Document Coversheet

Study Title: Exercise-induced Skeletal Muscle Exosomes Promote Adipocyte Lipolysis

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SIGNIFICANCE

A sedentary lifestyle contributes to or exacerbates numerous diseases that represent urgent public health problems, including heart failure, coronary heart disease, type 2 diabetes, breast and colon cancer [1,2]. Ding and colleagues have conservatively estimated the worldwide cost of inactivity in 2013 to be $53.8 billion [3]. It is known that exercise confers health benefits by enhancing the function of non-skeletal muscle tissues, but the molecular mechanisms underlying these beneficial effects are not fully understood [4]. The majority of research investigating the systemic benefits of exercise has focused on aerobic exercise, leading to a relative lack of information regarding the health benefits of resistance exercise (RE) beyond improvements in muscle function. The few studies that have examined the effects of RE on non-muscle tissues suggest that aerobic and resistance exercise may confer comparable metabolic benefits [5-9]. A better understanding of the relevant molecular mechanisms underlying the systemic response to RE will help to generate new therapeutic options for patients who have limited ability to exercise, while improving exercise responses in patients who are able.

In the past two decades, skeletal muscle has emerged as a secretory organ, producing myokines (e.g., irisin, GDF11, IL-6, BDNF, others) that may mediate the beneficial effects of exercise and ameliorate the negative effects of a sedentary lifestyle [10-12]. More recently, exosomes have emerged as potential mediators of inter-tissue signaling. Exosomes are small (~30–150 nm) membrane-bound vesicles that are secreted following the fusion of multivesicular late endosomes with the plasma membrane [13]. Elegant work by Valadi and colleagues demonstrated that exosomes contain nucleic acids (including miRNAs) that are delivered to recipient cells where they modulate mRNA stability [14]. Notably, exosomes are easily internalized by recipient cells, making them an attractive vehicle for drug delivery and gene therapy [15,16]. Subsequently, numerous groups have reported alterations in circulating miRNAs with various pathological conditions [17-19]. We and others have shown that exosomes are released from cultured rodent and human muscle cells [20-24]. Additionally, the muscle cell line C2C12-derived exosomes, rich in miR-133a, are capable of down-regulating target genes in NIH-3T3 cells [25]. In humans, exosomes containing miR-133b and miR-181a are released acutely following aerobic exercise [26]. Furthermore, miR-1, a muscle-specific miRNA, was increased in serum following downhill walking in humans [27]. A fascinating new study by Whitham and coworkers showed that acute aerobic exercise promoted the release of exosomes that were preferentially taken up by liver [28]. In contrast, our preliminary data show that RE induces a rapid systemic release of miR-1 containing exosomes that are preferentially delivered to adipose tissue (see Fig. 1-4).

The primary role of adipose tissue is to store excess energy as lipids, and it is the principal source of serum non-esterified fatty acids (NEFA) during times of energy demand (i.e., fasting or exercise). As such, the metabolic function of adipose is critical for determining whole body metabolic outcomes. Adipocyte lipolysis is driven by catecholamines (norepinephrine and epinephrine), which stimulate β-adrenergic receptors (β-AR; β-1, β-2, and β-3). β-AR agonism enhances insulin sensitivity, lean mass, energy expenditure, and thermogenesis [29-33]. A growing body of work indicates that adipocytes develop catecholamine resistance in the context of obesity and insulin resistance [34-41], potentially due to down-regulation of β-AR [35]. We and others have shown that adipose miRNAs are altered with obesity and insulin resistance [42-44]. Moreover, miRNAs in adipose have been shown to modulate β-AR signaling in vivo [45] and in vitro [46]. These data, along with our preliminary data, indicate that miRNA-mediated mRNA processing can improve adipose β-AR sensitivity, leading to the exciting hypothesis that exosomal delivery of miRNAs to adipose could be used to ameliorate metabolic disease.

Numerous studies in humans and animals have shown that aerobic exercise is beneficial to adipose tissue function and whole-body metabolism [10,11,47-49]. Both acute and chronic aerobic exercise enhance adipocyte catecholamine sensitivity in humans and animals [50-56]. Although relatively few studies have investigated whether adipose adrenergic signaling is affected by RE, it is known that a single bout of RE can increase circulating NEFA and resting energy expenditure and decrease respiratory quotient for up to 24 hours [57-61], indicative of increased adipocyte lipolysis and muscle fatty acid oxidation. Furthermore, the lipolytic response to RE is impaired in obese men [62,63]. Using synergist ablation, a model of RE in mice, we show that adipose transcriptional responses are exosome-dependent, and that serum exosomes enhance adipocyte catecholamine sensitivity and lipolysis for at least 24 hours (Fig. 6). To our knowledge, this is the first demonstration of a potential mechanism whereby RE imparts metabolic adaptations in adipose. Since adipose metabolic function is crucial for determining whole-body metabolic outcomes, the ability of RE-induced exosomes to improve adipose metabolism has significant clinical implications.

Scientific Premise. The premise that RE has a beneficial effect on adipose metabolism is strongly supported by the literature and by our preliminary data. We will test the highly novel hypothesis that RE promotes adipocyte catecholamine sensitivity via muscle-derived exosomal delivery of miR-1 to adipocytes, thereby enhancing β-AR signaling. We will begin by determining if the effect of RE-induced exosomes on adipocyte β-AR signaling are miR-1 dependent, and the specific mechanism of action of miR-1 in adipocytes. Next we will determine whether muscle fiber-derived exosomes specifically promote adipocyte lipolysis through enhanced β-AR signaling. Lastly, in order to obtain translational proof-of-principle, we will determine if in vitro mechanisms may be operating in humans by measuring serum exosome, muscle and adipose miR-1 levels following a single bout of RE, and testing the effects of the isolated exosomes on adipocyte catecholamine sensitivity. To our knowledge, we are the first group to mechanistically investigate the capacity of RE to enhance adipocyte catecholamine sensitivity. Furthermore, our preliminary data indicate a central role for exosomes and miRNAs, representing newly emerging and highly novel mechanisms of intercellular communication.

INNOVATION

The current proposal is highly innovative for the following reasons:

- The proposed mechanism in which RE regulates adipose metabolism through exosomal delivery of the muscle-specific miR-1 is completely novel and, to the best of our knowledge, never been described before.
- While aerobic exercise has been shown to influence the metabolic flexibility of adipose tissue, the finding that RE may also modulate the catecholamine sensitivity of adipose tissue provides a new mode of exercise that can be employed to potentially treat obesity; importantly, high-intensity exercise has been shown to have improved adherence compared to moderate-intensity training [64,65].
- Establishing the use of skeletal muscle-derived exosomes as a delivery platform to regulate adipose tissue metabolism represents a highly unique therapeutic strategy to treat obesity.
- A robust research design employing mouse and human studies to rigorously test our central hypothesis both mechanistically and if the proposed muscle-adipose crosstalk translates to humans.
- We have assembled a distinguished team of investigators with complementary expertise in the cellular and molecular mechanism regulating skeletal muscle plasticity, exercise physiology, adipose tissue biology and metabolism.

Collectively, these factors come together to produce a highly innovative research proposal that is expected to demonstrate a novel mechanism through which RE regulates adipose tissue metabolism, providing clinically significant insights into the potential pharmacological use of exosomes and/or miR-1 to augment or mimic the beneficial effects of RE on metabolic health and to treat obesity.

PRELIMINARY DATA

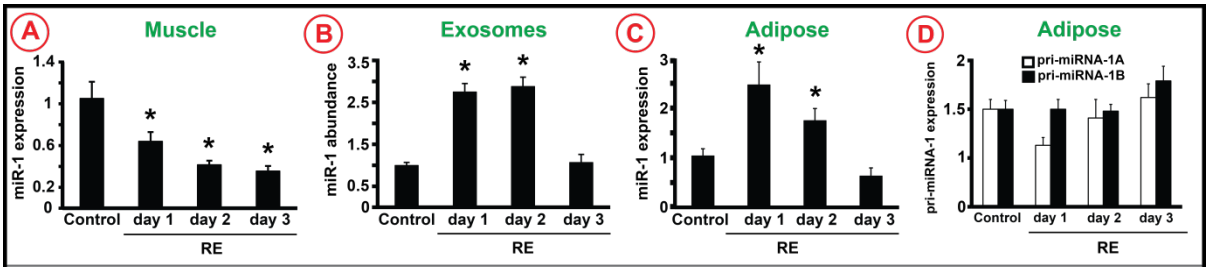

Figure 1. Delivery of muscle-specifc miR-1 to adipose by exosomes in response to resistance exercise. A, mouse plantaris muscle was subjected to synergist ablation, an established model of resistance exercise (RE), for 1-3 days. Muscle-specific miR-1 expression was significantly lower in the plantaris muscle in response to RE after 1, 2 or 3 days. **B**, exosomes isolated from serum of mice showed significantly higher miR-1 abundance after 1 and 2 days of RE. **C**, epidymal adipose miR-1 expression was significantly higher by ~2-2.5-fold after 1 and 2 days of RE. **D**, no change in primary miRNA-1A/B (pri-miRNA) expression in adipose in response to RE indicating the higher miR-1 expression shown in **C** was derived from an exogenous source, *i.e.*, exosomes. Data are presented as mean +/- SEM (n=7-8/group) with significant difference ($p < 0.05$) from Control indicated by an asterisk.

We recently reported that exosomes released from activated skeletal muscle stem cells deliver muscle-specific miR-206 to fibroblasts in response to synergist ablation, a well-accepted model of resistance exercise (RE) in rodents, that induces robust hypertrophy of the plantaris muscle [24, 66]. This discovery inspired us to further explore exosome release in response to RE and their impact on putative target tissues. In agreement with our previous findings, muscle-specific miR-1 levels were significantly lower by 40% in the

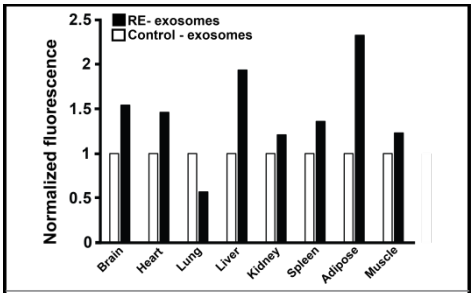

Figure 2. Enhanced uptake of exercise-induced exosomes by adipose. Exosomes were isolated from serum of mice that had either undergone sham (Control) or synergist ablation (Resistance Exercise,RE) for one day. Isolated exosomes were fluorescently labeled and then reintroduced to the circuation of a naive mouse by tail vein injection. Tissue was collected after 1 hr, lysed and fluorescence measured. Fluorescence signal was increased 2.3-fold in adipose following injection of labeled exosomes from exercised mouse compared to sham-control mouse.

plantaris muscle after a single day of RE and continued to decrease for the next two days (Fig. 1A) [67,68]. Given the relatively long half-life (5-9 days) of miRNAs, this rapid decrease in muscle miR-1 suggested the intriguing possibility that miR-1 was being exported out of the myofiber by exosomes [69]. As shown in Fig. 1B, exosomes isolated from serum of mice subjected to 1-3 days of RE had an increase in the abundance of miR-1 after 1 and 2 days, returning to baseline by day 3. We took advantage of the muscle specificity of miR-1 to determine if exercise-induced exosomes were being taken up by the liver, brain or adipose tissue. Whereas we did not detect a change in miR-1 abundance in the brain or liver (data not shown), we did observe ~2.5-fold higher miR-1 abundance in epididymal adipose after a single day of RE (Fig. 1C). Importantly, expression of primary miRNA-1 (both A and B) transcripts in adipose did not change in response to RE indicating the increase in miR-1 was from an exogenous source and not driven by transcription of the miR-1 gene within adipose cells (Fig. 1D).

To determine if RE-induced exosomes were preferentially taken up by adipose tissue, we isolated exosomes from serum of mice that had either undergone sham surgery (Control) or synergist ablation for a single day (RE). Isolated exosomes were fluorescently labeled, reintroduced separately into naive mice by tail vein injection with tissue collected one hour later. Tissue fluorescence was first normalized to amount of tissue (mg) and then normalized to Control exosome fluorescence. Although most tissues showed a relatively modest increase in fluorescence, epididymal adipose showed the largest (2.3-fold) increase in fluorescence following injection of RE-induced exosomes (Fig. 2). This finding suggests the intriguing possibility that RE-induced exosomes may affect many tissues throughout the body with adipose being a primary target.

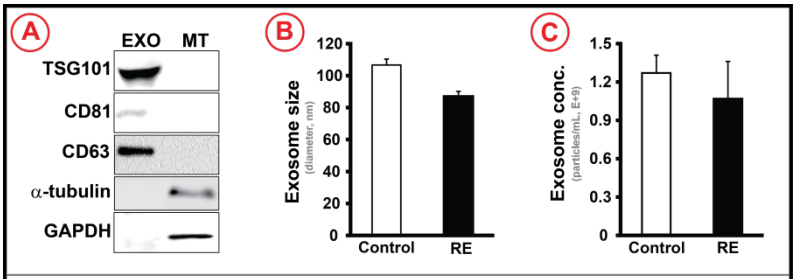

Figure 3. Exosome characterization following hypertrophic growth stimulus. The mouse plantaris muscle was subject to synergist ablation, a model of resistance exercise (RE), to induce a hypertrophic stimulus. **A**, Western blot analysis confirmed exosome isolation from mouse serum using the ExoQuick protocol based on detection of markers of extracellular vesicles Tsg101, CD81 and CD63. **B**, NanoSight analysis of exosomes showed a mean particle size of ~100 nm, indicative of exosomes, with a trend (p = 0.07) for decreased particle size following a single day of RE. **C**, Nanosight analysis showed no difference in exosome concentration between groups. Data are presented as mean +/- SEM (n = 6/group).

To confirm we had successfully isolated exosomes we performed Western blot analysis using antibodies against established markers of exosomes. As shown in Fig. 3A, we detected TSG101, CD81 and CD63 proteins only in the exosome sample, not in myotubes (MT); importantly, we did not detect the cellular proteins α-tubulin or GAPDH in the exosome sample demonstrating the specificity of exosome isolation protocol. Nanoparticle tracking analysis showed the isolated extracellular vesicles were within the range (73-139 nm) of exosomes (~30-150 nm) and that exosome serum concentration did not change in response to RE (Fig. 3B-C) [70].

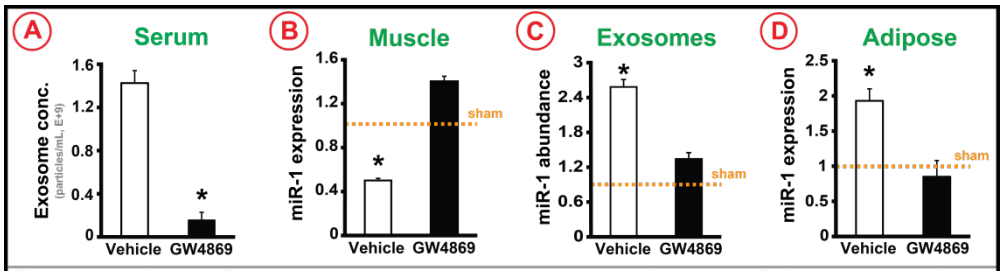

Figure 4. Inhibition of exosome release prevents increase in adipocyte miR-1 expression. Mice were treated with either vehicle (3% DMSO in sterile saline) or the neutral sphingomyelinase inhibtor GW4869 to prevent the release of exosomes. **A**, NanoSight analysis demonstrated that GW4869 treatment reduced serum exosome concentration by >90%. Blocking exosome release by GW4869 treatment prevented the resistance exercise-induced decrease in miR-1 in muscle (**B**); and the increase in miR-1 abundance in exosomes (**C**) and adipose(**D**). Data were normalized to the respective sham control (yellow dotted line) and presented as mean +/- SEM (n = 4-6/group) with significance (p < 0.05) denoted by an asterisk.

We next determined if the increase in adipose miR-1 abundance after a single day of RE was the result of exosome delivery. This was critical because of an alternative scenario in which miR-1 is released into circulation by damaged muscle (as the result of RE) and travels to adipose tissue bound to lipoproteins or

Ago2 [71]. Mice were treated with vehicle or GW4869 for seven days prior to either sham or synergist ablation surgery. GW4869 is an inhibitor of neutral sphingomyelinases which has been shown to effectively block the release of exosomes [72]. Nanoparticle tracking analysis confirmed GW4869 treatment effectively reduced serum exosome concentration by over 90% (Fig. 4A). GW4869 treatment prevented the decrease in skeletal muscle miR-1 expression in response to a single day of RE (Fig. 4B) indicating exosome release was necessary for the down-regulation of skeletal muscle miR-1 expression. Consistent with this finding, GW4869 treatment also prevented the increase in exosomal miR-1 abundance observed with RE and the subsequent increase in miR-1 abundance in adipose tissue (Fig. 4C-D). Together, these findings provide compelling evidence that miR-1 is exported from skeletal muscle by exosomes following RE and exosomal delivery of miR-1 is responsible for the increase in adipose miR-1 abundance.

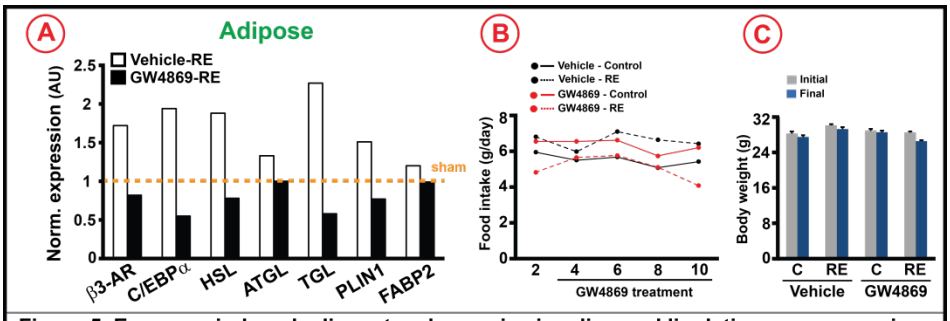

Figure 5. Exosome-induced adipocyte adrenergic signaling and lipolytic gene expression. **A**, microarray analysis of adipose showed increased adipocyte adrenergic signaling (β3-AR and C/EBPα) and lipolytic (HSL, ATGL,TGL, PLIN1 and FABP2) gene expression in response to one day of sham (Control) or synergist ablation (RE) which was prevented by blocking exosome release via GW4869 treatment. **B**, the calculated food intake/mouse/day; surgeries were performed on day 9 with serum collected on day 10. **C**, initial (grey bar) and final (blue bar) body weight for each group was unchanged throughout the experiment indicating the proposed exosome-induced increase in adipocyte lipolysis was not caused by a reduction in food intake.

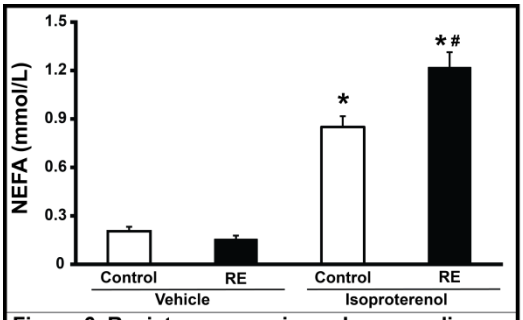

Figure 6. Resistance exercise enhances adipose catecholamine responsiveness. Adipocytes were isolated from epididymal adipose from either Control (sham surgery) or Resistance Exercise (RE,1 day of synergist ablation) mice. Adipocytes were incubated with either vehicle or 100 uM isoproterenol for one hour. Media non-esterified fatty acid (NEFA) concentration was determined for each condition. Data are present as mean +/- SEM (n=7/group). Asterisk, significantly different ($p < 0.05$) from control; hashtag, significantly different from isoproterenol control.

In an effort to identify how miR-1 might be affecting gene expression in adipose tissue, we performed a microarray analysis on adipose collected from vehicle or GW4869 treated mice after one day of either sham surgery or synergist ablation. Our initial search strategy was to identify genes whose expression was changed in response to RE in the vehicle group but were unchanged in the GW4869 group (relative to their respective sham). Using this strategy, we identified the up-regulation of genes involved in adrenergic signaling (β3-AR and C/EBPα) and lipolysis (HSL, ATGL, TGL, PLIN1 and FABP2) in adipose of vehicle-treated mice following RE; however, following GW4869 treatment, the expression of these same genes was either unchanged or down-regulated (Fig. 5A). To insure the up-regulation of lipolytic genes in adipose was not caused by a decrease in food intake, we measured food intake and body weight throughout the experimental period. As shown in Fig. 5B-C, food intake and body weight remained unchanged and, in particular, for the Vehicle-RE group. These microarray results suggest that lipolysis was activated following RE through β-adrenergic signaling and prevented when exosome release was blocked by GW4869 treatment.

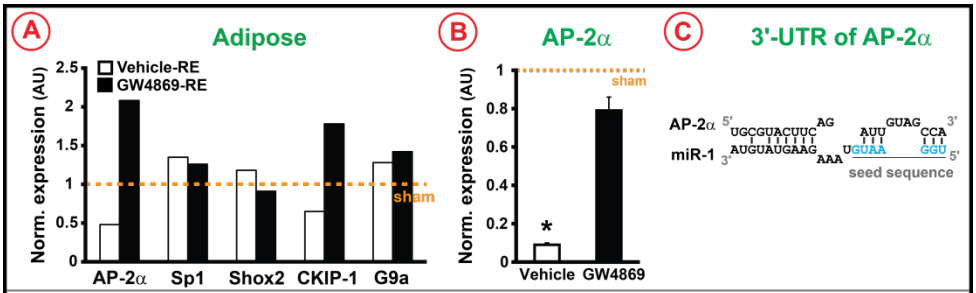

Figure 7. Exercise-induced exosome down-regulation of adipocyte AP-2α gene expression. **A**, microarray analysis of adipose tissue for known inhibitors of C/EBPα expression showed down-regulation of AP-2α and CKIP-1 expression following one day of synergist ablation (RE) which was prevented when exosome release was inhibited by GW4869 treatment. **B**, qPCR confirmed the 90% decrease in AP-2α expression with exercise that was blocked with GW4869 treatment. **C**, sequence alignment of miR-1 and predicted target binding site within the 3'-UTR of AP-2α.

To determine if RE in fact enhances catecholamine sensitivity of adipose tissue, we measured the ability of adipocytes isolated from mice subjected to either sham surgery (Control) or synergist ablation (RE) to release non-esterified fatty acids (NEFA) in response to 100 uM isoproterenol. As shown in Fig. 6, RE adipocytes released significantly more NEFA into the media than did Control adipocytes in response to

isoproterenol. These findings are consistent with the increase in the expression of β3-AR and lipolytic genes presented in Fig. 5.

We next hypothesized that RE-induced exosomal miR-1 was promoting adipose β-adrenergic and lipolytic gene expression by targeting a repressor of C/EBPα. Specifically, we developed a working model in which the down-regulation of a C/EBPα repressor by miR-1 would lead to an increase in C/EBPα expression. The up-regulation of C/EBPα would in turn drive β3-AR expression thereby enhancing catecholamine sensitivity of adipose and subsequently promoting lipolysis. Microarray analysis of adipose tissue (as described in Fig. 5) for five known inhibitors of C/EBPα expression showed ~50% lower expression of AP-2α and CKIP-1 in response to RE that was completely blocked by GW4869 treatment (Fig. 7A). qPCR analysis of adipose AP-2α expression found a significant 90% decrease in AP-2α expression following RE in the vehicle group that was prevented with GW4869 treatment (Fig. 7B). The RNAhybrid program identified a non-conventional miR-1 binding site within the 3'-UTR of the AP-2α transcript with mis-matches within the seed sequence but extensive homology at the 3' region (Fig 7C) [73]. The possibility that this site is functional is not without precedent as studies have provided evidence that miRNAs do not necessarily follow the "classical" rules dictated by canonical seed matches [74-76]. Based on the preliminary data presented in Fig. 6 and 7 we developed a working model that describes a novel mechanism whereby the exosomal release of miR-1 in response to RE is preferentially taken up by adipose tissue which in turn leads to enhanced catecholamine sensitivity through C/EBPα activation of β3-AR by miR-1 repression of AP-2α.

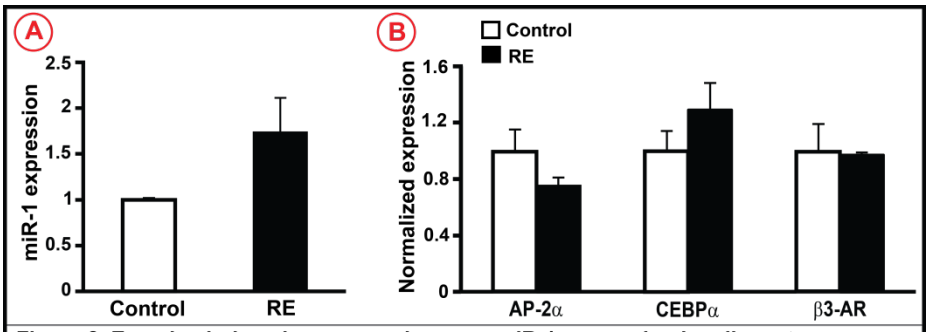

Figure 8. Exercise-induced exosomes increase miR-1 expression in adipocytes. **A**, exosomes isolated from serum of mice that have undergone sham surgery (Control) or synergist ablation (Resistance Exercise, RE) were incubated with 3T3-L1 differentiated adipocytes for 24 hr with miR-1 expression trending (p = 0.22) higher by 1.7-fold (n=3/group). **B**, compared to Control exosomes, RE-induced exosomes caused AP-2α expression to trend lower by 25% while trending to increase CEBPα expression by 29%; there was no change β3-AR expression. These preliminary data support the proposed use of in vitro model to investigate the metabolic and molecular response of adipocytes to exercise- and muscle-specific exosomes. Data are presented as mean +/- SEM.

To more specifically test the ability of RE-induced exosomes to alter adipose gene expression, we applied exosomes isolated from the serum of mice subjected to either sham surgery (Control) or synergist ablation (RE) to 3T3-L1 cells differentiated into adipocytes. Although we did not observe any significant change in gene expression following 24 hr incubation, we did see a trend for increased miR-1 and C/EBPα expression as well as decreased AP-2α expression (Fig. 8A-B). These trends in gene expression are consistent with our working model and suggest with a larger "n" we will likely observe significant changes in gene expression. These preliminary data further demonstrate that we have the technical expertise to perform the proposed in vitro experiments using isolated exosomes and adipocytes.

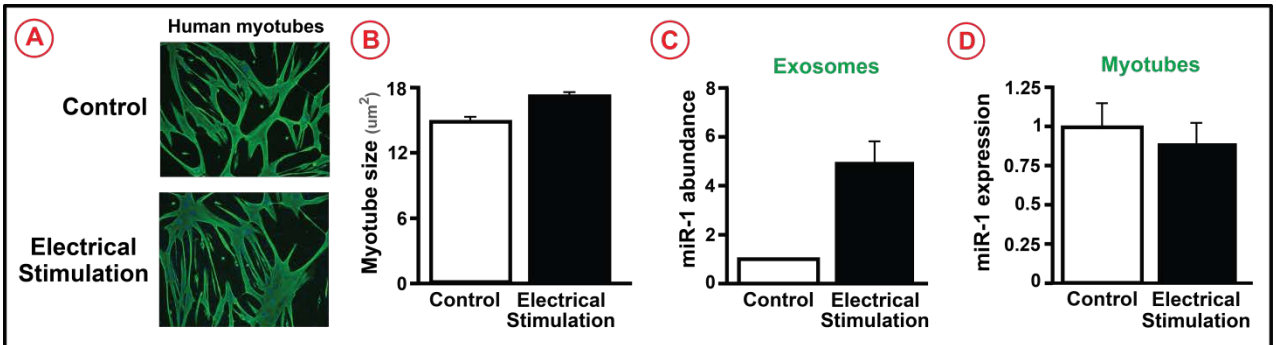

Figure 9. Increased exosomal miR-1 from human myotubes in response to electrical stimulation. A, human primary myoblasts (n=2) were differentiated for six days to form myotubes, then stimulated (12V, 1 Hz, 2 ms pulse) for 8 hr and subsequently processed 16 hr later for image analysis or RNA isolation with media collected for exosome isolation. **B**, myotube size was increased by ~15% in response to electrical stimulation as determined by MyoVision image analysis program. **C**, abundance of miR-1 of exosomes isolated from media of stimulated (Electrical Stimulation) myotubes increased 5-fold compared to exosomes from unstimulated (Control) myotubes. **D**, miR-1 expression of Electrical Stimulation myotubes decreased by 9% compared to Control myotubes. This preliminary data demonstrate that electrical stimulation of human myotubes induces the release of muscle-specific exosomes containing miR-1, thus mimicing the response of mouse skeletal muscle to hypertrophic stimulus induced by synergist ablation.

Given that there are no established skeletal muscle-specific exosomal proteins that would allow for the isolation of muscle-specific exosomes, we have

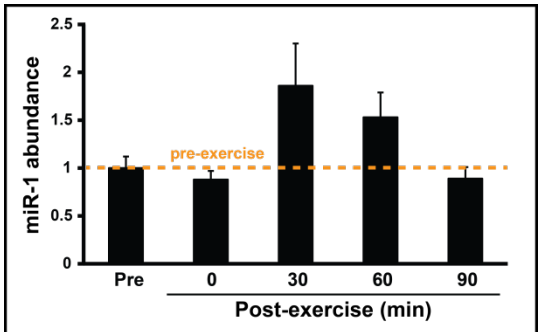

Figure 10. Human exosomal miR-1 abundance following a bout of resistance exercise. Subjects performed a bout of resistance exercise (knee extension) to failure. Blood was collected pre- and post-exercise (0, 30, 60 and 90 minutes) followed by immediate isolation of exosomes from plasma and qPCR analysis of exosomal miR-1 abundance. Following resistance exercise, exosomal miR-1 abundance was trending higher after 30 and 60 minutes post-exercise, returning to baseline by 90 min. Data are presented as mean +/- SEM (n = 10).

used miR-1 as a readout of muscle-derived exosomes. To more rigorously test our working hypothesis that skeletal muscle-derived exosomes alter adipose metabolism, we have developed an in vitro model of RE using human myotubes. As shown in Fig. 9A-B, electrical stimulation of human myotubes appeared to increase myotube size compared to unstimulated myotubes and, importantly, resulted in a 5-fold increase in the abundance of miR-1 in exosomes isolated from the culture media (Fig. 9C). Moreover, there was a small decrease (9%) in myotube miR-1 expression with electrical stimulation (Fig. 9D). Although we were unable to run statistics because of insufficient "n" (n=2), the overall trend in myotube size and both exosomal and myotube miR-1 abundance follows the same general pattern shown in Fig.1 for RE.

As an initial effort to test whether or not our working model translates into humans, we carried out a pilot study. Young, sedentary, adults (n=10, 5 females, 5 males) performed a bout of RE (knee extension) to failure. Blood was collected pre- and post-exercise (0, 30, 60 and 90 minutes). Exosomes were isolated from serum and miR-1 abundance quantified by qPCR. Thirty minutes post-exercise there was a trend for an increase in exosomal miR-1 abundance. This result suggests that both mice and humans respond to RE in a similar manner by releasing miR-1 into the circulation through exosomes. Further, this pilot demonstrates we have the necessary expertise and infrastructure to successfully conduct the human RE study proposed in Aim 3.

Collectively, we have provided strong preliminary data to support a novel mechanism in which skeletal muscle, in response to RE, regulates adipose metabolism through the exosomal delivery of muscle-specific miR-1. Also, our preliminary data clearly demonstrate that we have both intellectual and technical expertise to successfully perform the proposed experiments.

APPROACH

An overall strength of the proposed experimental design is a shared set of techniques which will facilitate the comparison of results from the mouse (Aim 1), cell culture (Aim 2) and human subjects (Aim 3). The shared set of techniques includes exosome isolation, exosome miRNA loss- and gain-of-function studies, gene expression analysis and catecholamine sensitivity assay. Each of these techniques will be briefly described below and subsequently referred to as the SOP section. Aim-specific methods will be provided in each aim.

Standard Operating Procedures (SOP)

Exosome isolation. Exosomes will be isolated using Exoquick-LP (mouse serum) or ExoQuick-TC (human plasma and culture media) according to the manufacturer's instructions. Briefly, following centrifugation (3000 *g* ,15 min) exosome precipitation solution will be added to filtered (0.22 um) supernatant, incubated overnight at 4^0C with ExoQuick solution and then centrifuged at 1,500 *g* for 5 minutes to remove all traces of fluid. The exosome pellet will be resuspended into 25ul of PBS. While the exosome field has yet to reach a consensus on the best method for isolating exosomes, ExoQuick has recently been shown to be as good (50-fold > yield), if not better, than traditional ultracentrifugation methods [77,78]. We will confirm exosome isolation by Western blot, nanoparticle tracking analysis (ZetaView) and transmission electron microscopy [78].

Exosome miRNA loss- and gain-of-function. Exosomes derived from Control or RE serum (~1 × 10^6 particles) will be combined with 20 pmol miR-1 mimic or inhibitor and 10 µl Exo-fect solution and then incubated at 37°C for 10 min. Transfected exosomes will be then mixed with 30 µl ExoQuick-TC and incubated at 4°C for 30 min. After centrifugation of samples (14,000 rpm for 3 min), the transfected exosomes will be suspended in PBS.

Gene expression analysis. Total RNA will be isolated from either skeletal muscle or adipose using Direct-zol with 0.5 µg of DNase-treated RNA used to make cDNA using the SuperScript™ VILO™ cDNA Synthesis Kit. Taqman gene expression assays for the following genes: β3-AR, C/EBP2α, HSL, ATGL, TGL, PLIN1, FABP2, FABP4 and AP-2α. miRNAs expression/abundance of muscle, adipose and exosomes will be determined using the miRCury RNA isolation kit and LNA™ Universal RT microRNA PCR system.

Catecholamine sensitivity assay. Lipolysis and anti-lipolysis will be measured in 3T3-L1 and ADHAS cells. Cells will be pre-treated for 5 min with adenosine deaminase and then treated with either 10 µM, 100 µM, or 1 mM isoproterenol, 0.5 nM insulin, both insulin and 100 µM isoproterenol, or DMEM only. The cells will be incubated at 37°C for 1 hr with gentle rocking, and the media collected and used for NEFA measurement as previously described by us [79]. To correct for cell surface area, cell diameters will be measured via microscopy [80,81].

3T3-L1 adipocyte culture. 3T3-L1 (CL-173, ATCC) adipogenic cells will be maintained in DMEM, 10% FBS and differentiated into adipocytes as previously described by us [80]. Briefly, 3T3-L1 cells will be grown to confluency, then placed in differentiation media (DM), composed of DMEM, 10% FBS, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), and 10 µg/ml insulin for four days. Cells will then be maintained in DM without dexamethasone and IBMX for an additional 3 days [82]. Differentiation to adipocytes will be assessed by Oil Red O staining and expression of the adipocyte-specific mRNA FABP4.

Adult-derived human adipocyte stem cells (ADHASC) culture. Pre-adipocytes were obtained from lipo-aspirate from healthy women undergoing liposuction. Lipo-aspirate was digested using collagenase type I (1 g/l KRB with 1% BSA) for 1 hr at 37°C with gentle shaking. The stromal-vascular fraction was separated from adipocytes by centrifugation, and plated in growth media (DMEM/Hams-F12, 10% fetal bovine serum, and 15 mmol/l HEPES). Following two passages, cells were cryopreserved. Differentiation, as previously described by us [79,82,83]; will be induced with DM containing 100 nmol/l insulin, 1.0 µmol/l dexamethasone, 0.25 mmol/l IBMX, 0.033 mmol/l biotin, 0.017 mmol/l pantothenate, and 1.0 µmol/l rosiglitazone for three days, followed by three additional days using DM without IBMX and rosiglitazone. Differentiation to mature adipocytes will be assessed by Oil Red O staining and FABP4 gene expression.

Specific Aim 1: Determine if exercise-induced exosomal miR-1 enhances adipocyte adrenergic signaling through activation of β3-AR expression.

Rationale. The rationale for this aim is based on our preliminary data showing that RE-induced exosomes containing muscle-specific miR-1 enhance the catecholamine sensitivity of adipocyte. Further, microarray analysis identified a potential underlying mechanism for this enhanced catecholamine sensitivity that involves C/EBPα activation of β3-AR expression via miR-1 down-regulation of AP-2α, a repressor of C/EBPα. The purpose of this aim is to determine if this proposed mechanism is functionally operative by investigating 1) how RE-induced exosomes alter adipose metabolism both in vivo and in vitro, 2) the necessity of exosomal miR-1 in regulating adipose metabolism using miR-1 loss- and gain-of-function studies and 3) validating miR-1 predicted target gene AP-2α. Given that our preliminary data were generated using male mice, for the proposed studies we plan to use both male and female mice to determine if sex has an influence on exosomal regulation of adipose catecholamine sensitivity.

Design. Based on preliminary data presented in Fig. 8A, we performed a power calculation to determine the number of mice required per group to detect significant ($p < 0.05$) difference between sham and synergist ablation groups. Male and female C57BL6/J mice (n=12/sex; 4 months of age) will undergo either sham (Control) or synergist ablation (RE) surgery which we have used extensively and previously described in detail [24,66,84-86]. The synergist ablation model has been successfully used for over half a century as a resistance exercise mimetic in rodents to uncover the molecular and cellular mechanisms regulating skeletal muscle hypertrophy [87]. One day post-surgery, skeletal muscle (plantaris), epididymal adipose and blood will be collected. Skeletal muscle and a portion (100 mg) of adipose tissue will be used to isolate total RNA to quantify miR-1 and gene expression by qPCR. Serum will be isolated from blood for exosome isolation as described in the SOP section.

Experiment 1.1. Control and RE exosomes will be incubated with 3T3-L1 adipocytes for 24 hr and then gene expression and catecholamine sensitivity measured as describe in the SOP.

Experiment 1.2. To determine if miR-1 is necessary and sufficient to enhance adipocyte catecholamine sensitivity, Control exosomes will be transfected with miR-1 mimic (Control-plus) and RE exosomes with anti-miR-1 oligonucleotide (RE-minus). These modified exosomes will be incubated with 3T3-L1 adipocytes for 24 hr and then gene expression and catecholamine sensitivity measured as described in the SOP section.

Experiment 1.3. Based on our preliminary data, we hypothesize that RE-induced exosomal miR-1 is repressing expression of AP-2α, a negative regulator of C/EBPα. To determine if AP-2α is a target gene of miR-1 we will generate a luciferase reporter gene harboring the 3'-UTR of AP-2α. We will assess the ability of miR-1 to

repress reporter gene expression; if so, then we will repeat the experiment with a mutant AP-2α 3'-UTR reporter gene in which the predicted miR-1 binding site is mutated as previously described by us [24].

Experiment 1.4. Microarray analysis of epididymal adipose showed increased expression of genes involved in β-adrenergic signaling and lipolysis in response to RE that was blocked by GW4869 treatment (see Fig. 5). The purpose of this experiment is to use qPCR to confirm the microarray results and, importantly, determine if the observed changes in gene expression and effects of GW4869 treatment are the same in female mice (n=12/sex).

Statistical Analysis. A two-factor ANOVA (treatment X sex) will be used to determine if a significant interaction exists between factors for each dependent variable under consideration. If a significant interaction is detected, Scheffé post-hoc comparisons will be performed to identify the source of significance with $p \leq .05$.

Expected Outcomes & Alternative Approaches. The preliminary data presented demonstrate that we have the necessary expertise to carry out the proposed experiments so we do not anticipate any technical issues. We expect to confirm the change in β-adrenergic signaling and lipolytic gene expression and the enhanced catecholamine sensitivity of adipocytes exposed to RE-induced exosomes. Moreover, we expect to confirm the basis for these exosome-mediated effects is the exosomal delivery of miR-1 to adipocytes. In particular, we expect to find that Control exosomes containing the miR-1 mimic will promote the same up-regulation in gene expression and enhanced catecholamine sensitivity as do RE exosomes. By contrast, we anticipate the introduction of an anti-miR-1 into RE exosomes will prevent the changes in gene expression and catecholamine sensitivity. What is more difficult to predict is whether or not AP-2α is a bona fide target gene of miR-1. The miR-1 binding site within the 3'-UTR of the AP-2α transcript does not conform to a "classical" miRNA binding site but the predicted thermodynamics of miR-1 binding of ~ΔG= -20 is relatively favorable so there is a reasonable chance we will see miR-1 regulation of AP-2α expression.

If we fail to demonstrate miR-1 regulation of AP-2α, yet show that miR-1 is necessary for mediating the effects of RE exosomes on adipocytes, then we will explore the possibility that miR-1 targets a different repressor of C/EBPα such as Sp1, Shox2, CKIP-1 or G9a. We did not initially pursue these other repressors because the change in their respective mRNA expression was not consistent with regulation by miR-1 (see Fig. 7); however, there is the possibility that miR-1 is regulating one of these repressors at the level of translation and not mRNA stability. Finally, if we do not confirm our working model proposing enhanced catecholamine sensitivity, we will begin to explore the possible influence of RE-induced exosomes on the browning of adipose tissue. While our microarray analysis showed a striking increase in β-adrenergic signaling and lipolytic gene expression, we did detect increased expression of genes known to be involved in browning (Prdm16, PGC-1α, UCP-1 and PPARγ), although not as robust. This finding is not surprising given that C/EBPα is known to be involved in regulating the conversion of white adipose tissue into brown adipose tissue , as well as many other signaling pathways [88]. Thus, we will pursue other mechanisms potentially regulated by C/EBPα if the results of proposed experiments do not support our working model.

Specific Aim 2: Determine if skeletal muscle fiber-derived exosomes promote adipocyte lipolysis through enhanced β-adrenergic signaling.

Rationale. Based on our miR-1 preliminary data we hypothesize that skeletal muscle-derived exosomes are responsible for promoting lipolysis in fat in response to RE; however, the lack of an accepted marker of skeletal muscle-specific exosomes prevents identification of the tissue source of serum exosomes. To address this limitation, and test our hypothesis, we have developed an in vitro model in which electrical stimulation of human primary myotubes promotes an increase in the abundance of exosomal miR-1 to a similar magnitude as that observed in vivo with synergist ablation (see Fig. 9). Using this model system, we will be able to specifically determine if skeletal muscle-derived exosomes are able to promote adipocyte lipolysis through miR-1 activation of β3-AR expression by targeting AP-2α expression. It will also enable detailed profiling of miRs in muscle-derived exosomes. An additional strength of this model system is that it will allow us to determine if what we have observed in mouse cells translates to human cells.

Design. Human primary myoblasts will be differentiated into myotubes as previously described by us [89,90]. Through the Center for Muscle Biology, directed by Dr. Peterson, primary myoblasts have been isolated, expanded and characterized from vastus lateralis biopsies obtained from more than 100 healthy volunteers (https://www.uky.edu/chs/muscle/bank). We will use 10 distinct primary myoblast lines derived from both male

(n=5) and female (n=5) donors. After 1 week of differentiation, myotubes will be electrically stimulated for 8 hr (12V, 1Hz, 2ms) using an Ionoptix C-Pace Electrical Pulse Stimulator. A total of four 6-well plates can be stimulated at a time. Cells will be harvested after an additional 16 hr with no stimulation for total RNA isolation to compare with cells cultured for that time period (24 hr) with no stimulation. One well of stimulated and non-stimulated myotubes will be fixed, visualized with myosin heavy chain immunocytochemistry and imaged for measurement of myotube diameter using automated image analysis to quantify the hypertrophic response (see Fig. 9). At the time of cell harvest, media will also be collected from both stimulated and non-stimulated myotubes for the isolation of exosomes as described in the SOP section.

Experiment 2.1. The purpose of this experiment is to confirm our preliminary results showing the down-regulation of miR-1 in myotubes in response to electrical stimulation and if there is a corresponding increase in muscle-specific exosomal miR-1 abundance. If this relationship is confirmed, we will next treat myotubes with GW4869 to block exosome release to determine if the down-regulation of myotube miR-1 expression with electrical stimulation is mediated by the exosomal export of miR-1 as shown in vivo (see Fig. 4). The expression/abundance of miR-1 will be determined by qPCR as described in the SOP section.

Experiment 2.2. Exosomes from non-stimulated and stimulated myotubes will be incubated with primary human adipocytes for 24 hr and then gene expression and catecholamine sensitivity measured as described in the SOP section. Adult-derived human adipocyte stem cells (ADHASC) were isolated previously from lipo-aspirates [91]. Dr. Kern's lab has expanded and frozen ADHASC from 6 different individuals available for these experiments. We will begin with one line which has been already shown to differentiate very reproducibly and determine the effect of exosomes derived from stimulated and non-stimulated myotubes on gene expression and catecholamine sensitivity as described in the SOP section.

Experiment 2.3. The purpose of this experiment will be to determine if muscle-specific exosomal miR-1 is necessary and sufficient to enhance adipocyte catecholamine sensitivity. Non-stimulated exosomes will be transfected with miR-1 mimic (Control-plus) while stimulated exosomes will be transfected with anti-miR-1 oligonucleotide (Stim-minus). These modified exosomes will then be incubated with primary human adipocytes for 24 hr and gene expression and catecholamine sensitivity measured as described in the SOP section.

Statistical Analysis. A two-factor ANOVA (treatment X sex) will be used to determine if a significant interaction exists between factors for each dependent variable under consideration. If a significant interaction is detected, Scheffé post-hoc comparisons will be performed to identify the source of significance with $p \leq .05$.

Expected Outcomes & Alternative Approaches. We do not anticipate any technical issues with the planned experiments as we have experience with all of the described techniques. We also do not expect there to be any sex difference in the response of myotubes to electrical stimulation in terms of miR-1 expression in both myotubes and exosomes as Nielsen and colleagues reported no difference in miR-1 expression between men and women [92]. Further, we think that blocking exosome release with GW4869 treatment will prevent the down-regulation of miR-1 as well as the increase in exosomal miR-1 abundance. Finally there is the expectation that muscle-derived exosomal miR-1 will effectively enhance the catecholamine sensitivity and lipolysis of adipocytes through the up-regulation of β3-AR signaling. Though somewhat minor, one concern we have is that myotube-derived exosomes will behave differently than exosomes isolated from serum; specifically, myotube-derived exosomes will not effectively deliver their cargo (i.e., miR-1) to adipocytes. To determine if this is the case, we will fluorescently label myotube-derived and serum-derived exosomes (see Fig. 2) to visualize their fusion kinetics with adipocytes. If we do find myotube-derived exosomes have limited fusion capability, we will switch to using primary myoblast cells derived from both male and female mice. We are confident these primary cell lines will produce functional exosomes as we have successfully used them to model how exosomes released from activated muscle stem cells are necessary for the proper remodeling of the extracellular matrix during muscle hypertrophy [24].

This aim also provides the opportunity to profile the miR content of muscle-specific exosomes. It is reasonable to expect that other muscle-specific miRs (myomiRs) are packaged into exosomes and delivered to other tissues. For example, muscle-specific miR-133a has been reported to regulate the expression of Prdm16 and brown adipogenesis [93].

Specific Aim 3: Determine if an acute bout of resistance exercise in humans promotes miR-1-mediated adipocyte lipolysis.

Rationale. Our preliminary data in mice suggest that RE enhances adipocyte catecholamine sensitivity through a novel mechanism involving the delivery of muscle-specific miR-1 to adipose via exosomes. The in vitro analyses in Aim 1 will explore this process mechanistically, with Aim 2 extending the analyses to primary human myotubes and adipocytes. The purpose of this aim is to determine if our working model translates to humans in vivo. Analyses of serum exosomes, muscle and adipose tissue isolated before and after a bout of RE will determine if miR-1 potentially mediates muscle-adipose crosstalk during exercise in humans. The ability of isolated exosomes to induce adipocyte lipolysis in vitro by enhancing catecholamine sensitivity through CEBPα activation of βAR-3 expression will be assessed.

Design. Relatively sedentary, generally healthy lean (BMI <25) and obese (BMI >30) men and women between 18-30 years of age without study contraindications will be recruited (see inclusion/exclusion criteria in Human Subjects section). Potential eligibility will be assessed by self-reported height and weight, and by reporting no participation in regular (>1 day per week) exercise for at least the past three months. All potential participants will arrive fasted to the Center for Clinical and Translational Sciences (CCTS). After giving written, informed consent, a detailed medical history form will be filled out to screen for eligibility. Measures of physical activity level will be collected before baseline blood, adipose, and muscle samples are taken. We intend to recruit an equal number of lean and obese participants (n=40 in each group, 20 men and 20 women). Power analysis based on the change we observed in miR-1 abundance in exosomes following a bout of knee extensions in a pilot study of 10 individuals, indicated that n=36 is required in each group. We are requesting n=40 in both the lean and obese groups based on an expected difference in means between 1 and 1.95 and an expected standard deviation of 0.45, with the power set at 0.8 and alpha at 0.05. This will accommodate 10% drop out prior to completing all biopsies.

Following a brief rest period, subjects will perform two lower body RE tasks to failure (knee extension and leg press) with blood samples drawn pre- and immediately, 30, 60 and 90 minutes post-exercise. Biopsies of the vastus lateralis and abdominal subcutaneous adipose tissue will be obtained pre- and post-exercise (at approximately 60 and 90 min post, respectively; see graphic abstract in the Human Subjects section).

Experiment 3.1. Exosomal, muscle, and adipose miR-1 abundance will be quantified, as well as miR-1A/B primary transcripts in muscle and adipose by qPCR as described in the SOP section.

Experiment 3.2. Isolated exosomes from the 30 and 60 minute time points will be applied to differentiated ADHASC for 24 hr, and lipolysis in vitro and the βAR-3 pathway gene expression assessed as described in the SOP section. Pre-RE exosomes will be transfected with miR-1 mimic (Pre-plus) and 30 minute post-RE exosomes with anti-miR-1 oligonucleotide (Post-minus) and compared to mock transfected exosomes from those time points. These modified exosomes will be incubated with differentiated adipocytes to parallel Aim 1 mouse Experiments 1.1 and 1.2.

Methods.

Resistance exercise (RE). Following determination of each subject's one repetition max (1-RM, see Human Subjects section for detailed description), subjects will perform two exercises, knee extension and leg press at 80% of 1-RM, designed to work the quadriceps. Subjects will perform three sets of eight repetitions, with a 90-120 second rest between sets, with a fourth set performed to failure. RE will be performed on pneumatic resistance devices (Keiser Sports Health Equipment, Fresno, CA).

Muscle biopsy. A muscle biopsy will be obtained from the vastus lateralis at baseline and approximately 1 hr post-exercise under local anesthetic (1% Xylocaine HCl, 3cc). A ¼ inch wide incision will be made and a sterile 5mm Berkstrom biopsy needle (Pelomi Industries, Denmark) inserted with suction to obtain a 100-200 mg muscle sample. Dr. Kern, study physician and co-investigator on this project, has extensive experience with this procedure. It is well-tolerated and volunteers routinely provide multiple samples [83,89,94-96]. Muscle is snap frozen in liquid nitrogen and stored at -80^{o}C until RNA isolation.

Adipose Biopsy. An abdominal subcutaneous fat biopsy sample will be obtained from the lower abdominal wall prior to and approximately 90 min post exercise. The adipose biopsy procedure is similar to the thigh muscle biopsy procedure, except that 1-2 g of adipose tissue is routinely obtained [42,79,94,97,98].

Human serum analyses. Glucose and insulin will be measured in the University of Kentucky CCTS Biochemical Analysis Laboratory [99,100] from baseline serum collected from fasting lean and obese participants

to calculate HOMA-IR (Glucose*Insulin/405) as a measure of muscle insulin sensitivity. NEFA and free glycerol will be measured [81] both at baseline and in serum collected at multiple time points following the resistance exercise bout, and used to calculate a simplified "anti-lipolytic index" (NEFA*Insulin), which indicates adipocyte insulin sensitivity based on insulin's ability to suppress lipolysis [101]. In humans, fasting serum norepinephrine is positively associated with isoproterenol ED_{50} in isolated adipocytes. Thus, elevated fasting catecholamines may be indicative of decreased adipose tissue catecholamine sensitivity [102]. We will also measure serum epinephrine and norepinephrine by HPLC (Vanderbilt University Hormone & Analytical Services Core) [103].

Statistical Analysis. The College of Health Sciences supports the effort of a biostatistician through the Office of Research to provide dedicated support to funded investigators. A one-way repeated measures ANCOVA adjusting by sex will be used to determine if a significant difference exists between the groups in response to RE for miR-1 and miR-1A/B primary transcripts. If a significant difference is detected, LSD *post-hoc* comparisons will be performed to identify the source of significance with $p \leq .05$. The Pearson correlation coefficient will be used to determine whether muscle, serum, and adipose miR-1 and miR-1A/B primary transcripts are correlated to tissue insulin sensitivity and fasting catecholamines at baseline. All statistical analyses will be performed using SAS© 9.4 (SAS institute, Cary NC).

Expected outcomes and alternative approaches. We expect that the bout of lower extremity RE will result in decreased miR-1 abundance in muscle at 60 minutes, a rapid and transient increase in exosomal miR-1, and a subsequent increase in adipose miR-1 abundance at 90 minutes. We do not anticipate changes in miR-1 primary transcript abundance in adipose tissue which would support our hypothesis that miR-1 is delivered via exosomes. miR-1 is highly expressed in muscle and we acknowledge that a single bout of RE may not significantly decrease muscle miR-1 expression, even if it is packaged and released in exosomes. Moreover, 60 minutes post-exercise may not be the appropriate time point to detect this change. Our pilot time course blood sampling study following RE suggested that miR-1 in serum exosomes is highest at 30 minutes and is beginning to idecrease by 1 hr post exercise. Thus, we expect delivery to adipose should be detectable at 90 minutes. We acknowledge that this time point may not enable us to capture any changes in downstream targets of miR-1, but will provide proof-of-principle for a large scale clinical exercise trial that would include additional post-exercise time points following an acute bout of RE, as well as a training component.

We expect that lean individuals will be more insulin sensitive than obese individuals, and that obese participants will display higher fasting NEFA in the context of higher fasting insulin (due to decreased adipose tissue insulin sensitivity). Including both lean and obese individuals will determine if adiposity affects the miR-1 acute response to RE. It is possible that release of miR-1 in exosomes from muscle will be lost or blunted in the obese cohort. However, we hypothesize that insulin sensitivity (assessed by HOMA-IR and the anti-lipolytic index) will be positively correlated to the serum miR-1 excursion following the RE bout in both populations. NEFA and glycerol will be measured both pre- and post-RE to determine whether changes in NEFA and glycerol from baseline are correlated with change in miR-1 in serum and/or adipose from baseline. We will also determine if the induction of adipocyte lipolysis in vitro is different in exosomes isolated from lean compared to obese participants following RE. Further, the in vitro studies will provide the opportunity to explore additional aspects of adipocyte metabolism that may be regulated by miRs, which will be informed by results of Aims 1 and 2. Overall, we expect results from this study to inform the design of a resistance exercise training trial in obese individuals. Moreover, the proposed studies are expected to provide the fundamental knowledge necessary to determine if exosomal miR-1 delivery to adipose tissue has the potential to form the basis for the development of a novel therapeutic strategy to treat obesity, particularly in individuals physically unable to exercise.

Table 1 provides our projected timeline for the proposed experiments. As discussed above, the preliminary data presented demonstrate that we have the necessary expertise to perform the proposed experiments so we do not anticipate any major delays; however, the experiments have been laid out to accommodate unforeseen delays such that the proposed experiments and analyses will be completed by the end of Year 5.

Table 1. Timetable for proposed experiments.