) from aPVAT, mPVAT, and GON depots were collected assess adipocyte phenotype gene expression using RT-qPCR. In all depots, there were no differences seen in the expression of *Pparg* while aPVAT and mPVAT had increased expression of *Cebpa* in co-cultured adipocytes over SVF. Co-culture adipocytes had a higher expression of *AdipoQ* and *TH* in aPVAT and *FABP4* in GON. Both PVAT co-cultures had higher expression of *Plin1*. Surprisingly, expression of *Tbx1*, a brite phenotype marker, was highest in the SVF of mPVAT and adipocytes differentiated by pharmacological induction. As expected, there was no Tbx1 expression in aPVAT (Figure 13).

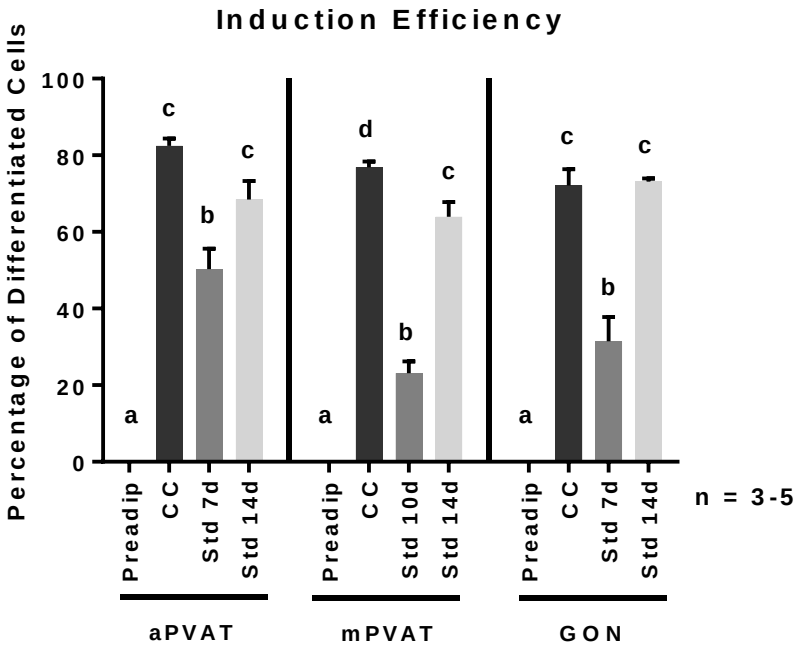

Figure 8: Mesenteric PVAT adipocytes have increased induction efficiency than those differentiated by pharmacological induction alone. Preadipocytes from aortic (aPVAT) and mesenteric (mPVAT) perivascular adipose tissue as well as those from the perigonadal (GON) adipose depot were stimulated to differentiate by co-culture or pharmacological induction protocols. Differentiated adipocytes were then stained with NucBlue™ and HCS LipidTox™ to identify nuclei and triacylglycerides respectively. Treatment abbreviations are as follows: Preadip = Preadipocytes, CC = Co-culture, Std = Pharmacological Induction for 7 (aPVAT and GON) or 10 days (mPVAT), Std 14d = Pharmacological Induction for 14 days. Values are percentage of differentiated cells ± SEM. Significant differences are indicated by letters a, b, c, d ($P < 0.05$).

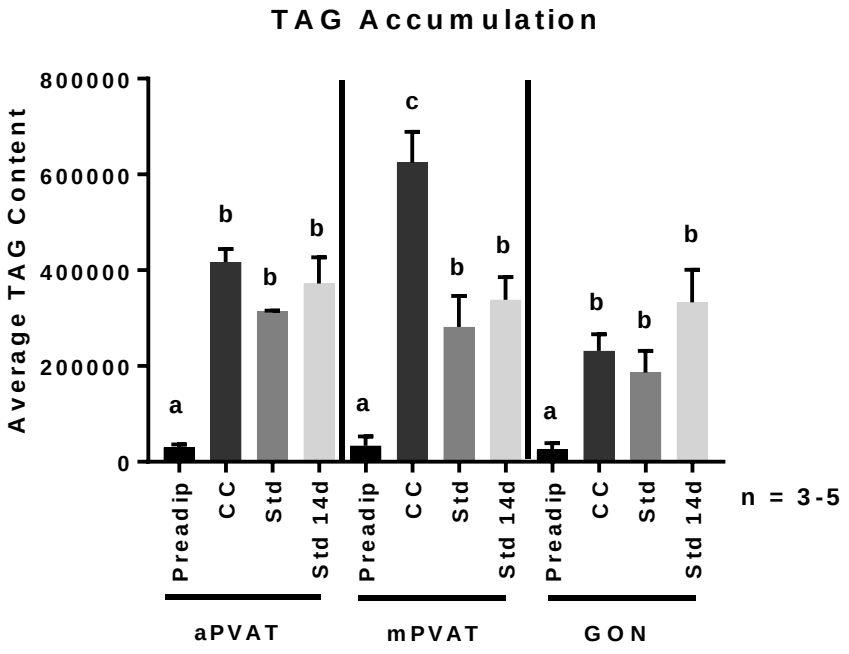

Figure 9: Mesenteric PVAT adipocytes differentiated by co-culture accumulate more triacylglycerides per cell than when induced by pharmacological induction alone. Preadipocytes from aortic (aPVAT) and mesenteric (mPVAT) perivascular adipose tissue as well as those from the perigonadal (GON) adipose depot were stimulated to differentiate by co-culture or pharmacological induction protocols. Differentiated adipocytes were then stained with NucBlue™ and HCS LipidTox™ to identify nuclei and triacylglycerides respectively. Treatment abbreviations are as follows: Preadip = Preadipocytes, CC = Co-culture, Std = Pharmacological Induction for 7 (aPVAT and GON) or 10 days (mPVAT), Std 14d = Pharmacological Induction for 14 days. Values are mean triglyceride fluorescence per adipocyte $\pm$ SEM. Significant differences are indicated by letters a, b, c ($P < 0.05$).

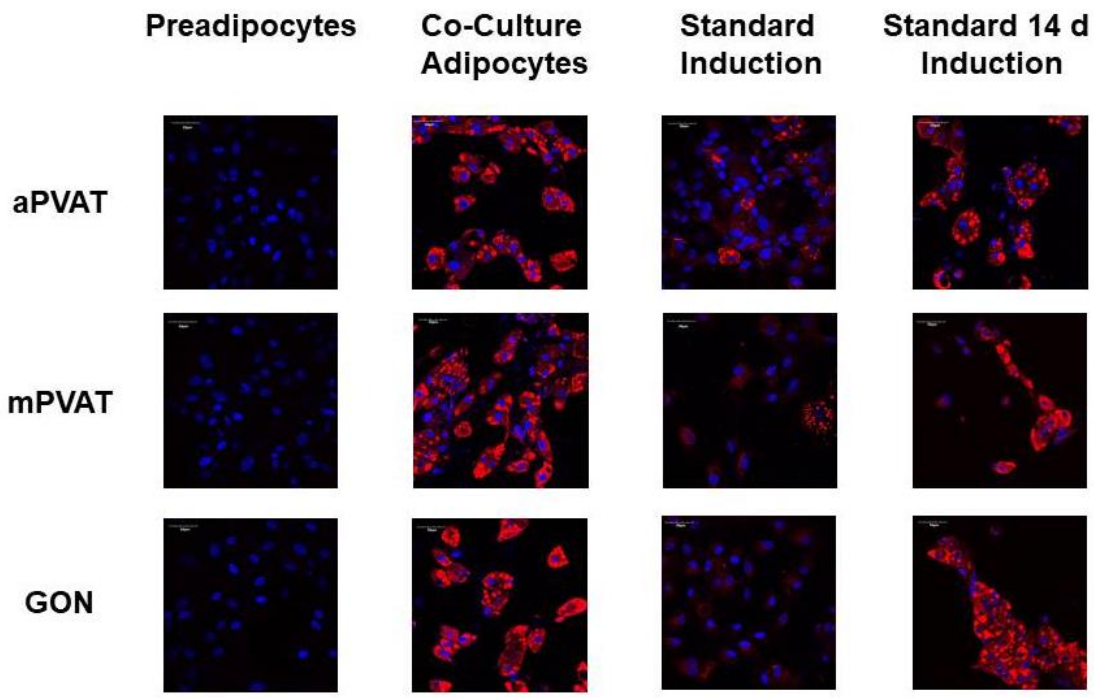

Figure 10: Co-culture increases triacylglyceride accumulation in culture.

Representative high resolution images of aortic (aPVAT) and mesenteric (mPVAT) PVATs and perigonadal adipose tissue (GON) as preadipocytes and differentiated by various cultures with nuclei stained with NucBlue™ (blue) and triacylglycerides stained with HCS LipidTOX™ (red). Scale bars represent 50 microns.

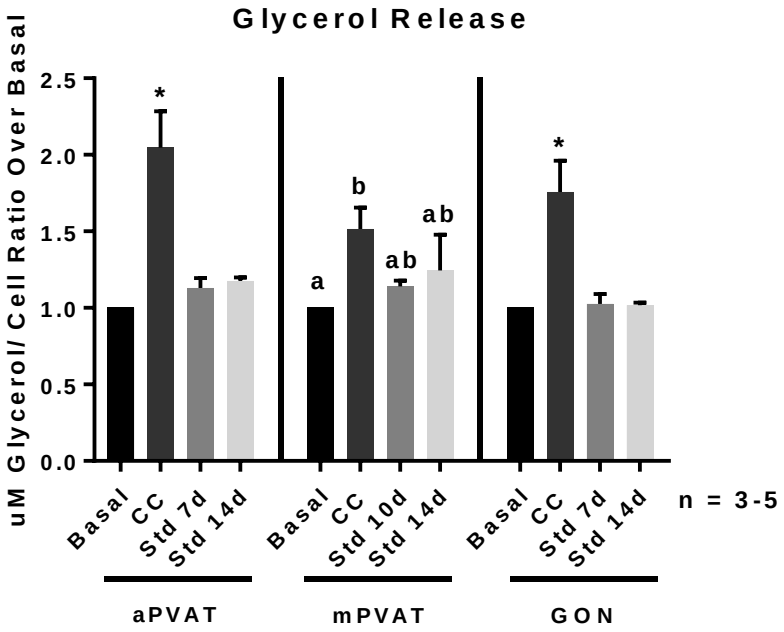

Figure 11: Co-culture produces aortic PVAT and gonadal adipocytes that undergo a higher rate of lipolysis with stimulate with a beta agonist. Adipocytes from aortic (aPVAT) and mesenteric (mPVAT) perivascular adipose tissue as well as those from the perigonadal (GON) adipose depots were differentiated by co-culture and pharmacological induction and were then stimulated to undergo lipolysis by a beta agonist and glycerol was measured. Treatment abbreviations are as follows: Basal = Basal lipolysis, CC = Co-culture, Std = Pharmacological Induction for 7 (aPVAT and GON) or 10 days (mPVAT), Std 14d = Pharmacological Induction for 14 days. Values are mean fold change over basal glycerol release $\pm$ SEM measured Glycerol Assay and adjusted to cell number. Significant differences are indicated by letters a, b and * ($P < 0.05$).

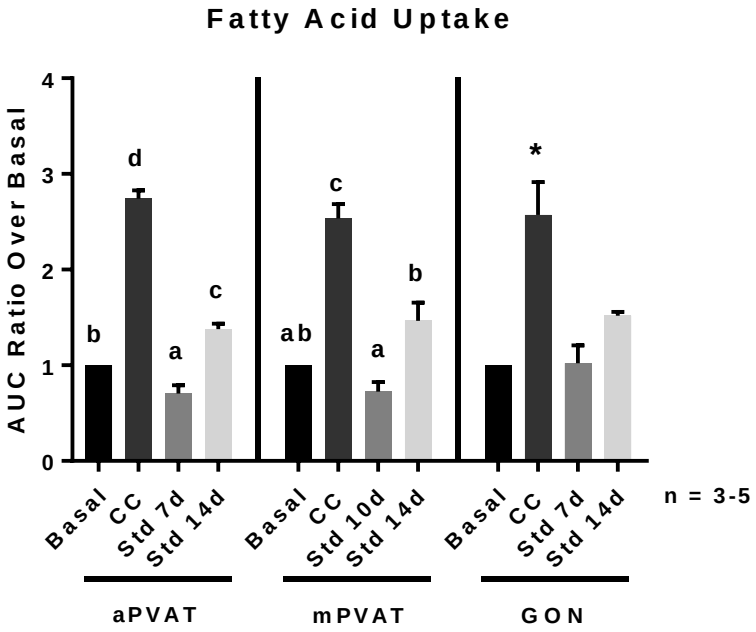

Figure 12: Co-culture differentiation produces adipocytes with a higher capacity to take up fatty acids than those adipocytes differentiated by pharmacological induction alone. Adipocytes from aortic (aPVAT) and mesenteric (mPVAT) perivascular adipose tissue as well as those from the perigonadal (GON) adipose depots were differentiated by co-culture and pharmacological inductions and then stimulated by insulin to take up fatty acid. Treatment abbreviations are as follows: Basal = Basal fatty acid uptake, CC = Co-culture, Std = Pharmacological Induction for 7 (aPVAT and GON) or 10 days (mPVAT), Std 14d = Pharmacological Induction for 14 days. Values are mean fold change over basal uptake $\pm$ SEM measured by QBT Fatty Acid Uptake Assay. Significant differences are indicated by letters a, b, c, d and * ($P < 0.05$).

Discussion

In this study we demonstrate that co-culture does improve differentiation and produces adipocytes that are phenotypically and functionally similar to primary adipocytes from the same depot. This co-culture method has been previously used in the differentiation of subcutaneous white preadipocytes (Stacey et al., 2009), but not before tested in the culture of preadipocytes of PVATs. Adipocytes from various depots express differences in adipogenic potential and susceptibility to apoptosis (Tchkonia et al., 2005). This may be reflected in the health of the PVAT primary adipocytes here as we didn't see any changes in viability even after 144 hours of co-culture. Co-culture increased the efficiency of differentiation in all depots. This indicates that the co-culture protocol does improve differentiation efficiency in time and in triglyceride accumulation. The primary adipocytes may have increased differentiation by signaling the need for energy storage and supplying a depot specific profile of fatty acids to preadipocytes.

Increased adipogenic efficiency in cell culture models of PVAT is extremely beneficial to in vitro assays that would assess PVATs ability to modulate vasculature tone. Our study tested the functionality of co-culture adipocytes by stimulated lipolysis and fatty acid uptake. After stimulation with isoproterenol, a beta agonist, adipocytes in all depots underwent significant lipolysis regardless of differentiation culture. Previous studies have determined that isoproterenol is a less effective beta agonist in lipolysis stimulation in mesenteric adipocytes (Umekawa et al., 1997). This may explain why mPVAT adipocytes had a lesser response over basal to the stimulated lipolysis than other depots. Previous studies demonstrate that adipocytes take up fatty acids through active transport (Ho et al., 2006; Stahl et al., 2002). Co-cultured adipocytes in all depots

showed increase fatty acid uptake in response to insulin stimulation. This indicates a more active hormone response in co-cultured adipocytes compared to those differentiated by pharmacological induction alone.

Conclusion

Our results demonstrate that the adipogenesis efficiency of preadipocytes is enhanced by co-culture with site specific primary adipocytes from aPVAT, mPVAT, and GON. This is evident by the lipid accumulation in cells that were differentiated by co-cultured compared to those that were induced with pharmacological induction media alone. We were able to determine the optimal exposure to primary adipocyte exposure and total differentiation time. Our data shows that maximum adipogenesis was achieved within 7 days in aPVAT and GON depots when co-cultured with primary adipocytes for 144 hours and 120 hours respectively. Increased adipogenesis in mPVAT required 10 days of total differentiation and a minimum of 120 hours of primary adipocyte exposure.

Once our co-culture protocol was optimized, we were able to assess its efficiency, as well as adipocyte functionality and phenotype. Our aPVAT and GON co-culture differentiated with similar efficiencies to that of the 14 day pharmacological inductions, while mPVAT co-culture had a higher efficiency than other pharmacological inductions. All induction cultures accumulated similar amounts of triacylglycerides in aPVAT and GON depots, but those co-cultured in the mPVAT depot accumulated a greater amount than standard pharmacological inductions. Co-cultures proved to be effective in differentiating adipocytes functional in lipolysis and lipogenesis in all depots. Our data indicates that co-culture is a more efficient method in differentiating PVATs than pharmacological induction alone.

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CHAPTER 4

Conclusion

PVAT secretory functions and its proximity to blood vessels make it an important regulator of vasculature function. PVAT expansion during obesity impairs its ability to regulate vessel function and thus impact the risk of cardiovascular disease development. The expansion of PVAT by hyperplasia is determined by the APC content in each depot. Due to phenotype variations in PVAT, adipogenic models must be depot-specific. Many methods have attempted to improve standard pharmacological inductions of adipogenesis, including conditioned media, polymers, matrices, and co-cultures, but do not represent the unique qualities expressed by PVATs. Isolating specific APC populations from various depots allow us to improve the adipogenic efficiency of PVATs in vitro. Using the magnetic activated cell sorting method allows for a more cost effective way isolate a specific group of adipocyte precursors and to induce differentiation. Utilizing co-culture protocols also prove to be more effective in differentiation PVAT adipocytes. Increased lipid accumulation over a shorter period of time enables a more efficient differentiation. Adipocytes differentiated by co-culture were also more responsive to external stimuli driving lipolysis and fatty acid uptake. Through our research, we have found that APC isolation and co-culture are the most efficient way to increase adipogenic efficiency in in vitro culture of PVAT.