Encapsulation of Genetically Modified Preadipocytes for Potential Treatment of Metabolic Disorders

DISSERTATION

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Abstract

Obesity is a worldwide epidemic that affects millions of people. The location of the excess fat dictates the health risk. Excess visceral fat increases the risk of fatal diseases like type II diabetes and certain cancers. Not all forms of obesity can be treated with traditional methods like diet, exercise, and drugs because of physiological differences between individuals. There is a need for new treatments that directly target visceral fat as well as treat the biochemistry that comprises the obesity disease.

This dissertation is designed to investigate potential treatments for obesity and metabolic diseases by using genetically modified adipocytes. Cell lines can be engineered to be thermogenic or to overproduce hormones that regulate metabolism. However, there is not currently a safe way to genetically modify preexisting adipocytes and transplantations of modified adipocytes require immunosuppression. We developed a solution to these problems by encapsulating engineered fibroblasts in an immunoprotective poly-L-lysine membrane, allowing for controlled delivery of genetically modified cells into the host’s tissue. We hypothesized that encapsulating stably transduced preadipocytes, designed to be more thermogenic or overproduce hormones that regulate metabolism, could attenuate weight gain and improve metabolic
markers when injected into the visceral fat pads of mice. For this reason, we investigated encapsulated genetically modified cells’ ability to improve glucose metabolism, metabolic rate, and attenuate weight gain in mouse models of obesity.

Chapter one is a literature review of the obesity epidemic, the difference between fat depots, and how microencapsulation technology could be applied to treating obesity related diseases.

In chapter two, we hypothesize that encapsulated Rptor deficient preadipocytes will have a thermogenic phenotype in vivo and improve symptoms of diet induced obesity. We find that these capsules attenuated weight gain briefly, but improved glucose metabolism and possibly fat metabolism through increased production of ATGL.

In chapter three, we hypothesized that increasing the ability of Rptor deficient fibroblasts to uptake nutrients could increase the rate of thermogenesis. These cells could be engineered to overproduce appetite suppressing hormones, amylin and leptin to compensate for increased appetite that comes with increased thermogenesis. The in vivo effects of these encapsulated cells were studied in mice fed a high fat diet.

In chapter four, we hypothesized that encapsulated adipocytes overproducing leptin could secrete leptin, improve metabolism, and attenuate weight gain in ob/ob mice, an obese mouse model lacking leptin. We discovered that cells constitutively produced leptin and were able to secrete leptin when encapsulated. In vivo, these capsules improve metabolic rate, glucose metabolism, and reduce resistin.

We previously showed that Aldh1a1−/− fibroblasts derived from subcutaneous fat are much more thermogenic than wild type fibroblasts from the same depot. In chapter
five, we isolated these fibroblasts from subcutaneous fat, immortalized the cells, and stably transduced them to overexpress a fluorescence protein in order for these cells to be used for a future encapsulation experiment in obese dogs.

Our preliminary data provides evidence, for the first time, that encapsulation of engineered preadipocytes may be a viable treatment option for metabolic diseases.
Dedication

This work is dedicated to God, my family, my friends, and graduate students everywhere
whose route to graduation is never quite as they planned.
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List of Abbreviations

ADH.................................................................Alcohol dehydrogenase
ALDH..............................................................Aldehyde dehydrogenase
A-PLL ............................................................Alginate-poly-L-lysine
ATGL..............................................................Adipose triglyceride lipase
ALDH1A1.........................................................Aldehyde dehydrogenase 1 isoform a1
BAT.................................................................Brown adipose tissue
C/EBP-β..........................................................CCAAT enhancer-binding protein beta
CS...........................................................................Calf serum
DIO.................................................................Dietary induced obesity
4E-BP1 ............................................................eukaryotic translation initiation factor 4E binding protein 1
FBS.................................................................Fetal bovine serum
Glut4..................................................................Glucose transporter type 4
HF...........................................................................High fat
IL-6......................................................................Interleukin-6
Lep .........................................................................Leptin
LPL.......................................................................Lipoprotein lipase
mTOR..................................................................Mammalian/mechanistic target of rapamycin
mTORC1................................................................mTOR complex 1
Pparg.............................................Peroxisome proliferator-activated receptor gamma
PRDM16 .................................................................PR domain containing 16
Pref1 .................................................................Preadipocyte factor-1
RA .................................................................Retinoic acid
RALD ...........................................................................Retinaldehyde
RAR ...........................................................................Retinoic acid receptor
RARE ........................................................................ Retinoic acid response element
Rptor...........................................................................Regulatory associated protein of mTOR
S6K1..............................................................................p70 ribosomal S6 kinase 1
SF ................................................................................ Subcutaneous fat
T2DM ................................................................................ Type II diabetes mellitus
TG ..................................................................................Triglyceride/triacylglycerol
TNF-α .................................................................Tumor necrosis factor alpha
UCP1 ............................................................................... Uncoupling protein 1
VF ...................................................................................Visceral fat
WAT ................................................................................ White adipose tissue
WT ..................................................................................Wild type (C56BL/6J mice)
Chapter 1

Literature Review

1.1 Obesity as an epidemic

Obesity and obesity related diseases are a global problem. Obesity affects 150 million people worldwide and is characterized as pandemic disease [1, 2]. In the United States alone, one third of the adult population and approximately 17% of children are obese [1]. Based on current trends researchers predict that in the United States obesity cases will increase by 65 million by the year 2030 [3]. This would result in 50% of the population being obese [3]. Increases in obesity will escalate spending on health care. This spike in health care costs will economically burden countries [3].

Obesity increases the risk of cardiovascular disease, certain cancers, type II diabetes (T2DM), and all-cause mortality [4, 5]. In North America, the European Union, China, and other countries, obesity is considered to be responsible for up to 70-90% of the adult cases of type II diabetes mellitus (T2DM) [1, 2, 6]. Obesity also increases the risk of certain cancers. For example, obesity has been linked to increasing breast cancer risk in many different populations of women [7-9]. It may be more frugal to combat a wide range of diseases by preventing their underlying cause, obesity.
1.2 Not all fat is created equal

The location of the fat dictates the risk of developing disease. Obesity primarily affects white adipose tissue (WAT). WAT provides insulation, warmth, and energy for the body. In adults, there are two major types of WAT, subcutaneous (SF) and visceral (VF) fat tissue. WAT is divided into these two categories based on the WAT’s developmental origin and location. SF, or peripheral fat, lies beneath the skin and in skeletal muscle. VF is located around internal organs and is also referred to as pericardial, perigonadal, and perirenal fat. In some studies, VF is referred to as abdominal or intra-abdominal fat. Abdominal or visceral obesity is characterized by greater fat mass in the abdomen [10, 11]. The unhealthy accumulation of fat mass is caused by an increase in the adipocyte’s size and number of adipocytes in these obese fat pads [4, 12]. Obesity in the wrong physiological location can have deleterious effects. However, some adipose tissue can be health promoting.

SF may have some health promoting effects. SF has been associated with lowering levels of triglycerides, glucose, and insulin in the blood [13, 14]. In allograft studies, SF transplantation into the VF depot reduces weight and improves insulin tolerance [13-15]. In contrast to SF, increasing the VF mass in the abdominal region is detrimental to a healthy metabolism.

Visceral obesity poses a great health risk to the individual. Visceral obesity has been linked to an increased risk of death from cardiovascular disease and certain cancers [4]. VF accumulation, even in non-obese people, increases the risk of premature death from all causes from 150 to 200% [4]. Other diseases are also affected by visceral
obesity. Visceral obesity increases the risk of T2DM [16]. In a Chinese male population, visceral fat was positively associated with carotid atherosclerosis [17]. Strikingly, this association was for both obese and non-obese individuals, giving further evidence that health risk is associated with visceral fat mass [17]. Biomarkers, such as high-sensitivity C-reactive protein, increase with diabetes, obesity, insulin resistance, and atherosclerosis [18]. Visceral fat mass is positively correlated with high-sensitivity C-reactive protein, which further establishes the link between visceral fat, diabetes, and atherosclerosis [18]. VF produces different pro-inflammatory cytokines than SF. VF has greater secretion of the pro-inflammatory cytokines TNF-α, IL-6, and resistin [19-21]. This creates a systemic and localized state of chronic low-grade inflammation that induces insulin resistance [22, 23]. Moreover, glucose sensitizing hormones, like adiponectin, that improve insulin sensitivity are secreted in higher levels by SF than VF [24]. When VF is transplanted into SF, the transplant had no effect, which further illustrates the unique properties of different fat depots [13].

In addition to WAT, brown adipose tissue (BAT) is the other predominant type of adipose tissue [14]. BAT is metabolically different from WAT, particularly different from harmful VF. In BAT, stored energy from nutrients (glucose and fatty acids) can be partially converted into heat in a process known as thermogenesis [25]. This process is driven by the mitochondrial uncoupling protein 1 (UCP1). BAT has numerous UCP1 positive mitochondria that dissipate energy. UCP1 is one major factor responsible for wasting energy in the form of heat [26]. Brown adipocytes have smaller lipid droplets than other adipocytes, which is most likely due to increased thermogenesis [14]. BAT
and skeletal muscle arise from a similar progenitor expressing myogenic factor 5 (Myf5) [27]. Neonates have notable amounts of BAT to support thermogenesis [28]. BAT mass declines with age. In adults, only small BAT depots are located in the neck and perivascular areas [29]. An elevated amount of BAT is associated with resistance to obesity and improved glucose sensitivity in human and mouse obesity models [25, 30].

Both BAT and SF are associated with improving metabolism [13]. WAT can also contain thermogenic adipocytes expressing Ucp1 and Ucp2 [31]. We describe these adipocytes as ‘thermocytes’ in this dissertation, while they have also been termed as ‘brown-like’, ‘beige’ or ‘brite’ adipocytes elsewhere [31-33]. White thermocytes and lipogenic adipocytes in WAT appear to originate from a similar precursor; however, they express distinct gene clusters [33]. In WAT, thermogenesis can be induced by either sympathetic nerve stimulation, prolonged cold exposure [34], or consumption of high-fat and spicy diets [35]. Induction of thermogenesis in WAT can change energy homeostasis and effectively decrease obesity and insulin resistance [36-38]. Notably, the increased proportion of thermogenic to lipogenic adipocytes in VF can decrease lipid accumulation in this deleterious depot [37]. Although thermogenesis in WAT offers a unique therapeutic opportunity to decrease abdominal fat, the current methods of inducing thermogenesis in WAT are not suitable for clinical applications.

1.3 Novel obesity therapies and their issues

There are several therapies currently being used to fight the obesity epidemic. Fat tissue can be surgically removed with benefits depending on the tissue. Surgically removing VF improves glucose metabolism in rodents [39, 40] and humans [41].
However, in humans, liposuction, which removes SF, has not been shown to improve metabolic markers like glucose tolerance [42]. These results are duplicated in lifestyle changes to treat obesity. Reducing SF by behavior modifications, diet, and exercise does not have the same beneficial effects that reducing VF does [43]. Behavior modifications, exercise, and a very low calorie diet (less than 500 kcal a day) can also reduce visceral fat and improve glucose metabolism [44]. Non-essential nutrients may also be able to reduce VF mass. In one study, quercetin was fed to C56BL/6J mice fed a western diet (diet high in sucrose, fat, and cholesterol) for 20 weeks [45]. At the end of the study the quercetin treated group had reduced VF accumulation and reduced plasma cholesterol, triglyceride, insulin, and glucose levels when compared to western diet control [45]. Likewise β-cryptoxanthin, a carotenoid, was shown to have similar function in human and mice studies and lowered the risk of diabetes and hypertension [46]. Drugs can also reduce visceral fat mass. In human studies, metformin has been shown to reduce visceral fat and weight, while improving insulin sensitivity [47-49]. It is also possible to increase UCP1 in patients. Some research has shown that dietary interventions in diabetic mice have successfully induced UCP1 [50].

Diet, exercise, or drugs to treat obesity are not always a possibility. The modern patient is unique and not all interventions are possible. Diet changes may not fully correct underlying metabolic problems that prevent an individual from losing weight. Exercise is not always a possibility if the patient is disabled. Moreover, drugs can have unwanted side effects like increased inflammation, nausea, and mood disorders [51].
Some researchers have investigated the transplantation of adipose tissue to address these problems and to make WAT thermogenic to promote weight loss.

Transplantation of brown adipocytes has been successful in immunocompromised models. Brown adipocytes were injected into nude mice and increased UCP1 expression within WAT [14]. Tseng et al treated C3H10t1/2 multipotent progenitor cells with bone morphogenetic protein 7, a growth factor, and then injected the cells into athymic mice [52]. The injection successfully produced brown fat tissue expressing UCP1 [52]. Seale al injected *Pparg* Fibroblasts, expressing both PPARγ2 and either PRDM16 or PPARγ2, into athymic mice and brown fat tissue was formed expressing UCP1 [27, 53]. Lastly, Kajimura et al retrovirally transduced mouse embryotic fibroblasts with retroviral PRDM16 and/or C/EBP-β [54]. These cells were injected into athymic mice resulting in brown fat tissue formation, UCP1 expression, and greater glucose uptake than surrounding tissue [54]. However, BAT transplantations have been less successful because the transplants take on the original phenotype of the host animal’s tissue over time [14]. The BAT transplantations begin to “whiten” and have a reduced thermogenic effect. Developing a method to transplant subcutaneous or brown adipose tissue may beneficial in treating obesity related diseases (7). Nevertheless, thermogenic fat cells have been successfully transplanted into mice models.

A novel approach is to combat visceral fat at the cellular level through transplanting tissues or thermogenic cells. However, these cells were all injected into athymic mice and into the SF. Human patients will mostly have a fully functioning immune system. In order to be applicable to humans, implanted cells must avoid rejection
by the immune system to function in vivo. Furthermore, it is impractical to transplant fat tissue into obese patients. This requires donor samples, invasive surgery, high surgical costs, increased risks of infection, and complications from the surgery. Developing a method to place the thermocytes into the deleterious VF, protect the thermocytes, reduce side effects and unwanted harmful effects of major surgery could result in a more beneficial and effective therapy.

1.4 Alginate-poly-L-lysine encapsulation technology

Encapsulation procedures useful for encapsulating cells have been around since 1964 when Chang et al invented a semipermeable microcapsule [55]. These capsules could be viable for delivery of drugs or cells to target areas because the capsule provides protection to the encapsulated material, but allows for small molecule transfer in and out of the capsule [56]. Using the Zhang et al procedure, cells can be encapsulated by suspending them in sodium alginate [56, 57]. The sodium alginate hardens in the presence of calcium chloride. Poly-L-lysine coats the hardened sodium alginate forming a layer around the sodium alginate. The sodium alginate is then dissolved leaving the cells unharmed wrapped in a semipermeable poly-L-lysine membrane. The poly-L-lysine membrane allows for the exchange of molecules all the while protecting the encapsulated cells from the body’s immune system [37, 57, 58]. Cell using alginate-poly-L-lysine (A-PLL) encapsulation has been shown to protect the cells from the body’s immune system in both human and animal models [37, 59, 60]. Sodium alginate is comprised of primarily two acids, guluronic and mannuronic acid [61]. Alginate with a higher portion of mannuronic acid used to make A-PLL capsules generated the weakest immune system
response [61]. If possible, high mannuronic acid alginate should then be used for most encapsulation procedures [61]. Encapsulated cells in A-PLL can be injected into the body and interact with the surrounding tissue. The capsules are on the micro scale (approximately 400 µm in diameter) and are easily injected into the host. Because of this delivery system it has been utilized for treatment of primarily type I diabetes.

1.5 A-PLL encapsulation use in various models

Type I diabetes is caused by a dysfunction of the pancreatic beta cells that no longer properly produce insulin, unlike T2DM where the disease is characterized by high blood sugar because of insulin resistance. To solve this issue, researchers attempted to use encapsulation to provide a way to introduce functioning beta cells into the host so that insulin could be made endogenously. Early studies showed that islet cells could survive and secrete insulin after A-PLL encapsulation [62, 63]. In a simple encapsulation model not using poly-L-lysine, porcine islets cells encapsulated in BaCl₂ alginate capsules were able to improve glucose metabolism in mice [64]. These results were confirmed in xenograft models. Rat islet cells encapsulated using A-PLL survived for 220 days in diabetic mice [63]. Similar results were found when A-PLL capsules containing islet cells were injected into diabetic dogs [65]. These dogs were able to survive independent of insulin injections for up to 172 days [65]. Xenograft models were also successful in monkeys. Porcine islet cells encapsulated in A-PLL were able to normalize glucose metabolism and keep fasting blood sugar normal for up to 2 years [66]. These some of these monkeys did not develop hyperglycemia before the end of the study and examining the internal organs did not show a harmful effect from the
microcapsules [66]. Xenografts have been successful in humans. Clinical trial in 4 patients with type 1 diabetes, encapsulated islet cells lead to a decrease in exogenous insulin dependence and the cells did not lead to an immune system response [60]. These functioning islet cells survived for up to 9.5 years in the human subject [67].

A-PLL encapsulation has been used to treat other diseases. In a very small study (n=2) db/db rats, a model of T2DM, cells transduced to overexpress glucagon-like peptide 1 were encapsulated in a bioisolator tube and surgically implanted into the host [59]. In the pharmaceutical industry, capsules may provide a novel way to deliver drugs into the patient. To potentially treat hepatitis C, ribavirin, a drug that has severe side effects was encapsulated in a biodegradable poly-L-lysine based capsule. Ribavirin was able to be slowly excreted from these capsules offering a possibly safer option [68]. To our knowledge capsulation studies in obesity have been limited to the ones described in this dissertation. Most other encapsulation studies have not used A-PLL capsules to treat obesity or obesity related diseases. Our lab has explored using A-PLL capsules to treat obesity in one previous study involving vitamin A metabolism.

1.6 Encapsulation of Ald1a1−/− fibroblast to treat DIO

Vitamin A metabolism begins via the oxidation of the vitamin A metabolite, retinol (ROL), to retinaldehyde (Rald) by the alcohol dehydrogenase (ADH) family of enzymes [69]. Several microsomal enzymes of the short chain dehydrogenase (SDR/RDH) family convert ROL to Rald in vitro [69, 70]. This conversion of retinol to Rald is a reversible step, carried out by ADHs, RDHs, DHRS9, and some aldo-keto
reductases like AKR1B1 [71, 72]. The cytosolic aldehyde dehydrogenase 1 enzyme family (ALDH1, alias RALDH) catalyzes the final irreversible step in retinoic acid (RA) production by oxidating Rald to RA [69, 73].

RA induces differentiation of cells and regulates gene transcription, signaling events, and post-translational modification of proteins [74]. All isomers of RA are high affinity ligands for the retinoic acid receptor (RAR) [75]. RAR can heterodimerize with retinoic X receptor (RXR), another nuclear receptor that binds to 9-\textit{cis} RA. This heterodimer then binds to RA response elements (RARE) and regulates gene expression [75]. Furthermore, RA regulates expression of peroxisome proliferator–activated receptor γ (\textit{Pparg}) [76], the master regulator of adipogenesis, via activation of transcription factor \textit{Zfp423} [75, 77]. This makes inhibiting RA production within VF a potential therapeutic target.

In contrast, Rald suppresses adipogenesis by inhibiting PPARγ and RXR activation [78]. PPARγ, RAR, and RXR regulate numerous differentiation processes and immune response [74, 75]. The ALDH1 family is comprised of three enzymes: ALDH1A1, ALDH1A2, and ALDH1A3 [73, 79]. ALDH1 enzymes share similar properties, such as conversion of Rald to RA but have unique biochemical and physiological functions [79]. ALDH1A1 is the primary isoform in female mice adipose tissue and producer of RA [36, 77].

In vitro, \textit{Aldh1a1} expression is increased during adipocyte differentiation and contributes to ~70% RARE activation [77]. Knocking out \textit{Aldh1a1} in mice demonstrates how prevention of lipogenic adipogenesis in adipocyte cultures results in the acquisition
of thermogenic characteristics [37]. Additionally, differentiated \textit{Aldh1a1}^{−/−} cells compared to WT or 3T3-L1 adipocytes display higher expression of Ucp1, Pgc-1α, and CoxIV as well as down-regulation of lipogenic genes \textit{Pparg}, \textit{Fas} and \textit{Fabp4} [37, 77]. These results are in agreement with findings in vivo, where thermogenic remodeling occurs in the VF of \textit{Aldh1a1}^{−/−} females [36]. In our previously published study, \textit{Aldh1a1}^{−/−} cells were isolated from embryos of \textit{Aldh1a1}^{−/−} mice and stably transduced with green fluorescent protein (GFP) [37]. WT and \textit{Aldh1a1}^{−/−} fibroblast cell lines were encapsulated using A-PLL and injected into both VF pads of female mice fed a high fat (HF) diet [37]. The implantation of \textit{Aldh1a1}^{−/−} cells into the VF of WT female mice induces similar thermogenic modification of adipocytes as seen in whole body \textit{Aldh1a1} deficient female mice [37]. This increased thermogenesis in VF results in the loss of VF mass in treated females despite consumption of a high-fat diet [37]. However, this technology has some drawbacks.

Humans, similar to mice, exhibit enhanced lipolysis in the presence of Rald than RA \textit{in vitro} [36]. Stromal vascular cells isolated from obese women, but not men, had significantly greater expression of \textit{Aldh1a1} suggesting that ALDH1A1 function in the regulation of adipocyte biology is conserved between species [36]. It seems plausible that \textit{Aldh1a1} deficiency in human omental fat or implantation of \textit{Aldh1a1}^{−/−} deficient pre-adipocytes could serve as a potential sex-specific therapeutic strategy to combat android obesity in women. It is also necessary to devise weight loss therapies that also work in males and not only females. The increase in the thermogenic capacity of cells could be achieved by ectopic expression of genes participating in thermogenesis via UCP1,
signaling pathways regulating multiple proteins supporting thermogenesis, transcriptional pathways inducing thermogenic differentiation programs.

Therefore, it is necessary to investigate another thermogenic cell line model to see if it is as effective as Aldh1a1−/− to induce thermogenesis and combat obesity in a different manner.

1.7 Target: Raptor (regulatory associated protein of mTOR)

Mammalian target of rapamycin or mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that can regulate cell growth, survival, and transcription in response to changes in nutrients, cell stressors, and growth factors. There are two mTOR complexes, mTORC1 and mTORC2. Regulatory associated protein of mTOR (raptor, gene symbol Rptor), mTOR, the adaptor protein mLST8, and a proline-rich Akt substrate of 40 kDa (PRAS40) comprise mTORC1 [80]. mTORC1 is inhibited by the DEP domain-containing mTOR-interacting protein (DEPTOR) [81]. mTORC1 can be pharmalogically inhibited by the immunosuppressant and anticancer drug rapamycin [82]. Protein synthesis is regulated through mTORC1 by the phosphorylation of eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), a repressor of mRNA translation, and by the activation and phosphorylation of p70 ribosomal S6 kinase S6K1 [83, 84]. mTORC1 is a critical signaling pathway that regulates translation of 15% of cellular proteins, and decreases longevity in mammals [82, 85, 86]. Rapamycin-insensitive companion of mTOR (Rictor), mTOR, mLST8, and Sin1 comprise mTORC2. The function of mTORC2 is not well understood, but it phosphorylates protein kinase B
mTORC1 is the complex sensitive to rapamycin, whereas, mTORC2 is not [80]. mTORC1 is the component of mTOR that regulates survival and growth responses in response to changes in nutrients, cell stressors, and growth factors. Nojima et al showed that raptor is a regulatory protein of mTORC1 that functions as a scaffold holding the complex together [87]. Rapamycin disrupts the interaction of raptor with mTORC1 inhibiting the complex [80]. 4E-BP1 represses translation by binding to eIF4E. Hyperphosphorylation of 4E-BPs weakens the interaction between 4E-BPs and eIF4E, resulting in dissociation of the 4E-BPs from eIF4E and initiating translation [83]. Inhibition of mTORC1 has been associated with energy restriction and attenuation of tumor growth [88]. Harrison et al showed that inhibiting mTOR with rapamycin in mice increased life span [89]. Moreover, the study provided evidence that it is possible to regulate life span in mammals by pharmacological interventions [89]. Specifically, this most likely is due to inhibition of raptor. Knock outs of raptor, specifically in adipocytes, inhibit mTORC1 signaling and subsequent downstream products such as S-phase kinase-associated protein 1(S6K) [90]. Knocking out S6K mimics calorie restriction [80] and could increase life span [88, 91, 92]. Rapamycin also decreases expression of 2 late-phase adipocyte differentiation transcription factors, PPARγ and C/EBPα [93]. Furthermore, knocking out S6K in a mouse model positively effects insulin sensitivity and protects against obesity [91]. S6K1-deficient mice are glucose tolerant and are protected against obesity, which further suggests participation of mTOR in insulin signaling and fat formation [86, 91].
Because S6K is downstream of the mTOR1 complex, raptor may be a good target for thermogenic cells. It has been shown by Polak et al that conditionally knocking out raptor expression in adipose tissue increases UCP1, GLUT1, and insulin sensitivity [94]. Furthermore, the knockout mice had lower cholesterol levels when compared to the wild type control [94]. The raptor knockout also inhibited S6K, via phosphorylation of S6K [94]. Thus, a raptor deficiency in adipocytes can improve blood glucose levels, an underlying cause of diabetes.

Cells with a genetic mutation knocking out raptor will inhibit mTORC1. If raptor is knocked out, then metabolism should increase because of increased UCP1, GLUT1, and insulin sensitivity [94]. Therefore, cells deficient in raptor will provide an energy outlet via thermogenesis for high blood glucose and excess energy making it a viable therapy for diabetic patients. The increase in thermogenesis could be the mechanism for increased life span and attenuated tumor growth because energy is wasted and not used for cell division.

We developed adipocyte encapsulation procedure [95] for transplantation of genetically engineered cells in adipose tissue without immune rejection [37]. In chapter 2, we explore the effects of encapsulated Rptor knocked down fibroblasts in mice fed a HF diet.

### 1.8 Target: Leptin

Leptin was selected as a candidate to fight obesity through endocrine, paracrine, and autocrine mechanisms. The adipokine leptin has been recently considered as a
hormone candidate reducing obesity [96] and managing diabetes in the absence of insulin or in addition to insulin [97]. Leptin mediates its therapeutic effects on energy homeostasis through the hypothalamus and selected action on specific neurons [98-105]. Leptin induces hypothalamic secretion of the appetite suppressing corticotrophin-releasing hormone [102]. Leptin stimulation releases sympathetic neurotransmitters, norepinephrine and epinephrine, that increase activation of lipolysis and thermogenesis in WAT and brown adipose tissues (BAT) [105, 106]. Leptin works synergistically with insulin to promote thermogenesis in WAT and increasing energy expenditure through hypothalamic neurons [107]. Cumulatively, these effects reduce obesity in mouse models of obesity and in human patients with homozygous Lep mutations or congenital Lep deficiency [108-110]. Leptin’s simulation of the hypothalamus increases glucose uptake in BAT, heart muscle, and skeletal muscle, but not WAT [103, 104]. Leptin and insulin also synergize to increase glucose uptake in these tissues in part via suppression of glucagon effects [103]. These responses make leptin a promising candidate to work alongside insulin therapies for treatment of diabetes and obesity.

A primary model for development of leptin therapies is the ob/ob mouse (Lep\textsuperscript{ob}, or ob), a homozygous mutant in the leptin (Lep or ob gene) gene. In the absence of functioning leptin, the ob/ob mouse rapidly develops obesity and hyperglycemic conditions similar to T2DM [96, 111-113]. Reintroduction of leptin by injection of exogenous recombinant leptin, adenovirus transduction of leptin expression in tissues, or transplantation of tissues producing leptin attenuate weight gain, improve glucose tolerance, decrease appetite, and increase metabolic rate in ob/ob mice [96, 114-117].
However, these methods of leptin delivery cannot be directly translated into therapies for humans. Leptin replacement therapy is necessary in patients with homozygous $Lep$ mutations or acquired leptin dysfunction that produces congenital and acquired generalized lipodystrophy [108-110]. Treating these patients with recombinant leptin through injections produces short lasting effects and requires repetitive doses. The supra-physiological increase in leptin in the circulation followed by regular injections is associated with serious side effects [51]. One study reports that endogenous production of leptin works better than insulin injections [118]. However, adenovirus carrier of a functioning $Lep$ gene [118, 119] or transplantation of tissues or cells expressing functional leptin [15, 117, 120] pose risks of infection, immune rejection of transplant, or potential genetic viral contamination that are hazardous to human health. Transplantation models also require drugs to suppress the host’s immune system. To improve transplant survival, Oosman et al, suspend leptin-producing intestinal cells in alginate beads; however, this treatment also requires immunosuppression to be viable [117].

Recently we developed adipocyte encapsulation procedure [95] for transplantation of genetically engineered cells in adipose tissue without immune rejection [37]. The encapsulating poly-L-lysine generates a porous membrane that protects the encapsulated cells from the host’s immune system as well as allows small molecules, like hormones, to diffuse out of the capsule. In chapter 4, we showed that treatment with encapsulated leptin-producing adipocytes provides an effective solution that improves glucose tolerance in genetic and diet-induced mouse models of obesity. Encapsulated leptin-
producing adipocytes reveal novel aspect of leptin signaling including suppression of resistin in \(ob/ob\) mice.

1.9 Summary

Obesity is a pandemic that will increase health care costs because of increased risk of disease. Traditional treatment options do not always work and may not target the detrimental abdominal fat depot. To combat this threat, novel treatment options need to be investigated to prevent obesity in a depot and biochemically specific manner. A-PLL encapsulation offers a unique delivery method to introduce drugs or specifically designed fat fighting cells. This method has been shown to allow the diffusion of small molecules through the capsule membrane, but protecting the cells within the A-PLL capsule. Further research is needed to see if A-PLL capsules can be used a therapy for obesity and obesity related disease.
Chapter 2

Encapsulation of Rptor knockdown fibroblasts for treatment of DIO

2.1 Introduction

The inhibition of mTORC1 has been shown to increase lifespan and reduce adiposity in mice. The mTORC1 pathway has been suggested as a viable target for obesity therapy. Rptor, when removed from mTORC1, causes the complex to fall apart and not function. Polak et al showed that an adipose specific knock out of Rptor remodeled WAT producing a lean phenotype with greater expression of Ucp1 [94]. In Rptor knockdown 3T3-L1 cells, adipose triglyceride lipase (ATGL), the rate limiting enzyme in lipolysis, was increased [121]. These knockdown cells were not reported to overexpress Ucp1 as in the adipose specific knock out [94, 121]. Studies in human adipocytes have shown that inhibition of mTORC1 may contribute to insulin resistance, contrary to animal studies [122, 123]. These studies in human adipocytes of both non-diabetic and diabetic subjects have shown that dysregulated feedback to mTORC1 leads to insulin resistance [122, 123]. These studies indicate that there may be differences, in regards to mTORC1, between species. However, the human cells used in these studies were not immortalized. Non-immortalized cells begin the process of dying, which affects the ability of the cell to properly utilize glucose like a normal cell would. Even though
inhibition of mTORC1 may be species specific, more research is still needed. Therefore, targeting mTORC1 and specifically RPTOR may be viable as a therapeutic strategy for combating obesity and insulin related diseases until the species differences are fully elucidated.

Pharmacological mTOR inhibition, through rapamycin, inhibits both mTORC1 and mTORC2 [124]. Although in adipose tissue mTORC1 participates in the development of obesity, in other tissues mTORC1 has an anti-obesity effect. In the muscle tissue of mice, mTORC1 is necessary for mitochondria production through PGC1alpha [125]. A RAPTOR deficiency in muscle tissue leads to stunted muscle growth and impaired glucose tolerance [125]. Pharmacological inhibition of mTORC1 can inhibit a variety of tissues and have opposite effects in each tissue. Therefore, a tissue specific strategy is more viable to minimize side effects. A possible strategy for tissue-specific and disease-specific targeting of mTORC1 involves transplantation of encapsulated RPTOR deficient cells. However, it is unclear whether encapsulated RPTOR deficient cells can also exert in vivo thermogenic or insulin sensitizing responses. Encapsulating genetically modified cells foreign to the host in a porous poly-L-lysine membrane will protect the encapsulated cells from the host’s immune system. The semi-porous capsule enables an influx of nutrients and efflux of catabolic products which promotes long-term survival of the transplant. Our previous study investigated the role of encapsulated aldehyde dehydrogenase 1a1 knockout thermocytes [37]. Encapsulated thermocytes attenuated lipid accumulation in the abdominal fat depot in wild-type female mice fed a high fat diet. This may have been due to thermogenic
remodeling of the fat tissue surrounding the encapsulated thermocytes [37]. This previous study showed that genetically engineered thermogenic fibroblasts were capable of surviving and functioning in the adipose tissue in an allotransplantation model [37]. There currently are no studies investigating the role of A-PLL encapsulated cells as a therapeutic approach to treating T2DM. This study is to characterize a RPTOR deficient fibroblast cell line and its role in glucose metabolism in vitro and in vivo.

2.2 Materials and methods

Chemicals and Reagents

Unless otherwise noted, we purchased reagents from Sigma-Aldrich (St. Louis, MO), cell culture media from Invitrogen (Carlsbad, CA). Primary antibodies β-actin was obtained from Cell Signaling Technology (Danvers, MA). UCP1 was purchased from ABCAM (Cambridge, MA). Secondary antibodies were purchased from LI-COR Biosciences (Lincoln, Nebraska).

Cell line engineering

To create a Rptor deficient (knockdown) (3T3\(^{RptorKD}\)) or the scrambled shRNA control 3T3-L1 (3T3\(^{Ctrl}\)) preadipocyte cell line, we performed stable transfection of shRNA set against Rptor (RIKEN cDNA 4932417H02 gene, alias name raptor, Rptor, GFP positive, puromycin resistant, GeneCopoeia, Rockville, MD), or shRNA scrambled control (GFP positive, puromycin resistant, GeneCopoeia, Rockville, MD) packed in a lentivirus using
a Lenti-Pac FIV Expression Packaging Kit (GeneCopoeia, Rockville, MD). Briefly, lentiviral titers were produced using Lenti-Pac™ Lentiviral Packaging Kits (GeneCopoeia, Rockville, MD). A total of 1.3–1.5 × 10^6 of the HEK293Ta Lentiviral Packaging cells (GeneCopoeia, Rockville, MD) were plated in a 10-cm dish 2 days before transfection in DMEM (10 mL), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS). Puromycin resistant lentiviral shRNA against Rptor cDNA clone (2.5 μg; GeneCopoeia, Rockville, MD), 5 μL Lenti-Pac FIV mix, and 15 μL of EndoFectin–Lenti were diluted into 200 μL Opti-MEMI (Invitrogen, Grand Island, NY). The DNA–EndoFectin–Lenti complex was incubated at room temperature for 15 min and then added directly to the cells. Cells were incubated in a CO₂ incubator at 37°C for 8–14 h. TiterBoost reagent (GeneCopoeia, Rockville, MD, 0.2%) was added to the culture medium. Culture medium containing either the Rptor shRNA or control pseudovirus vector was collected 48 h post transfection after being centrifuged at 2000 rpm for 15 min. and used for the transfection. Murine 3T3-L1 preadipocytes were grown in a 6-well plate until 80% confluent. Then they were transfected with 1 mL of the appropriate vector-containing supernatant, 0.5 μL Polybrene (Millipore, Billerica, MA) in 0.5mL 10% calf serum (CS). 24 h post transfection, cells were replaced with a standard culture medium (DMEM containing 10% CS and 1% penicillin-streptomycin). At 90% confluence, cells were plated into 96-well plate with puromycin (1.0 mg/mL, Invitrogen, Grand Island, NY). The survived cells were plated in 96 well to achieve a density of single cell per well. Stably transfected Rptor knocked down clone, 3T3^{RptorKD}, or scrambled control, 3T3^{Ctrl}, were derived from a single cell and green fluorescence was confirmed by
fluorescence microscope. Expression of Rptor was measured by RT-PCR. The clone least expressing Rptor was selected for 3T3 RpKD.

**mRNA analysis**

mRNA was purified from adipocytes or adipose tissue according to the manufacturer’s instructions (Qiagen, Germantown, MD) and quantified using 7900HT Fast Real-Time PCR System and TaqMan fluorogenic detection system (Applied Biosystems, Grand Island, NY). Validated primers were also purchased from Applied Biosystems (Grand Island, NY). Comparative RT-PCR was performed in triplicate, including no-template controls. Expression was calculated using the comparative Ct method normalized to 18S or Tata box protein (TBP).

**Cell differentiation**

All preadipocytes (3T3 RpKD, 3T3 Ctrl, 3T3-L1) were cultured in DMEM medium containing 10% calf serum. Lipogenic differentiation medium contained 10% FBS, 10µg/mL insulin, 1µM dexamethasone, 0.5mM 3-isobutyl-1-methyl xanthine. Medium was replaced every 48 hours with DMEM containing 10% FBS, 10µg/mL insulin, and continued for 7 days.

**Glucose Uptake and TG Assay**

Glucose uptake experiments were performed in differentiated or non-differentiated cells. 10,000 of each type of cell were incubated overnight in growth medium. Cells were
washed with PBS and treated with 2-NBDG (a fluorescently-labeled deoxyglucose analog, 100 μg/mL, Cayman Chemical) and insulin (10 μg/mL, bovine, Sigma) in DMEM (glucose and phenol red free, Gibco, Invitrogen, Carlsbad, CA) for 15 min at 37°C. Excess 2-NBDG was removed by washing three times with PBS. Fluorescence was read at 485/535 nm (excitation/emission).

TG were measured according to manufacturer’s instructions using a calorimetric kit L-Type Triglyceride M Kit (Wako Diagnostics; Richmond, VA).

**Encapsulation**

The phase microencapsulation technique has been performed as described in [57]. In short, the cell suspension (1 x 10^6 cells/ml in 2% sodium alginate solution) was extruded through a 0.4-mm needle into a 100mM CaCl₂ solution, using Encapsulation Unit (Nisco Engineering, AG, Switzerland) at 5.4kV to form calcium alginate gel beads. Microbeads were solidified for 20 min, and then incubated with 0.05% (w/v) poly-L-lysine (MW 20,700) to form alginate-poly-L-lysine membrane around the surface. The core was liquefied using 50mM sodium citrate. Capsules’ properties were characterized before [57]. Encapsulated preadipocytes were maintained in culture under standard conditions for up to 30d.

**Animal studies and metabolic measurements**

All experimental protocols were approved by the Institutional Animal Care and Use Committee.
Six 3-month-old wild type C57BL/6J (WT) female mice were randomly assigned into two groups (n=3 each) and fed ad libitum a high-fat diet (HF, 45% kcal from fat, D12451, Research Diets Inc., New Brunswick, NJ) for 78 days. All mice had similar weights before treatments. After injection of mice into both visceral (peri-ovarian) depots following treatment groups were studied:

1. Control treatment group (WT[Emp]) Female WT mice were injected with vehicle (0.3 mL of acellular (empty) capsules in 0.5 mL of sterile phosphate buffer (PBS) per visceral depot).

2. Encapsulated 3T3-L1 group (WT[RpKD]) (n=5 at the conclusion of the study). Female WT mice were injected with encapsulated 3T3-L1 preadipocytes (0.3*10⁶ 3T3RpKD cells in 0.3 mL of capsules in 0.5 mL of PBS per visceral depot).

Both groups had similar weights (P<0.43) before treatment of 42.6 and 39.2 g for WT[Emp] and WT[RpKD] respectively. After injection, mice continued on a similar HF diet for 40 days. Food intake and weight was monitored. Two weeks prior to the end of the study, metabolic parameters in the treated mice were measured by indirect calorimetry. Food intake and weight was monitored. Metabolic parameters in the treated mice were measured by indirect calorimetry (CLAMS, Columbus Instruments, Columbus, OH) at ambient temperature (22°C) with 12 hours light/dark cycles. Animals were fed the same HF chow diet and water provided ad libitum. Mice were placed individually and allowed to acclimate to the metabolic cages. O₂ consumption, CO₂ production, energy expenditure, and locomotor activity were measured for 48 hours. Based on these data,
respiratory quotient or exchange ratio (CO₂/O₂) and Δ heat values were calculated by CLAMS.

For glucose tolerance tests (GTT) and insulin tolerance tests (ITT) mice were fasted overnight. They were injected with a single intraperitoneal glucose dose (0.001 g glucose/g body weight) for GTT. Blood was obtained from mouse tails. Blood glucose was measured by One Touch Ultra glucometer (LifeScan). For mice were fasted overnight. Mice allowed recovered for at least one week, then ITT test was performed using a single intraperitoneal insulin dose (0.5 mU of insulin/g body weight). Blood glucose was measured.

**Organ collection, lipid extraction, and western blot analysis**

Mice were anesthetized. Blood was collected by cardiac puncture into EDTA-containing tubes. Plasma was prepared by centrifugation. One whole subcutaneous and visceral fat pad were dissected and homogenized in RIPA buffer with protease inhibitors. Aliquot from fat pad homogenates were used for protein (Bicinchoninic Acid Kit, Thermo Scientific, Waltham, MA) measurements, and Western blots. For Western blot analyses, tissue lysates were separated on 10% acrylamide gel under reducing conditions. After transfer to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Billerica, MA), proteins were analyzed using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Images were quantified by ImageJ software.

**Immunohistochemistry**
Subcutaneous and visceral fat pads were embedded in paraffin. Sections (14µm) were stained with hematoxylin and eosin (H&E) using a modified hematoxylin QS procedure [126] followed by dehydration in graded alcohol [127].

**Statistical analysis**

Data are shown as mean±SD of experiments that were performed at least with n=3 in vitro and n=3 in vivo unless otherwise noted. Group comparisons were performed using the two-tailed Student’s *t*-test with a *P* value less than 0.05 to be considered significant unless otherwise indicated.

**2.3 Results**

To create a *Rptor* knockdown cell line, 3T3-L1 preadipocytes were stably transfected with shRNA against *Rptor* (3T3*RptorKD*). *Rptor* expression was knocked down by 95.6% when compared to scrambled control (3T3*Ctrl*) (Fig. 2.1A). To show that decreased *Rptor* expression increases thermogenesis, cells were cultured, differentiated, and lysed with RIPA buffer and inhibitors. The triglyceride to protein concentration ratio from the lysates was calculated to be 0.0052 ± 0.002 and 0.0010 ± 0.0001 (g/mL to g/mL) for 3T3*Ctrl* and 3T3*RptorKD* respectively. The ratio of triglyceride concentration to protein concentration was greater for 3T3*Ctrl* than 3T3*RptorKD* (*P*<0.005) (Fig. 2.1B). To measure if glucose metabolism was accelerated in the absence of *Rptor*, fluorescence glucose uptake tests were performed in both differentiated and non-differentiated cells. Non-differentiated 3T3*RptorKD* was able to take up 45.3% more glucose than control (*P*<0.001).
In differentiated cells, 3T3\textsuperscript{RpKD} was able to uptake 144\% (\(P<0.05\)) and 125\% (\(P<0.05\)) more glucose than 3T3-L1 and 3T3\textsuperscript{Ctrl} respectively (Fig. 2.1D).

Mice were fed a HF diet and then injected with either acellular capsules (empty, WT\textsuperscript{Emp}) or 3T3\textsuperscript{RpKD} capsules (WT\textsuperscript{RpKD}). Food intake and weights were recorded. Weights were normalized to 100\% on injection day. Three days after injection, the percent body weight of WT\textsuperscript{RpKD} was less than WT\textsuperscript{Emp} (Fig. 2.2A). Percent body weight was reduced by 6.9\% for WT\textsuperscript{RpKD} and the control WT\textsuperscript{Emp} percent body weight was only reduced by 0.6\%, showing a minimal effect of the injection of empty capsules. Weights were statistically the same at the end of the study 40 days later (Fig. 2.2C). Encapsulated 3T3\textsuperscript{RpKD} cells were found inside of capsules embedded in the VF (Fig. 2.2B). The border of the capsule glowed green under fluorescence because of the GFP positive 3T3\textsuperscript{RpKD}.

Two weeks prior to sacrifice, mice were placed in metabolic cages. The total activities of the mice were not different over a 24 hour period (Fig. 2.2A). WT\textsuperscript{Emp} had a greater average VO\textsubscript{2} than WT\textsuperscript{RpKD} for both the dark and light cycles (3323 \pm 419 vs. 2919 \pm 195 mL/kg/h and 2913 \pm 252 vs. 2482 \pm 177 mL/kg/h, \(P<0.001\)) (Fig. 2.2B). This coincided with a greater average respiratory exchange ratio (RER) for WT\textsuperscript{Emp} during the light and dark cycles than WT\textsuperscript{RpKD} (0.78 \pm 0.02 vs. 0.75 \pm 0.02 and 0.83 \pm 0.025 vs. 0.81 \pm 0.025, \(P<0.001\)) (Fig. 2.2C). The average metabolic rate was higher for WT\textsuperscript{Emp} than WT\textsuperscript{RpKD} for both the dark (15.86 \pm 1.96 kcal/kg/h vs. 13.85 \pm 0.91 kcal/kg/h, \(P<0.001\)) and light (14.12 \pm 1.28 kcal/kg/h vs. 11.95 \pm 0.88 kcal/kg/h, \(P<0.001\)) phases (Fig. 2.2D).
GTT and ITT tests were performed on mice one week prior to sacrifice. $WT^{RpKD}$ had a lower concentration of glucose than $WT^{Emp}$ (334 ± 48 mg/dL vs. 490 ± 29 mg/dL, $P<0.01$) 30 min after the mice received a standardized dose of glucose injected intraperitoneally (Fig. 2.3A). AUC, normalized to percent of control ($WT^{Emp}$), for GTT were statistically lower for $WT^{RpKD}$ (76.0 ± 7.62 %) than $WT^{Emp}$ (100 ± 9.40 %). ITT tests were not significantly different at any time points.

SF masses were not different for $WT^{Emp}$ (2.3 ± 0.7 g) and $WT^{RpKD}$ (2.6 ± 0.4 g) (Fig. 2.5A). Likewise, VF masses were not different for $WT^{Emp}$ (4.4 ± 1.4 g) and $WT^{RpKD}$ (4.8 ± 1.0 g) (Fig. 2.5B). The ratio of triglyceride concentration to protein concentration were the same for $WT^{Emp}$ (4.71 ± 2.1) and $WT^{RpKD}$ (4.46 ± 3.1) SF depots (Fig. 2.5C). The ratio of triglyceride concentration to protein concentration was higher for $WT^{Emp}$ than $WT^{RpKD}$ (9.95 ± 1.56 vs. 1.8 ± 0.43, $P<0.001$) in the VF depot (Fig. 2.5D). The ratio of ATGL normalized to beta-actin was not changed in SF (Fig. 2.5E). There was more ATGL in the VF of $WT^{RpKD}$ than $WT^{Emp}$ (0.38 ± 0.05 vs. 0.24 ± 0.06, $P<0.05$).

2.4 Discussion

A previous study that knockout $Rptor$ in adipose tissue generated a lean thermogenic phenotype [94]. We encapsulated $Rptor$ knockdown 3T3-L1 preadipocytes ($3T3^{RpKD}$) and injected them into mice fed a HF diet. We successfully knocked $Rptor$ down (Fig. 2.1A) and these cells had a more thermogenic profile than scrambled control because less triglycerides were in $3T3^{RpKD}$ compared to scrambled control. Glucose
metabolism was improved in 3T3<sup>RpKD</sup>. 3T3<sup>RpKD</sup> had greater glucose uptake than control in both differentiated and non-differentiated conditions (Fig. 2.1C-D).

To see if Rptor knockdown cells had a thermogenic effect in vivo, 3T3<sup>RpKD</sup> was successfully encapsulated and injected into mice fed a HF diet. Proper injection was confirmed from histological VF images showing GFP positive capsules (Fig. 2.2B). These capsules attenuated weight gain initially (Fig. 2.2A). It could have been that food intake increased to compensate for increases in thermogenesis or metabolism (Fig. 2.2D). At the end of the study, weights were the same signifying that encapsulated 3T3<sup>RpKD</sup> did not have a long term effect on body weight.

Surprisingly, WT<sup>Emp</sup> had a higher metabolic rate and RER (Fig. 2.3C-F). Encapsulated 3T3<sup>RpKD</sup> did not improve metabolic rate as was initially expected. However, even with a lower resting metabolic rate, WT<sup>(RpKD)</sup> had better glucose metabolism than WT<sup>Emp</sup>, as shown by better GTT (Fig. 2.4A,C). These data are consistent with the greater glucose uptake for 3T3<sup>RpKD</sup> in vitro. However, Insulin sensitivity remained unchanged (Fig. 2.3B,D). There could be a more localized effect of the capsules rather than a systemic effect.

To see the localized changes, fat pads were examined. Fat pad mass was similar between groups (Fig. 2.5A,B). Strikingly, triglyceride levels standardized to protein were lower in the VF depot of WT<sup>(RpKD)</sup> indicating that these capsules could be having a localized effect (Fig. 2.5D). It was shown previously that ATGL is over expressed in a 3T3-L1 Rptor knockdown model [121]. Here we show that ATGL is present in greater amounts in the VF of WT<sup>(RpKD)</sup> (Fig. 2.5F). Unfortunately, UCP1 was not detected in
vitro or in vivo (data not shown). Increasing \textit{Ucp1} mRNA expression by knocking out \textit{Rptor} in adipose tissue has only been reported in Polik et al [94]. Protein levels of UCP1 were not reported in either of the two cited studies [94, 121]. To our knowledge, increased \textit{Ucp1} expression has not been reported using in vitro knockdown models. Encapsulated 3T3\textsuperscript{RpKD} were not sufficient to produce the same lean phenotype as the adipose specific knockout in Polik et al [94].

For the first time, we show that encapsulated fibroblasts with impaired mTOR function because of a knockdown of \textit{Rptor} are capable of remodeling the VF depot. These encapsulated adipocytes can survive, increase ATGL, and increase TG catabolism. However, this is independent of better insulin sensitivity or increases in metabolic rate. Future studies should look at improving the weight reducing and appetite suppressing effects to prevent overeating because of increased thermogenesis.
Fig. 2.1 Rptor knockdown decreases triglycerides and improves glucose uptake

3T3-L1 cells were knocked down by a lentiviral stable transduction of shRNA against Rptor or with a scrambled control vector. (A) Rptor expression for 3T3\textsuperscript{RDKD} and scrambled control (3T3\textsuperscript{Ctrl}) was measured by RT-PCR normalized to 18s (n=3, \(P<0.001\)). (B) Cells were differentiated for 7 d, lysed in RIPA buffer with protease and phosphatase inhibitors. The ratio of triglyceride to protein concentration from the cell lysates was calculated (unitless, n=4, \(P<0.005\)). (C) Glucose uptake was performed using fluorescently labeled glucose analog in non-differentiated cells. (n=8, \(P<0.005\)). (D) Glucose uptake was performed using fluorescently labeled glucose after lipogenic differentiation for 7 d. Rptor knockdown increased glucose uptake when compared to 3T3-L1 and 3T3\textsuperscript{Ctrl} (n=6). All data are represented as mean ± standard deviation. Significant different comparisons are shown by a black line.
Fig. 2.2 Encapsulated Rptor deficient cells attenuate weight gain and survive injection

(A) Both groups (n=3) were fed a high fat diet and injected on day 76 with either empty capsules (WT\[^{Emp}\]) or 3T3\[^{RptKd}\] (WT\[^{RptKd}\]). Mice weights were normalized to their individual weights just prior to injection (g of mouse/g of mouse on injection day x 100) to accurately show weight changes relative to the mouse’s original weight. Three days after injection normalized weight was lower for WT\[^{RptKd}\] (n=3, *P<0.05). (B) A capsule (green C) is surrounded by adipocytes (green A). Under fluorescence, the capsule glows from the presences of functional GFP from the encapsulated 3T3\[^{RptKd}\]. (C) Average weights in grams at sacrifice. Weights were not statistically different between groups. (D) Mice were housed in groups. The food intake is recorded as g/mouse/day (n=1). All data are shown as mean ± standard deviation.
Fig. 2.2

A

Injected

Weight (%)

125

100

50

0 50 50 75 100

Time (days)

WT\(^{RpKO}\)

WT\(^{Emp}\)

\(P < 0.05\)

B

e-cells

C

A

A

A

A

C

A

A

A

A

30 µm

C

Weight (g)

40

30

WT\(^{Emp}\)

WT\(^{RpKO}\)

D

Food Intake (g/mouse/day)

4

2

0

75 85 95

Time (days)

Injected

WT\(^{RpKO}\)

WT\(^{Emp}\)
Mice were placed in metabolic cages for 48 h. After acclimation, metabolic parameters were measured. (A) Total activity, as measured by the sum of the x,y, and z axis, was not statistically different between groups (n=3). (B, C, D) VO₂, RER, and metabolic rate was higher in WT[^3T3] than WT[^RpKD] for both the light and dark time cycles (n=3, P<0.001). (E) WT[^3T3] (black open circles) had a greater RER than WT[^RpKD] (red filled circles) at various time points (n=3, * P<0.05). WT[^3T3] (black open circles) had a greater metabolic rate than WT[^RpKD] (red filled circles) at various time points (n=3, * P<0.05). All data in A-D are represented as mean ± standard deviation. Data points in E and D are means.
Fig. 2.4 Rptor deficient fibroblast capsules improve glucose tolerance

Mice were starved overnight (12 h) during the dark phase. (A) Blood glucose was injected intraperitoneally into each mouse and blood glucose readings were taken at the indicated time points. WT[RpKD] (black filled square) had a statistically lower blood glucose level than WT[3T3] at the 30 min time point (n=3, * P<0.01). (B) Blood glucose was measured after an intraperitoneal injection of insulin. WT[RpKD] and WT[3T3] were statistically the same at all time points. (C) Area under the curve (AUC), calculated using a trapezoidal approximation of the integral, is represented at percent of the control for GTT. AUC for WT[RpKD] was less than control (n=3, P<0.05). (D) AUC represented at percent of the control for GTT. AUC was the same for both groups. Data points are represented as mean ± standard deviation.
Fig. 2.5 *Rptor* deficient fibroblast capsules increase ATGL in vivo

Whole SF and VF pads were removed and homogenized in RIPA buffer with protease and phosphatase inhibitors. (A, B) SF and VF pads were weighed prior to homogenization. SF and VF mass did not differ between groups (n=3). (C) Triglyceride (TG) and protein concentration was measured in SF homogenates using a colorimetric kit. The SF triglyceride to protein concentration ratio (unitless) did not differ. (D) Likewise, TG and protein concentration was measured in VF homogenates. WT[^RpkD] had a statistically lower VF triglyceride to protein concentration ratio (unitless) when compared to WT[^Emp] (n=3, * P<0.05). (E) SF protein lysates were stained for ATGL and were normalized to beta-actin. There was no significant difference between groups for relative ATGL levels. (F) VF protein lysates were stained for ATGL and were normalized to beta-actin. WT[^RpkD] had a statistically higher ATGL than WT[^Emp] (n=3, * P<0.05). All data are shown as mean ± standard deviation.
Chapter 3

*Rptor* knockdown cells further engineered to treat models of obesity: A pilot study

3.1 Introduction

In mice, *Rptor* deficient adipocytes were shown in the literature to be thermogenic [94, 121]. It is possible to engineer cell lines using lentiviral based stable transduction. Using this technology, we engineered *Rptor* deficient 3T3-L1 fibroblasts, which showed promise in our previous studies mentioned in chapter two. To create an engineered cell line that maximized health promoting effects, we targeted two metabolic mechanisms: nutrient transport pathways and appetite suppression.
**Transport Pathways**

Thermogenic cells can utilize two principal energy sources: lipids and glucose. Firstly, burning unnecessary lipids is important to patients suffering from obesity. In order for thermogenic cells to have increased fatty acid utilization, fatty acids must first be transported into the cell. Lipoprotein lipase (LPL) may function as a fatty acid delivery mechanism for the thermogenic cells. LPL resides in the cell membrane in a complex with proteoglycanes and is responsible for regulating triglyceride levels by hydrolyzing plasma triglycerides found in lipoproteins. Dysregulation of this protein may result in diabetes, atherosclerosis, and possibly cancer [128]. For example, one polymorphism, a missense mutation in exon five (I225N), renders LPL ineffective and causes diabetic lipaemia [129]. LPL may also play a role in satiety. LPL cleaves circulating triglycerides and forms free fatty acids, which can send signals to the brain [130]. In a neuron-specific LPL-deficient mouse model, mice became obese and had impaired satiety signal to the brain [130]. LPL overexpression could increase the energy consumption of thermogenic cells by increasing catabolism of triglycerides. However, it has been reported that LPL is sufficient to cause accumulation of LDL, resulting in a greater problem for atherosclerosis patients [131]. However, this was a mechanistic study in vitro that only looked at lipoprotein accumulation and not metabolism. On the other hand, ApoE<sup>−/−</sup> and LDL receptor deficient mice, which develop atherosclerosis, are protected from atherosclerosis if they express LPL. Furthermore, it has been discovered that some hypertriglyceridemic patients without autoimmune disease have anti-LPL antibodies [132]. Therefore, triglyceride levels increase because LPL is
destroyed by the immune system. If encapsulated cells overexpressing LPL are localized to visceral fat tissue, then the encapsulated cells are expected to increase lipid consumption and catabolism in this tissue without raising hepatic LDL production.

In a similar fashion, glucose transport may be increased into a thermogenic cell via increased expression of glucose transporter 4 (GLUT4). GLUT4 locates to the cell’s surface in response to insulin. Diabetic patients are insulin insensitive primarily due to the malfunction of GLUT4. This could be because GLUT4 is downregulated in type 2 diabetes patients [133]. Furthermore, adipose tissue specific GLUT4 deficient mice develop insulin insensitivity [134]. In GLUT4 muscle specific knock out model, GLUT4 overexpression in the adipose tissue is sufficient to reverse insulin insensitivity [135]. Overexpressing GLUT4 could increase the uptake of glucose into the encapsulated cell. The increased influx of glucose into the thermogenic cell should increase the glucose consumption capacity of the cell, possibly, with subsequent lipogenesis and uncoupled lipid oxidation.

**Appetite suppression**

Previous data showed that when thermogenic capsules were injected into mice fed a high fat diet, the mice initially lost weight ([37] and chapter 2). However, the weight was regained. The weight gain was possibly due to an increase in food consumption to compensate for increased thermogenesis. Amylin (also known as islet amyloid polypeptide) and leptin are appetite suppressing hormones that signal the body when it is full and prevent a person from constantly eating [136, 137]. Leptin and amylin work
synergistically to suppress appetite and reduce obesity because amylin functions as a
short-term satiety signal and leptin functions as a long-term satiety signal [137].

We investigated the role of engineered cells in glucose uptake in vitro and in a
preliminary study of encapsulated thermocytes overexpressing the appetite suppressing
hormones, leptin and amylin, in WT mice fed a HF diet.

3.2 Materials and methods

**Chemicals and Reagents**

Unless otherwise noted, we purchased reagents from Sigma-Aldrich (St. Louis, MO), cell
culture media from Invitrogen (Carlsbad, CA).

**Cell line engineering**

The generation of the *Rptor* knockdown cell line (3T3*RptorKD*) was described in chapter 2.
For this study, multiple different cell lines were created using lentiviral transduction from
GeneCopoeia, (Rockville, MD). GeneCopoeia (Rockville, MD) provided all ORF cDNA
vectors to be packed in lentiviral titers. To create the overexpressing *Lpl* thermocyte, we
used an ORF cDNA *Lpl* vector (catalog number EX-Mm20200-Lv72 CMV promoter,
neomycin resistant, mCherry labeled C-terminus) to generate viral titers and stably
transduced 3T3*RptorKD* (*RptorKD*LPL+). To create an overexpressing *Glut4* thermocyte, we
used an ORF cDNA *Glut4* vector (catalog number EX-Mm30073-Lv72, CMV promoter,
neomycin resistant, mCherry labeled C-terminus) to generate viral titers and stably
transduced 3T3<sup>RpKD</sup> (<i>RpKD<sup>Glut4</sup></i>). To create an appetite suppressing thermocyte, we used an ORF cDNA <i>Iapp</i> (amylin) vector (EX-Mm03193-Lv62, CMV promoter, neomycin resistant, eCFP C-terminus labeled) and ORF cDNA <i>Lep</i> (leptin) vector (catalog number EX-Mm03615-Lv09, CMV promoter, neomycin resistant, eYFP C-terminus labeled) to generate viral titers and stably transduced 3T3<sup>RpKD</sup> (LepAR). We performed stable transductions using a Lenti-Pac FIV Expression Packaging Kit (GeneCopoeia, Rockville, MD). Briefly, lentiviral titers were produced using Lenti-Pac™ Lentiviral Packaging Kits (GeneCopoeia, Rockville, MD). A total of 1.3–1.5 × 10<sup>6</sup> of the HEK293Ta Lentiviral Packaging cells (GeneCopoeia, Rockville, MD) were plated in a 10-cm dish 2 days before transfection in DMEM (10 mL), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS). Neomycin resistant lentiviral ORF cDNA clone (2.5 μg; GeneCopoeia, Rockville, MD), 5 μL Lenti-Pac FIV mix, and 15 μL of EndoFectin–Lenti were diluted into 200 μL Opti-MEMI (Invitrogen, Grand Island, NY). The DNA–EndoFectin–Lenti complex was incubated at room temperature for 15 min and then added directly to the cells. Cells were incubated in a CO<sub>2</sub> incubator at 37°C for 8–14 h. TiterBoost reagent (GeneCopoeia, Rockville, MD, 0.2%) was added to the culture medium. Culture medium containing the appropriate pseudovirus vector was collected 48 h post transfection after being centrifuged at 2000 rpm for 15 min. and used for the transfection. Murine 3T3<sup>RpKD</sup> preadipocytes were grown in a 6-well plate until 80% confluent. Then they were transfected with 1 mL of the appropriate vector-containing supernatant, 0.5 μL Polybrene (Millipore, Billerica, MA) in 0.5 mL 10% calf serum (CS). 24 h post transfection, cells were replaced with a standard culture medium (DMEM
containing 10% CS and 1% penicillin-streptomycin). At 90% confluence, cells were plated into 96-well plate with neomycin (1.0 mg/mL, Invitrogen, Grand Island, NY). The survived cells were plated in 96 well to achieve a density of single cell per well. Stably transfected cells were derived from a single cell and green fluorescence was confirmed by fluorescence microscope. Expression of Lpl, Glut4, Iapp, and Lep was measured by RT-PCR.

**mRNA analysis**

mRNA was purified from adipocytes or adipose tissue according to the manufacturer’s instructions (Qiagen, Germantown, MD) and quantified using 7900HT Fast Real-Time PCR System and TaqMan fluorogenic detection system (Applied Biosystems, Grand Island, NY). Validated primers were also purchased from Applied Biosystems (Grand Island, NY). Comparative RT-PCR was performed in triplicate, including no-template controls. Expression was calculated using the comparative Ct method normalized to 18S.

**Cell differentiation**

All preadipocytes were cultured in DMEM medium containing 10% calf serum. Lipogenic differentiation medium contained 10% FBS, 10μg/mL insulin, 1μM dexamethasone, 0.5mM 3-isobutyl-1-methyl xanthine. Medium was replaced every 48 hours with DMEM containing 10% FBS, 10μg/mL insulin, and continued for 7 days.
**Glucose Uptake and TG assay**

Glucose uptake experiments were performed in differentiated or non-differentiated cells. 10,000 of each type of cell were incubated overnight in growth medium. Cells were washed with PBS and treated with 2-NBDG (a fluorescently-labeled deoxyglucose analog, 100 μg/mL, Cayman Chemical) and insulin (10 μg/mL, bovine, Sigma) in DMEM (glucose and phenol red free, Gibco, Invitrogen, Carlsbad, CA) for 15 min at 37˚C. Excess 2-NBDG was removed by washing three times with PBS. Fluorescence was read at 485/535 nm (excitation/emission).

TG were measured according to manufacturer’s instructions using a calorimetric kit L-Type Triglyceride M Kit (Wako Diagnostics; Richmond, VA).

**Encapsulation**

The phase microencapsulation technique has been performed as described in [57]. In short, the cell suspension (1 x 10^6 cells/ml in 2% sodium alginate solution) was extruded through a 0.4-mm needle into a 100mM CaCl₂ solution, using Encapsulation Unit (Nisco Engineering, AG, Switzerland) at 5.4kV to form calcium alginate gel beads. Microbeads were solidified for 20 min, and then incubated with 0.05% (w/v) poly-L-lysine (MW 20,700) to form alginate-poly-L-lysine membrane around the surface. The core was liquefied using 50mM sodium citrate. Capsules’ properties were characterized before [57]. Encapsulated preadipocytes were maintained in culture under standard conditions for up to 30d.
Animal studies and metabolic measurements

All experimental protocols were approved by the Institutional Animal Care and Use Committee.

Thirteen 2-month-old wild type C57BL/6J (WT) female mice were assigned into three groups and fed ad libitum a high-fat diet (HF, 45% kcal from fat, D12451, Research Diets Inc., New Brunswick, NJ) for 132 days. After injection of mice into both visceral (peri-ovarian) depots following treatment groups were studied:

1. Control treatment group (WT$^{[\text{Emp}]}$) Female WT mice were injected with vehicle (0.3 mL of acellular (empty) capsules in 0.5 mL sterile phosphate buffer (PBS) per VF depot).

2. Encapsulated 3T3$^{RpKD}$ preadipocytes group (WT$^{[RpKD]}$) (n=4 at the conclusion of the study). Female WT mice were injected with encapsulated 3T3$^{RpKD}$ preadipocytes (0.3*10$^6$ cells in 0.3 mL of capsules in 0.5 mL of PBS per visceral depot).

3. Encapsulated LepAR preadipocytes group (WT$^{[\text{LepAR}]}$) (n=4 at the conclusion of the study) Female WT mice were injected with encapsulated 3T3$^{RpKD}$ preadipocytes (0.3*10$^6$ LepAR in 0.3 mL of capsules in 0.5 mL of PBS per visceral depot)

All three groups had dissimilar average weights of 24.1 ± 1.3, 29.8 ± 2.5, and 31.9 ± 5.9 g ($P$<0.05) before treatment for WT$^{[\text{Emp}]}$, WT$^{[RpKD]}$, and WT$^{[\text{LepAR}]}$ respectively. Weights were normalized to the weight of the mouse at injection (g of mouse / g of mouse on injection day x 100). After injection, mice continued on a similar HF diet for 239 days. Food intake and weight was monitored for 119 days post injection. There was a significant delay in sacrificing the mice due to unforeseen circumstances.
1 month prior to the completion of the study glucose tolerance testers were performed (GTT) mice were fasted overnight. They were injected with a single intraperitoneal glucose dose (0.001g glucose/g body weight) for GTT. Blood was obtained from mouse tails. Blood glucose was measured by One Touch Ultra glucometer (LifeScan).

**Organ collection, lipid extraction, and western blot analysis**

Mice were anesthetized. Blood was collected by cardiac puncture into EDTA-containing tubes. Plasma was prepared by centrifugation. One whole subcutaneous and visceral fat pad were dissected and homogenized in RIPA buffer with protease inhibitors. Aliquot from fat pad homogenates were used for protein (Bicinchoninic Acid Kit, Thermo Scientific, Waltham, MA) measurements, and Western blots. For Western blot analyses, tissue lysates were separated on 10% acrylamide gel under reducing conditions. After transfer to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Billerica, MA), proteins were analyzed using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Images were quantified by ImageJ software.

**Immunohistochemistry**

Subcutaneous and visceral fat pads were embedded in paraffin. Sections (14µm) were stained with hematoxylin and eosin (H&E) using a modified hematoxylin QS procedure [126] followed by dehydration in graded alcohol [127].
Statistical analysis

Data are shown as mean±SD of experiments that were performed at least with n=3 in vitro and n=3 in vivo unless otherwise noted. Group comparisons were performed using the two-tailed Student’s t-test with a P value less than 0.05 to be considered significant unless otherwise indicated.

3.3 Results

*Glut4* or *Lpl* or both was stably transduced into 3T3*RpKD*. To verify that clones were stably transfected, mRNA levels were measured in non-differentiated clones with RT-PCR normalized to 18s. *Glut4* was stably transduced into 3T3*RpKD* fibroblasts (Fig. 3.1A). Clones overexpressing *Glut4*, *RpKD*\textsuperscript{*Glut4*+} and *RpKD*\textsuperscript{*Glut4*/LPL+}, have 300 times higher *Glut4* expression than *RpKD*\textsuperscript{LPL+} not overexpressing *Glut4* (P<0.005). *Lpl* expression was 1.6 times greater in *RpKD*\textsuperscript{LPL+} than *RpKD*\textsuperscript{*Glut4*+} and 1.4 times greater in *RpKD*\textsuperscript{*Glut4*/LPL+} than *RpKD*\textsuperscript{*Glut4*+}. Glucose uptake was performed using a fluorescently labeled glucose analog (2-NBDG) in these cells and normalized to *RpKD*\textsuperscript{LPL+}. *RpKD*\textsuperscript{*Glut4*+} uptook 188 ± 40 % more glucose than *RpKD*\textsuperscript{LPL+}. *RpKD*\textsuperscript{*Glut4*/LPL+} uptook 160 ± 110 % more glucose than *RpKD*\textsuperscript{LPL+}.

To generate a leptin and amylin producing raptor deficient preadipocyte line, 3T3-L1 were stably transduced to overproduce leptin and amylin (Fig. 3.2A,B). *Iapp* expression was 52 times greater in LepAR than 3T3-L1. *Lep* expression was 29 times greater in LepAR than 3T3-L1. Glucose uptake was not changed with transduction of *Lep* and *Iapp* into 3T3*RpKD*. Glucose uptake is represented as percent up take relative to
the glucose uptake of 3T3<sup>Ctrl</sup> 3T3<sup>RpKD</sup> uptake of 145 ± 31 % was statistically the same as the glucose uptake of LepAR at 134 ± 19 % (<i>P</i><0.29) (Fig. 3.2C).

When LepAR cells were encapsulated and injected into WT mice fed a HF diet, these mice had lower normalized weights on days 9, 45, 51, and 93 than mice injected with empty capsules (<i>P</i><0.05) (Fig. 3.3A). Injections did not change average food intake per day (Fig. 3.3B). Weight was not statistically different at sacrifice (data not shown). There were no differences in VF or SF mass at sacrifice between groups (data not shown). GTT were performed and the AUC was not different between all groups (Fig. 3.3C). Plasma TG levels were lower for WT<sup>(RpKD)</sup> than WT<sup>LepAR</sup> (12 ± 7 vs 29 ± 7 mg/dL, <i>P</i><0.05).

In SF, the ratio of TG concentration to protein concentration did not differ between groups (Fig. 3.4A). TG concentration to grams of SF did not differ as well (Fig. 3.4B). In the VF, WT<sup>(RpKD)</sup> had a lower TG concentration to protein concentration than WT<sup>Emp</sup> and WT<sup>LepAR</sup> (0.58 ± 0.08 vs 0.74 ± 0.07 and 1.16 ± 0.39, <i>P</i><0.05) (Fig. 3.4C). TG concentration to grams of VF did not differ (Fig. 3.4D).

### 3.4 Discussion

Clones (<i>RpKD</i><sup>Glut4+</sup>, <i>RpKD</i><sup>Glut4/LPL+</sup>, and <i>RpKD</i><sup>LPL+</sup>) were generated to create a more thermogenic cell line that could improve glucose and fat metabolism by increasing the rate at which nutrients enter the thermocyte. Open reading frames of <i>Lpl</i> and <i>Glut4</i> were stably transduced into 3T3<sup>RpKD</sup> to overproduce LPL and GLUT4 (Fig. 3.1A,B). The cell lines with a <i>Glut4</i> transduction to constitutively express <i>Glut4</i> were able to take in a
greater amount of glucose in the presence of insulin than control (Fig. 3.1C). However, these cell lines were abandoned from future study for a couple of reasons. Multiple transductions may damage the host cell and the cell could lose it’s thermogenic phenotype. LPL functions on cell’s surface. We were unsure if the A-PLL membrane of the capsule would prevent LPL from functioning correctly because of LPL’s large size (55 kDa). It was also unclear if the 3T3_RpKD cell line was thermogenic enough to handle an influx of nutrients since UCP1 overproduction has not been shown in this cell line in our hands. Therefore, it was determined to use the LepAR cell line for in vivo experiments because of the possible appetite suppressing properties of the leptin and amylin.

The LepAR cell line overexpresses two appetite suppressing hormones, Lep and Iapp (amylin) and was derived from the 3T3_RpKD, which were shown to have Rptor knocked down in chapter 2 (Fig. 3.1A,B). LepAR was as effective at taking up glucose as 3T3_RpKD (Fig. 3.2C). When LepAR was encapsulated in poly-L-lysine and injected into WT mice fed a HF diet, that group’s normalized weight was lower than WT^{Emp} over the course of the study (Fig. 3.3A). However, food intake was not lower showing that leptin and amylin may have not been functioning properly, not overexpressed, or unable to be secreted from the capsule (Fig. 3.3B). GTT were not changed unlike the previous study (Fig. 3.3C). Interestingly, WT^{RpKD} had lower TG levels in the plasma (Fig. 3.3D) than the WT^{LepAR} group and also a lower TG level in the VF than other groups when normalized to protein (Fig. 3.4C). ATGL is responsible for TG catabolism. The lower levels of TG in the VF of WT^{RpKD} could be because of the greater ATGL expression.
These data are consistent with previous data reported on higher ATGL in the VF of mice injected with \textit{Rptor} deficient fibroblasts in chapter two.

However, there were several fatal flaws in this study. The mice were not the same weight on the injection day. These mice were housed in groups and could not be changed because food intake and weight gain rates may change when mouse housing is disrupted [138]. There also may have been issues with the LepAR cell line. Expression levels of \textit{Lep} and \textit{lapp} were only done in with a sample size of two and never repeated. Leptin and amylin protein production in LepAR was never quantified. Leptin and amylin were tagged with a fluorescent tag on the C-terminus. This tag could have prevented the leptin and/or amylin from diffusing through the capsule’s membrane due to increased size. The fluorescent tag could have also interfered with the functionality of the leptin and/or amylin. Lastly, mice were sacrificed 239 days after injection due to extenuating circumstances. This delayed GTT and other assays. The effect of the capsules could have been missed because of the delay in sacrificing the mice. The study on the encapsulation of fibroblasts overproducing leptin in the next chapter hoped to fix these errors.
Fig. 3.1 Glu4 and Lpl stably transduced into Rptor deficient fibroblasts

Glu4 or Lpl or both was stably transduced into 3T3<sup>RpKD</sup>. mRNA levels were measured in non-differentiated clones with RT-PCR normalized to 18s. (A) Glu4 was stably transduced into 3T3<sup>RpKD</sup> fibroblasts. Clones overexpressing Glu4 have higher Glu4 expression than clones not overexpressing Glu4 (n=3, P<0.005). (B) Lpl expression was greater in clones overexpressing Lpl (n=3, P<0.05 or P<0.005 where indicated). (C) Glucose uptake was performed using a fluorescently labeled glucose analog (2-NBDG) and represented as percent fluorescence normalized to RpKD<sup>LPL+</sup>. Clones transduced with Glu4 uptook more glucose than RpKD<sup>LPL+</sup>, a non-Glu4 overexpressing fibroblast (n=3, P<0.005, P<0.001 where indicated). All data are represented as mean ± standard deviation. Statistical comparisons between groups are shown with a black line.
Fig. 3.2 *Rptor* deficient fibroblasts engineered to overexpress *Lapp* and *Lep*

Cells were lipogenically differentiated and *Lep* or *lapp* expression levels were measured via RT-PCR and normalized to 18s. (A) *Lep* expression is shown after lipogenic differentiation for 3T3-L1 and LepAR (n=2). (B) *lapp* levels are shown after lipogenic differentiation for 3T3-L1 and LepAR (n=2). (C) Glucose uptake was performed using a fluorescently labeled glucose analog (2-NBDG) and represented as percent fluorescence normalized to 3T3^Ctrl^. 3T3^RpKD^ and LepAR glucose uptake was greater than control (n=8, *P*<0.001). All data are represented as mean ± standard deviation. Statistical comparisons between groups are shown with a black line.
Acellular (WT\[^{\text{Emp}}\]), 3T3\(^{RpKD}\) (WT\(^{RpKD}\)), or LepAR (WT\(^{\text{LepAR}}\)) fibroblasts were encapsulated in A-PLL and injected in WT mice fed a HF diet. (A) Weight is represented as percent of weight on injection day. Data points are means. WT\(^{\text{LepAR}}\) normalized weight was lower than WT\(^{\text{Emp}}\) at indicated time points (n=4 for WT\(^{\text{Emp}}\), n=5 for WT\(^{\text{LepAR}}\). *\(P<0.05\)). (B) Average food intake (g/day) did not change after injection for any treatment group (paired t-test). (C) The area under the curve (AUC) from glucose tolerance tests (GTT), represented as percent of control (WT\(^{\text{Emp}}\)), did not differ. (D) WT\(^{\text{RpKD}}\) had a lower concentration of TG in the plasma than WT\(^{\text{LepAR}}\) (\(P<0.05\)). Data in B-D are represented as mean ± standard deviation. Statistical comparisons between groups are shown with a black line.
Fig. 3.4 TG levels in adipose tissue

Whole SF for VF pads were removed, weighed, and lysed with RIPA buffer plus phosphatase and protease inhibitors. Protein concentrations were measured using the BCA assay and TG concentrations were measured using a commercially available kit. (A) The ratio of TG concentration to protein concentration (unitless) in SF was not different between groups. (B) Likewise the ratio of TG (mg/mL) to SF mass (g) was not different between groups. (C) WT[RpKD] had a lower ratio of TG concentration to protein concentration in VF (n=4 for WT[RpKD] and WT[Emp], n=5 for WT[LepAR], P<0.05). (D) The ratio of TG (mg/mL) to VF mass (g) was not different between groups. All data are represented as mean ± standard deviation. Statistical comparisons between groups are shown with a black line.
Chapter 4

Encapsulation of Leptin overproducing fibroblasts for treatment of obesity in mice

4.1 Introduction

Obesity affects 150 million people worldwide and is characterized as pandemic disease [1, 2]. In North America, European Union, China, and other countries, obesity is considered to be a major risk factor for up to 70-90% of adult type II diabetes mellitus (T2DM) cases [1, 2, 6]. The treatment of diabetes with insulin induces an accumulation of lipids in adipose, muscle, and other peripheral tissues [97]. These effects reduce the efficacy of insulin therapy and are responsible for the detrimental side effects increasing cardiovascular mortality in patients with diabetes [139]. There is a critical need to develop therapies simultaneously improving lipid and glucose homeostasis for treatment of obesity and diabetes [97].

The adipokine, leptin, has been recently considered as a hormone candidate for managing diabetes in the absence of insulin or in addition to insulin [96]. Leptin mediates its therapeutic effects on energy homeostasis through the hypothalamus and selected action on specific neurons [98-105]. Leptin induces hypothalamic secretion of the appetite suppressing corticotrophin-releasing hormone [102]. Leptin stimulation releases
the sympathetic neurotransmitters, norepinephrine and epinephrine, that increase activation of lipolysis and thermogenesis in WAT and BAT [105, 106]. Leptin works synergistically with insulin to promote thermogenesis in WAT by increasing energy expenditure through hypothalamic neurons [107]. These leptin stimulated effects reduce obesity in mouse models of obesity, human patients with homozygous Lep mutations, and patients with congenital Lep deficiencies when leptin is administered through injections [108-110]. Leptin’s simulation of the hypothalamus increases glucose uptake in BAT, heart muscle, and skeletal muscle, but not WAT [103, 104]. Leptin and insulin also synergize to increase glucose uptake in these tissues in part via suppression of glucagon’s effects [103]. These responses make leptin a promising biological adjuvant for insulin therapy for treatment of diabetes and obesity.

A primary model for development of leptin therapies is the ob/ob mouse (Lep\textsuperscript{ob}, or ob), a homozygous mutant in the leptin (Lep or ob gene) gene. In the absence of functioning leptin, the ob/ob mouse rapidly develops obesity and hyperglycemic conditions similar to T2DM [96, 111-113]. Reintroduction of leptin by injection of exogenous recombinant leptin, adenovirus transduction restoring leptin expression in tissues, or transplantation of tissues producing leptin has the beneficial effects of attenuating weight gain, improving glucose tolerance, decreasing appetite, and increasing metabolic rate in ob/ob mice [96, 114-117]. However, these methods of leptin delivery cannot be directly translated into therapies for humans. Leptin replacement therapy is necessary in patients with homozygous Lep mutations or acquired leptin dysfunction that produces congenital and
acquired generalized lipodystrophy [108-110]. Treating these patients with recombinant leptin through injections produces short-lasting effects and requires repetitive doses. The supra-physiological increase in leptin in the circulation followed by regular injections is associated with serious side effects [51]. One study reports that endogenous production of leptin works better than insulin injections [118]. However, adenovirus carrier of a functioning \textit{Lep} gene [118, 119] or transplantation of tissues or cells expressing functional leptin [15, 117, 120] pose risks of infection, immune rejection of the transplant, or potential genetic viral contamination that could be hazardous to human health. Transplantation models also require drugs to suppress the host’s immune system. To improve transplant survival, Oosman et al, suspend leptin-producing intestinal cells in alginate beads; however, this treatment also requires immunosuppression [117].

Recently, we employed nanotechnology to develop a procedure of adipocyte microencapsulation [95] for transplantation of genetically engineered cells in adipose tissue without immune rejection [37]. The encapsulating poly-L-lysine generates a nanoporous membrane that protects the encapsulated cells from the host’s immune system as well as allows small molecules, like hormones, to diffuse out of the capsule. Here we showed that treatment with encapsulated leptin-producing adipocytes provides an effective solution that improves glucose tolerance in genetic and diet-induced mouse models of obesity. Encapsulated leptin-producing adipocytes also reveal novel aspects of leptin signaling including suppression of resistin in \textit{ob/ob} mice.
4.2 Materials and methods

Chemicals and Reagents

Unless otherwise noted, we purchased reagents from Sigma-Aldrich (St. Louis, MO), cell culture media from Invitrogen (Carlsbad, CA). Primary antibodies β-actin was obtained from Cell Signaling Technology (Danvers, MA). UCP1 was purchased from ABCAM (Cambridge, MA). Secondary antibodies were purchased from LI-COR Biosciences (Lincoln, Nebraska).

Cell line engineering

To derive a leptin overexpressing 3T3-L1 preadipocyte cell line, we performed a stable transfection of 3T3-L1 using an untagged overexpressing leptin clone (GeneCopoeia, Rockville, MD, catalog number EX-Mm03615-Lv67, CMV promoter, puromycin resistant) packaged in a Lenti-Pac FIV Expression Packaging Kit (GeneCopoeia, Rockville, MD) according to manufacturer’s instructions. Briefly, lentiviral titers were produced using Lenti-Pac™ Lentiviral Packaging Kits (GeneCopoeia, Rockville, MD). A total of $1.3\times10^6$ to $1.5\times10^6$ of the HEK293Ta Lentiviral Packaging cells (GeneCopoeia, Rockville, MD) were plated in a 10-cm dish 2 days before transfection in DMEM (10 mL), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS). Puromycin resistant lentiviral ORF leptin cDNA clone (2.5 μg; GeneCopoeia, Rockville, MD), 5 μL Lenti-Pac FIV mix, and 15 μL of EndoFectin–Lenti were diluted into 200 μL Opti-MEMI (Invitrogen, Grand Island, NY). The DNA–EndoFectin–Lenti complex was
 incubated at room temperature for 15 min and then added directly to the cells. Cells were incubated in a CO₂ incubator at 37°C for 8–14 h. TiterBoost reagent (GeneCopoeia, Rockville, MD, 0.2%) was added to the culture medium. Culture medium containing leptin pseudovirus vector was collected 48 h post transfection after being centrifuged at 2000 rpm for 15 min. and used for the transfection. Murine 3T3-L1 preadipocytes were grown in a 6-well plate until 80% confluent. Then they were transfected with 1 mL of the vector-containing supernatant, 0.5 μL Polybrene (Millipore, Billerica, MA) in 0.5 mL 10% calf serum (CS). 24 h post transfection, cells were replaced with a standard culture medium (DMEM containing 10% CS and 1% penicillin-streptomycin). At 90% confluence, cells were plated into 96-well plate with puromycin (1.0 mg/mL, Invitrogen, Grand Island, NY). The survived cells were plated in 96 well to achieve a density of single cell per well. Stably transfected overexpressing leptin clones (3T3\textsuperscript{Lep}) were derived from a single cell and were tested for leptin mRNA expression by RT-PCR and leptin release by ELISA (ALPCO, Salem, NH).

**mRNA analysis**

mRNA was purified from adipocytes or adipose tissue according to the manufacturer’s instructions (Qiagen, Germantown, MD) and quantified using 7900HT Fast Real-Time PCR System and TaqMan fluorogenic detection system (Applied Biosystems, Grand Island, NY). Validated primers were also purchased from Applied Biosystems (Grand Island, NY). Comparative real-time PCR was performed in triplicate, including no-template controls. Expression was calculated using the comparative Ct method.
normalized to 18S or Tata box protein (TBP).

**Cell differentiation**

All preadipocytes (3T3<sup>Lep</sup> and parent 3T3-L1 cells) were cultured in DMEM medium containing 10% calf serum. Lipogenic differentiation medium contained 10% FBS, 10µg/mL insulin, 1µM dexamethasone, 0.5mM 3-isobutyl-1-methyl xanthine. Medium was replaced every 48 hours with DMEM containing 10% FBS, 10µg/mL insulin, and continued for 7 days.

**Encapsulation**

The phase microencapsulation technique has been performed as described [57]. The cell suspension (1 x 10<sup>6</sup> cells/ml in 2% sodium alginate solution) was extruded through a 0.4-mm needle into a 100mM CaCl<sub>2</sub> solution, using Encapsulation Unit (Nisco Engineering, AG, Switzerland) at 5.4kV to form calcium alginate gel beads. Microbeads were solidified for 20 min, and then incubated with 0.05% (w/v) poly-L-lysine (MW 20,700) to form alginate-poly-L-lysine membrane around the surface. The core was liquefied using 50mM sodium citrate. Capsules’ properties were characterized before [57]. Encapsulated preadipocytes were maintained in culture under standard conditions for up to 30d.

**Animal studies**

All experimental protocols were approved by the Institutional Animal Care and Use
In mouse study one, five week old ob/ob male mice (n=21) were housed individually and randomly assigned into three groups and fed *ad libitum* a standard chow diet (irradiated 7912, Harlan Laboratories, Indianapolis, IL) for 72 days. The following treatment groups were studied:

1. Control treatment group (OB\[^{Emp}\]) (n=5 at the conclusion of the study). Male ob/ob mice were injected with vehicle (0.3 mL of acellular (empty) capsules in 0.5 mL of sterile phosphate buffer (PBS) per visceral depot).

2. Encapsulated 3T3-L1 group (OB\[^{3T3}\]) (n=5 at the conclusion of the study). Male ob/ob mice were injected with encapsulated 3T3-L1 preadipocytes (0.3*10^6 3T3-L1 cells in 0.3 mL of capsules in 0.5 mL of PBS per visceral depot).

3. Encapsulated 3T3\[^{Lep}\] group (OB\[^{Lep}\]) (n=7 at the conclusion of the study). Male ob/ob mice were injected with encapsulated 3T3\[^{Lep}\] preadipocytes overexpressing leptin (0.3*10^6 3T3\[^{Lep}\] cells in 0.3mL of capsules in 0.5 mL of PBS per visceral depot).

All mice assigned to OB\[^{Emp}\], OB\[^{3T3}\], and OB\[^{Lep}\] groups had similar weights before treatments of 56.3, 56.8, and 58.0 g, respectively(P<0.80, ANOVA). After injection, mice continued on a similar chow diet for 72 days and then sacrificed. Food intake and weight was monitored. On day 28, mice were removed from standard housing and placed in metabolic cages for two days, then returned to individual housing. Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed three weeks prior to sacrifice. DEXA was performed on OB\[^{Emp}\], OB\[^{3T3}\], and OB\[^{Lep}\] just prior to sacrifice.
using the GE Lunar Prodigy (Fairfield, CT). Percent body fat was measured using enCORE software (Fairfield, CT). On day 72, mice were anesthetized and sacrificed. Blood was collected by cardiac puncture into EDTA-containing tubes. Plasma was prepared by centrifugation. One whole subcutaneous and visceral fat pad were dissected as shown in [36]and homogenized in RIPA buffer with protease inhibitors.

**In mouse study two,** eight C57BL/6 (WT) five week old male mice were fed a high-fat (HF) diet (45% kcal from fat, D12451, Research Diets Inc., New Brunswick, NJ) for 218 days. Mice were housed together in two groups and food intake and weight was recorded. Treatments were:

1. Control treatment group (WT\textsuperscript{Emp}) (n=3). Body weight at the beginning of study was 56.3±2.9g. WT male mice were injected with vehicle (0.3 mL of acellular (empty) capsules in 0.5 mL sterile phosphate buffer (PBS) per visceral depot);

2. [3T3\textit{Lep}] treatment group (WT\textsuperscript{[Lep]}) (n=5). Body weight at the beginning of study was 58.0±3.3g WT male mice were injected with encapsulated 3T3\textit{Lep} preadipocytes overexpressing leptin (0.3*10\textsuperscript{6} 3T3\textit{Lep} cells in 0.3 mL of capsules in 0.5 mL of PBS per visceral depot).

After injection, mice continued on the same HF chow diet for 70 days and glucose and insulin tolerance tests were performed. Prior to sacrifice, the three heaviest mice in the WT\textsuperscript{[Lep]} group died of age-related issues before the end of study.
**Metabolic measurements**

In study one, metabolic parameters in the treated mice were measured by indirect calorimetry (CLAMS, Columbus Instruments, Columbus, OH) at ambient temperature (22°C) with 12 hours light/dark cycles. Animals were fed the same diet and water provided *ad libitum*. Mice were placed individually and allowed to acclimate to the metabolic cages. O$_2$ consumption, CO$_2$ production, energy expenditure, and locomotor activity were measured for 24 hours. Based on these data, respiratory quotient or exchange ratio (CO$_2$/O$_2$) and Δ heat values were calculated by CLAMS.

**Glucose and insulin tolerance tests**

For GTT and ITT, mice were fasted overnight. They were injected with a single intraperitoneal glucose dose (0.001g glucose/g body weight) for GTT. Blood was obtained from mouse tails. Blood glucose was measured by One Touch Ultra glucometer (LifeScan). For mice were fasted overnight. Mice allowed recovered for at least one week, then ITT test was performed using a single intraperitoneal insulin dose (1mU of insulin/g body weight). Blood glucose was measured.

**Western blot analysis**

Aliquots from fat pad homogenates were used for protein (Bicinchoninic Acid Kit, Thermo Scientific, Waltham, MA) measurements, and Western blots. For Western blot analyses, tissue lysates were separated on 10% acrylamide gel under reducing conditions. After transfer to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Billerica,
MA), proteins were analyzed using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Images were quantified by ImageJ software.

**ELISA assays**

Leptin (mouse/rat) ELISA (ALPCO, Salem, NH) was performed on media samples and plasma samples. 3T3-L1 and 3T3<sup>Lep</sup> were differentiated as described (n=5). 2 mL of fresh media was added to the differentiated cells and incubated for 48 h. Media samples were collected and leptin (mouse/rat) ELISA (ALPCO, Salem, NH) was performed according to manufacturer’s instructions. 3T3-L1 and 3T3<sup>Lep</sup> were encapsulated in poly-L-lysine as described. 200μL of encapsulated 3T3-L1 ([3T3-L1]) or encapsulated 3T3<sup>Lep</sup> ([3T3<sup>Lep</sup>]) were added to 2mL of fresh cell culture medium (DMEM medium containing 10% CS). After 24 h incubation, media samples were taken, centrifuged, and filtered to remove r capsules. Leptin (mouse/rat) concentrations in the media were measured by ELISA (ALPCO, Salem, NH) according to the manufacturer’s instruction. Insulin concentration in plasma was determined using the Rat/Mouse Insulin ELISA Kit (EMD Millipore Corporation, St. Charles, MO). Resistin concentration in plasma was determined using Quantikine ELISA Mouse Resistin Immunoassay (R&D Systems, INC., Minneapolis, MN). Hemolyzed plasma samples were excluded from measurements according to manufacturer’s recommendations.

**Statistical analysis**

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4.3 Results

*Encapsulated adipocyte overexpressing Lep secrete leptin in vivo and in vitro*

The generated *Lep* (3T3\textsuperscript{Lep}) cell lines expressed approximately 90,000 times greater levels of *Lep* compared to 3T3-L1 preadipocytes (Fig. 4.1A) and approximately 90,000 times greater *Lep* when differentiated (Fig. 4.1B). We validated differentiation using markers of preadipocytes *Pref1* [140] (Fig. 4.1C), and differentiated adipocytes *Pparg*. Differentiated 3T3-L1 decreased expression of *Pref1* by 98.4% (*P*<0.02). Both 3T3-L1 and 3T3\textsuperscript{Lep} adipocytes differentiated for 6 days expressed similar levels of *Pref1* (Fig. 4.1D). 3T3\textsuperscript{Lep} had a statistically insignificant greater expression of *Pref1* (19%, *n*=4, *P*<0.06) than 3T3-L1. *Pparg* was slightly downregulated in differentiated 3T3\textsuperscript{Lep} adipocytes (33% of the expression in 3T3-L1, *P*<0.001) (Fig. 4.1E). To determine if *Lep* was translated into leptin, 3T3-L1 and 3T3\textsuperscript{Lep} were differentiated for 7 days, and the supernatant media on the seventh day was analyzed for secreted leptin. 3T3\textsuperscript{Lep}, but not 3T3-L1, released leptin into media (63.4 ± 13 ng/mL vs. 0.01 ± 0.01 ng/mL, *n*=4, *P*<0.001) (Fig. 4.1F). To validate the release of leptin from encapsulated 3T3\textsuperscript{Lep} preadipocytes, 3T3-L1 and 3T3\textsuperscript{Lep} were encapsulated in poly-L-lysine (described as \([3T3-L1], [3T3\textsuperscript{Lep}]\) where parenthesis indicate encapsulation). After encapsulated cells
were cultured overnight, media was collected and analyzed for secreted leptin. These encapsulated cells were from the same batch that was used in vivo. Similar to non-encapsulated 3T3-L1 and 3T3\textsuperscript{Lep} adipocytes, [3T3\textsuperscript{Lep}], but not [3T3-L1], produced leptin (Fig. 4.1G). The concentration of leptin produced by encapsulated adipocytes was less than those of cultured preadipocytes (0.611 ng/mL vs 63.4 ng/mL) due to the smaller number of encapsulated cells in the well and shorter incubation time (Fig. 4.1F vs Fig. 4.1G). These capsules (Fig. 4.2) were capable of survival and production of leptin in vivo. Average plasma leptin concentrations were measured 72 days post capsule injection in OB\textsuperscript{[Emp]}, OB\textsuperscript{[3T3]}, and OB\textsuperscript{[Lep]} to be $0 \pm 0$, $2.59 \pm 4.5$, and $122 \pm 63$ pg/mL, respectively (Fig. 4.3A). Plasma leptin levels were statistically greater in OB\textsuperscript{[Lep]} than the other two groups ($P<0.01$). However, OB\textsuperscript{[Lep]} had lower plasma leptin levels when compared to WT mice fed a HF diet (0.22 \pm 0.62 ng/mL vs. 1.66 \pm 0.08 ng/mL) (Fig. 4.3B). OB\textsuperscript{[Lep]} have 7.4\% the plasma leptin of a WT mouse fed a HF diet.

Leptin treatment by encapsulated adipocytes attenuates weight gain, reduces food intake, and increases BAT and metabolic rate.

To test metabolic effects of stable leptin produced from its physiological side in adipose tissue, we injected three groups of ob/ob mice with encapsulated acellular capsules (OB\textsuperscript{[Emp]}), encapsulated 3T3-L1 preadipocytes (OB\textsuperscript{[3T3]}), and encapsulated 3T3\textsuperscript{Lep} (OB\textsuperscript{[Lep]}) in both visceral fat pads. After treatment, we monitored kinetics of weight for OB\textsuperscript{[Emp]}, OB\textsuperscript{[3T3]}, and OB\textsuperscript{[Lep]} were recorded (Fig. 4.3C, pre-injection period is shown in Fig. 4.4A). Weights were normalized to the weight before treatment in each mouse. We showed that treatment with OB\textsuperscript{[Lep]} transiently decreased weight on days 2
and 7 post injection compared to OB\[^\text{Emp}\] (Fig. 4.3C). The weight reduction was coincident with transient suppression of appetite in OB\[^{\text{Lep}}\] compared to OB\[^{\text{Emp}}\]. Food intake for OB\[^{\text{Lep}}\] was statistically lower on day 9 post injection than OB\[^{\text{Emp}}\] (Fig 4.3D). Also, Encapsulation of 3T3-L1 attenuated food intake and weight gain when compared to OB\[^{\text{Emp}}\]. Weight for OB\[^{3T3}\] was less than OB\[^{\text{Emp}}\] on days 14 and 21 post injection (Fig. 4.4C). Likewise, food intake for OB\[^{3T3}\] was less than OB\[^{\text{Emp}}\] on days 14 and 21 post injection (Fig. 4.4D). At the end of study (72 days post-injection) food intake, body weight (Fig. 4.5A), percent body fat measured by DEXA (Fig. 4.5B), SF, VF, and liver weight were not altered by any treatment (Fig. 4.6A-D). Adipose tissues were dissected as in Yasmeen et al [36]. However, we found that OB\[^{\text{Lep}}\] treatment increases BAT mass. OB\[^{\text{Lep}}\] had greater normalized BAT weight (0.013 ± 0.004) when compared to OB\[^{\text{Emp}}\] (0.008 ± 0.002) (Fig. 4.6E). OB\[^{\text{Lep}}\] had 75.2% more BAT than OB\[^{\text{Emp}}\] control. This increase in BAT did not cause an increase in UCP1 (Fig. 4.6F). UCP1 normalized to beta-actin was the same for OB\[^{\text{Emp}}\], OB\[^{3T3}\], and OB\[^{\text{Lep}}\]. To test the differences in metabolic parameters, six mice from OB\[^{\text{Emp}}\], OB\[^{3T3}\], and OB\[^{\text{Lep}}\] were placed into metabolic cages. Activity did not differ between groups (Fig. 4.7A). OB\[^{\text{Lep}}\] had a greater average metabolic rate of 10.43 ± 1.05 and 8.92 ± 0.66 kcal/h/kg for the dark and light cycles when compared to 7.99 ± 0.57 and 7.36 ± 0.62 kcal/h/kg for OB\[^{\text{Emp}}\] (P<0.001) (Fig. 4.7D,F). OB\[^{\text{Lep}}\] had a higher metabolic rate than OB\[^{3T3}\] for the dark cycle (10.43 ± 1.05 vs 9.31 ± 0.66, P<0.005) but not the light cycle (8.92 ± 0.66 vs. 8.75 ± 0.60, P<0.16) (Fig. 4.8D,F). OB\[^{\text{Lep}}\] had a greater respiratory exchange ratio (RER) of 0.93 ± 0.01 for dark and 0.90 ± 0.01 for light than OB\[^{\text{Emp}}\] (0.90 ± 0.01 and 0.87 ± 0.01) and OB\[^{3T3}\](0.91
± 0.01 and 0.88 ± 0.01) for both the dark and light cycles (Fig. 4.7C,E and Fig. 4.8C,E) suggesting that glucose is effectively utilized in OB\[^{[Lep]}\].

Leptin treatment by encapsulated adipocytes and improves glucose tolerance, resistin and insulin

To access leptin’s role in glucose metabolism, we performed glucose tolerance and insulin tests in studied groups. The glucose tolerance test responses in OB\[^{[Emp]}\] and OB\[^{[3T3]}\] were similar (Fig. 4.9C and Fig. 4.10A). The leptin treatment in OB\[^{[Lep]}\] resulted in better glucose tolerance tests than OB\[^{[Emp]}\] and OB\[^{[3T3]}\] (Fig. 4.9A,C and Fig. 4.10C). OB\[^{[Lep]}\] glucose levels were lower than OB\[^{[Emp]}\] at the 30, 90, and 120 min time points (Fig. 4.9A). OB\[^{[Lep]}\] glucose levels were statistically lower than OB\[^{[3T3]}\] at the 15, 60, 90 min time points (Fig. 4.10C). OB\[^{[Lep]}\] (78 ± 14 %) was more sensitive to glucose than OB\[^{[Emp]}\] (100 ± 5 %) and OB\[^{[3T3]}\] (108 ± 20 %) as measured by the AUC (% AUC of control) for glucose tolerance tests (Fig. 4.9C). Insulin tolerance tests were not different between OB\[^{[Emp]}\], OB\[^{[3T3]}\], OB\[^{[Lep]}\] groups (Fig. 4.9B,D and Fig. 4.10B,D). In WT mice, WT\[^{[Lep]}\] had statistically lower blood glucose levels at 15, 30, 90, 120 min than WT\[^{[Emp]}\] (Fig. 4.11A). WT\[^{[Lep]}\] (70 ± 14 %) was more sensitive to glucose than WT\[^{[Emp]}\] (100 ± 14 %) as measured by the AUC (represented as % AUC of control) for glucose tolerance tests (Fig. 4.11C). Insulin tolerance tests for WT mice were not changed (Fig. 4.11B,D)

Increased resistin levels is associated with increased insulin resistance. To elucidate the link between improved glucose tolerance and leptin, we measured insulin and resistin. Average insulin levels at sacrifice were 19.1 ± 5.7, 42.5 ± 28.9, 75.4 ± 43.9 ng/mL for OB\[^{[Emp]}\], OB\[^{[3T3]}\], and OB\[^{[Lep]}\] respectively (Fig. 4.12A). OB\[^{[Lep]}\] average insulin
concentration was statistically greater than OB\textsuperscript{Emp} (Fig. 4.12A). Glucagon levels of 1.11 ± 0.67, 0.86 ± 0.29, and 1.80 ± 0.87 ng/mL for OB\textsuperscript{Emp}, OB\textsuperscript{[3T3]}, and OB\textsuperscript{[Lep]} respectively were not statistically different (Fig. 4.12B). Resistin is decreased in the presence of leptin (Fig. 4.12C). Resistin concentration of 11.5 ± 3.1 ng/mL for OB\textsuperscript{[Lep]} was less than the 16.5 ± 3.2 and 16.9 ± 2.0 ng/mL for OB\textsuperscript{Emp} and OB\textsuperscript{[3T3]}, respectively.

4.4 Discussion

Two decades of research have established leptin as a key adipokine regulating energy balance through the central nervous system and endocrine pathways [97, 141]. The leptin stimulated effects on hypothalamus include: regulation of appetite, increase in metabolic rate, lipolysis in WAT and thermogenesis [97]. These effects offer potential for an intervention therapy against obesity. Recently studies showed that hypothalamic action of leptin on BAT and muscles can mediate lifesaving and antidiabetic actions not only in insulin-resistant, but also in insulin deficient rodents [142]. These studies provide a rationale for the translation of leptin as a complementary therapy to traditional insulin treatments for type I and type II diabetes. Potentially, this complementary therapy may improve the treatment of these diseases [141]. However, the therapeutic application of leptin is limited by detrimental effects associated with daily leptin injections [51]. Injection site reactions often include inflammation, erythema and ecchymoses, whereas the systemic spike of plasma leptin injections can lead to hypoglycemia, and other complications [51]. Notably, hyperleptinemia has multiple deleterious effects [143, 144], including epigenetic transgenerational effects and that promote obesity in offspring [145].
Leptin injections were approved in 2014 for the treatment of congenital leptin deficiency and generalized lipodystrophy in USA and other countries; however, the current methods of leptin delivery impede its use for general population of patients with diabetes [145]. Physiologically, leptin is predominantly produced in circadian fashion in WAT [146, 147]. In our study, we mimic physiological leptin production by employing encapsulated adipocytes producing leptin and by implanting these capsules in a physiological location, the visceral adipose tissue. We showed that a sub-physiological concentration of leptin produced by these engrafts can effectively improve glucose tolerance in mice with genetic and diet-induced obesity.

The functions of leptin were established on ob/ob mice with systemic leptin deficiency and validated with the systemic injections of leptin or adenovirus producing leptin [116-119]. Majority of systemic leptin effects, including weight loss, thermogenesis, appetite suppression, and improved glucose metabolism were recapitulated by direct stimulation hypothalamus with leptin. Although leptin is widely established as adipokine found in all fat depots, it also expressed in heart, intestine and some other tissues [147]. However, the contribution of these tissue and adipose depot to the leptin responses has not been examined. Some insight into the tissues-specific effects of leptin was gained from these implantation studies. Implants of intestinal cells overexpressing leptin showed effective appetite and weight loss in ob/ob mice [117]. However, failed to induce these effect in WT mice with high-fat diet-induced obesity and insulin-resistance [117]. However, the data from this study need to be interpreted with caution because leptin production was induced with a drug. Moreover, genetically-
different implants in this study were susceptible to rejection by the host’s immune system, although they were introduced bound into an alginate polymer [117]. Other leptin replacement therapies have attempted to investigate endogenous leptin production by the transplantation of tissue [15, 117, 120]. However, these methods also induce an immune response and it is difficult to translate them for patient treatment. Encapsulation into alginate-poly-L-lysine can overcome the immune response due to the small pores size protecting from immunoglobulin influx [37, 67]. Xenografts of porcine beta islets were able to survive and function in a human patient for over 9.5 years without immune rejection [67]. Encapsulation of adipocytes in our study, allow the effects of leptin produced constitutively in visceral location to be studied. In this pilot study, we introduced only 0.6 million cells per visceral depot and achieved only 10% of level observed in obese WT mice. Under these conditions a limited leptin secretion from the capsules was not sufficient to reduce appetite and weight loss for the duration of the study. However, it had a profound effect on glucose metabolism due to its systemic effects and effects in adipose tissue. This result also demonstrates that encapsulated adipocytes were able to evade the immune system and survive and produce leptin for at least 72 days.

We attributed transiently reduced weight and lower food intake when compared to empty capsule injected control to a combination of factors. First factor was a response to the injections since a significant decrease in food intake and weight was seen in all groups of mice during the first 5 days post treatment. The second factor was related to implanted 3T3-L1 adipocytes. Food intake and weight was significant in OB$^{[Lep]}$ and
OB\(^{3T3}\) compared to empty groups and seen between days 5-9. This transient effect could be attributed to leptin as well as to the implantation of 3T3-L1 cells, because similar effects were reported after cross-transplantation of adipose tissue from different depots in \(ob/ob\) mice [15]. 3T3-L1 adipocytes could also secrete some leptin [148, 149] in the physiological VF environment producing these paracrine signals. The lack of the constitutive suppression of appetite and weight gain on our study could be due to the sub-physiologic leptin concentrations in plasma from small amount of encapsulated cells. It is also possible that leptin produced in VF stimulate afferent neural responses that are not involved in the regulation of appetite and weight, because neural effects on weight loss seem to be uncoupled from other leptin-dependent effects [150].

The primary outcome of leptin production in encapsulated adipocytes on our study was long-lasting improvement of glucose tolerance. This improvement was achieved by low, sub-physiological circulating levels of leptin. Three different mechanisms were at work and could be accounted for improved glucose uptake and utilization. We found: 1) increased BAT mass; 2) increased insulin; and 3) reduced resistin concentrations in plasma. Although WAT mass and liver fat accumulation was not influenced by leptin in \(ob/ob\) mice in our study, BAT mass was markedly greater in OB\(^{Lep}\) mice and associated with a higher basal metabolic rate. The greater RER in OB\(^{Lep}\) mice suggested the better glucose utilization that was demonstrated using GTT test. Recent studies showed that activation of \(\gamma\)-aminobutyric (GABA) and pro-opiomelanocorticicotropin (POMC) neurons by leptin can induce BAT formation and function that increased glucose utilization [97]. The stimulation of innervation can
potentially lead to improve secretion of insulin from pancreas, because studies in leptin-deficient β-cells suggest only a moderate direct effect of leptin in these cells [151]. Insulin secretion increases after long term leptin administration [116]. Thus, both sub-physiological concentration of leptin from physiological location and systematic injections of high leptin concentrations are similarly effective in improving of insulin secretion.

Our study revealed also a new endocrine effect of leptin produced in VF location. OB\[^{Lep}\] mice secreted significantly less resistin compared to other treated groups. Resistin is an adipokine that was identified in visceral adipocytes as a key cytokine inducing insulin resistance [152]. Resistin deficiency in \( ob/ob \) mice and DIO mice have been shown to improve glucose tolerance [153]. Resistin is widely implicated in the development of and progression of insulin resistance in rodents and humans [152]. An antagonism between resistin and leptin secretion from adipocytes has been previously demonstrated in vitro [154]. Here we provide evidence that secretion of leptin from the small subset of encapsulated adipocytes is sufficient to mediate a long term decrease in resistin concentrations in plasma. The reduction of resistin has more beneficial outcome than the complete removal of resistin, because the complete removal of resistin decreases metabolic rate [153].

Our study shows that encapsulated adipocytes can produce leptin over 72 days. Although amount of capsules could be optimized, we showed that sub-physiological levels of leptin produce array of beneficial effects, notably long term improved glucose tolerance. Leptin treatment considered to be ineffective in general population of patients.
with obesity and/or type 2 diabetes due to development of leptin resistance and hyperleptinemia, in addition to insulin resistance [155]. This effect is also present in WT with diet-induced obesity (DIO) [155]. Previous strategies to improve glucose tolerance in DIO mice were ineffective [117]. However, more research is still needed to maximize the appetite suppressing and weight loss effects of encapsulated cells that may include higher leptin production in adipocytes or development and delivery of other encapsulated leptin-producing cells, e.g. intestinal cells. In our study leptin, produced by encapsulated adipocytes in physiological location led to long lasting improvement in glucose tolerance. Notably, this effect was achieved in severely obese old mice. The therapeutic effects and biocompatibility of encapsulated adipocytes suggest that this delivery method offers advantages over standard delivery methods including injections, adenovirus delivery, or transplantation of tissue. Encapsulation is a minimally invasive and cost-effective procedure for delivery of leptin at a constant rate over prolonged period of time from months [37] to years [67] as well as to the termination of this procedure [37]. Our data prove a principle that encapsulation of leptin-producing adipocytes could be a feasible translational approach for controlled delivery of genes into specific tissue.
3T3-L1 cells were stably transduced to overexpress Leptin. (A) In non-differentiated preadipocytes, Leptin expression was determined using RT-PCR and normalized to TBP. Leptin was detected in 3T3\textsuperscript{Lep} and was not detected in 3T3-L1. Leptin expression normalized to TBP was 90,000 times greater in 3T3\textsuperscript{Lep} ($n=4$, $P<0.001$). (B) In non-differentiated preadipocytes, Leptin expression was detected in 3T3\textsuperscript{Lep} and was not detected in 3T3-L1. Leptin expression normalized to TBP was 90,000 times greater in 3T3\textsuperscript{Lep} ($n=4$, $P<0.001$). (C) Pref1 is downregulated after differentiation in 3T3-L1 adipocytes ($n=3$, $P<0.02$). (D) Pref1 expression is not different in differentiated 3T3-L1 and 3T3\textsuperscript{Lep} ($n=4$, $P<0.06$). (E) Pparg was downregulated in 3T3\textsuperscript{Lep} when compared to 3T3-L1 ($n=4$, $P<0.001$). (F) Media samples from cell cultures of 3T3\textsuperscript{Lep} had leptin in the media. Media samples from cell cultures of 3T3-L1 did not significant levels of leptin in the media ($n=4$, $P<0.001$). (G) Likewise encapsulated 3T3-L1 ([3T3-L1]) and 3T3\textsuperscript{Lep} ([3T3\textsuperscript{Lep}]) were placed in fresh media overnight. [3T3\textsuperscript{Lep}], but not [3T3-L1], were able to secrete leptin into the media ($n=4$, $P<0.001$).
Fig. 4.2 Capsules

Capsules are shown containing 3T3-L1 or 3T3^{Lep} cells.
Fig. 4.3 Capsules secrete leptin in vivo, attenuate weight gain and food intake

Plasma samples were taken at sacrifice and assayed for leptin using an ELISA. (A) OB\textsuperscript{Lep} had significant circulating levels of leptin in the plasma. OB\textsuperscript{Emp} did not have any detectable leptin. OB\textsuperscript{3T3} only had one sample with detectable leptin due to most likely errors in the standard curve (n=7 for OB\textsuperscript{Lep}, n=3 for OB\textsuperscript{3T3}, n=4 for OB\textsuperscript{Emp}, \(P<0.01\)). Plasma samples from OB\textsuperscript{Lep} and WT mice fed a HF diet were analyzed for leptin (B) OB\textsuperscript{Lep} had less plasma leptin than WT mice (n=7 for OB\textsuperscript{Lep}, n=5 for WT, \(P<0.01\)). Mice were housed individually. Food intake and weights were recorded on a weekly basis. Weight was normalized to weight of the mouse on injection day and data are expressed as percent weight relative to the injection day. (C) OB\textsuperscript{Lep} had lower normalized weight than OB\textsuperscript{Emp} on two days (n=7 for OB\textsuperscript{Lep}, n=6 for OB\textsuperscript{Emp}, \(P<0.05\)). Likewise, food intake was normalized to the average grams of food consumed per day up to injection day. Data are expressed as percent of average food intake relative to the injection day. (D) OB\textsuperscript{Lep} had lower normalized food intake than OB\textsuperscript{Emp} on day 9 (n=7 for OB\textsuperscript{Lep}, n=6 for OB\textsuperscript{Emp}, \(P<0.05\)). All data are represented as mean ± standard deviation. Statistical comparisons between groups are shown with a black line or asterisk.
Fig. 4.4 Cellular capsules attenuate weight gain and food intake

Food intake and mouse weights were recorded on a weekly basis. Food intake and weight were normalized to food intake and weight on injection day. Data are expressed as a percent of the value for the injection day. Pre-injection period is shown. On the injection day (indicated with an arrow), mice were injected with acellular, [3T3-L1], or [3T3-Lep]. (A) OB\(^{Lep}\) had lower normalized weight than OB\(^{Emp}\) on two days (n=7 for OB\(^{Lep}\), n=6 for OB\(^{Emp}\), *P<0.05). (B) OB\(^{Lep}\) had lower normalized food intake than OB\(^{Emp}\) on day 9 (n=7 for OB\(^{Lep}\), n=6 for OB\(^{Emp}\), *P<0.05). (C) OB\(^{3T3}\) had lower normalized weight than OB\(^{Emp}\) on day 14 and 21 (n=6 for OB\(^{Lep}\), n=6 for OB\(^{Emp}\), *P<0.05). (D) OB\(^{3T3}\) had lower normalized food intake than OB\(^{Emp}\) on day 14 and 21 (n=6 for OB\(^{Lep}\), n=6 for OB\(^{Emp}\), *P<0.05). All data are represented as mean ± standard deviation.
Fig. 4.5 Weight and percent body fat
Weights were measured at the conclusion of the study (day 72). DEXA scans of each mouse were performed just prior to sacrifice. (A) All groups had similar weights at sacrifice. (B) Percent body weight was the same for all groups. (C) Sample DEXA images of tissue and bones are shown. All data are represented as mean ± standard deviation.
Fig. 4.6 Leptin increases BAT

Whole fat pads were removed, weighed, and homogenized in RIPA buffer with protease and phosphatase inhibitors. (A) Average normalized SF weight (g of SF/g of mouse) was not different between groups. (B) Average normalized VF weight (g of VF/g of mouse) was not different between groups. (C) Average normalized liver weight (g of liver/g of mouse) was not different between groups. (D) OB\textsuperscript{[Lep]} had statistically greater BAT tissue than OB\textsuperscript{[Emp]} (n=7 for OB\textsuperscript{[Lep]}, n=5 for OB\textsuperscript{[Emp]}, P<0.05). (E) OB\textsuperscript{[Lep]} had statistically greater ratio of BAT to VF than OB\textsuperscript{[Emp]} (n=7 for OB\textsuperscript{[Lep]}, n=5 for OB\textsuperscript{[Emp]}, P<0.05). (F) UCP1 levels in mice were not different as measured by western blot normalized to beta-actin. All data are represented as mean ± standard deviation. Statistical comparisons between groups are shown with a black line.
Fig 4.6

A

Normalized SF

OB\(^{\text{Emp}}\)  OB\(^{\text{[3T3]}\)  OB\(^{\text{[Lep]}\)

B

Normalized VF

OB\(^{\text{Emp}}\)  OB\(^{\text{[3T3]}\)  OB\(^{\text{[Lep]}\)

C

Normalized Liver

OB\(^{\text{Emp}}\)  OB\(^{\text{[3T3]}\)  OB\(^{\text{[Lep]}\)

D

Normalized BAT

OB\(^{\text{Emp}}\)  OB\(^{\text{[3T3]}\)  OB\(^{\text{[Lep]}\)

E

Ratio of BAT to VF

OB\(^{\text{Emp}}\)  OB\(^{\text{[3T3]}\)  OB\(^{\text{[Lep]}\)

F

UCP1/Beta Actin

UCP1
Fig. 4.7 Metabolic data for OB^{Lep} and OB^{Emp}

28 days after injection, mice were placed in metabolic cages for 48 hours. (A) Activities were not different between groups. (B) Average VO\textsubscript{2} for both the dark and light cycle was greater in OB^{Lep} (n=6, \(P<0.001\)). (C) Average RER for both the dark and light cycle was greater in OB^{Lep} (n=6, \(P<0.001\)). (D) Average metabolic rate (kcal/h/kg) for both the dark and light cycle was greater in OB^{Lep} (n=6, \(P<0.001\)). (E) Kinetic data for RER vs time (h) for OB^{Lep} (filled red circles) and OB^{Emp} (open black circles) is presented. Points are taken 22min apart with arrows indicating the dark (D) and light (L) phases (n=6). (F) Kinetic data for metabolic rate (kcal/h/kg) vs time (h) for OB^{Lep} (filled red circles) and OB^{Emp} (open black circles) is presented. Points are taken 22min apart with arrows indicating the dark (D) and light (L) phases (n=6). All data in A-D are represented as mean ± standard deviation. Data points in E and F are means. Statistical comparisons between groups are shown with a black line.
Fig 4.7

A

Average XYZ activity

0 1000

D L

B

VO₂ (mL/kg/h)

0 3000

D L

C

Average RER

0.80 1.00

D L

P < 0.001

D L

P < 0.001

D L

P < 0.001

D L

P < 0.001

D L

E

RER

0.8 1.0

0 6 12 18 24

D L

F

Metabolic rate (Kcal/kg/h)

0 10 20

0 6 12 18 24

D L
Fig. 4.8 Metabolic data for OB$^{[3T3]}$ and OB$^{[Lep]}$

28 days after the injection, mice were placed in metabolic cages for 48 hours. (A) Activities were not different between groups. (B) Average VO$_2$ for only the dark cycle was greater in OB$^{[Lep]}$ (n=6, $P<0.001$) (C) Average RER for both the dark and light cycle was greater in OB$^{[Lep]}$ (n=6, $P<0.001$) (D) Average metabolic rate (kcal/h/kg) for only the dark phase was greater in OB$^{[Lep]}$ (n=6, $P<0.001$) (E) Kinetic data for RER vs time (h) for OB$^{[Lep]}$(filled red circles) and OB$^{[3T3]}$(open black circles) is presented. Points are taken 22min apart with arrows indicating the dark (D) and light (L) phases (n=6). (F) Kinetic data for metabolic rate (kcal/h/kg) vs time (h) for OB$^{[Lep]}$(filled red circles) and OB$^{[Emp]}$(open black circles) is presented. Points are taken 22min apart with arrows indicating the dark (D) and light (L) phases (n=6). All data in A-D are represented as mean ± standard deviation. Data points in E and F are means. Statistical comparisons between groups are shown with a black line.
Fig. 4.8

A

Average XYZ activity

B

VO₂ (mL/kg/h)

C

Average RER

D

Average Metabolic Rate (kcal/kg)

E

RER

F

Metabolic rate (kcal/kg)

- OB₁
- OB₁[Lept]
Glucose tolerance tests (GTT) or insulin tolerance tests (ITT) were performed by fasting mice overnight then injecting them with a standardized dose of glucose or insulin. Blood glucose levels were measured at the indicated time points. (A) Average blood glucose (mg/dL) of OB^{Lep} was less than OB^{Emp} at the indicated time points (n=5, * P<0.05). (B) ITT tests were statistically the same. (C) The area under the curve (AUC) for GTT, measured as percent of OB^{Emp}, was less for OB^{Lep} than other treatment groups (n=5, P<0.05). (D) AUC for ITT were statistically the same for all groups. AUC was calculated using the trapezoidal rule. All data are represented as mean ± standard deviation. Statistical comparisons between groups are shown with a black line or asterisk.
Glucose tolerance tests (GTT) or insulin tolerance tests (ITT) were performed by fasting mice overnight then injecting them with a standardized dose of glucose or insulin. Blood glucose levels were measured at the indicated time points. (A) Average blood glucose (mg/dL) of OB\(^{3T3}\) was the same as OB\(^{Emp}\) at all time points (n=5). (B) ITT tests were statistically the same. (C) Average blood glucose (mg/dL) of OB\(^{3T3}\) was greater than OB\(^{Lep}\) at all time points (n=5, *P<0.05). (D) ITT tests were statistically the same. All data are represented as mean ± standard deviation. Statistical comparisons between groups are shown an asterisk.
Glucose tolerance tests (GTT) or insulin tolerance tests (ITT) were performed by fasting mice overnight then injecting them with a standardized dose of glucose or insulin and blood glucose levels were measured. (A) Average blood glucose (mg/dL) of WT\(^{\text{Lep}}\) was less than WT\(^{\text{Emp}}\) at the indicated time points (n=5, * P<0.05). (B) ITT tests were statistically the same. (C) The area under the curve (AUC) for GTT, measured as percent of WT\(^{\text{Emp}}\), was less for WT\(^{\text{Lep}}\) than WT\(^{\text{Emp}}\) (n=5, P<0.05). (D) AUC for ITT were statistically the same for all groups. AUC was approximated by using the trapezoidal rule. All data are represented as mean ± standard deviation. Statistical comparisons between groups are shown with a black line or asterisk.
Fig. 4.12 Glucose metabolism hormones

Plasma insulin, glucagon, and resistin concentrations were measured using commercially available ELISA kits. (A) Average plasma insulin (ng/mL) was greater in OB\[^{[Lep]}\] than OB\[^{[Emp]}\] (n=7 for OB\[^{[Lep]}\], n=4 for OB\[^{[Emp]}\], P<0.02) (B) Treatments did not cause differences in plasma glucagon (ng/mL). (C) Average plasma resistin (ng/mL) was less in OB\[^{[Lep]}\] than OB\[^{[Emp]}\] and OB\[^{[3T3]}\] (n=7 for OB\[^{[Lep]}\], n=4 for OB\[^{[Emp]}\], n=3 OB\[^{[3T3]}\], P<0.02). All data are represented as mean ± standard deviation. Statistical comparisons between groups are shown with a black line.
Chapter 5

Reproducing GFP labeled Aldh1a1⁻/⁻ fibroblasts for use in future encapsulation experiments

5.1 Introduction

In our previous work in Yang et al, we showed that A-PLL capsules containing GFP labeled Aldh1a1⁻/⁻ fibroblasts were capable of attenuating weight gain in female mice fed a high fat diet [37]. Moreover, I showed that these capsules had a browning effect and induced UCP1 in the VF, a tissue that is not normally UCP1 positive [37]. These capsules were determined to be present in the VF depot because GFP was detected via immunoblotting and also fluorescence. Because of these promising results, this technology is needed to be further developed and studied in xenograft models.

Unfortunately, the original Aldh1a1⁻/⁻ (KO), Aldh1a1⁻/⁻ labeled with GFP (GKO), C57BL/6J (WT), and WT labeled with GFP (GWT) preadipocytes fibroblast lines were lost. It was necessary to immortalize new cell lines and stably transduce them with functioning GFP for use in future experiments.
5.2 Materials and methods

Chemicals and Reagents

Unless otherwise noted, we purchased reagents from Sigma-Aldrich (St. Louis, MO), cell culture media from Invitrogen (Carlsbad, CA). Primary antibodies β-actin and GFP were obtained from Cell Signaling Technology (Danvers, MA). UCP1 was purchased from ABCAM (Cambridge, MA). Secondary antibodies were purchased from LI-COR Biosciences (Lincoln, Nebraska).

Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee.

Aldh1a1<sup>−/−</sup> mice (KO) were provided by Dr. Duester (Sanford-Burnham Medical Research Institute). Their construction and characterization was described before [156]. C57BL/6J (WT) mice were used as control.

Cell line engineering

To immortalize WT and KO fibroblasts, SF pads were removed from young (4 week old) female Aldh1a1<sup>−/−</sup> mice and C57BL/6J (WT). SF pads were digested in collagenase type I solution (Invitrogen, Life Technologies, Carlsbad, CA) using a modified fibroblast isolation procedure described in [157]. Mature adipocytes were separated from WT and Aldh1a1<sup>−/−</sup> preadipocyte fibroblast cell lines. WT and Aldh1a1<sup>−/−</sup> preadipocyte
fibroblasts were immortalized by constant passaging following a classical protocol by Green & Meuth [158].

To derive the GWT and GKO fibroblast cell line, we performed a stable transfection of a GFP expressing lentiviral vector (GeneCopoeia, Rockville, MD, GFP positive, CMV promoter, puromycin resistant) packaged in a Lenti-Pac FIV Expression Packaging Kit (GeneCopoeia, Rockville, MD) according to manufacturer’s instructions. Briefly, lentiviral titers were produced using Lenti-Pac™ Lentiviral Packaging Kits (GeneCopoeia, Rockville, MD). A total of 1.3–1.5 × 10⁶ of the HEK293Ta Lentiviral Packaging cells (GeneCopoeia, Rockville, MD) were plated in a 10-cm dish 2 days before transfection in DMEM (10 mL), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS). Puromycin resistant lentiviral ORF leptin cDNA clone (2.5 μg; GeneCopoeia, Rockville, MD), 5 μL Lenti-Pac FIV mix, and 15 μL of EndoFectin–Lenti were diluted into 200 μL Opti-MEMI (Invitrogen, Grand Island, NY). The DNA–EndoFectin–Lenti complex was incubated at room temperature for 15 min and then added directly to the cells. Cells were incubated in a CO₂ incubator at 37°C for 8–14 h. TiterBoost reagent (GeneCopoeia, Rockville, MD, 0.2%) was added to the culture medium. Culture medium containing leptin pseudovirus vector was collected 48 h post transfection after being centrifuged at 2000 rpm for 15 min and used for the transfection. Murine 3T3-L1 preadipocytes were grown in a 6-well plate until 80% confluent. Then they were transfected with 1 mL of the vector-containing supernatant, 0.5 μL Polybrene (Millipore, Billerica, MA) in 0.5mL 10% calf serum (CS). 24 h post transfection, cells were replaced with a standard culture medium (DMEM containing 10% CS and 1%
penicillin-streptomycin). At 90% confluence, cells were plated into 96-well plate with puromycin (1.0 mg/mL, Invitrogen, Grand Island, NY). The survived cells were plated in 96 well to achieve a density of single cell per well. Stably transfected overexpressing GFP clones (GWt and GKo) were derived from a single cell and were tested for green fluorescence using a fluorescence microscope and using immunoblotting with GFP primary antibody (Cell Signaling Technologies, Danvers, MA).

**mRNA analysis**

mRNA was purified from adipocytes or adipose tissue according to the manufacturer’s instructions (Qiagen, Germantown, MD) and quantified using 7900HT Fast Real-Time PCR System and TaqMan fluorogenic detection system (Applied Biosystems, Grand Island, NY). Validated primers were also purchased from Applied Biosystems (Grand Island, NY). Comparative real-time PCR was performed in triplicate, including no-template controls. Expression was calculated using the comparative Ct method normalized to 18S or Tata box protein (TBP).

**Cell differentiation**

All fibroblasts (WT, KO, and GFP labeled clones) were cultured in DMEM medium containing 10% calf serum. Lipogenic differentiation medium contained 10% FBS, 10µg/mL insulin, 1µM dexamethasone, 0.5mM 3-isobutyl-1-methyl xanthine. Medium was replaced every 48 hours with DMEM containing 10% FBS, 10µg/mL insulin, and continued for 7 days.

**Microscope and imaging**

Pictures were taken using an Olympus IX71 fluorescence microscope with a DP72
camera. Green fluorescence was generated by a mercury lamp and using the proper filter.

5.3 Results

GFP was confirmed in $^G$WT and $^G$KO by viewing green fluorescence using a fluorescence microscope. Pictures are displayed in (**Fig. 5.1**). To confirm that stable transduction did not alter gene expression of these fibroblasts, mRNA analysis was performed. $Aldh1a1$ was expressed in WT, but not KO or $^G$KO (cycle 40 of RT-PCR) (**Fig. 5.2A**). $Ucp1$ was not expressed (cycle number 40) in WT, but was expressed in both KO and $^G$KO (**Fig. 5.2B**). Expression levels of $Ucp1$ in KO and $^G$KO were the same. Expression of $Ucp1$ in $^G$KO was insignificantly approximately 33% lower than the expression in KO ($P<0.39$). However, this was due to the high variability of the KO samples ($\pm 60\%$ standard deviation for KO and $\pm 5\%$ standard deviation for $^G$KO).

5.4 Discussion

GFP, a jelly fish protein that has green fluorescent properties, is not found in the mammalian genome. Stable transduction of GFP into KO and WT fibroblasts was successful, as shown by green fluorescence that the foreign GFP produces (**Fig. 5.1**). To determine that the stable transduction did not change the phenotype of these cells, RT-PCR was performed and $Aldh1a1$ and $Ucp1$ were measured. $Aldh1a1$ remained knocked out in KO and $^G$KO. $Aldh1a1$ was expressed in WT (**Fig. 5.2A**). Moreover, transduction did not change $Ucp1$ expression in $^G$KO (**Fig. 5.2B**). Because the biochemistry is not significantly altered in these cells, $^G$WT and $^G$KO can be used in the continuation of previous work [37]. For this reason, these cells were encapsulated and are currently being studied in a xenograft canine model.
Fig. 5.1 Microscope images

3T3-L1, \(^G\text{WT}\), and \(^G\text{KO}\) fibroblasts were viewed under a fluorescent microscope. Images were taken under visible light and with fluorescence.
**Fig. 5.2 mRNA expression in GFP labeled immortalized fibroblasts**

(A) *Aldh1a1* expression was measured by RT-PCR and normalized to TBP. WT cells expressed *Aldh1a1* while KO and GKO did not (n=3, P<0.001). (B) *Ucp1* expression was measured by RT-PCR and normalized to TBP. *Ucp1* was upregulated in KO and GKO, but not in WT (n=3, P<0.005). All data are represented as mean ± standard with a black line indicating statistical comparisons.

![Graph A](image1.png)

![Graph B](image2.png)
Chapter 6

Epilogue

Obesity is a pandemic that will cause health care costs to rise. Depending on the circumstance, traditional methods, such as diet and exercise may not be sufficient to treat obesity or the obesity related disease in a depot specific and disease specific fashion. This dissertation used genetically modified preadipocytes encapsulated in A-PLL to treat mouse models of obesity. We hypothesized that capsules could provide a more physiological method for introducing genetically modified cells into physiologically relevant locations. This type of delivery and treatment could be therapeutically better than traditional methods under certain circumstances.

In our original work, we showed that Aldh1a1-/- adipocytes, when encapsulated and injected into VF attenuated weight gain [37]. Strikingly, these capsules caused a “browning effect” in the surrounding VF. The normally UCP1 negative VF adipocytes became UCP1 positive [37]. These encapsulated cells most likely secreted an adipokine that remodeled the surrounding tissue. The remodeling could be useful for treatment of metabolic diseases that are caused by increased visceral fat, such as type II diabetes. The browning of this tissue could make the fat depot less deleterious. When BAT tissue is transplanted into the visceral depot, there is weight loss and improved glucose sensitivity [14]. We showed the same effect with capsules. However, these results were in female
mice. There could be sex specific differences that render this therapy ineffective in male mice due to different hormonal effects. Moreover, the weight loss effect was transient and was not sustained. This therapy in conjunction with appetite suppression could have a synergistic effect that produces long-term weight loss results. We tried to see if a more effective thermogenic cell line existed, which is why we encapsulated \textit{Rptor} deficient 3T3-L1 for treatment in mice fed a HF diet.

In chapter two, we investigated the role of knocking down \textit{Rptor} in 3T3-L1. Adipose specific knockouts of \textit{Rptor} produce a lean phenotype in male mice. In our previous studies, it was discovered that \textit{Aldh1a1}\textsuperscript{-/-} female mice are leaner than WT female mice to a greater extent than their male counterparts. \textit{Rptor} deficient cells may be a more potent cell line to use that would not be sex specific, which was a possible flaw in the previous Yang et al study. However, encapsulated \textit{Rptor} knockdown cells only reduced weight initially and that weight was quickly regained. Surprisingly, metabolic rates were lower for the mice injected with \textit{Rptor} knockdown capsules. Glucose tolerance tests were better for the knockdown group and the capsules successfully remodeled the VF to produce ATGL. Unfortunately, UCP1 was not detected as in the Polak et al study [94]. It is my opinion that this cell line does not have therapeutic promise after investigating it further in chapter three.

Chapter three provided the basis for the experiments in chapter four. We hypothesized that when mice are injected with encapsulated thermogenic cells, the mice eat to compensate for the increased energy expenditure. We engineered a \textit{Rptor} deficient 3T3-L1 cell line that overexpressed the appetite suppressing hormones leptin and amylin.
However, these cells were never confirmed to secrete amylin and leptin after encapsulation. The amylin and leptin contained fluorescent tags that could interfere with secretion through the poly-L-lysine membrane or prevent proper interaction with leptin and amylin with their appropriate receptors. The in vivo experiment was unremarkable and flawed because of extenuating circumstances delaying GTT, metabolic cages, and sacrifice. However, we learned that for future experiments untagged cytokines should be used to ensure proper function and secretion from the capsule.

In chapter 4, 3T3-L1 cells overproducing leptin was confirmed in vitro and in vivo. For the first time A-PLL encapsulation was used to protect engineered 3T3-L1 cells overexpressing leptin for 72 days. [3T3-L1] and [3T3\textsuperscript{Lep}] were both able to attenuate weight gain and reduce appetite transiently. Leptin was detected in the plasma of OB\textsuperscript{Lep}, which means that leptin was successfully secreted from the capsules. However, the concentration was on about 10% of the normal leptin levels. Future experiments can be done with greater concentrations of cells/capsules to hopefully improve the concentration. Even this little amount of leptin was able to suppress resistin release from adipocytes. The suppression of resistin may explain why OB\textsuperscript{Lep} had the best GTT. Furthermore, BAT was greater in these mice suggesting that leptin stimulated BAT formation through sympathetic innervation. Encapsulation of 3T3-L1 cells overproducing leptin may be developed into a future therapy for patients with lipodystrophy. Leptin injections are already being used in this population which may cause detrimental side effects like nausea and inflammation [51]. [3T3\textsuperscript{Lep}] may provide a more physiological way of producing leptin that limits these side effects. Our
preliminary research provides a proof of concept that can further be developed into therapies for this suffering population.

Lastly, our Aldh1a1<sup>−/−</sup> fibroblast line was lost. I immortalized a Aldh1a1<sup>−/−</sup> fibroblast cell line as well as a WT fibroblast line isolated from the SF of WT mice. I stably transduced these lines with GFP. These cells were encapsulated by Dr. Yang and injected into dogs. Unpublished results show that these dogs lost weight after being injected with capsules containing Aldh1a1<sup>−/−</sup> labeled with GFP cells more so than dogs injected with capsules containing WT labeled with GFP cells. This study provides the first evidence that xenograft transplants of encapsulated cells can attenuate weight gain.
References


