Distinct adipocyte progenitor cells are associated with regional phenotypes of perivascular aortic fat in mice

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Et al.
Brief Communication

Distinct adipocyte progenitor cells are associated with regional phenotypes of perivascular aortic fat in mice

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ABSTRACT

Objective: Perivascular adipose tissue depots around the aorta are regionally distinct and have specific functional properties. Thoracic aorta perivascular adipose tissue (tPVAT) expresses higher levels of thermogenic genes and lower levels of inflammatory genes than abdominal aorta perivascular adipose tissue (aPVAT). It is not known whether this distinction is due to the in-vivo functional environment or to cell-autonomous traits that persist outside the in-vivo setting. In this study, we asked whether the progenitor cells in tPVAT and aPVAT have cell-autonomous traits that lead to formation of regionally distinct PVAT.

Methods: We performed microarray analysis of thoracic and abdominal peri-aortic adipose tissues of C57Bl/6J mice to define gene expression profile of each depot. To derive adipocyte progenitor cells, C57Bl/6J mice were sacrificed and thoracic and abdominal aorta fragments were embedded in Matrigel and cultured under pro-angiogenic conditions. Adipogenesis was induced using the Ppar-γ agonist rosiglitazone, a thiazolidinedione (TZD). TZD-induced adipocyte populations were analyzed using immunofluorescence and qRT-PCR.

Results: Microarray analysis showed that tPVAT expressed higher levels of transcription factors related brown adipose tissue development compared to aPVAT. Classic brown adipose tissue (BAT) genes such as Ucp-1, Prdm16, Dio2, Scc27a displayed a concordant trend of higher level expression in tPVAT, while white adipose tissue (WAT) genes such as Hoxc8, Nnat, Snug, and Mest were expressed at a higher level in aPVAT. The adipokines resistin and retinol binding protein 4 were also higher in aPVAT. Furthermore, adipocyte progenitors from abdominal and thoracic aortic rings responded to TZD with expression of canonical adipocyte genes Acrp30, Pisin1, and Glut4. Adipocytes differentiated from thoracic aorta progenitors displayed markedly higher induction of Ucp-1 and Cidea.

Conclusions: Thoracic aorta PVAT expresses higher levels of brown adipocyte transcription factors than aPVAT. Precursor cells from the thoracic aorta give rise to adipocytes that express significantly higher levels of Ucp-1 and Cidea ex vivo, suggesting that progenitor cells in tPVAT and aPVAT have cell-autonomous properties that dictate adipocyte phenotype.

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Keywords Perivascular adipose tissue; Adipocyte precursors; Progenitors; Ucp-1; Adipogenesis; Aorta

1. INTRODUCTION

Perivascular adipose tissue (PVAT) is a newly recognized adipose depot with highly active endocrine and paracrine functions. PVAT secretes a multitude of adipokines and other factors that have important physiological and pathophysiological implications for the adjacent blood vessels [1–6]. Recent studies show that murine thoracic PVAT produces numerous factors, including adiponectin, leptin, H2S, and other unidentified factors, collectively referred to as adipocyte-derived relaxing factors, that influence vascular function [7–13]. These secreted factors can facilitate a vasodilatory effect through an endothelium-dependent mechanism via nitric oxide (NO) synthesis and release or an endothelium-independent mechanism via the generation of hydrogen peroxide [10,14]. On the other hand, in obese humans or mice, PVAT has been shown to release factors to promote inflammation, vasoconstriction, or vascular smooth muscle cell proliferation that are detrimental to vascular function [15,16]. Therefore, the physiological importance of PVAT in vascular homeostasis is undisputed and is an area of intense investigation. Among the most studied murine PVAT is adipose tissue surrounding the aorta. Along the aorta, the phenotype of the PVAT is unique depending on anatomical location [17]. The levels of inflammatory genes and markers of immune cell infiltration are greater in aPVAT than in tPVAT [18]. The aPVAT phenotype is more pro-inflammatory.
and atherogenic than the tPVAT phenotype and may explain why aneurysms more frequently occur in the abdominal aorta [17–19]. PVAT surrounding the murine thoracic aorta (tPVAT) exhibits phenotypic features of brown adipose tissue (BAT), and that surrounding the abdominal aorta PVAT (aPVAT) is more similar to white adipose tissue (WAT) [17–19]. While adipose tissue (WAT) classically functions to store excess energy in the form of triglyceride, in contrast, brown adipose tissue (BAT) oxidizes fatty acids to generate heat. “Beige” adipocyte exists in WAT and can be induced by cyclic adenosine monophosphate (cAMP) to express thermogenic genes to resemble BAT [20]. Although it is clear that there are inherent phenotypic differences between IPAT and aPVAT, it is unclear whether these differences are the result of extrinsic anatomical factors or intrinsic cell autonomic properties. For example, higher beta-adrenergic signaling could induce expression of brown adipocyte genes even if precursors are inherently the same existing in different environments [21]. In contrast, the cells could be intrinsically different due to transcriptional programing and would develop into distinct adipocyte in an environment-independent manner.

In this study, we sought to answer this question by analyzing transcriptomic data from peri-aortic adipose tissue and studying progenitor cells that give rise to new adipocytes from different regions of mouse aorta. For this purpose, we used the traditional ex vivo aortic ring assay to study progenitor cells arising from thoracic and abdominal aortic regions. Originally, Nicosia and Ottinetini designed the ex vivo aortic ring assay to study angiogenesis [22]. Cells arising from aortic rings form tubular structures and luminate. Since its first introduction, this method has been widely used to study angiogenesis. Adipocyte progenitor cells are known to reside in the vasculature of the adipose tissue [23–26]. The vasculature, such as the aorta, harbors PVAT progenitor cells. As such, we used the peroxisome proliferator-activated receptor gamma (Ppar-γ) agonist Rosiglitazone, a thiazolidinedione (TZD), to stimulate adipogenesis in precursor cells emerging from aortic explants from thoracic and abdominal aortic rings. We observed that cells emerging from the aortic ring take on adipocyte morphology, express adipocyte markers, and secrete adiponectin. Furthermore, we found that TZD-induced IPAT expresses higher levels of hallmark brown adipocyte genes, uncoupling protein 1 (Ucp-1) and cell death activator CIDE-A (Cidea), than aPVAT. Our microarray analysis showed that transcription factors relating to brown adipocyte development are elevated in IPAT compared to aPVAT. Taken together, our results demonstrate that adipocytes residing in IPAT and aPVAT have cell-autonomous characteristics that dictate phenotypic development.

2. MATERIAL AND METHODS

2.1. Microarray analysis

All animal experiments in this work has been approved by the Institutional Animal Care and Use Committee of University of Massachus-etts Medical School. Briefly, C57B1/6J mice were sacrificed using CO2 and cervical dislocation. The heart and vasculature were removed and the endothelial sprouts remaining in the Matrigel were isolated. The endothelial sprouts were species matched Alexa Fluor 594, and Alexa Fluor 488 (Molecular Probes). Secondary antibodies were polyclonal Guinea Pig Perilipin (Fitzgerald Industries International, 20R-PP004, 1:200). Secondary antibodies were polyclonal Guinea Pig Perilipin (Fitzgerald Industries International, 20R-PP004, 1:200). Secondary antibodies were polyclonal Guinea Pig Perilipin (Fitzgerald Industries International, 20R-PP004, 1:200). Secondary antibodies were polyclonal Guinea Pig Perilipin (Fitzgerald Industries International, 20R-PP004, 1:200). Secondary antibodies were polyclonal Guinea Pig Perilipin (Fitzgerald Industries International, 20R-PP004, 1:200).

2.2. Aortic ring assay

CS7B1/6J mice were sacrificed as above. After cutting side branches throughout, the aorta was excised from the root to the femoral bifurcation. PVAT was removed under a dissecting microscope leaving the tunica externa intact. The thoracic aorta was determined to be segments from the aortic arch to the diaphragm and the abdominal aorta was determined to be segments from below the diaphragm to femoral bifurcation. The aortic segments were washed with EGM-2 media and cut into 1 mm ring segments. Each aortic ring segment was embedded in a singular well of a 96-well plate containing 40 μL of Matrigel (BD Discovery Labware). After aorta was embedded in the 96-well plate, the plate was allowed to stand for 30 min in the 37 °C so that the Matrigel would solidify. The cells were fed with 200 μL of EGM-2 media, which was changed every other day. The mouse aorta segments were mechanically excised, and the endothelial sprouts remaining in the Matrigel were isolated using Dispase II (Roche, 2.4 U/mL), centrifuged, and RNA extracted from the pellet using an Ambion RNA extraction kit. Probes used are specified in Table 5 of Appendix.

2.3. Staining and analysis

Pieces of aortic rings were embedded in Growth Factor Reduced Matrigel (BD Biosciences) in 35 mm glass-bottom culture dishes (MatTek Corporation), and cultured in EGM-2 media for 14 days. After 14 days in culture with and without rosiglitazone, explants were fixed in 4% Formaldehyde (Ted Pella, Inc.) in PBS for 15 min and permeabilized in 0.5% TX-100 in PBS for 30 min. The primary antibodies used to characterize the origin of cells growing from the explants were mouse Cdx3 (BD Pharmingen, clone MEC 13.3, 1:50), mouse Cdx4 (BD Pharmingen, clone RAm34, 1:50), monoclonal mouse Adiponectin (Peirc, PA-054, 1:200), and polyclonal Guinea Pig Perilipin (Fitzgerald Industries International, 20R-PP004, 1:200). Secondary antibodies were species matched Alexa Fluor 594, and Alexa Fluor 488 (Molecular Probes; Molecular Probes). Negative controls treated with irrelevant mouse IgG instead of primary antibody were processed simultaneously. All sections were counterstained with DAPI (Molecular Probes.)

2.4. Western blotting

The mouse aorta fragments were mechanically excised and the sprouts remaining in the Matrigel were isolated using Dispase II (Roche, 2.4 U/mL). Cell lysates were prepared using the Complete
Lysis-M kit (Roche). Protein concentration was measured using the Beckman DU-640B Spectrophotometer. Equal amounts of protein (20 µg) and conditioned media (40 µl) were loaded and analyzed by western blot in accordance with standard procedure. The primary antibodies used were polyclonal Guinea Pig Perilipin (Fitzgerald Industries International, 20R-PP004, 1:2000) and goat polyclonal to beta actin (Abcam, ab8229, 1:5000) for cell lysate and rabbit polyclonal anti-Adiponectin (Pierce, PA1-054, 1:10000) for media. The secondary antibodies were goat anti-rabbit (Promega), rabbit anti-goat (Abcam), and goat anti-guinea pig antibody (Millipore).

3. RESULTS

It was previously reported that tPVAT resembles BAT and aPVAT resembles more of WAT [17–19]. To understand the mechanism by which they differ in phenotype, we performed transcriptome analysis of both tissues. Unsupervised hierarchical clustering analysis (HCA) demonstrated that tPVAT and aPVAT clustered based on anatomical location (Figure 1). Consistent with results previously described, microarray analysis showed that tPVAT has a gene expression profile resembling BAT.
genes such as *Ucp-1*, PR domain containing 16 (*Pdrm16*), deiodinase (*Dio2*), fatty acid transporter (*Scd27a*) at higher levels than aPVAT (Supplementary Table 1). Quantitative RT-PCR confirmed that tPVAT expressed *Ucp-1* at a higher level than aPVAT (Supplementary Figure 1). Abdominal PVAT expressed highly enriched WAT genes such as *Hoxc8*, *Nnat*, *Sncg* and *Mest*. Expression of the adipokines, resistin (*Retn*) and retinol binding protein 4 (*Rbp4*) were also higher in aPVAT. Interestingly, among the most upregulated genes in tPVAT compared to aPVAT were genes associated with muscle structure and function such as actin, myosin and troponin (Supplementary Table 1).

Gene set enrichment analysis (GSEA) revealed that expression of genes regulating myogenesis was the most upregulated pathway in tPVAT. Cholesterol homeostasis and well as fatty acid metabolism were among downregulated pathways in tPVAT compared to aPVAT (Figure 1). Microarray analysis of tPVAT and aPVAT also revealed that several transcription factors involved in the development of brown adipocytes from precursor cells such as bone morphogenetic protein 7 (*Bmp7*), early B-Cell factor 2 (*Ebf2*), euchromatic histone-lysine N-methyltransferase 1 (*Ehmt1*), *Pdrm16* and peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (*PGC-1α*) were expressed at a higher level in tPVAT compared to aPVAT (Figure 1.) Interestingly, myogenic factor 5 (*Myf5*), a gene involved in the lineage of muscle and brown adipocyte, and *Ppar-γ* were not significantly elevated in the tPVAT compared to aPVAT. Increased expression of the transcription factors relating to brown adipocyte development suggest differences in progenitor cells residing in tPVAT and aPVAT.

As microarray data show that transcription factors involved in the development of brown adipocytes were upregulated in tPVAT compared to aPVAT, we investigated perivascular adipose progenitor cells residing in the mouse aorta. Previous studies have shown that progenitor cells reside in the vasculature of the adipose tissue [23–26]. As such, we embedded thoracic aortic rings devoid of PVAT in Matrigel and treated them with the TZD rosiglitazone, a PPAR-γ agonist, to induce adipocyte formation. We found that in the presence of TZD, cells arising from the aortic rings can accumulate lipid droplets (Figure 2). The morphology of cells is no longer spindle-like, rather they resemble multilocular fat cells. Oil-Red-O staining clearly identified lipid droplets in cells coming from the aorta.

To confirm that these cells are adipocytes accumulating lipid droplets, we used immunofluorescence and real-time PCR to confirm expression of adipocyte genes. We found that after 14 days in the presence of TZD, expression of the endothelial specific genes *Cd31* and *Cd34* is decreased (Figure 3). Conversely, expression of the adipocyte specific genes adiponectin (*Acrp30*) and perilipin (*Plin1*) are increased. In particular, the pattern of expression of the Plin1 in these cells is consistent with an adipocyte identity. Approximately 20% of cells differentiated into adipocytes, as assessed by presence of Plin1 relative to Dapi (data not shown). Quantitative PCR also demonstrated a decrease in the endothelial specific markers, *Cdhs5* and *Cd34*, and an increase in the adipocyte markers, *Acrp30*, *Glut4*, *Cidea*, and *Ucp-1* (Figure 3). We further tested conditioned media of sprouts growing from aorta explants for the secretion of *Acrp30*. We confirmed that these TZD-induced fat cells are secreting Acrp30, a canonical adipokine, after approximately 13 days in culture (Figure 3).

After successfully inducing perivascular adipocyte formation in *ex vivo* aortic rings, we used our model to compare adipocyte progenitor cells from tPVAT and aPVAT. We examined the growth of capillary sprouts from the thoracic and abdominal regions of aorta (Figure 4). TZD increased the expression of *Acrp30*, *Glut4*, *Plin1*, *Cidea*, and *Ucp-1* in cells arising from the abdominal and thoracic aortic rings. Interestingly, *Acrp30*, *Glut4*, and *Plin1* were not differentially expressed in cells arising from aPVAT and TZD explants. However, TZD-induced adipocytes arising from the thoracic aorta had much higher expression of both *Cidea* and *Ucp-1* (Figure 4), hallmark brown adipocyte genes. The levels of *Ucp-1* and *Cidea* expression in TZD-induced adipocytes was not as high as that in interscapular BAT (Figure 4).

Figure 2: Oil-Red-O staining of cells arising from the thoracic aorta in the presence of TZD (Rosiglitazone). Representative photomicrographs of capillary sprouts growing from thoracic aorta explants in the absence (A, B, and C) and in the presence of TZD (D, E and F) stained with Oil-Red-O for identification of lipid droplets. Positive Oil-Red-O staining is observed only in the presence of TZD. Scale bars are 1000 µm, 500 µm and 100 µm, respectively.

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4. DISCUSSION

Our transcriptome analysis of tPVAT and aPVAT suggests that progenitors in tPVAT are transcriptionally primed to develop into brown adipocytes. In previous work, Ebf2 was found to be selectively expressed in brown preadipocytes. When ectopically expressed in myoblasts or white preadipocytes, Ebf2 recruited Ppar-γ to brown-selective binding sites and reprogrammed cells to a brown adipocyte fate [28]. Furthermore, Tseng and colleagues have found that Bmp7 activates a full program of brown adipogenesis including induction of early regulators of brown adipocyte fate such as Prdm16 and Pgc-1α [29]. Ehmt1 complexes with Prdm16 to activate the brown adipocyte transcriptional program [30]. In our analysis, expression of Prdm16, Ehmt1, Bmp7, and Pgc-1α were all significantly upregulated in tPVAT compared to aPVAT; and there was a non-significant trend for increased Ebf2 expression in tPVAT. Hoxc8, which is a homeobox gene thought to target and repress CCAAT/enhancer binding protein beta, a master regulator of the brown-adipocyte gene program, is increased in aPVAT compared to tPVAT [31]. In summary, many of the transcription factors that have been implicated in brown adipocyte formation are upregulated in tPVAT compared to aPVAT. Our results suggest that the adipocyte precursor cells that reside in the thoracic and aorta are distinct and have cell-autonomous traits that dictate adipocyte phenotype.

Pathway analysis of the transcriptome data using the Broad Institute Molecular Signature Database: Hallmark Gene Set showed that the myogenesis pathway is most upregulated pathway in tPVAT compared to aPVAT (Figure 1). Furthermore, genes relating to skeletal and smooth muscle structure and function among the genes that are most upregulated (Supplementary Table 3 and Figure 2). Fitzgibbons and colleagues have found that gene of skeletal muscle function are higher in BAT that tPVAT [19]. Timmons et al. have also found a number of muscle related genes that are expressed in BAT [32]. Brown adipocyte precursors are thought to share a common ancestor with skeletal...
muscle cell that express Myf5 [33]. Alternatively, beige adipocyte precursors are thought to be distinct from brown adipocyte precursors, in that they derive from a non-Myf5 expressing cells of smooth muscle origin [34]. Chang et al. demonstrated that mice with conditional knockout of Ppar-γ in smooth muscle cells are devoid of PVAT, suggesting that PVAT is derived from a smooth muscle cell origin [35]. In our prior microarray analysis, expression of the beige cell specific gene Slc27a1 tended to be higher in tPVAT than BAT, but not significantly [19]. In our current analysis, the muscle related genes upregulated included that of smooth and skeletal muscle (Supplementary Figure 2 and Table 3). Therefore, the precise origin of tPVAT is controversial and remains to be determined. Nonetheless, our transcriptome analysis suggests that the progenitor cells in tPVAT are transcriptionally activated to take on a "browner" phenotype in comparison to aPVAT.

We induced formation of adipocytes from the thoracic and abdominal aortic rings of mice to further study adipose progenitor cells from different areas of the aorta. We found that progenitor cells from each area of the aorta give rise to distinct adipocytes even when removed from the in vivo environment. Preadipocytes from thoracic aortic rings give rise to adipocytes that express higher levels of Ucp-1 and Cidea.
BAT specific genes. TZDs are known to induce \textit{Ucp-1} expression, but the induction is much higher in the iPVAT compared to aPVAT \cite{36}. Results from our \textit{ex vivo} aortic ring experiment support the hypothesis that progenitor cells from thoracic and abdominal aorta have cell-autonomous trait that dictate their development into distinct adipocytes. The progenitor cells maintain their inherent identity even when removed from their \textit{in vivo} environment. This distinction in their identity is supported by our microarray data which shows upregulation of BAT specific transcription factors expression in iPVAT compared to aPVAT. Although the aortic ring assay is widely used to study angiogenesis, this the first time that it has been used to study PVAT. Angiogenesis and adipogenesis are closely related \textit{in vivo} \cite{37}. This model allows the cross talk between endothelial cells and adipocytes to be maintained. We found that expression of endothelial cells specific genes was decreased, and adipocyte specific gene expression was increased upon stimulation with TZD. This likely reflects TZD favoring the adipocyte progenitor cells to differentiate and proliferate. The limitations to this system is that it is technically difficult to remove all adipose tissue from the mouse aorta. Thus, it is not completely clear if progenitor cells are arising from adipose tissue depots or from the aorta per-se. Nevertheless, the aortic ring assay is a useful model to study the development and characteristics of PVAT.

Understanding the identity of the PVAT progenitors and the adipocytes they give rise to is key for manipulation of the adipocyte phenotype to improve metabolic and vascular health. Ohman et al. have shown visceral adipose tissue transplantation to the carotid artery in mouse lead to endothelial cell dysfunction and accelerated atherosclerosis compared to subcutaneous adipose transplantation. Furthermore, disruption of PVAT formation disturbs intravascular thermoregulation in mice and lead to increased atherosclerosis \cite{35,38}. In humans, gene expression profile of PVAT surrounding coronary artery (PVAT-CA) and PVAT surrounding the internal thoracic arteries (PVAT-IMA) are unique \cite{39}. Interestingly, patients with coronary artery disease have upregulation of inflammatory and lipid metabolism pathways in gene expression patterns of PVAT-CA compared to PVAT-IMA, implicating adipose tissue inflammation and lipid metabolism as key factors in the development of atherosclerosis \cite{39}. Thus, studying the various phenotypes of PVAT is critical for the understanding and treatment of vascular disease, such as atherosclerosis.

In conclusion, we used transcriptome analysis and the aortic ring assay to show that progenitor cells from iPVAT are transcriptionally primed to develop into more thermogenic adipocyte in comparison with aPVAT. Our study highlights the phenotypic differences between progenitors of aPVAT and iPVAT. To the best or our knowledge, this is the first time the aortic ring assay has been used to study perivascular adipose tissue and their progenitors.

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\section*{CONFLICT OF INTEREST}
None declared.

\section*{APPENDIX A. SUPPLEMENTARY DATA}
Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2017.12.014.

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