Published in final edited form as:

Cardiovasc Drugs Ther. 2018 October ; 32(5): 519–530. doi:10.1007/s10557-018-6813-y.

Rab27a regulates human perivascular adipose progenitor cell differentiation

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Abstract

Purpose: Perivascular adipose tissue (PVAT) surrounds blood vessels and regulates vascular tone through paracrine secretion of cytokines. During conditions promoting cardiometabolic dysfunction such as obesity, cytokine secretion is altered towards a proinflammatory and proatherogenic profile. Despite the clinical implications for cardiovascular disease, studies addressing the biology of human PVAT remain limited. We are interested in characterizing the resident adipose progenitor cells (APC) because of their potential role in PVAT expansion during obesity. We also focused on proteins regulating paracrine interactions, including the small GTPase Rab27a, which regulates protein trafficking and secretion.

Methods: PVAT from the ascending aorta was collected from patients with severe cardiovascular disease undergoing coronary artery bypass grafting (CABG). Freshly-isolated PVAT was digested and APC expanded in culture for characterizing progenitor markers, evaluating adipogenic potential and assessing the function(s) of Rab27a.

Results: Using flow cytometry, RT-PCR, and immunoblot, we characterized APC from human PVAT as negative for CD45 and CD31, and expressing CD73, CD105, and CD140A on the cell surface. These APC differentiate into multilocular, UCP1-producing adipocytes *in vitro*. Rab27a was detected in interstitial cells of human PVAT *in vivo* and along F-actin tracks of PVAT-APC *in vitro*. Knockdown of Rab27a using siRNA in PVAT-APC prior to induction resulted in a marked reduction in lipid accumulation and reduced expression of adipogenic differentiation markers.

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Conflicts of interest: All authors confirm that they have no conflicts of interest to report.

Ethical approval: This study utilized human tissue that was procured via our Maine Medical Center Biobank, which provides de-identified samples. This study was reviewed and deemed exempt by our Maine Medical Center Institutional Review Board. The BioBank protocols are in accordance with the ethical standards of our institution and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

This article does not contain any studies with animals performed by any of the authors.

Informed consent: Informed consent was obtained from all individuals for de-identified use of their samples for research purposes.

Conclusions: PVAT-APC from CABG donors express common adipocyte progenitor markers and differentiate into UCP1-containing adipocytes. Rab27a has an endogenous role in promoting the maturation of adipocytes from human PVAT-derived APC.

Keywords

Perivascular; Adipose; Cardiovascular; Disease; Rab27a; Progenitor

Introduction

Perivascular adipose tissue (PVAT) is a specialized adipose depot surrounding blood vessels that functions to regulate vascular tone through paracrine signaling. In metabolically-healthy individuals, PVAT promotes vasodilation and inhibits inflammation through release of adipose-derived relaxing factors and anti-inflammatory cytokines (e.g. NO and IL-10). Indeed, PVAT deficient mice generated by PPAR γ -specific deletion in SM22 α positive cells, display enhanced atherogenesis [1]. Vasoprotective properties of PVAT are lost during obesity, likely due to adipocyte hypertrophy, hypoxia and inflammation [2]. Increased aortic PVAT volume during obesity correlates with visceral fat mass in humans and is linked to coronary artery disease and arterial calcification. Additionally, PVAT around coronary arteries from patients with a myocardial infarction is twice as thick as coronary PVAT from healthy individuals [3]. The heterogeneity and anatomical location of PVAT impacts the local microenvironment and vascular function. PVAT surrounding the coronary artery exhibits significantly increased production of inflammatory cytokines IL-1b, IL-6 and Leptin compared to PVAT surrounding the athero-resistant internal thoracic artery [4]. Likewise, retention of PVAT on blood vessels during bypass surgeries, the so called “no-touch” procedure, increases graft patency [5] and reduces the risk of post-procedural cerebrovascular events [6]. These studies emphasize the regulatory effect of PVAT on vascular tone, and highlight the need to understand mechanisms regulating its expansion.

Adipose tissue is rich in mesenchymal-like, multi-potent stem/progenitor cells harboring broad regenerative capacity and therapeutic potential [7]. Recent breakthroughs in regenerative medicine show promising ability for ASC to regenerate damaged cardiomyocytes, endothelium and vascular smooth muscle [8] via paracrine signaling from ASC or by direct differentiation of ASC. ASC are also potent regulators of angiogenesis, and contribute to adipose tissue homeostasis, expansion and inflammation, thus mediating central physiological functions related to cardiometabolic health. ASC proliferation gives rise to identical daughter cells that maintain pluripotency, and progeny can continue dividing indefinitely to maintain the progenitor cell pool or terminally differentiate to committed adipocytes in response to environmental and biological inputs [9]. There is no consensus on a defined set of markers for identifying adipose progenitor cells [10]. However, human adipose progenitor cells have been shown to express CD34 [11], CD73 [12], CD90 [12], CD105 [11, 13], CD140A [14], and CD271 [15].

Mechanisms regulating ASC differentiation to adipocytes have been extensively studied [16], but surprisingly little is known about ASC proliferation during obesity [17]. Autocrine secretion of Pref-1 from ASC regulates their self-renewal [9], and Pref-1 null mice develop

obesity because of enhanced differentiation and maturation of ASC [18]. Likewise, obesity increases proliferation of human-derived ASC [19], suggesting an important role for ASC in obesity and cardiometabolic health. Given that PVAT expands during obesity, regulates vascular function and exacerbates cardiovascular disease, it is critical to identify mechanisms regulating PVAT-ASC differentiation. We are not aware of any published studies characterizing the adipose progenitor cell derived from human PVAT, thus our study is unique.

Rab27a is a member of the small GTPase family of Rab proteins that coordinate intracellular sorting and trafficking of cargo proteins [20]. Widely expressed, Rab27a regulates immune, metabolic and blood vessel function [21–23], and is linked to several human cancers. Unlike most Rab proteins, which share complementary and/or overlapping functions, loss of Rab27a is linked to disease. Humans born with a null mutation in Rab27a will develop Griscelli Syndrome [24], a fatal condition early in childhood characterized by hyperactivation of the immune system, demyelination of nerves, clotting defects, and hypopigmentation of skin and hair. Mice harboring a null mutation in Rab27a (*ashen*) are viable, but phenocopy several attributes of Griscelli Syndrome [25]. Mechanistically, Rab27a regulates Golgi-to-plasma membrane vesicle trafficking, and exosome secretion [26], although non-secretory functions have been reported [21, 27].

Descriptive and mechanistic studies on human PVAT are extremely limited, in part, due to the highly invasive nature of procurement. Furthermore, the progenitor cell population has not been characterized, and mechanisms that regulate PVAT-APC cell function are unknown. Additionally, there have been no reports of Rab27a expression or function in any adipose depots. In this work, we obtain human PVAT from the ascending aorta of patients with coronary artery disease undergoing coronary artery bypass grafting (CABG). From this tissue, we sought to identify APC by cell surface progenitor marker expression and test the ability of APC to undergo adipogenesis. Further, we made the novel discovery that Rab27a is expressed in human PVAT and PVAT-APC from CABG patients, and is required for their adipogenic differentiation *in vitro*; thus identifying a new function for Rab27a in regulating adipogenesis. This work provides new mechanistic insight into the regulation of PVAT expansion.

Materials and Methods

Donor samples

PVAT is discarded tissue during bypass surgery and has been approved as non-human subjects research by the Maine Medical Center Institutional Review Board. PVAT was collected from the ascending aorta of patients with coronary artery disease undergoing CABG (Table 1). Once removed from the donor, PVAT was placed into a tube with ice cold-DMEM and immediately transferred to the Maine Medical Center Biobank for documenting and de-identification. Tissue was picked up from the Biobank within 2h of resection and transferred to the lab on wet ice. PVAT was separated into multiple segments for fixing and tissue processing for paraffin embedding, whole tissue lysis, or digest for stromal cell explant. Subcutaneous adipose tissue was collected from the abdominal region of obese donors undergoing liposuction.

Tissue Digest and Cell Explant

Preparation of Collagenase solution —Collagenase I solution was prepared by dissolving 10mg collagenase I (Worthington Biochemical Corporation, cat# LS004196, lot# 46K16856) into 5ml of buffer (123mM NaCl, 5mM KCl, 1.3mM CaCl₂, 5mM glucose, 100mM HEPES, 4% BSA, pH=7.4) and incubating for 45 minutes. Collagenase solution was sterile filtered (0.22 micron pore size) and pre-warmed to 37°C while the tissue was prepared.

Tissue Digest —An approximately 400mg piece of human PVAT was rinsed in HBSS containing 2× pen/strep on ice for 10 minutes. PVAT transferred to a 60mm petri dish and minced in 1ml of collagenase solution using surgical scissors until no chunks remained. Minced tissue slurry was transferred to a 5ml tube containing 4ml of pre-warmed collagenase and incubated on its side at 37°C for 30 minutes with moderate rotation. After incubation, the 5ml cell solution was transferred to a 50ml conical tube and diluted with 45ml of ice cold HBSS containing 2× pen/strep and centrifuged at 300×g for 10 minutes.

Cell explant —After centrifugation, the supernatant was removed and cell pellet (PVAT stromal cells) resuspended in 25ml HBSS to wash. Cells were centrifuged again at 300×g for 10 minutes and supernatant removed. Pellet was re-suspended in 2ml of DMEM F12 containing 10% FBS and 100µg/ml primocin (Invivogen cat #ant-pm-1) and 50ng/ml bFGF (Peprotech, cat #100–18C) and plated to one well of a 12-well culture dish coated with 0.02% gelatin. Confluency was established after 3–4 days and cells passaged for experimentation. All experiments were performed with cells between passages 3–5.

Adipogenic Induction

PVAT-APC were grown to confluency and incubated in adipocyte induction medium (7.5ml growth media, 40.8ml high glucose DMEM, 0.5mM IBMX, 1µM dexamethasone, 5µM rosiglitazone, 33µM biotin, 100nM insulin, 20µM pantothenic acid and 20U/ml penicillin G, 20µg/ml streptomycin and 50ng/ml Amphotericin B (1× pen/strep, HyClone cat #SV30079.01) for 72h at 37°C. After 72h, the cells were washed 2× in PBS and incubated in adipogenic maintenance media (induction media minus IBMX and rosiglitazone) for the duration of the experiment, with media changes every other day.

Protein Isolation

Whole adipose tissue was ground in 200µl radioimmunoprecipitation assay (RIPA) buffer supplemented with 1% w/v SDS and 1× protease/phosphatase inhibitors (Cell Signaling Technology cat #5872S) using a microcentrifuge tube pestle. Lysates were then sonicated using output power 3 and duty cycle at 30 for 10 pulses on ice and proteins precipitated with 4 volumes of ice-cold 100% acetone and incubation at –20°C for 1h. Samples were centrifuged at 4°C for 20 minutes at 12,000×g, supernatant poured off, pellets re-suspended in 70% acetone to wash, and centrifuged for 5 minutes at 10,000×g. Supernatant was removed and pellets allowed to dry at room temperature for 30 minutes before resuspending in RIPA buffer. Pellets were sonicated as indicated above to solubilize. Cultured cells were lysed in RIPA supplemented with 1× protease/phosphatase inhibitors without acetone precipitation. Protein was quantified using the DC protein assay (BioRad cat #5000111) and

denatured by heating at 95°C for 15 minutes in Laemmli sample buffer supplemented with 100mM DTT.

Flow Cytometry

Passage 4 human PVAT-derived APC were detached using Accutase solution (VWR Cat #10210–214) for 2 minutes at 37°C, added to 10ml of PBS and centrifuged for 5 minutes at 300×g. Cell pellets were re-suspended in FACS buffer (2mM EDTA, 0.5% BSA in PBS) and divided into FACS tubes (~75K cells/ml). Fcγ receptors were blocked by addition of 1μg of Human Fc Block- (BD Biosciences cat #564219) to each tube for 10 minutes at room temperature. For labeling cells, 1μg of each fluorochrome-conjugated antibody was added to each tube (1μg/ml) and incubated at 4°C for 40 minutes with occasional mixing. Antibodies used were all from Biolegend (CD105-PeCy7 cat #323218 clone 43A3, CD73-FITC cat #344016 clone AD2, and CD90-APC, cat #328113, clone 5E10). Fluorescence-minus one controls were included by excluding one fluorochrome from each condition. Unstained cells were labeled with DAPI for live/dead staining. Cells were washed 2× in FACS buffer and analyzed using a Miltenyi Biotec MACSQuant equipped with three lasers (405nm, 488nm and 638nm). Data were analyzed and histograms generated using Flowjo_V10.

RT-PCR

Total RNA was isolated from whole tissue using the miRCURY RNA Isolation Kit (Exiqon cat #588705, product now discontinued). RNA was normalized to 25ng/μl in RNase free water and 10μl (250ng) used for generating cDNA. DNA was removed and cDNA synthesized simultaneously using iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad cat #1725034) according to the manufacturer's protocol. PCR amplification was carried out using 0.5μg template DNA with Promega 2× PCR master mix (cat #M7502) with cycling conditions: 95°C for 2 minutes followed by 40 cycles of 95°C for 15s, 58°C for 20s and 72°C for 20s. PCR products were separated on a 1.5% TAE-agarose gel containing. Positive control was pooled cDNA from human MEC1, MCF7 and THP1 cells, negative control was water loaded in place of template.

Immunoblot

For immunoblotting, 25–50μg of protein was loaded onto a 10% TGX gel (Bio-Rad) and separated at 200 volts for ~30 to 40 minutes. Protein was transferred to a PVDF membrane using Bio-Rad Trans-blot Turbo (1.5amps, 15 volts for 30 minutes). Membranes were blocked using 5% w/v non-milk dissolved in PBS with 0.1% tween-20 (PBS-T) for 30 minutes and incubated with primary antibody overnight at 4°C. Primary antibodies and dilutions used were as follows: from Cell Signaling Technologies - ACTB 1:5000 (#4970S), C/EBPα 1:750 (#8178S), FASN 1:1000 (#3180S), PLIN1 1:4000 (#9349S), PPARG 1:500 (#2443S), TUBA1B 1:2500 (#2125S); from Santa Cruz Biotechnology - CD73 1:1000 (#sc-398260), CD140A 1:1000 (#sc-398206), Rab5 1:750 (#sc130010), Rab27a 1:1000 (#sc136996); from R&D Systems - UCP1 1:750 (#MAB6158-SP); and from Abcam - FABP4 1:2500 (#ab92501), PGC1A 1:2000 (#ab54481). The next day, the membranes were washed 3× for 15 minutes each in PBS-T and incubated in HRP-conjugated secondary antibody from Cell Signaling Technology (#7074S and #7076S) diluted 1:4000 in PBS-T supplemented with 5% w/v milk for 1h. Membranes were washed 3× for 15 minutes each

and developed using Immobilon forte chemiluminescent reagent (Millipore cat #WBLUF0100). Signal was detected using HyBlotCL® autoradiography films (Denville #E3212–1001371) in a dark room and developed on a Konica developer (SRX-101A).

Immunohistochemistry and immunofluorescence

Immunohistochemistry —Formalin fixed, paraffin embedded PVAT samples were sectioned at 5µm and rehydrated in AmeriClear- followed by decreasing ethanol concentrations. Antigen retrieval was performed using 0.01M sodium citrate and heat, followed by quenching of endogenous peroxidases with 3% H₂O₂. Sections were permeabilized with 0.5% Triton X-100 and blocked for 2h at room temperature (blocking solution was 2% BSA and 2% goat serum in TBS). Sections were incubated overnight at 4°C in primary antibody diluted in blocking solution, washed 3× in TBS-T, and detected using SignalStain® Boos IHC Detection Reagent and SignalStain® diaminobenzidine substrate (Cell Signaling Technologies). IHC primary antibody dilutions were as follows: CD105 1:200 (SCBT, sc-18838), CD140A 1:200 (SCBT, sc-398206), Rab27a 1:50 (SCBT, sc-81914) and concentration matched IgG1 Isotype Control (CST, 5415). Sections were counterstained with hematoxylin to visualize nuclei.

Confocal Immunofluorescence —For tissue, sections were processed and labeled with primary antibody as described above. Primary antibody dilutions were as follows: Rab27a 1:50 (SCBT, sc-74586), PLIN1 1:4000 (CST, 9349), and concentration matched IgG (CST, 3900) and IgG1 (CST, 5415) Isotype Controls. After washing 3× in TBS, sections were incubated with Highly Cross Adsorbed IgG (H+L) alexa-fluor conjugated goat anti-mouse or anti-rabbit secondary antibody (ThermoFisher Scientific A-11037 and A32723) in TBS-T for 1h at room temperature, followed by 2 additional washes in TBS-T. To quench auto fluorescence, sections were incubated with True View Autofluorescence Quenching Kit (Vector Labs SP-8400) for 2 minutes according to the manufacturer's protocol and counter stained with 1µg/ml DAPI for 5 minutes. Vectashield Hard Set Anti-fade mounting medium was used for cover slipping and to preserve fluorescence. Images were captured using a Leica TCS SP8 laser scanning confocal with a 20×/0.75 air objective.

Oil Red O Staining

Oil red O (ORO) stock solution was prepared by mixing 0.35g ORO (Sigma-Aldrich #O-0625) into 100ml of 100% isopropanol. To prepare ORO working solution, stock solution was diluted 3 parts ORO to 2 parts nanopure H₂O and filtered through a 0.2µm filter. 10% formalin-fixed cells were dehydrated in 60% isopropanol with two 5 minute washes, and cells incubated in ORO working solution for 10 minutes at room temperature. Wells were washed using nanopure H₂O and cells imaged. To elute ORO from the cells, 100% isopropanol was added to the wells for 5 minutes and collected to measure absorbance at 490nm. Total protein was collected by lysing the cells in RIPA after elution of ORO.

Results

Identification of progenitor cells from human aortic PVAT

Adipose tissue is a rich source of multipotent progenitor cells [28]. The progenitor population from human PVAT has not been defined, and it is unclear if cells with adipogenic potential can be harvested from human PVAT. Histologically, human aortic PVAT from CABG patients has large adipocytes with collagen-rich, cellular interstitial regions between lipid-laden adipocytes (Fig. 1a, left, arrows). CD105 and CD140A are markers of adipocyte progenitor cells [10, 14], and we tested for their localization in human PVAT. We detected strong CD105 and CD140A protein in the interstitial regions of human aortic PVAT (Fig. 1, columns 2 and 3, arrows) while tissue stained with isotype-matched IgG were negative (Fig. 1, right). Next, we tested if CD105+/CD140A+ progenitor cells could be explanted from human PVAT and cultured *in vitro*. Aortic PVAT (~450mg) was digested in collagenase I and plated on 0.02% gelatin-coated plates in DMEM F12 with 10% FBS and 50ng/μl bFGF. By day 3, explanted cells were spread on the substrate, and by day 7, were proliferating rapidly (Fig. 2b). To determine their identity, we performed RT-PCR on explanted cells from two different female donors. Consistent with *in vivo* staining (Fig. 1a), explanted cells expressed *CD140A* and *CD105*, albeit levels of expression were variable between donors (Fig. 1c). Strong expression of progenitor markers *CD90* and *CD73* was also detected, while cells were negative for the leukocyte marker *CD45*. Intriguingly, we detected expression of preadipocyte commitment marker *ZFP423*, early phase adipogenic differentiation marker *PPARG*, and thermogenic marker *UCP1*, while late phase differentiation marker *C/EBPA* was absent (Fig. 1c). Consistent with transcript expression, we detected CD140A by immunoblot (Fig. 1d) and cell surface expression of CD73, CD105 and CD90 by live cell flow cytometry (Fig. 1e–g, green peak, stained cells; blue peak, unstained; grey peak, fluorescence-minus-one control). These data are consistent with the identification of these cells derived from the stromal fraction of human PVAT as APC, due to their expression of markers of adipocyte progenitors and committed pre-adipocytes [11–13, 29].

Human PVAT-APC exhibit adipogenic potential

Given the expression of pre-adipocyte commitment markers from explanted APC, we wondered if they have the potential to generate adipocytes *in vitro*. Human PVAT-APC were induced to differentiate towards an adipogenic lineage for 7 days using previously established methods [30], and neutral lipid accumulation visualized by oil red O (ORO) staining [31]. Compared to non-induced PVAT-APC, induced cells showed accumulation of multilocular adipocytes (Fig. 2a). The multilocular phenotype persisted through 28 days of differentiation (data not shown). Immunoblot of non-induced and induced cells at 7 and 14 days showed robust activation of PPARγ (early), C/EBPα (late) and FABP4 (terminal) adipogenic differentiation markers and lipid-droplet associated protein PLIN1 (Fig. 2b) demonstrating strong adipogenic induction. Interestingly, despite the white fat-like appearance of human PVAT (Fig. 1a), UCP1 positive adipocytes were detected present in human thoracic aortic PVAT [32]. Consistent with this, we detected UCP1 protein in induced human aortic PVAT-APC at 7 and 14 days of differentiation (Fig. 2b), suggesting the potential for PVAT-APC to form thermogenic brown-like adipocytes *in vitro*. Although PLIN1 is mainly associated with lipid droplets, endoplasmic localization has been reported

[33]. We labeled cells using PLIN1 antibody and visualized PLIN1 localized predominantly to lipid droplets (Fig. 2c) indicating association with developing droplets. Collectively, our data demonstrate that explanted PVAT-APC differentiate into multilocular, UCP1-producing adipocytes.

Rab27a is expressed in human PVAT and suppressed during adipogenesis

PVAT modulates vascular and metabolic function by paracrine secretion of growth factors and adipokines in the local environment [34–36]. We searched for candidate secretory proteins enriched in PVAT compared to subcutaneous white adipose tissue (WAT) by MS/MS of whole tissue lysates. Using this approach, we identified numerous secretion-associated proteins that appeared to be enriched in human PVAT compared to including human subcutaneous white adipose tissue, including synaptotagmin-1, myosin-9, Rab11 and Rab27 (data not shown). Importantly, Rab27a is a well-established regulator of protein secretion and degradation [20, 27, 37], and regulates the function of multiple systems [38], including pancreatic beta cell release of insulin [22], VEGFR1 degradation during angiogenesis [39], cancer stem cell self-renewal [40], initiation of clotting cascades [41] and lytic granule exocytosis from cytotoxic T-cells [42]. Rab27a expression and function has not been studied in adipose tissue, and given its prominent role in regulating secretion, we validated expression in human PVAT. Rab27a protein was detected in human PVAT in the interstitial cells using immunohistochemistry (Fig. 3a, top) and confocal immunofluorescence (Fig. 3a, bottom, arrows), suggesting Rab27a is expressed in some interstitial progenitor cells of human PVAT. Further, immunoblot of tissue lysates from PVAT and WAT revealed detectable, albeit variable, Rab27a expression in PVAT but not WAT (Fig. 3b). UCP1 protein was also detected in PVAT but not WAT, confirming previous reports [32]. We also verified expression of Rab27a in explanted human PVAT-APC, and found that it colocalized along F-actin tracks (Fig. 2c, arrows), which is consistent with active secretory function for Rab27a [43] in these cells. Negative controls were stained with fluorophore conjugated secondary antibody only. Next, we tested Rab27a levels during adipogenic differentiation of human PVAT-APC for 7 and 14 days. Immunoblot of PVAT-APC whole cell lysates revealed high levels of Rab27a in non-induced cells that was completely suppressed by adipogenic differentiation (Fig. 3d). The related secretory Rab protein Rab5 was detectable throughout differentiation, indicating a regulated and specific suppression of Rab27a. Thus, Rab27a is produced in human PVAT and PVAT-APC, and is selectively suppressed during adipogenic differentiation.

Rab27a is a novel regulator of adipogenesis in human PVAT-APC-

Since Rab27a is expressed in PVAT-APC, but suppressed during differentiation, we hypothesized that it functions to regulating the phenotype and identity of APC prior to differentiation. To test this, PVAT-APC were transfected with a scrambled non-targeting (ntRNA) or siRNA specific for Rab27a (siRab27a) for 48h prior to adipogenic induction. After 48h, cells were induced to an adipogenic lineage for 3 days, and maintained in adipogenic maintenance medium for 5 days (total 8 days) before visualizing lipid mobilization. Intriguingly, ORO staining revealed a reduction in lipid accumulation in siRab27a APC as compared to ntRNA (Fig. 4a). Non-induced cells from both conditions were negative for lipid accumulation (Fig. 4a, right column). The amount of ORO in the

cells (Fig. 4b) and the number of ORO positive cells (Fig. 4c) were significantly reduced in siRab27a cells, suggesting impaired lipid accumulation and adipogenic differentiation respectively. Given these observations, we hypothesized that knockdown of Rab27a in PVAT-APC prior to adipogenic induction may impair their differentiation. To test this, we analyzed protein expression by immunoblot from non-induced and induced cells transfected with ntRNA or siRab27a. Rab27a knockdown was confirmed in non-induced and induced cells, and as expected, significant loss of progenitor markers CD140A and CD73 upon adipogenic induction (Fig. 4d, bottom). In line with reduced ORO staining in siRab27a cells (Fig. 4a–c) we noted increased levels of the mitochondrial biosynthesis protein PGC1A, and marked reductions in fatty acid synthase (FASN), perilipin1 (PLIN1), C/EBPA and FABP4 in induced cells with Rab27a knockdown (Fig 4d and e–j). These data suggest that Rab27a expression in human PVAT-APC is required to prime the cells for adipogenesis.

Discussion

PVAT is a critical regulator of vascular tone and function. Pathological conversion of PVAT contributes to vasoconstriction, vascular inflammation and metabolic dysfunction. Studies on human PVAT are extremely limited, likely due to the invasiveness of procurement from major vessels. The adipogenic potential and mechanisms involved with regulating differentiation of PVAT-APC remain undefined and a thorough investigation of PVAT-APC will advance our understanding of the development of cardiometabolic dysfunction.

There has been increased attention to anatomical and molecular features of PVAT that may potentially assist in the understanding of cardiovascular disease. Coronary computed tomography angiography identified a significant increase in PVAT volume surrounding the coronary artery in patients with localized vasospasm[44]. In addition, patients with abdominal aortic aneurysms had local necrotic and highly inflamed PVAT compared to PVAT surrounding aortae from patients without vascular disease[45]. One study compared PVAT surrounding the heart (atherosusceptible) and PVAT surrounding the internal mammary artery (atheroresistant) in males undergoing coronary artery bypass grafting (CABG) surgery for advanced atherosclerotic disease[46]. That study showed that leptin levels were lower in PVAT from the internal mammary artery compared to PVAT surrounding the aortic root and coronary arteries. Conversely, PVAT around the heart was more vascularized with more macrophages. A recent study compared gene expression of PVAT around human internal mammary arteries versus coronary arteries in patients with or without cardiovascular disease [47] and found that differentially expressed genes high in cardiovascular disease patients included those involved in inflammation and lipid metabolism. Other adipokines with enrichment in PVAT/epicardial adipose tissue in coronary artery disease include IL-1 β , TNF α , leptin, visfatin, resistin, and IL-6, whereas adiponectin and migration inhibitory factor were decreased [48]. These studies provide strong impetus for the continued molecular characterization of mature and progenitor cells within PVAT.

In this report, we obtain human PVAT from the ascending aorta of patients undergoing coronary artery bypass grafting to assess progenitor marker expression and test the hypothesis that Rab27a is a novel regulator of APC differentiation. Through a combination

of flow cytometry, immunoblot, PCR and immunohistochemistry, we establish a baseline characterization of PVAT-APC as CD45-/CD31-/CD73+/CD90+/CD105+/CD140A with robust adipogenic potential. Expression of a CD73, CD90, CD105 and CD140A positive progenitors within a CD45-/CD31- population from adipose tissue has been reported in numerous adipose depots [11, 12, 14, 49], and indicates that human PVAT, like classical adipose depots, is a rich source of progenitor cells, likely harboring broad regenerative capacity [50, 51]. Furthermore, we identified Rab27a as highly expressed in human PVAT compared to SWAT. Since there are no reports of Rab27a in adipose, the impact of this finding is unclear. Interestingly, PVAT-APC expression of Rab27a regulates their ability to undergo adipogenesis, and siRNA-mediated knockdown of Rab27a caused impaired adipogenesis and lipid mobilization. Rab27a has been shown to regulate progenitor cell self-renewal of breast and colon cancers via effects on secretion [40, 52]. Given these findings, it is feasible to speculate that Rab27a may regulate PVAT expansion during obesity.

The Rab-family of small GTPases are trafficking and secretory mediators that play vital roles in metabolic function [53, 54]. In 3T3-L1 cells, Rab18 controls the balance between insulin-induced lipogenesis and β -adrenergic-induced lipolysis [55]. Likewise, Rab4 regulates the subcellular localization [56] and Rab11 the endocytic trafficking [57] of the glucose transporter protein GLUT4, in 3T3-L1 adipocytes, however further studies are needed to test these theories in primary human APC lines and in adipose tissue. A recent study comparing gene expression profiles of human PVAT to subcutaneous WAT using genome wide expression analysis identified genes involved in vascular regulation and inflammation as upregulated in human PVAT compared to SWAT [58]. Intriguingly, Rab27a was detected in the analysis as upregulated in human PVAT, but was not the focus of the research. Our data in Fig. 3b confirm higher expression of Rab27a in human PVAT compared to SWAT at the protein level. Given that Rab27a controls secretory vesicle trafficking in numerous cell types, and that PVAT regulates vascular tone via paracrine secretion, it is tempting to speculate that Rab27a in PVAT influences the local vascular environment through effects on cytokine secretion.

We demonstrated impaired adipogenesis of PVAT-APC with Rab27a knockdown (Fig. 4). This effect on adipogenesis was observed in all donor cells tested (Table 1), regardless of gender, BMI, age and cardiometabolic disease status, suggesting it may represent a generalizable mechanism controlling PVAT-APC differentiation in patients with cardiovascular disease. A limitation to our study is the small sample size (n=9 CABG patients). Despite obtaining consistent findings on both progenitor marker expression and the effects of Rab27a knockdown, it is imperative to extend this work out to larger sample sets, and include PVAT-APC from healthier donors absent coronary artery disease; and determine the potential impact of confounding variables like diabetes and medications at the time of procurement. It's imperative to determine if Rab27a is a marker of metabolically-impaired PVAT, or is constitutively expressed regardless of its metabolic state. In addition, how does Rab27a exert its effects on PVAT and the adjacent vessel, and does it have different functions in healthy and diseased PVAT?

In summary, this work provides seminal research on the progenitor cell population residing in human PVAT and identifies Rab27a as a critical mediator of PVAT-APC differentiation.

These findings provide a significant advancement of our understanding of human PVAT and the mechanisms potentially regulating cardiovascular disease progression.

Funding:

This research was supported by NIH grant R01HL141149 (L. Liaw, PI) and American Heart Association grant 17GRNT33670972 (L. Liaw, PI). JB was partially supported by a pilot project from NIH grant 5P30GM106391, which also supported the Progenitor Cell Analysis Core, which was used for flow cytometry (R. Friesel PI). This work was also supported by our Histopathology and Histomorphometry Core, which is supported by NIH grants P20GM121301 (L. Liaw, PI), P30GM106391 (R. Friesel, PI), and U54GM115516 (C. Rosen, PI).

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Figure 1. Identification of adipocyte progenitor cells from human aortic PVAT

a) Immunohistochemical analysis of human aortic PVAT. Trichrome: blue=connective tissue, purple=nuclei. b) Phase contrast microscopy of progenitor cells explanted from the stromal vascular fraction of PVAT from a CABG patient at days 3 and 7 in culture. c) RT-PCR for the indicated transcripts from passage 1 PVAT-APC. d) Immunoblot for the indicated proteins from passage 2 PVAT-APC. e-g) Histograms of PVAT-APC analyzed by flow cytometry for CD73 (e) CD105 (f), and CD90 (g) from donor R17-1092. Blue, DAPI stained; grey, FMO negative control; green, labeled cells. Results are representative of results from 5 separate donors.

Figure 2. Human PVAT-APC differentiate to adipocytes *in vitro*

a) Oil red O (ORO) staining of lipids in non-induced (top) and induced (bottom) at day 7. b) Immunoblot for the indicated proteins of non-induced and induced PVAT-APC for 7 and 14 days. c) Confocal immunofluorescent analysis for the indicated proteins in non-induced and induced human PVAT-APC. Arrows, PLIN1 surrounding lipid droplets.

Figure 3. RAB27A is expressed in human PVAT and suppressed during adipogenic differentiation

a) Immunohistochemistry (top, brown Rab27a staining) and confocal immunofluorescence (bottom, green Rab27a staining) for the indicated proteins from sections of human PVAT. Nuclei were stained with DAPI. Negative controls were stained using isotype-matched IgG control antibodies. b) Immunoblot of human PVAT and subcutaneous WAT whole tissue for the indicated proteins. c) Confocal immunofluorescence microscopy of PVAT-APC. RAB27a is labeled in green, actin filaments are detected by phalloidin (red), and nuclei are stained with DAPI. Negative control is stained using fluorophore conjugated secondary antibody. d) Immunoblot for the indicated proteins from PVAT-APC as indicated.

Figure 4. RAB27A regulates adipogenic differentiation of human PVAT-APC

a-c) ORO staining (a), quantification of ORO incorporation (b) and % of ORO positive cells (c) from control (ntRNA) or *RAB27A* knockdown (siRAB27A) PVAT-APC induced to differentiate for 8 days. Negative control, non-induced cells from each condition stained with ORO. Statistics: Shown are means +SD. Students *t*-test with Tukey's post-hoc analysis, *n*=8 CABG patients. d-j) Representative immunoblot (d) and quantification of protein levels (e-j) of non-induced and induced human PVAT-APC with and without RAB27A knockdown.

Table 1.

Characteristics of PVAT from donors used for this report.

donor ID	age	sex	BMI	Medical History (all have coronary artery disease)	diabetic
R17-0609	71	F	36.4	Aortic valve stenosis, congestive heart failure, hypertension	N
R17-0809	33	F	33.3	Mitral valve prolapse/regurgitation, atrial fibrillation,	N
R17-1066	73	F	38.7	Hypertension, hyperlipidemia,	Y
R17-1092	59	F	32.5	Hyperlipidemia, heart murmur, hypertension	Y
R17-1240	71	M	25.4	Hyperlipidemia, hypertension, aortic stenosis	N
R17-1440	71	M	28.0	Hyperlipidemia, myocardial infarction, peripheral vascular disease, mitral regurgitation	Y
R17-1787	58	F	25.3	Aortic stenosis, hypercholesterolemia, valvular disease	N
R17-1865	65	F	33.0	Hypertension, congestive heart failure, mitral regurgitation	Y
R18-0500	66	M	28.7	Hyperlipidemia, hypertension	Y

Table 2.

Primers used for RT-PCR.

Target	Forward 5'- 3'	Reverse 5'- 3'	Tm (°C)
<i>CD45</i>	AGTACAGACGCCTCACCTTC	TGGCTGTACTCCTCTCTCCT	58
<i>CD73</i>	GAAGGCCTTTGAGCATAGCG	TAACTGGGCACTCGACACTT	58
<i>CD90</i>	AGTACGAGTTCAGCCTGACC	CCCTCGTCCTTGCTAGTGAA	58
<i>CD105</i>	CCATTGTGACCTTCAGCCTG	CTTGGATGCCTGGAGAGTCA	58
<i>CD140A</i>	ATGGATTAAGCCGGTCCCAA	TAAATGGGGCCTGACTTGGT	58
<i>C/EBPA</i>	CAAGGCCAAGAAGTCGGTG	GGTCATTGTCACTGGTCAGC	58
<i>PPIA</i>	CTCGAATAAGTTTGACTTGTGTTT	CTAGGCATGGGAGGGAACA	58
<i>PPARG</i>	GACCACTCCCACTCCTTTGA	GAGATGCAGGCTCCACTTTG	58
<i>UCP1</i>	TTAGCAGATGACGTCCCCTG	CACTAACGAAGGACCAACGG	58
<i>ZFP423</i>	CAAGAACATTCCACTGGCCC	TGGAGAACTTGAGGTCGCAT	58