ELECTROSPUN PLGA MICROFIBERS FOR LOCALIZED DELIVERY OF SMALL MOLECULES TO INDUCE BROWN ADIPOGENESIS

By

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ABSTRACT

Obesity and its related disorders have been on the rise in the last few decades. Current prescribed treatments range from diet and physical activity for low risk patients to pharmacological intervention and surgical procedures for higher risk patients. In the past few years, with the discovery of its presence in adult humans, brown adipose tissue has emerged as an interesting tool for reducing the risk of developing obesity or improving weight reduction in overweight or obese individuals. This study aims to create a system to deliver drugs specifically to white fat pads to increase brown fat differentiation, thus increase overall energy expenditure and improve energy metabolism in obesity and other metabolic disorders. The designed model system encapsulates rosiglitazone, a small molecule anti-diabetic drug, within electrospun poly(D,L-lactide-co-glycolide) (PLGA) microfibers. Drug release is sustained over a period of 35 days from microfibers with 0.5% w/w rosiglitazone loaded. In vitro experiments with the microfibers in a Transwell system demonstrate that 0.5% rosiglitazone was an optimal concentration for stimulating brown adipocyte differentiation. Furthermore, the rosiglitazone released from the microfibers was able to stimulate differentiation of white adipose-derived stem cells towards a brown adipocyte lineage. These results provide promising steps towards developing a therapy to create a localized depot of brown fat in situ that can be easily administered in the clinic to supplement current obesity treatment and prevention techniques.

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1. Introduction

Obesity is a disease with widespread prevalence in the United States and worldwide, with 73% of men and 64% of women in the United States are categorized as overweight or obese. If trends continue with these high percentages, total healthcare costs attributable to obesity could reach $861 to $957 billion by 2030, which would account for 16-18% of US health expenditures. This disease is staggering because of the excess morbidity associated with obesity in terms of risk factor development and incidence of type II diabetes, cardiovascular disease end points, cancer, end-stage renal disease, joint disease, and many other diseases. [1]

Adipose tissue is central in the understanding of the metabolic imbalance associated with the development of obesity. Traditionally, white adipose tissue (WAT) is thought of as an energy reservoir for the body by storing triglycerides and releasing fatty acids when needed. Now, it is known that white adipose tissue has extremely important functions as an endocrine organ that regulates the metabolism of other fat cells and cells located in the brain, liver, muscle or pancreas. [2,3] Obesity, a state of energy imbalance associated with overnutrition, impairs systemic metabolic homeostasis and elicits stress. Simultaneously, there is an inflammatory process in WAT, which increases levels of proinflammatory cytokines, thus increasing macrophage presence in WAT. Also in response to obesity-related stress, components of the autonomic nervous system are activated to increase levels of glucocorticoid, thus furthering the increase of WAT mass and perpetuating the vicious cycle of obesity.[4,5] Although the exact cause of the development of obesity is not certain, there is a clear link between an increase in visceral fat and obesity and obesity-related diseases. Also evident is the fact that obesity is a multi-faceted disease, and there are many potential targets for treatment of the associated symptoms or prevention of disease

progression. Our strategy will focus on targeting a different type of adipose tissue, brown adipose tissue (BAT), whose function in thermogenesis burns off energy as heat, instead of storing it as WAT does.

This thesis describes the design and initial evaluation of a delivery system to release small molecule drugs directly into the fat pad to target white adipose-derived stem cells. The intended outcome would be the development of a localized brown fat depot within WAT that would work to offset the energy imbalance created in obesity. Chapter 2 reviews the current literature on brown adipose tissue and current targets and strategies for utilizing the powerful tissue. In Chapter 3, we evaluate the in vitro aspects of rosiglitazone-embedded PLGA microfibers, including macroscopic features, release kinetics, and the effect on differentiation for both white and brown adipose-derived stem cells (ASCs). Finally, implanting the microfibers and microparticles into an animal model illustrates the capability of the delivery system in vivo.

2. Brown Adipose Tissue

2.1 Introduction

Adipose tissue is comprised of both white adipose tissue and brown adipose tissue (BAT). These two types of tissue differ greatly in both structure and function. White adipocytes, the major cell type in white fat, contain one large lipid droplet, which takes up the majority of the space within the cell. The primary function of white fat is to store energy. Brown adipocytes, present in BAT, contain multiple lipid droplets and most notably, a high number of mitochondria, giving the tissue a brown color. Brown fat holds an important role in thermogenesis. A protein unique to brown adipocytes, uncoupling protein 1 (UCP1), uncouples oxidative phosphorylation, so heat is produced instead of ATP.[6] The two tissue types serve two opposing functions, while white fat stores energy, brown fat dissipates energy and acts as a metabolic sink.

Until recent years, it was thought that in humans, BAT was biologically irrelevant in adult humans and only utilized in newborns for thermogenesis. However, through the use of positron emission tomography and radiotracers that measure the metabolic activity of tissues, major depots of active BAT were found in the cervical-supraclavicular region of adult humans.[7] Also interestingly, the amount of BAT present is correlated with age and health, as younger and leaner individuals tend to have more brown fat. Because of its function of burning energy for thermogenesis, brown adipose tissue is now thought to play an important role in offsetting the energy imbalance that occurs in obesity. Brown fat is also shown to affect glucose homeostasis and insulin sensitivity, which are both crucial factors in diabetes and other metabolic diseases.[4,8]

With recent advances in the field, brown fat is now an extremely attractive tool to combat diseases such as obesity and diabetes through its function as a metabolic sink.

2.2 Potential targets for inducing brown adipose tissue

To better utilize brown fat as a weapon against obesity and other metabolic imbalances, the origin and control of BAT development must be better understood. BAT is differentiated from multipotent mesenchymal stem cells, such as the adult stems found in adipose tissue, adipose-derived stem cells (ASCs). Interestingly, there are two different types of brown adipocytes with distinct developmental lineages. The "classical" brown adipocytes from interscapular depots originate from myoblastic-like Myf5+ precursors. The "inducible" or "beige" brown adipocytes found scattered in white adipose tissue posses many of the same characteristics of classical brown adipocytes but come from a non-Myf5 lineage.[9]

2.2.1 Transcription factors

In order to start the differentiation process, insulin or IGF-1 stimulates a transcriptional cascade. Then PRDM16 is a key transcription co-factor that controls the development of classical brown fat. [10] This interacts with transcription factors PPARg and C/EPBb and their coactivators PGC-1a/b.

2.2.2 Proteins and Small Molecules

Some members of the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families have been identified to trigger brown adipocyte development. In particular, BMP-7 is a powerful inducer of BAT and increases mitochondrial biogenesis and expression of UCP1.[11]

Several small molecules have been shown to promote brown adipose tissue growth and differentiation. Beta-adrenergic agonists have been shown to stimulate development of "inducible" brown adipocytes in white fat deports. Examples of such drugs are norepinephrine and isoproterenol. Rosiglitazone is an antidiabetic drug that binds to the PPAR receptors in fat

cells and makes cells more responsive to insulin, as well as inducing the white-to-brown conversion through PRDM16.[9] Lithocholic acid (LCA) is a bile acid that is a potent agonist of TGR5. This molecule has many functions including stimulating the conversion of T4 into active T3, which is known to induce BAT formation, and increases uncoupling in BAT, thus increasing energy expenditure.[12] However, these, along with other promising drug candidates have shown to cause multiple life-threatening adverse effects if introduced systemically to other parts of the body.[13,14]

2.3 Current strategies

Currently, there are two approaches being employed for stimulating BAT volume and activity. The first strategy considers in vivo pharmacological approaches using small molecules and/or growth factors to stimulate BAT. The second strategy entails an ex-vivo cell-based approach, differentiating progenitor cells in vitro and then implanting them into patients seeking weight loss. [13] The in vivo development of BAT also provides a method for in vitro culture where small molecules can be tested and evaluated for their ability to stimulate BAT activity. Several small molecules, such as L-796569 or CL-316243, have been tested to increase overall body energy expenditure. While these beta-3 agonists have been shown to increase lipolysis and energy expenditure, the effect is lost over 28 days of treatment. This may be due to the fact that these agonists have poor oral bioavailability or unfavorable pharmacokinetics.[5] Additionally, it has been shown that systemically increasing the amount of brown fat in the body can actually be harmful, as a study found that cold activation of mice with increased brown fat were at higher risk of heart disease due to increased plaque in blood vessels.[15] These studies together indicate that orally introducing a small molecule or protein to increase or activate brown fat may not be the safest therapy. The toxicity challenge of these small molecules is the impetus for the present studies investigating local delivery.

Ex vivo strategies are currently dependent on the understanding of the development of brown fat, both classical and inducible. The envisioned therapy would be isolating the adult stem cells present in white fat after a liposuction procedure, then inducing brown adipocyte differentiation, then transplanting those same cells back into the original host for in-vivo growth. The intention would be to create a localized depot of brown fat, thus avoiding the systemic complications of using small molecules. However, the ex vivo approach requires manipulation outside of the body, which introduces complications that can affect the sterility of the cells. Therefore, in vivo strategies are considered more promising due to their ease of application and minimized external manipulation.

In this thesis, I will explore an in vivo approach to increase BAT mass and activity in adult humans. I have developed a synthetic delivery system to locally induce brown fat to avoid the harmful effects of the small molecules on other parts of the body. The delivery system can be used in conjunction with a liposuction procedure, as the separated cells can be re-introduced along with the delivery system to supplement tissue development.

3. PLGA Microfiber Implant to Induce Browning

3.1 Introduction

As obesity and type II diabetes have become such staggering problems, the focus must be turned to treatment options for these multi-faceted diseases. The first line of treatment prescribed is diet and exercise. However, recent studies have shown that the biggest predictor of weight loss is adherence to a certain diet, and on average, patient adherence wanes after 3-6 months. Furthermore, moderate-intensity physical activity for 60 minutes most days of the week can prevent or mitigate weight regain, but both diet and exercise are dependent on the patient's own dedication.[16] The next line of treatment is drug treatment, which would be prescribed in conjunction with diet and exercise for higher risk patients. Recent weight loss medications include Lorcaserin (Belviq), which is a selective serotonergic 2 receptor agonist meant to decrease food intake, and Bupropion SR/naltrexone SR (Contrave), which is a dopamine and norepinephrine reuptake inhibitor and opioid receptor antagonist intended to reduce energy intake, increase energy expenditure, and block compensating mechanisms that would otherwise prevent long-term weight loss. While these drugs sound promising, there are several safety concerns including cardiovascular risk from increased heart rate and blood pressure.[17,18] The most severe cases of obesity must resort to surgical treatments including the LAP-BAND and gastric bypass. Other surgeries, such as liposuction, simply remove the excess fat, but do not change the body's ability to gain or lose weight. [19,20]

Brown adipose tissue can be a powerful tool used in conjunction with these treatments to combat obesity because of its increased and essentially inefficient energy expenditure properties. The uncoupled oxidative phosphorylation that allows for thermogenesis effectively creates a

metabolic sink. BAT has an enormous potential in this capacity because it is estimated that 40-50 grams of stimulated BAT could utilize up to 20% of basal caloric needs. [21,22] Therefore, I aim to create a delivery system for the fat pad to locally stimulate adipose progenitor cells to differentiate towards a brown adipocyte lineage. As a model system, I will use the drug, rosiglitazone, but the rosiglitazone can easily be substituted with other small molecule drugs.

First I will explore the effect of the rosiglitazone released from the PLGA microfibers on cells in in vitro culture. Next I will evaluate the possibility of using the microfibers as a cell delivery system as well. Therapeutically, the cells would be isolated from a patient's lipoaspirate, then spun down onto the microfibers, then placed into the fat pad. This would supplement the number of cells that would be affected by the drugs in the microfibers. Finally, I will apply the system to an animal model, both as a subcutaneous implantation and implantation directly into a fat pad. In this in vivo model, I will also test an alternative formulation of microparticles containing rosiglitazone. The microparticles are injectable, making it more clinically easy to administer, and there is a faster release rate due to the higher surface to volume ratio.[23] Similar approaches have been taken to promote differentiation of adipose-derived stem cells for applications in soft tissue reconstruction.[24,25] My goal is to apply this to brown fat differentiation and create a system to deliver drugs specifically to fat pads to increase brown fat differentiation (**Fig 3.1**), thus increasing overall energy expenditure and improve energy metabolism in obesity and diabetes.

3.1.1 Electrospinning

Electrospinning is a widely used process that produces ultra-fine fibers from a polymer solution. This is achieved by ejecting the polymer solution from a syringe at a controlled speed, using a syringe pump, into a high-voltage electrostatic field. The electrically charged jet of polymer solution is shot towards a collecting screen, and the solution jet evaporates, thus forming a web of fibers.[26] The benefit of electrospinning is that it creates a microstructure of fibers, which

is morphologically similar to the extracellular matrix (ECM) of natural tissue. This means that such a structure would be beneficial for cell attachment and proliferation.[27] This technique is also beneficial for drug delivery applications because the encapsulation of the drugs allows for sustained delivery so therapeutic concentrations of the drug can be delivered over longer periods of time compared to a bolus injection. Also, the thin fiber geometry means a higher surface to volume ratio, allowing for the drug to be released in a shorter time frame.[26]

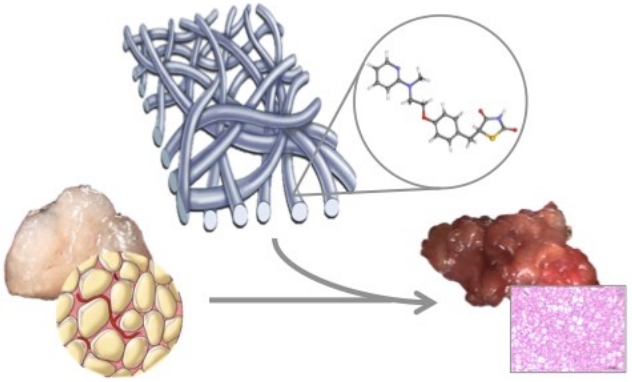

Figure 3.1 Schematic of the rosiglitazone-embedded microfiber delivery system. Rosiglitazone loaded into the microfibers will aid in the conversion of progenitor cells in white adipose tissue to brown adipose tissue .

3.2 Materials and Methods

Materials were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

3.2.1 Electrospun fibers

Electrospun fibers were fabricated with a 30 w/v% solution of poly(D,L-lactide-*co*-glycolide) (50:50, PLGA) dissolved in dichloromethane (DCM). Rosiglitazone was embedded in the fibers by dissolving them in the same solution at specific w/w percentages with the PLGA. The solution was loaded into a 3 mL glass syringe fitted with an 18-gauge needle with a 90-

degree angle and fixed onto the syringe pump (KdScientific). The polymer was ejected at a rate of 0.1 mL/min into an electric field of 14-15 kV, produced by a high voltage power supply (Gamma High Voltage Research). Collected fibers were lyophilized for 4 days, then stored at -20°C until further use.

3.2.2 Material characterization

Drug release profile

A 12 mm (diameter) biopsy punch (Acupunch) was used to cut pieces of 0%, 0.05%, 0.1%, 0.2%, 0.5%, 1% w/w of rosiglitazone (Cayman Chemical) in the PLGA nanofibers. Each piece was placed in a 24-well plate (BD) with 1 mL of phosphate-buffered saline pH 7.4 (PBS) (Gibco). At every timepoint, the PBS was removed and collected and a fresh 1 mL was pipetted into the well. Timepoints were taken at days 1, 2, 3, 4, 7, 9, 11, 14, 16, 18, 21, 35, and 49. The rosiglitazone was extracted from the PBS by adding 10 mL of ethyl acetate. After vigorous agitation, the aqueous and organic layers were separated by centrifugation. The aqueous layer was removed and the organic phase was dried under vacuum. The rosiglitazone was then resuspended in 250 µL of methanol. The concentration of rosiglitazone was determined by high performance liquid chromatography (HPLC) (Autosampler 2707 Waters Corporation) with a C18 column (4.6x250 mm, 5 um, Agilent Technologies). The mobile phase consisted of 75% acetonitrile and 25% 10 mM ammonium acetate, pH 5.5. The flow rate was 0.8 mL/min and the retention time was 5.1 minutes.

Scanning electron microscopy (SEM)

Dried samples were mounted on stubs using a conductive carbon adhesive. Samples were sputter-coated in platinum then imaged using a Leo 1550 Field Emission Scanning Electron Microscope (Leo/Zeiss).

3.2.3 Adipose-derived stem cell isolation

Subcutaneous white fat and interscapular brown fat were dissected out of euthanized Sprague-Dawley female rats. For each of these tissue types, adipose-derived stem cells (ASCs) were isolated by digestion with 1 mg/mL collagenase I (Worthington) in DMEM-F12 (Gibco) for 1.5 hours on an orbital shaker at 37°C. The resulting cell suspension was centrifuged and filtered through 70 μm and 40 μm cell strainers. ASCs were seeded at 5,000 cells/cm^2 and cultured in DMEM-F12 supplemented with 10% fetal bovine serum (Thermo Scientific HyClone) and 1% penicillin streptomycin (Life Technologies). Cells were used between passages 4-6 for all studies.

3.2.4 In vitro cell studies

2D adipogenesis

White and brown ASCs were seeded separately in 6-well plates at 120,000 cells/well. White adipogenic differentiation was stimulated using a white adipogenic differentiation media containing 1 μM dexamethasone, 200 μM indomethacin, 500 μM methylisobutylxanthine, 10 μg/ml insulin, 1% penicillin/streptomycin, and 10% FBS in high glucose (4.5 g/L) DMEM. Brown adipogenic differentiation was stimulated using a brown adipogenic differentiation media containing dexamenthasone, indomethacin, methylisobutylxanthine (IBMX), insulin, rosiglitazone, and triiodothyronine (T3). White ASCs were differentiated using the white adipogenic media ("White"); white ASCs were differentiated using the brown adipogenic media ("White → Brown"); and brown ASCs were differentiated using the brown adipogenic media ("Brown"). Samples were collected at day 0, 7, 14, 21, and 28 for RT-PCR analysis, and histological analysis was performed at day 28.

2D differentiation study with microfibers

To evaluate the effect of the fibers on cell differentiation, brown ASCs were seeded on a 24-well plate at 30,000 cells/well, and four 6 mm diameter pieces of 0%, 0.05%, 0.1%, 0.2%,

0.5%, 1% w/w of rosiglitazone in the PLGA nanofibers were placed in the top chamber of a 6.5mm 3.0 μm pore size polystyrene Transwell (Corning). Cells were cultured in brown adipogenic media without either rosiglitazone or T3 (add media composition). Media was changed every 2-3 days for 3 weeks.

3D cell seeding on microfibers

One million cells were seeded directly onto the fibers to see if the fibers could be used as a cellular delivery vehicle as well. Each of the cell types (rat white ASCs, rat brown ASCs, and human white ASCs) was tested. Brown adipogenic media without either rosiglitazone or T3 was used. Media was changed every 2-3 days for 3 weeks.

Histological analysis

For 2D cultures, cells were fixed in 10% formalin for 30 minutes. Rabbit anti-UCP1 antibody (Abcam) was used to probe for the presence of UCP1, the key defining protein for brown differentiation. This was followed by incubation with FITC conjugated goat anti-rabbit IgG (H+L) secondary antibody (Life Technologies) for visualization. The nuclei were stained with a Hoescht stain. To look at adipogenesis, the cells were stained with Oil Red O, which stains lipids a dark red.

For 3D cultures, harvested constructs were fixed in 10% formalin overnight then dehydrated using graded ethanol solutions (70%, 80%, 95%, 100%) then finally with xylene. The samples were then infiltrated with paraffin at 60°C overnight, and then embedded and mounted into paraffin blocks. The paraffin block was sectioned using a microtome into 5 μm sections and mounted onto microscope slides. The slides were dried in a 40°C oven overnight. For immunohistochemistry, the slides were de-paraffinized and rehydrated in xylene, then graded ethanol solutions. Antigen retrieval was performed by immersing the slides in a citrate buffer and heating them in a vegetable steamer for 30 minutes. The slides were then blocked with 1% bovine serum albumin in phosphate-buffered saline with 1% Tween-20 for 1 hour. The primary antibody

was incubated with the slides overnight at 4°C. To probe for UCP1, a rabbit anti-UCP1 antibody (ab 10983, Abcam) was used. Following the primary incubation, endogenous peroxidase quenching is performed using a methanol and hydrogen peroxide solution. The primary antibody was dectected using an HRP polymer conjugated secondary antibody from the SuperPicture Polymer Detection Kit DAB, Rabbit (Life Technologies). The slides were then dehydrated, cleared and cover-slipped with Permount for imaging.

For Oil Red O staining, the harvested constructs were fixed in 10% formalin overnight. The following day, the constructs were infiltrated with 20% sucrose and then OCT (Tissue-Tek), then finally embedded in OCT. The blocks were cryosectioned into 10 μm sections and mounted onto microscope slides. Oil Red O staining was performed on the slides, which were then cover-slipped using glycerin jelly.

RT-PCR

Samples for determine gene expression levels were prepared with Trizol Reagent (Invitrogen). RNA extraction was carried out according to the manufacturer information with the addition of 1 μg of glycogen to each sample to aid in RNA precipitation. RNA was resuspended in DEPC water and quantified using a Nanodrop (Thermo Scientific). cDNA was synthesized using the SuperScript RT III kit (Invitrogen) according to manufacturer information. RT-PCR was carried out with Power SYBR Green (Applied Biosystems) on a StepOne Plus system (Applied Biosystems). Relative quantitation was done using the ΔΔCt method with beta actin as the housekeeping gene. Primer sequences used are listed in Table 4.1 (rat) and 4.2 (human).

Table 3.1 RT-PCR Primer sequences for rat

Gene	Forward Sequence 5' – 3'	Reverse Sequence 5' – 3'
Adiponectin	TGGTCCCTCCACCCAAGGAAA	ACACCTGCGTCTCCCTTCTCT
PPARG	TCAAAAGCCTGCGGAAGCCC	TGGGCGGTCTCCACTGAGAATAA
FABP4	GCCTTTGTGGGGACCTGGAAA	GCGAAGCCAACTCCCACTTCTT
UCP1	TGGTGAGTTCGACAACTTCC	GTGGGCTGCCCAATGAATAC

cidea	TGGAAAAGGGACAGAAATGG	TCTTCTGTGTCACCCAGTGC
PGC1a	TATGGAGTGACATAGAGTGAGCT	CCACTTCAATCCACCCAGAAAG
PRDM16	TAGAAAAGCGGAAGGTGGCG	CTGACATCTGGGGGTGCAAC
BActin	CGGTCAGGTCATCACTATCGGCA	GCCACAGGATTCCATACCCAGGA

Table 3.2 RT-PCR Primer sequences for human

Gene	Forward Sequence 5' – 3'	Reverse Sequence 5' – 3'
Adiponectin	CTG TTG CTG GGA GCT GTT CT	CCCTTAGGACCAATAAGACCTGG
PPARG	AGGAGAAGCTGTTGGCGGAGA	TGCTTTGGTCAGCGGGAAGG
FABP4	ACAGGAAAGTCAAGAGCACCATAACC	TGACGCATTCCACCACCAGTTT
UCP1	TCCAGGTCCAAGGTGAATGC	ACTAGGTGCTGTTTCTTTCCCT
cidea	TAAGCGAGTCCTGTTCACCC	GCATCCAGAGTCTTGCTGATG
PGC1a	CCTGCATGAGTGTGTGCTCT	CAGCACACTCGATGTCACTCC
PRDM16	ACCCCATCTACAGCAGGGTA	TACCTGCACGTGTATCGCTC
BActin	GGCACCCAGCACAATGAA	GCTAACAGTCCGCCTAGAAGC

3.2.5 In vivo browning study

All animal studies were conducted with approval by the Johns Hopkins Animal Care and Use Committee.

PLGA microparticles

PLGA microparticles were created by dissolving 1 mg rosiglitazone and 200 mg PLGA in 2 mL dichloromethane to create 0.5% w/w rosiglitazone loaded PLGA microparticles. This solution was poured drop-wise into 50 mL of 1% polyvinyl alcohol, and an emulsion was formed by stirring the solution at 4000 RPM. After 15 minutes, this solution was poured into 100 mL of 0.5% polyvinyl alcohol. This solution was stirred at 4000 RPM for 2 hours. The solution was then centrifuged to isolate the resulting microparticles. The microparticles were washed with deionized water three times, and then lyophilized for 4 days. Before surgery, the microparticles were resuspended in PBS at a concentration of 25 mg/mL.

Animal surgery

Retired breeder Sprague-Dawley rats (approximately 9-10 months old) were ordered from Charles River Laboratories. Twelve mm diameter rosiglitazone-embedded PLGA microfibers (0.5% RSG) seeded with brown ASCs and rosiglitazone-embedded PLGA microfibers (0.5% RSG) seeded with white ASCs were implanted subcutaneously in the upper back of the rats. Rosiglitazone-embedded PLGA microfibers (0.5%) without cells were implanted into the dorsolumbar fat pads on either side of the rat. A small incision was made in each fat pad, and the microfiber was implanted within. Empty PLGA microfibers were used as a negative control. Rosiglitazone-embedded PLGA microparticles (0.5% RSG) were loaded into syringes at 20 mg/mL with PBS. 200 μL of each solution (4 mg of microparticles) were injected into the dorsolumbar fat pad using an 18-gauge needle. Wounds were closed using 4-0 nylon sutures (Medline). One of the rats with the 0.5% RSG implants was sacrificed at 1 week post-implantation to evaluate the initial response to the implants. The remaining rats were sacrificed at 3 weeks post-implantation.

Histological analysis

All specimens were fixed in 10% formalin overnight. Specimens for paraffin embedding were fixed with 10% formalin, dehydrated through graded ethanol solutions, cleared in xylene, and then embedded in paraffin. Samples were sectioned at 5 μm thickness. Hematoxylin and eosin staining was used to examine the cellularity and tissue around each implant. Immunostaining for UCP1 was performed with rabbit anti-UCP1 antibody (Abcam) with HRP conjugated secondary antibody, as described previously. For Oil red O staining of lipids, specimens were fixed in 10% formalin and infiltrated with graded sucrose solutions, and then embedded in OCT. Samples were cryosectioned at 10 μm thickness to see new adipose tissue formation.

3.3 Results

3.3.1 Characterization of rosiglitazone-embedded PLGA microfibers

Rosiglitazone-embedded PLGA microfibers were created using electrospinning techniques. Scanning electron microscopy revealed that the microfibers had an even size distribution with random alignment of the fibers (**Fig 3.2**). Rosiglitazone-embedded PLGA microparticles were created using a double emulsion technique in polyvinyl alcohol. The SEM image shows that the particles have a varied size distribution from 5 – 100 µm diameter. The release profile of the microfibers was analyzed using the 0.5% rosiglitazone-embedded PLGA microfibers. Approximately 30% of the rosiglitazone is released in the first 7 days. The release slightly plateaus until day 16, where the release begins to increase. This is likely due to the start of the degradation of the PLGA microfiber.

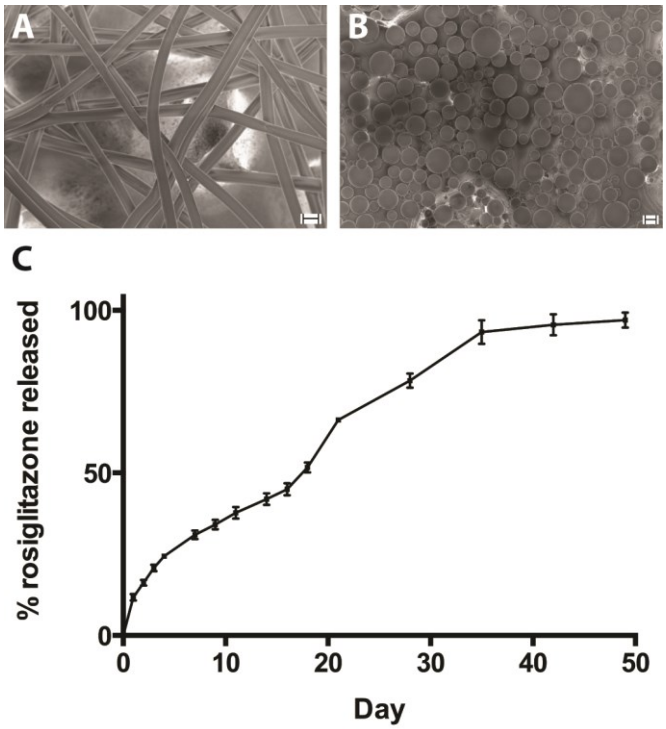

Figure 3.2 Characterization of rosiglitazone-embedded microfibers and microparticles. SEM image of the microfibers (A) showed an even size distribution of the diameter of the fibers around 30 µm. SEM image of the microparticles (B) show a varied size distribution from 5-100 µm. Scale bar is 20 µm. The release profile of the microfibers (C) shows that rosiglitazone is being released over a period of 28 days.

3.3.2 2D adipogenesis of white and brown ASCs

After 28 days of differentiation in white adipogenic media, the white ASCs in white adipogenic media showed clear adipogenesis from the presence of lipid droplets in the Oil Red O staining, and the immunostaining shows the presence of PPARG, a protein that regulates fatty acid storage and glucose metabolism. The white ASCs differentiated in brown adipogenic media (containing rosiglitazone and triiodothyronine) have distinct lipid droplet staining, as well as the presence of both PPARG and UCP1 (**Fig 3.3**). This showed that the white ASCs had the capacity to differentiate towards a brown-like lineage, because UCP1 is one of the key defining proteins of brown adipocytes. UCP1 allows for the thermogenic mechanism in brown adipocytes by disrupting the proton gradient formed in oxidative phosphorylation. The brown ASCs differentiated in brown media have a clear brown phenotype because of the presence of lipid droplets, PPARG, and most importantly, UCP1.

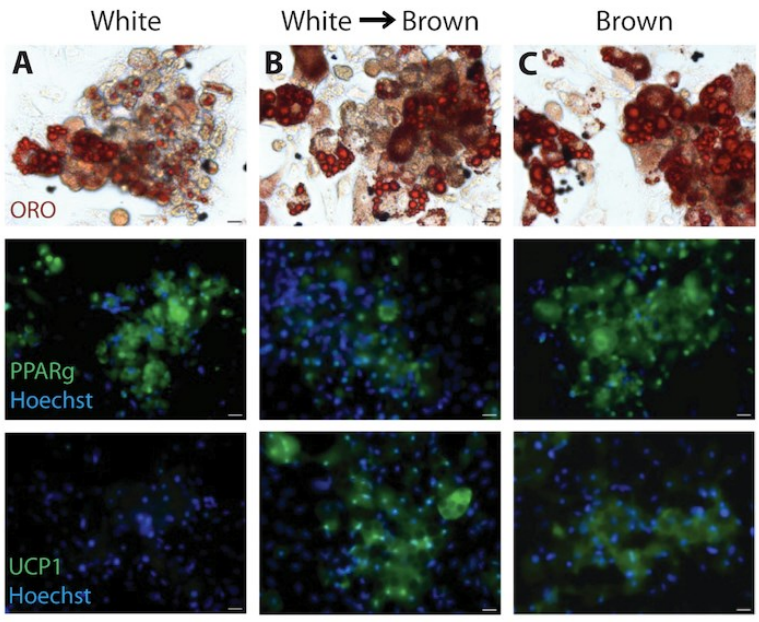

Figure 3.3 Adipogenesis of white and brown ASCs. White ASCs differentiated in white adipogenic media (A) show lipid formation and PPARG expression, but very little UCP1 expression. White ASCs differentiated in brown adipogenic media (B) show lipid formation, PPARG and UCP1 expression, very similar to the brown ASCs differentiated in brown adipogenic media (C). Scale bar is 20 µm.

The gene expression of the cell populations was measured over time using RT-PCR. Adiponectin is secreted exclusively from adipose tissue, and modulates glucose regulation and fatty acid oxidation. PPARG, peroxisome proliferator-activated receptor gamma, regulates fatty acid storage and glucose metabolism, and genes activated by PPARG stimulate lipid uptake and adipogenesis. FABP4, fatty acid binding protein 4, is a carrier protein for fatty acids. UCP1, uncoupling protein 1, disrupts the proton gradient formed from oxidative phosphorylation to allow for the thermogenic properties of brown fat. Cidea, cell death-inducing DFFA-like effector a, is found predominantly in brown adipose tissue and is thought to regulate UCP1. [9,15,28,29]

Each of the cell populations showed a dramatic increase in expression of adiponectin, PPARG and FABP4, which is consistent with adipogenesis (**Fig 3.4**). The key differences between the three populations were in the expression of brown fat genes. The white ASCs differentiated in white media showed a slight increase in both UCP1 and cidea over time when compared to white ASCs. However, both the white → brown and brown cell populations showed great increases in both UCP1 and cidea expression. The white ASCs differentiated in brown media showed a large initial spike in UCP1 and cidea expression, while the brown ASCs differentiated in brown media had a gradual increase over the course of differentiation.

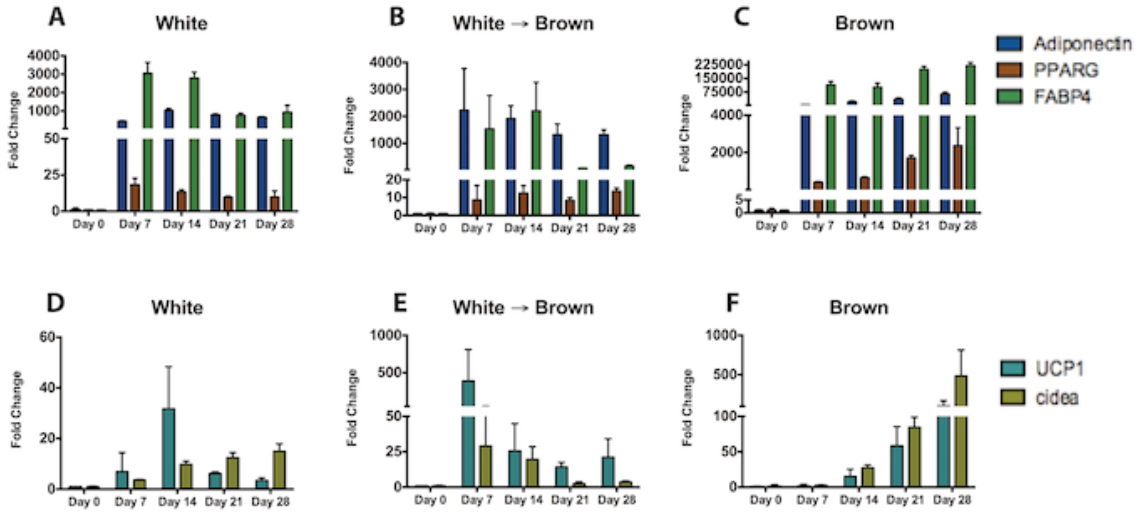

Figure 3.4 Time course of gene expression during adipogenesis. Fold change is calculated to day 0, and beta actin is used as the housekeeping gene. Each of the populations show a great increase in adipogenic genes (A-C). In contrast, the white population (D) only shows a slight increase in brown adipogenic genes over time, compared to the other two populations. The white ASCs differentiated in brown media (E) have a large initial increase in brown adipogenic genes, but tapers off throughout the course of differentiation. The brown population (F) shows a continuing increase over time of brown adipogenic genes.

3.3.3 In vitro effect of rosiglitazone-embedded microfibers

Brown adipose-derived stem cells cultured with PLGA-rosiglitazone microfibers in a Transwell set-up differentiated towards a brown fat lineage in media not supplemented with the typical drugs for brown fat differentiation (**Fig 3.5**). The positive immunostaining for UCP1 confirms that the ASCs have differentiated down a brown lineage. The RT-PCR data shows that the gene expression levels are relatively not as high as the positive control, but still at least 2-fold higher than the negative control. From the data shown, the 0.5% RSG fibers overall show the greatest extent of brown differentiation. There is increased expression of UCP1, cidea, and PGC1a, as well as positive immunostaining for UCP1. It is possible that the higher concentrations of rosiglitazone are detrimental to the cells, as the 1% RSG fibers show less positive UCP1 immunostaining than the 0.5%.

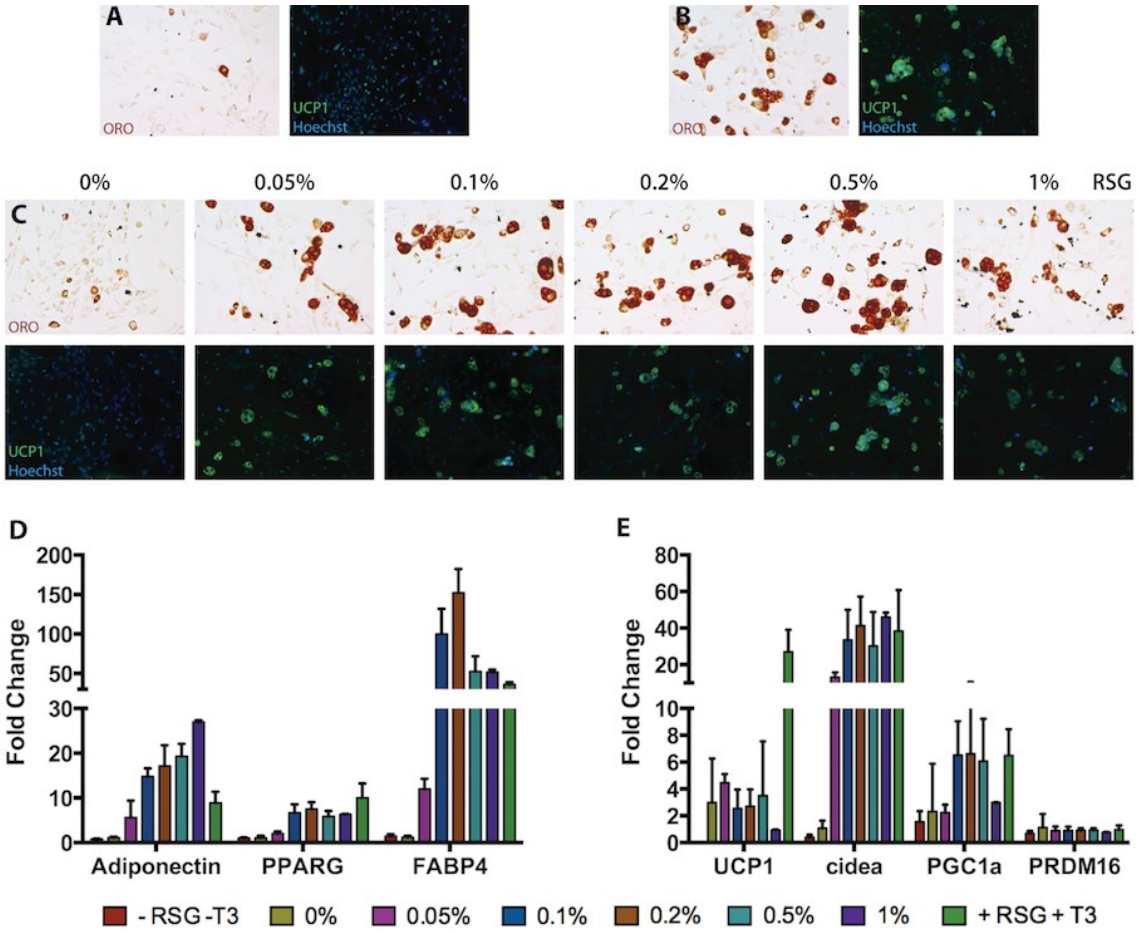

Figure 3.5 Rosiglitazone (RSG) embedded in PLGA nanofibers stimulates brown adipogenic differentiation. Oil Red O staining and UCP1 immunostaining of the cells in the presence of rosiglitazone-embedded PLGA nanofibers (C) shows the brown phenotype as compared to the negative control without fibers (A). The 0.5% RSG fibers showed similar staining to the positive control (B), cells without fibers but with T3 and RSG supplemented in the media. RT-PCR for adipogenic genes (D) shows adipogenic differentiation. RT-PCR for UCP1 and cidea (E) show increased expression of these upregulated genes in brown adipocytes in the presence of nanofibers.

3.3.4 In vitro cell seeding on RSG-embedded microfibers

One million cells were seeded directly on RSG-embedded microfibers, to test the possibility of using the fibers as a cell delivery vehicle as well. The white ASCs had significantly increased expression of PPARG, cidea and PGC1a, showing that the fibers were able to induce

differentiation towards a brown phenotype (**Fig 3.6**). The brown ASCs did not show a significant increase in gene expression when seeded on rosiglitazone-embedded microfibers.

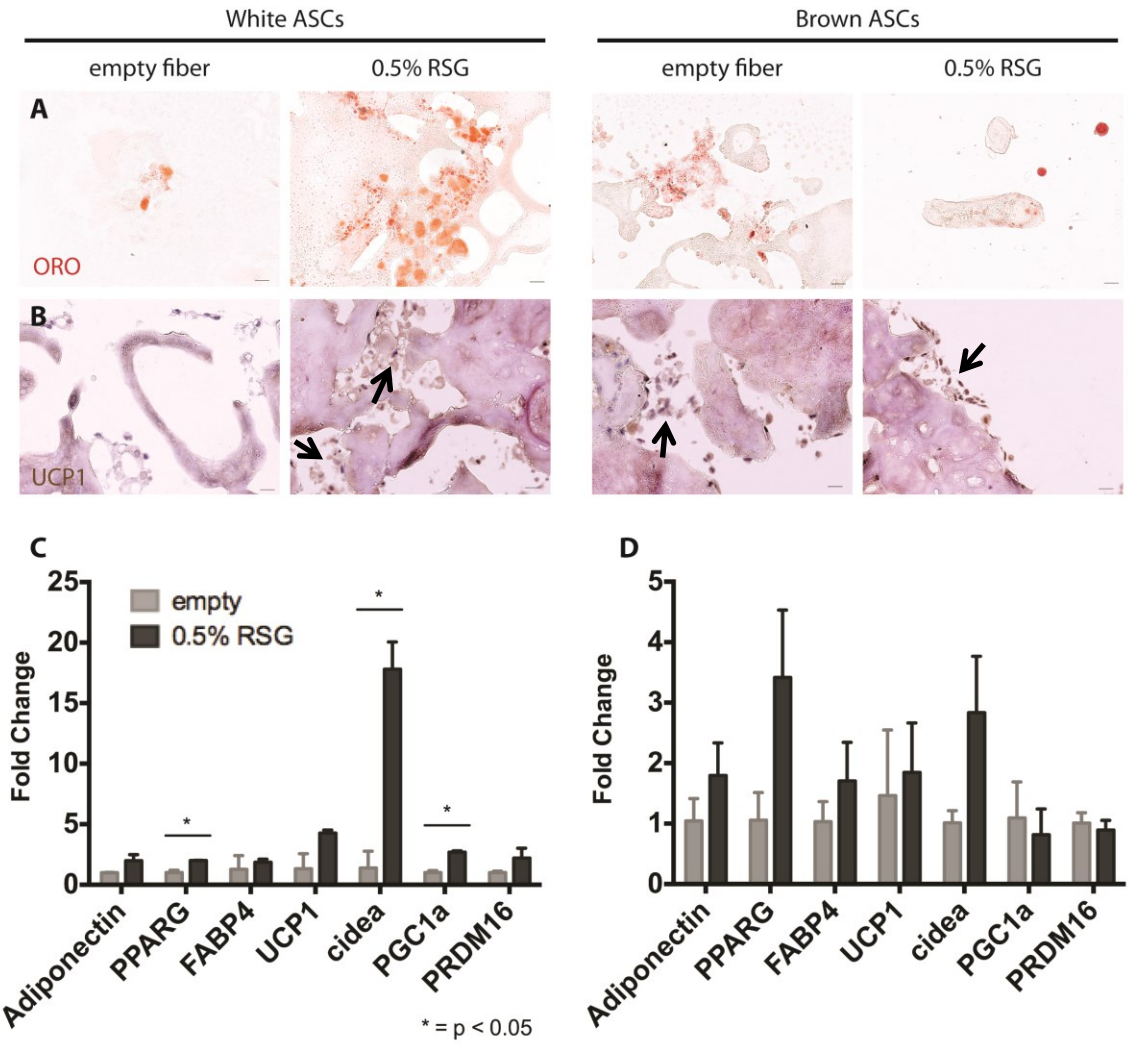

Figure 3.6 Histology and gene expression of cells seeded on rosiglitazone-embedded microfibers. Cells show increased lipogenesis (Oil Red O, A) and UCP1 expression (UCP1 immunostaining, B) on fibers loaded with 0.5% rosiglitazone. Arrows point to cells with positive UCP1. Images taken at 40x, scale bar is 20 um. White ASCs seeded on 0.5% rosiglitazone-embedded microfibers (C) show increased expression of several genes as compared to cells seeded on empty microfibers. Gene expression is also increased with brown ASCs (D) on rosiglitazone-embedded microfibers.

While the microfibers are stimulating a differentiation towards a brown lineage, the microfibers are not ideal for cell viability. In culture, the cells contracted and curled up the

microfibers, leaving little surface area for cell attachment. This may explain the decreased effect of the rosiglitazone in the microfibers on the brown ASCs as compared to the 2D study, as the brown ASCs contracted the microfibers more severely than the white ASCs.

3.3.5 In vivo effect of RSG-embedded microfibers

RSG-embedded microfibers with white and brown ASCs seeded on top were implanted subcutaneously in 9-10 month old Sprague-Dawley rats. After a 1-week pilot study, the microfibers showed good integration with the host tissue, with no major inflammatory response (**Fig 3.7**). Further 3-week studies will be needed to evaluate the functionality of the microfiber implants in stimulating brown fat development.

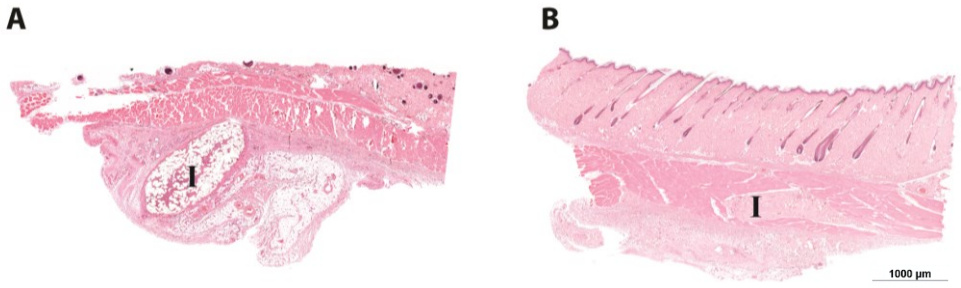

Figure 3.7 RSG-embedded microfibers after 1 week of subcutaneous implantation. Implants integrated well with host tissue with both white ASCs seeded on top (A) and brown ASCs (B). Images taken at 2.5x (then merged), scale bar is 1000 um. "I" indicates the RSG-embedded microfiber implant.

4. Discussion and Future Work

In recent years, brown fat has become increasingly popular as a target for fighting obesity and diabetes. Multiple in vivo pharmacological approaches and ex vivo transplantation approaches have been considered. In this thesis, we explore a novel system that uses a pharmacological approach, but aims to mitigate the harmful systemic effects by locally delivering these small molecule drugs. The result would be a small brown fat depot created within white adipose tissue that would work to offset the energy imbalance caused by obesity and its associated complications.

The in vitro work presented in this thesis first demonstrates the white adipose-derived stem cells isolated from rat tissue have the capability to differentiate towards a brown lineage. Next, it is clear that the PLGA microfibers can be used to effectively deliver a small molecule, rosiglitazone. Both the immunostaining and the PCR from the 2D and 3D studies confirm that the rosiglitazone released from the microfibers was able to induce the differentiation of both brown and white ASCs.

Future studies for this work would include optimizing the properties of the PLGA delivery system. Mechanical testing to study the interactions between the PLGA microfibers or microparticles with adipose tissue would provide useful information on the biocompatibility of the delivery system. The drug release profile could also be fine-tuned by manipulating both the size of the fibers and the amount of drug loaded. Several additions can also be added to this system to enhance its function. To improve cell viability and proliferation to create a localized brown fat depot, the system could be used in conjunction with decellularized adipose ECM. Decellularized tissues are a matrix of proteins and sugars that maintain biochemical cues to support cell migration into the matrix and cell proliferation and viability within the matrix. [30] The

adipose ECM could be implanted with the microfibers and cells to support cell growth and differentiation, or the adipose ECM could be injected with the PLGA microparticles as well. Another enhancement to the system would be the addition of multiple drugs within one implant. The drugs could also be released with different kinetics to support different functions. For example, the initial drugs released could be molecules such as rosiglitazone or lithocholic acid, which promote brown fat differentiation; then as a delayed release, drugs that activate brown fat, such as isoprenaline, could be released to get maximal activity of the newly created brown fat depot. Such orchestrated drug delivery systems have been designed for a variety of different applications. [31–33] With these improvements, we hope to design a robust system to create a localized depot of brown fat from adipose-derived stem cells within white fat.

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