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Adipocyte exosomes induce transforming growth factor beta pathway dysregulation in hepatocytes: a novel paradigm for obesity-related liver disease

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ABSTRACT

Background: The pathogenesis of nonalcoholic fatty liver disease (NAFLD) has been attributed to increased systemic inflammation and insulin resistance mediated by visceral adipose tissue (VAT), although the exact mechanisms are undefined. Exosomes are membrane-derived vesicles containing messenger RNA, microRNA, and proteins, which have been implicated in cancer, neurodegenerative, and autoimmune diseases, which we postulated may be involved in obesity-related diseases. We isolated exosomes from VAT, characterized their content, and identified their potential targets. Targets included the transforming growth factor beta (TGF-β) pathway, which has been linked to NAFLD. We hypothesized that adipocyte exosomes would integrate into HepG2 and hepatic stellate cell lines and cause dysregulation of the TGF-β pathway.

Methods: Exosomes from VAT from obese and lean patients were isolated and fluorescently labeled, then applied to cultured hepatic cell lines. After incubation, culture slides were imaged to detect exosome uptake. In separate experiments, exosomes were applied to cultured cells and incubated 48-h. Gene expression of TGF-β pathway mediators was analyzed by polymerase chain reaction, and compared with cells, which were not exposed to exosomes.

Results: Fluorescent-labeled exosomes integrated into both cell types and deposited in a perinuclear distribution. Exosome exposure caused increased tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and integrin αvβ-5 expression and decreased matrix metalloproteinase-7 and plasminogen activator inhibitor-1 expression in to HepG2 cells and increased expression of TIMP-1, TIMP-4, Smad-3, integrins αvβ-5 and αvβ-8, and matrix metalloproteinase-9 in hepatic stellate cells.

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Conclusions: Exosomes from VAT integrate into liver cells and induce dysregulation of TGF-β pathway members *in vitro* and offers an intriguing possibility for the pathogenesis of NAFLD.

1. Introduction

The pathogenesis of nonalcoholic fatty liver disease (NAFLD) in patients with obesity has long been attributed to increased systemic inflammation and insulin resistance mediated by visceral adipose tissue (VAT), though the exact mechanisms have yet to be defined [1–3]. This hypothesis is based on the findings that VAT is more metabolically active than subcutaneous adipose tissue, and many of the mediators secreted from visceral adipose are pro-inflammatory cytokines [4–9]. Indeed a significant body of work supports a role for VAT in the pathogenesis of NAFLD, with increased visceral adiposity clearly associated with NAFLD [4,10,11]. Despite the strong correlation between visceral adiposity and NAFLD, no direct link between adipocytes and hepatocyte dysfunction has been demonstrated.

Expanding research in the field of exosomes may explain the mechanism by which VAT exerts its effects on liver tissue in NAFLD. Exosomes are membrane-derived vesicles with a lipid bilayer, 40–120 nm in size, which contain microRNA (miRNA), messenger RNA, and proteins. They are derived from the endolysosomal pathway and have been isolated from nearly every cell type. Exosomes have been implicated in immune surveillance, tissue maintenance and repair, and in the development and progression of cancers, autoimmune diseases, and neurodegenerative diseases [12–16]. One mechanism by which exosomes may mediate function is by transporting miRNAs that act as posttranscriptional regulators of messenger RNA expression, and as such have the ability to induce significant fold changes in the protein products of a large number of genes [17,18].

In an attempt to better understand how exosomes from adipocytes may be implicated in disease pathogenesis in patients with morbid obesity, we isolated and characterized the content of exosomes from VAT of obese and lean female adolescents in an earlier separate study (article under review). These experiments evaluated exosomes from 12 adolescent females; 7 obese, and 5 lean. Only miRNAs were found, and using Pathway Analysis software (Ingenuity Systems, Redwood City, CA), we identified potential targets of those miRNAs that were differentially expressed between exosomes from lean and obese VAT. The transforming growth factor beta (TGF-β) pathway was ranked second of those pathways predicted to be targeted by the differentially expressed miRNAs. Alterations in the TGF-β pathway have been linked to the development of NAFLD. Pathway activation results in increased extracellular matrix production (ECM) by hepatic stellate cells (HSC), and also inhibits production of matrix-degrading proteases [18–21]. Both hepatocytes and HSC are integral to the development of NAFLD, though via different mechanisms. Activation of HSC triggers ECM production, whereas injury to hepatocytes may be a trigger for the initial activation of the TGF-β pathway [22–25]. If adipocyte exosomes could induce TGF-β pathway dysregulation in liver cells, they could be implicated in the pathogenesis of NAFLD. Thus, we hypothesized that exosomes from VAT would be capable of integrating into hepatocyte and HSC lines *in vitro*, and would cause dysregulation of TGF-β pathway mediators.

2. Materials and methods

2.1. Adipose collection

VAT was collected from mixed race female adolescents ($n = 4$), aged 15–17 y, body mass index (BMI) 35–46 who underwent laparoscopic sleeve gastrectomy for the treatment of morbid obesity at our institution by removing the greater omentum that was part of the stomach specimen. VAT was also collected from one lean adolescent female, aged 16 y, BMI 25, who underwent laparoscopic cholecystectomy. The study was approved by the institutional review board, and informed assent and consent were obtained from all subjects and legal guardians, respectively. Samples were obtained in the operating room and immediately stored in phosphate buffered saline (PBS).

2.2. Exosome culture and isolation

Exosomes were isolated from VAT using the protocol established by Deng, *et al.* [26,27]. Samples were dissected into ~4-mm cubes, cultured in 12-well plates containing 3 mL/well of serum-free Dulbecco modified Eagles medium (Life Technologies, Carlsbad, CA) with 50 μg/mL gentamicin (Sigma–Aldrich, St. Louis, MO), and cultured in a 37°C incubator in an atmosphere of 5% CO_2 and/or 95% air for 1 h. Exosomes were isolated from supernatant using ExoQuick-TC Precipitation Solution (System Biosciences, Mountain View, CA) and stored in PBS at −80°C.

2.3. Cell culture

The human hepatoma cell line HepG2 was purchased from American Type Culture Collection (Manassas, VA); the human HSC line HHSteC was purchased from ScienCell Research Laboratories (Carlsbad, CA). HepG2 cells were cultured in eagle minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin solution (10,000 IU/mL; 10,000 μg/mL), all purchased from American Type Culture Collection. HHSteC cells were cultured in Stellate Cell Medium with 2% fetal bovine serum and 1% penicillin–streptomycin solution (10,000 IU/mL; 10,000 μg/mL) (ScienCell Research Laboratories). HHSteC cells were cultured in poly-D-lysine-coated 75 cm^2 culture flasks (Corning Incorporated Life Sciences, Tewksbury, MA). All cells were cultured in an incubator at 37°C and 5% CO_2 and/or 95% air atmosphere.

2.4. Microscopy

Exosome integration into cells was evaluated using a protocol modified from Chiba, *et al.* [28]. Exosomes from VAT were separately labeled with PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma–Aldrich), and labeled exosomes were added to the cell culture media at 1 and

0.1% dilutions. HepG2 and HHSteC cells were cultured to 75% confluence on 8-well chamber slides (EMD Millipore, Billerica, MA), with slides for HHSteC cells coated with poly-L-lysine (Sigma–Aldrich). Cultured cells were washed with PBS and 0.5 mL of exosome media was added to each well. After 48 h of incubation, slides were washed with PBS and fixed in 4% paraformaldehyde solution for 10 min. Nuclei were counter stained with ProLong Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (Life Technologies, Grand Island, NY). Slides were viewed on an Olympus BX61 upright bright field and/or fluorescent imaging microscope (Olympus America, Center Valley, PA).

2.5. Cell exposure

HepG2 and HHSteC cells were cultured to 75% confluence before exosome exposure. Media were prepared with exosomes at 1 and 0.1% dilutions. Cultured cells were washed three times with PBS, then 15 mL of exosome media was added to each flask. HepG2 cells were exposed to adipocyte exosomes from four obese donors and one lean donor; HHSteC cells were exposed to exosomes from only one obese donor. Of the four obese donors used in this experiment, two were included in the earlier experiments to characterize exosome miRNA content. The other two donors were collected after the characterization experiments were completed. Exosome media from each donor was applied to three flasks at each concentration, with three flasks receiving exosome-free media as controls for the HepG2 cells. Five replicates were used for the HHSteC cell exposure. Cells were incubated for 48 h, at, which time they were again washed three times with PBS and cells were harvested with TRI reagent (Molecular Research Center, Cincinnati, OH). RNA was isolated according to standard trizol protocol and clean-up was performed with the RNeasy Mini Kit (Qiagen, Valencia, CA). Final RNA concentration was assessed using NanoDrop 8000 (Thermo Fisher Scientific, Wilmington, DE).

2.6. Semiquantitative real-time polymerase chain reaction

Complimentary DNA was generated from 1000 ng of total RNA using SuperScript II Reverse Transcriptase (Invitrogen, Grand Island, NY). Polymerase chain reaction (PCR) was performed using 5 μL of complimentary DNA mixture with iQ SYBR Green Supermix (BioRad, Hercules, CA) on an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA) with SDS 2.4 software. Each sample was analyzed in at least duplicate. Gene expression results were analyzed with analysis of variance and Student *t*-test, with *P* values <0.05 considered significant. Results were analyzed separately at each exosome dilution, as well as in pooled comparisons of all samples for each exosome donor and compared with controls.

3. Results

3.1. Microscopy

After exposure to PKH26-labeled exosomes, red fluorescence was seen in a perinuclear pattern in HepG2 and HHSteC cells at all concentrations (Figs. 1 and 2, respectively). No red fluorescence was present in cells exposed to control media without exosomes.

3.2. HepG2 PCR

Analysis of gene expression changes at each exosome dose revealed significant increases in tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) for cells exposed to obese donors when the data are pooled (0.1%: 3.24 ± 1.54 $P = 0.008$, 1%: 3.18 ± 1.34, $P = 0.003$), as well as significant increases for obese donor 4 at 0.1% (4.69 ± 0.003, $P = 0.01$), and for obese donors 1, 3, and 4 at 1% (2.64 ± 1.14, $P = 0.04$; 4.78 ± 0.66, $P = 0.004$; 3.36 ± 0.27, $P = 0.01$, respectively). Plasminogen activator inhibitor-1 (PAI-1) expression was significantly decreased for obese donor 2 at the 1% dose (0.32 ± 0.06, $P = 0.03$). There were no significant differences between doses for any mediator assessed, and fold changes tended to be similar between doses. Therefore, we then performed analysis on pooled doses for each donor. This analysis demonstrated significant changes in expression of TIMP-1, PAI-1, matrix metalloproteinase-7 (MMP-7), and integrin αvβ-5 in cells exposed to exosomes from obese patients but not in the single lean donor (Table 1). Overall, we found increased expression of TIMP-1 (3.21 ± 1.38, $P = 0.026$) and integrin αvβ-5 (1.44 ± 0.49, $P = 0.027$) and decreased expression of MMP-7 (0.66 ± 0.33, $P = 0.03$) and PAI-1 (0.70 ± 0.27, $P = 0.003$). When results were stratified into individual exosome donors, some, though not all, of the results did reach statistical significance, likely due to a limited number of replicates per exosome donor or individual variations in the exosome contents. However, fold changes were generally in the same direction and trended toward significance. Complete results are shown in Table 1.

In cells exposed to exosomes from our single lean donor, there was a significant increase in TIMP-1 expression at the 0.1% exposure, though not at the 1% exposure or when doses were pooled (0.1%: 7.51 ± 1.05 $P = 0.002$, 1%: 0.93 ± 1.48, $P = 0.29$, pooled 5.32 ± 3.87, $P = 0.19$). There were no significant changes in other mediators assessed.

3.3. Hepatic stellate cell PCR

Exposure to adipocyte exosomes from one obese donor induced increased expression at both 0.1 and 1% exposures for TIMP-4 (0.1%: 1.43 ± 0.47, $P = 0.02$, 1%: 1.42 ± 0.33, $P = 0.0009$), integrin αvβ-5 (0.1%: 1.69 ± 0.46, $P = 0.0005$, 1%: 1.29 ± 0.26, $P = 0.01$), and integrin αvβ-8 (0.1%: 2.06 ± 0.90, $P = 0.013$, 1%: 1.59 ± 0.67, $P = 0.02$). Expression of TIMP-1, Smad-3, and MMP-9 was increased at the 0.1% exposure only (TIMP-1: 2.07 ± 0.72, $P = 0.0007$, Smad-3: 1.27 ± 0.22, $P = 0.02$, MMP-9: 2.13 ± 0.39, $P = 0.01$), though trended toward significance at the 1% dose in TIMP-1 (1.51 ± 0.57, $P = 0.09$) and Smad-3 (1.19 ± 0.23, $P = 0.06$), without significant changes in other mediators assessed. Complete results are detailed in Table 2.

4. Discussion

4.1. Adipocyte exosomes integrate into HepG2 and HHSteC cells

Our work is the first to demonstrate that adipocyte-derived exosomes are capable of integrating into HepG2 cells, even

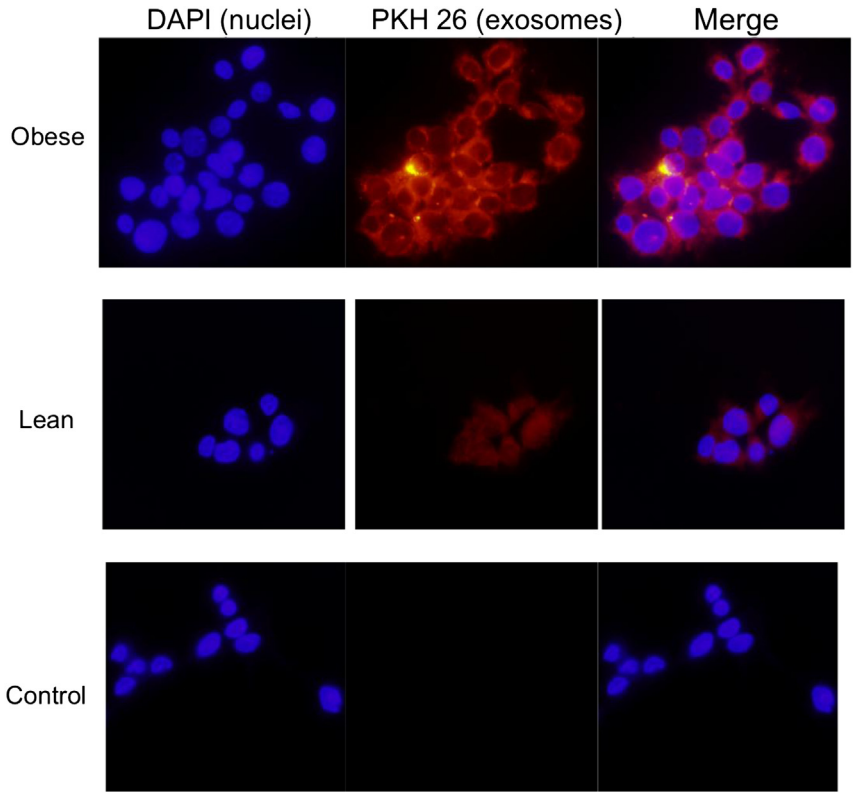

Fig. 1 – Immunofluorescence microscopy images of HepG2 cells after exposure to fluorescent-labeled exosomes.

when diluted from full strength. Limited work has demonstrated that tumor-derived exosomes are capable of integrating into HepG2 cells, though these experiments were performed using labeled exosomes at concentrations that are likely orders of magnitude larger than those present in normal circulation [28,29]. To our knowledge, we are also the first to demonstrate that exosomes from any source integrate into HHSteC cells. The ramifications of these findings are

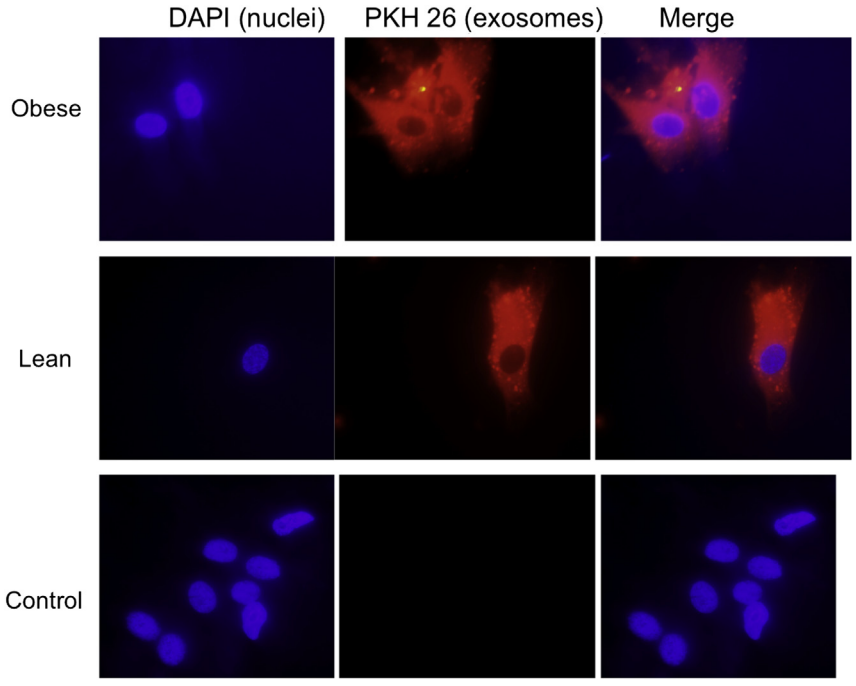

Fig. 2 – Immunofluorescence microscopy images of HHSteC cells after exposure to fluorescent-labeled exosomes.

Table 1 – Changes in expression of TGF-β pathway mediators in HepG2 cells exposed to visceral adipocyte exosomes (Data represent mean fold change ± standard deviation).

Mediator	0.1% dilution		1% dilution		Both doses	
	Fold change	P	Fold change	P	Fold change	P
TIMP-1						
Obese 1	2.82 ± 1.91	0.24	2.64 ± 1.14	**0.04**	2.73 ± 1.41	**0.02**
Obese 2	1.74 ± 0.83	0.31	2.01 ± 0.91	0.16	1.94 ± 0.76	0.08
Obese 3	3.13 ± 1.21	0.08	4.78 ± 0.66	**0.004**	4.36 ± 0.99	**0.002**
Obese 4	4.69 ± 0.003	**0.01**	3.36 ± 0.27	**0.01**	4.02 ± 0.78	**0.004**
All obese	3.24 ± 1.54	**0.008**	3.18 ± 1.34	**0.003**	3.21 ± 1.38	**0.026**
Lean 1	7.51 ± 1.05	**0.002**	0.93 ± 1.48	0.29	5.32 ± 3.87	0.19
MMP-7						
Obese 1	0.44 ± 0.07	0.46	0.41 ± 0.20	0.41	0.42 ± 0.14	**0.0008**
Obese 2	0.84 ± 0.003	0.6	0.51 ± 0.39	0.69	0.51 ± 0.39	0.40
Obese 3	0.56 ± 0.34	0.62	1.07 ± 0.23	0.85	0.95 ± 0.16	0.62
Obese 4	0.61 ± 0.28	0.51	0.97 ± 0.31	0.74	0.85 ± 0.31	0.41
All obese	0.50 ± 0.10	0.31	0.74 ± 0.38	0.70	0.66 ± 0.33	**0.03**
Lean 1	0.96 ± 0.23	0.55	1.17 ± 0.78	0.94	1.07 ± 0.23	0.80
PAI-1						
Obese 1	0.50 ± 0.10	0.06	1.08 ± 0.26	0.80	0.79 ± 0.36	0.24
Obese 2			0.32 ± 0.06	**0.03**	0.32 ± 0.06	**0.03**
Obese 3	0.50 ± 0.18	0.17	0.67 ± 0.13	0.15	0.60 ± 0.16	0.08
Obese 4	0.82 ± 0.14	0.38	0.76 ± 0.22	0.29	0.79 ± 0.17	0.30
All obese	0.62 ± 0.20	0.09	0.74 ± 0.32	0.18	0.70 ± 0.27	**0.003**
Lean 1	1.00 ± 0.05	0.99	1.11 ± 0.19	0.66	1.06 ± 0.13	0.80
Integrin αvβ-5						
Obese 1	1.60 ± 0.24	0.14	1.79 ± 0.21	0.12	1.71 ± 0.22	0.14
Obese 2			0.94 ± 0.48	0.69	0.94 ± 0.48	0.69
Obese 3	1.31 ± 0.42	0.19	0.95 ± 0.38	0.74	1.04 ± 0.35	0.96
Obese 4	2.00 ± 0.11	0.13	1.34 ± 0.45	0.43	1.68 ± 0.48	0.11
All obese	1.75 ± 0.32	0.13	1.26 ± 0.49	0.53	1.44 ± 0.49	**0.027**
Lean 1	1.17 ± 0.29	0.56			1.17 ± 0.29	0.56

Bold values indicate statistical significance.

potentially far-reaching, as we have shown that at physiologic concentrations adipocyte exosomes integrate into two cell types that are essential for the development of NAFLD. If the exosome contents once internalized cause dysregulation in these cell lines, a novel mechanism by which adipocytes may mediate liver disease could be implied.

4.2. *Obese adipocyte exosomes induce TGF-β pathway dysregulation in HepG2 cells*

Our results show that exposure to exosomes from VAT of obese adolescent females causes significant increases in expression of TIMP-1 and integrin αvβ-5, as well as significant decreases in expression of MMP-7 and PAI-1. The gene expression data are of particular interest in that they offer direct evidence of dysfunctional ECM regulation caused by adipocyte exosome delivery to HepG2 cells. TIMP-1 is a potent inhibitor of MMP activity, and is elevated in liver fibrosis in animal models and humans [30,31] while contrarily MMP-7 acts to degrade ECM. MMP-7 activity is increased in liver fibrosis due to hepatitis C, as well as in fibrosing disorders of other organ systems [30,32–36], which may be a physiologic response to increased ECM deposition and is in contrast with the data we present here. In a normal homeostatic state, TIMP-1 and MMP-7 are in relative balance and maintain appropriate ECM turnover. The increase of TIMP-1 expression with concomitant decease in MMP-7 expression in our experiments would result in ECM production that is relatively unchecked by MMP-7 degradation, rendering a profibrotic state in the cells [37]. Similar in function to TIMP-1, PAI-1 is a serine protease inhibitor that inhibits ECM degradation by blocking uPA and/or tPA-dependent activation of MMPs [38–40]; abnormalities in PAI-1 activity have been implicated in fibrosis of the skin, lung, kidney, heart, and liver [40]. Decreases in PAI-1 activity in liver disease are associated with mitigation of fibrosis [41]. The decrease in PAI-1 expression seen in our experiments could be the result of natural feedback mechanisms in an attempt to restore equilibrium in ECM turnover, although the overall balance between TIMP-1, PAI-1, and MMP-7 protein production would ultimately determine

Table 2 – Changes in expression of TGF-β pathway mediators in HHSteC cells exposed to visceral adipocyte exosomes (Data represent mean fold change ± standard deviation).

Subject	0.1% dilution		1% dilution	
	Fold change	P	Fold change	P
TIMP-1	2.07 ± 0.72	0.0007	1.51 ± 0.57	0.09
TIMP-4	1.43 ± 0.47	0.02	1.42 ± 0.33	0.0009
Smad-3	1.27 ± 0.22	0.02	1.19 ± 0.23	0.06
Integrin αvβ-5	1.69 ± 0.46	0.0005	1.29 ± 0.26	0.01
Integrin αvβ-8	2.06 ± 0.90	0.013	1.59 ± 0.67	0.02
MMP-9	2.13 ± 0.39	0.01	1.32 ± 0.62	0.38

whether ECM degradation is increased or decreased in these hepatic cells after exosome exposure.

The integrins are a group of cell adhesion molecules, which are capable of binding to and activating latent TGF-β complexes [42–44]. Increased expression of these molecules has been demonstrated in many fibrotic liver diseases [39,45–47], though the role of integrin αvβ-5 specifically in the liver remains unexplored. Given its activity to induce fibrosis via the TGF-β pathway in other organ systems, it is likely that integrin αvβ-5 contributes via a similar mechanism in liver disease [42–45]. The increased expression seen after exosome exposure from obese donors in the HepG2 cells further suggests a role for the TGF-β pathway in the pathogenesis of NAFLD, and is more evidenced that the adipocyte exosomes may cause dysregulation in the liver. Although the fold changes induced by the exosomes may seem small when compared with those induced by toxins such as ethanol or lipopolysaccharide, the small fold changes when extrapolated over the huge volume of hepatocytes in the liver could potentially induce substantial changes in overall protein expression.

4.3. *Lean visceral adipocyte exosomes do not induce TGF-β pathway dysregulation in HepG2 cells*

Exposure to exosomes from visceral adipocytes of a lean female induced significant gene expression changes in TIMP-1 at a 0.1% dilution only. It is possible that adipocyte exosomes from lean patients may be able to induce hepatocyte dysregulation, only to a lesser degree than those from obese patients. It is also possible that the dramatic increase in TIMP-1 expression seen at the 0.1% dilution (7.51 ± 1.05, $P = 0.002$) is an anomaly related to the limited scope of this initial experiment. Evaluation of visceral adipocyte exosomes from additional lean donors would be required to best assess whether exosomes from visceral adipocytes from lean donors differ in content from those from obese donors, or whether it is merely a dose phenomenon where patients with obesity shed more visceral adipocyte exosomes to the liver.

Taken together, these data offer additional support for the hypothesis that VAT is the driving force behind the development of NAFLD in obesity. The development of visceral adiposity is associated with macrophage migration and change to a more metabolically active phenotype of the adipocytes [7,8,48]. As VAT becomes more active, we would expect that to be reflected in the content of its secreted exosomes. As such, we would expect that exosomes from obese patients would exert a greater effect on liver cells than those from lean patients, which is consistent with our results.

4.4. *Adipocyte exosomes induce TGF-β pathway dysregulation in HHSteC cells*

Exposure to exosomes from obese adipose tissue induced increases in expression of TIMP-1, TIMP-4, Smad-3, integrin αvβ-5, integrin αvβ-8, and MMP-9, all of which are intimately involved in the development of fibrosis in liver disease and show increased expression in human studies and experimental models [38,42,45,46,49,50]. In contrast to the HepG2 experiments, the differences in gene expression are more pronounced at the 0.1% dose than at the 1% dose in HHSteC cells. This may be artifact related to the number of replicates performed, or may also represent negative feedback mechanisms that are triggered with increased exosome exposure. Only two mediators showed similar expression changes in both cell lines; TIMP-1 and integrin αvβ-5. The differences in mediators expressed between the two cell types is likely reflective of the complex interplay at work in the pathogenesis of NAFLD, but obviously more donor exposures to the HSC would be needed before drawing any significant conclusions. We were unable to expose the HHSteC cells to more obese donors due to the cost of working with these specialized cells. Due to the limited amount of VAT from our lean donor, we were also unable to expose stellate cells to lean exosomes.

5. Conclusions

In summary, the results of these experiments offer novel insights into the pathogenesis of NAFLD and the potential role of visceral adipocyte exosomes in other obesity-related diseases. These data are limited by the relatively small number of replicates per exposure, which was necessary due to the limited supply of exosomes and the limited number of patient donors. Research into exosome-mediated effects, both *in vitro* and *in vivo*, remains extremely limited, and we are unsure of the roles of ethnicity, age, or gender on the effect of exosome exposure either *in vitro* or *in vivo*. Our future experiments will focus on these factors, increasing replicates for the donors we have, and increasing the number of lean and obese exosome donors in general. It will also be important to assess protein expression changes in the cells to ensure that the gene dysregulation translates into altered protein production. Our continuing work will explore the contributions of BMI and ethnicity to the exosome-mediated changes in TGF-β pathway genes in hepatic cells in primary culture in addition to the immortalized cell lines, with a hope to uncover the direct link between obesity and NAFLD.

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Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in the article.

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