MSc Thesis

Genetic Editing of Adipocytes by CRISPR/Cas9 Delivery Particles for *Ex Vivo* Therapeutic Approaches to Type 2 Diabetes

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ΜΔΕ Διπλωματική Εργασία

Γενετική Τροποποίηση Λιποκυττάρων με Σωματίδια Μεταφοράς CRISPR/Cas9 για *Ex Vivo* Θεραπευτικές Προσεγγίσεις για τον Διαβήτη Τύπου 2

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ABSTRACT

Obesity and type 2 diabetes are major health threats with increasing prevalence affecting productive age groups in the developed countries. So far, there are no therapeutic approaches that target the causative mechanisms for type 2 diabetes in routine clinical practice. All pharmaceutical components that are administered daily throughout life, aim to lower blood glucose levels targeting different mechanisms of glucose synthesis, cell uptake and secretion. However, high blood glucose levels that cause the devastating cardiovascular complications of diabetes, seem to be the end-result of still unknown pathogenic mechanisms, occurring in patients with type 2 diabetes. Yet, remarkable progress has been made towards the understanding of insulin resistance pathogenesis, which is an important aspect of the efforts towards the development of more efficient therapeutic approaches.

Our group has recently demonstrated that suppression of *de novo* lipogenesis by deletion of the enzymatic complex fatty acid synthase (FASN) in white adipose tissue results in "browning", improvement of cold adaptation and whole-body metabolism. Furthermore, white adipose tissue derived from FASN knock-out mice has been shown to have a beneficial impact on glucose homeostasis, when transplanted into metabolically healthy, chow-fed wild type mice.

While exploring the role of the DNL pathway, and glucose metabolism in our lab, we have also made a conscious effort to continue to develop novel therapies to target adipocytes and diabetes *in vitro* and *in vivo*. To address this, we developed a powerful technology for gene deletion utilizing CRISPR/Cas9 genome editing called CRISPR delivery particles (CriPs), composed of Cas9, sgRNA and the amphipathic peptide Endoporter. The advantage to the CriPs is the possibility to be systemically administered *in vivo* or in cell cultures *ex vivo* to introduce gene deletions with a simple non-viral, plasmid-free system. This system is not cell type-specific, thus, we can efficiently introduce Cas9 endonuclease and single guide RNA to delete a target gene in many cell types such as macrophages and primary pre-adipocytes, without the adverse effects of plasmid or virus – mediated Cas9 and sgRNA engineered cells. Results presented here show that significant depletion of the FASN protein was obtained upon treating isolated adipocytes with CriPs containing sgRNA targeting the *Fasn* gene. Up to 50% loss of FASN protein was obtained in these experiments.

The purpose of this research project is to exploit new data supporting the beneficial effects of *de novo* lipogenesis suppression and improvement in glucose tolerance by using the CriPs technology available to advance a novel therapeutic approach for type 2 diabetes with a significant curative impact. The steps required to achieve this goal consist of improving the genomic editing efficiency of CriPs to target mature adipocytes, deleting FASN in these cells, followed by transplanting

the genetically modified cells back into mice, in order to improve glucose tolerance and reverse insulin resistance. Results presented here show successful transplantation of brown adipose tissue or immortalized brown adipocytes into the visceral region of recipient mice to achieve improvement in glucose tolerance. Such transplants will serve as controls for future experiments designed to test whether FASN depletion in primary white adipocytes will promote glucose tolerance when similarly transplanted into mice. If successful, this would be a promising novel and long-lasting therapeutic approach to improve energy homeostasis in patients with type 2 diabetes.

ΠΕΡΙΛΗΨΗ

Η παχυσαρκία και ο διαβήτης τύπου 2 αποτελούν μείζονα υγειονομική απειλή για με αυξανόμενο επιπολασμό που πλήττει τις παραγωγικές ηλικίες. Μέχρι στιγμής, δεν υπάρχει αιτιολογική θεραπευτική προσέγγιση στην καθημερινή κλινική πράξη. Τα φαρμακευτικά σκευάσματα τα οποία χορηγούνται καθημερινά και δια βίου, στοχεύουν στη μείωση των επιπέδων γλυκόζης στο αίμα παρεμβαίνοντας στους μηχανισμούς σύνθεσης, κυτταρικής πρόσληψης και απέκκρισης της γλυκόζης. Ωστόσο, τα υψηλά επίπεδα γλυκόζης που προκαλούν τις σοβαρές καρδιαγγειακές επιπλοκές του διαβήτη τύπου 2, φαίνεται να είναι το τελικό αποτέλεσμα ενός μέχρι στιγμής ανεξιχνίαστου παθογενετικού μηχανισμού. Εντούτοις, αξιοσημείωτη πρόοδος έχει σημειωθεί για την κατανόηση της παθογένεσης της αντίστασης στην ινσουλίνη, καθώς αυτό προ-απαιτείται για την ανάπτυξη αποτελεσματικών και μόνιμων θεραπευτικών προσεγγίσεων.

Η ομάδας μας έδειξε πρόσφατα ότι η καταστολή της *de novo* σύνθεσης λιπαρών οξέων με τη γενετική απαλοιφή του ενζυμικού συμπλέγματος συνθάση των λιπαρών οξέων (FASN) στον λευκό λιπώδη ιστό, προκαλεί τη μετατροπή του σε φαιό, βελτίωση της προσαρμογής στο κρύο και του συστηματικού μεταβολισμού. Επιπροσθέτως, λευκό λίπος στο οποίο έχει εκλεκτικά απαλειφθεί το γονίδιο FASN όταν μεταμοσχεύθηκε σε μεταβολικά υγιή, αγρίου τύπου ποντίκια που τρέφονταν με κανονική διατροφή, είχε θετικό αποτέλεσμα στην ομοιόσταση της

γλυκόζης.

Παράλληλα με τη διερεύνηση του ρόλου του μονοπατιού της *de novo* λιπογένεσης και του μεταβολισμού της γλυκόζης στο εργαστήριό μας, καταβάλουμε σημαντικές προσπάθειες να αναπτύξουμε καινοτόμες θεραπείες για τον διαβήτη στοχεύοντας στα λιποκύτταρα *in vivo* και *in vitro*. Σε αυτό το πλαίσιο, αναπτύξαμε μία ισχυρή τεχνολογία για απαλοιφή γονιδίων αξιοποιώντας την τεχνολογία CRISPR/Cas9 για την επεξεργασία γονιδιώματος, τα CRISPR σωματίδια ενδοκυττάριας μεταφοράς (CriPs), που αποτελούνται από Cas9 ενδονουκλεάση, sgRNA και το αμφιπαθητικό πεπτίδιο Endoporter. Το πλεονέκτημα των CriPs έγκειται στη δυνατότητά τους να χορηγούνται συστηματικά *in vivo,* ή σε κυτταρικές καλλιέργειες *ex vivo* και να προκαλούν τη διαγραφή γονιδίων με ένα απλό σύστημα χωρίς την συμμετοχή ιών και πλασμιδίων. Αυτό το σύστημα δεν προορίζεται για συγκεκριμένο κυτταρικό τύπο, κι επομένως, είναι εφικτό να εισαγάγουμε αποτελεσματικά την Cas9 ενδονουκλεάση και οδηγό – RNA σε διαφόρων τύπων κύτταρα όπως μακροφάγα και πρόδρομα προ-λιποκύτταρα, για να διαγράψουμε ένα γονίδιο-στόχο χωρίς τις παρενέργειες της μεσολαβούμενης από πλασμίδια ή ιούς έκφρασης Cas9 και sgRNA μέσα στα κύτταρα. Εδώ, παρουσιάζονται αποτελέσματα που δείχνουν σημαντική μείωση της FASN σε επίπεδο πρωτεΐνης κατόπιν εφαρμογής CriPs με sgRNA που στοχεύει το γονίδιο FASN σε απομονωμένα πρόδρομα λιποκύτταρα. Σε αυτά τα πειράματα, σημειώθηκε μέχρι 50% απώλεια της

πρωτεϊνης FASN.

Ο στόχος αυτής της ερευνητικής εργασίας είναι να αξιοποιήσει τα νέα δεδομένα για την καταστολή της *de novo* λιπογένεσης που βελτιώνει την ανοχή στη γλυκόζη με τη διαθέσιμη τεχνολογία των CriPs, για την ανάπτυξη μίας νέας θεραπευτικής προσέγγισης για τον διαβήτη τύπου 2 με αξιοσημείωτα κλινικά αποτελέσματα. Τα απαιτούμενα βήματα για την επίτευξη αυτού του στόχου περιλαμβάνουν τη βελτιστοποίηση των CriPs στη διαγραφή γονιδίων στα πρόδρομα ώριμα λιποκύτταρα για την απαλοιφή της FASN σε αυτά, και ακολούθως τη μεταμόσχευσή τους πίσω σε ποντίκια, ώστε να βελτιώσουμε την ομοιόσταση της γλυκόζης και να αναστρέψουμε την αντίσταση στην ινσουλίνη. Τα αποτελέσματα που παρουσιάζονται εδώ δείχνουν επιτυχή μεταμόσχευση φαιού λιπώδους ιστού ή αθανατοποιημένων φαιών λιποκυττάρων στο σπλαχνικό λίπος ποντικών-ξενιστών για να επιτευχθεί βελτίωση της ανοχής στη γλυκόζη. Αν είναι επιτυχές αυτό, θα θεμελίωνε μία υποσχόμενη και μακροπρόθεσμη θεραπεία για τη βελτίωση της ενεργειακής ομοιόστασης στους ασθενείς με τύπου 2 διαβήτη.

LIST OF ABBREVIATIONS

AUC = Area under the curve

BAT = Brown adipose tissue

Cas9 = CRISPR – associated protein 9

CriPs = CRISPR/Cas9 delivery particles

CRISPR = Clustered regularly interspaced short palindromic repeats

Ctrl = Control

DNL = *De novo* lipogenesis

DSB = Double – stranded breaks

EP = Endoporter

EtOH = Ethanol

FASN = Fatty acid synthase

GFP = Green fluorescent protein

GTT = Glucose tolerance test

HDR = Homology directed repair

HFD = High fat diet

IBA = Immortalized brown adipocytes

idFASNKO = inducible FASN knock out

IVT = *In vitro* transcription

iWAT = inguinal white adipose tissue

KO = Knock out

NHEJ = Non-homologous end joining

PAM = Protospacer - adjacent motif

PBS = Phosphate-buffered saline

RNP = Cas9-sgRNA ribonucleoprotein

SC = Subcutaneous

sgRNA = Single guide RNA

TAM = Tamoxifen

TH = Tyrosine hydroxylase

UCP1 = Uncoupling protein 1

Vsc = Visceral

WAT = White adipose tissue

WT = Wild type

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INTRODUCTION

Type 2 Diabetes

Insulin resistance is one of the major acquired metabolic medical problems of the Western population. According to the CDC 2017 statistical report, type 2 diabetes is the 7th leading cause of death in the United States with 30.2 million Americans diagnosed, out of whom 28 million represent the economically productive population. [1] Thus far, the therapeutic strategy employed aims to lower blood glucose levels. However, this strategy has only managed to delay the complications of type 2 diabetes. [2] The need for a more promising curative approach to be developed is becoming more urgent with the increase of incidence of the disease. Progress towards this direction is expected to have a positive impact in the lives of millions of people who suffer or who are highly predisposed to develop type 2 diabetes. Over the past couple of decades, adipose tissue has been demonstrated to play a critical role in the development and progression of insulin resistance. Despite the negative connotations adipocytes have in relation to the pathogenic mechanisms underlying diabetes, there are new advances that support the idea that adipocytes can be exploited positively for possible curative approaches for the disease. [3, 4, 5, 6, 7] Adipose tissue in humans is composed of several different types of fat cells including white, brown and beige and all have distinct morphology and function. White adipocytes that comprise the majority of adipose tissue are mainly lipogenic in

nature while brown adipocytes that are present in much lower numbers are thermogenic in nature. Interestingly, brown adipose tissue (BAT) is more abundant during infancy in dorsal, perirenal, etc. sites [8] and progressively disappears with aging. It has been lately shown that metabolically healthy individuals do have active BAT, in contrast to the older paradigm that human adults completely lacked BAT.[9, 10, 11, 12, 13, 14, 15, 16] In brown adipocyte mitochondria, unlike in white adipocytes, the electron transport chain is physiologically uncoupled from ATP production and primarily leads to heat production. This phenomenon is mediated by thermogenic uncoupling protein 1 (UCP1) which is abundantly expressed in brown adipocyte mitochondria. [17]

De novo lipogenesis in adipocytes and glucose homeostasis

De novo lipogenesis (DNL) is the pathway of fatty acid synthesis from acetyl CoA and takes place in liver and adipose tissue. In the adipocytes, the lipids produced via DNL regulate several functions of the tissue [18], energy homeostasis and whole-body metabolism. [19, 20] Additionally, DNL intermediate products such as acetyl-CoA, malonyl-CoA and palmitate have been shown to regulate a wide range of cell processes. [21, 22] Among these, there are post-translational modifications (eg. acetylation, malonylation, palmitoylation) related to histone regulation, protein functions and gene expression. (Fig. I)[23, 24, 25] Apparently, nutritional state, insulin sensitivity and obesity strongly interact with DNL pathway in adipocytes through

mechanisms that have not been completely understood.[21, 29, 30, 31, 32, 33] Previous data have demonstrated that blocking DNL, by conditionally knocking-out (KO) the enzyme complex fatty acid synthase (FASN) that catalyzes the formation of palmitate from malate in adipocytes, results in browning the white adipose tissue (WAT) and significantly ameliorates glucose tolerance even in chow-fed, metabolically healthy mice.

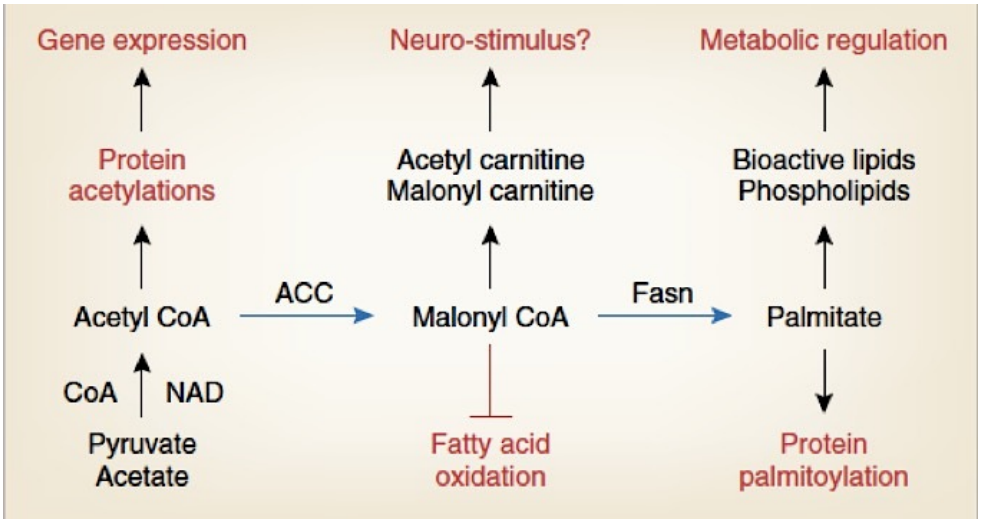

Figure I. De novo **lipogenesis in adipocytes. DNL regulates several functions of adipose tissue, energy homeostasis and whole-body metabolism. DNL Intermediate products such as acetyl-CoA, malonyl-CoA and palmitate have been shown to regulate a wide range of cell processes. These include post-translational modifications related to histone regulation, protein functions and gene expression.** **[33, modified]**

It has been previously demonstrated that suppression of the fatty acid synthesis pathway via *Fasn* deletion in white adipocytes stimulates neuronal signaling to

regulate the thermogenic programming in WAT. [34] Recently, further insight was gained into the effects of DNL disruption in adipocytes. Both in BAT and iWAT sympathetic neuronal activity is increased upon DNL disruption but only FASNKO in WAT can stimulate sympathetic neuronal outgrowth and promote thermogenesis in both types of adipose tissue (Fig. II). [35] Preliminary data produced by our group show that transplantations of FANSKO in WAT into wild type (WT), chow-fed, metabolically healthy mice, displayed improved glucose tolerance combined with local browning of their adipose tissue in the area surrounding the transplant.

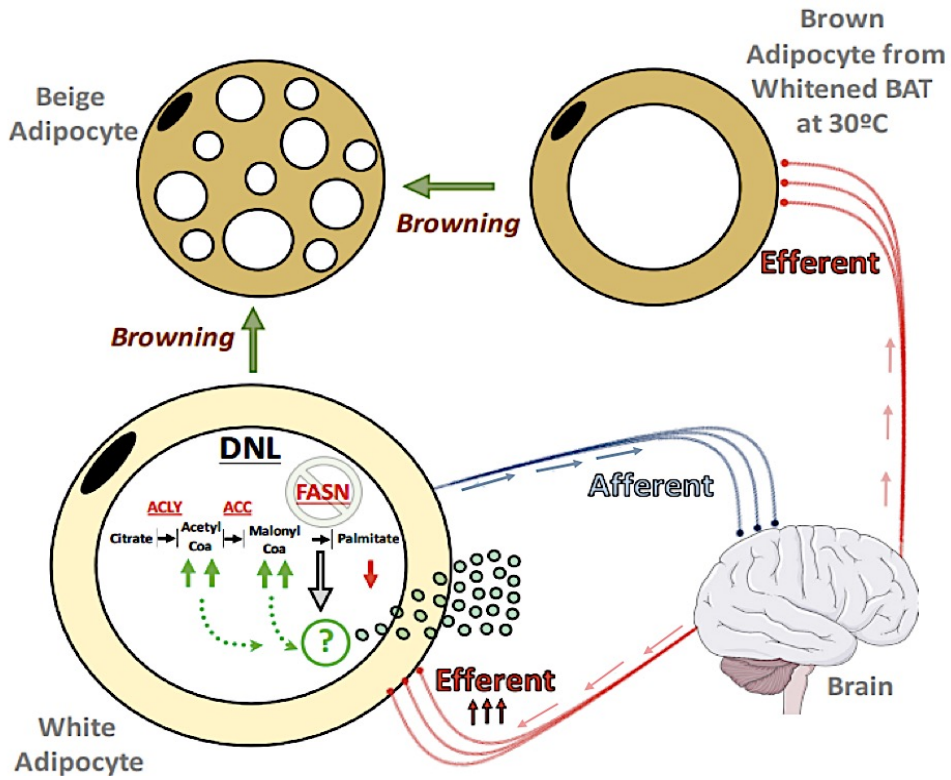

Figure II. **Proposed model of the mechanism that DNL inhibition by FASNKO increases neuronal activity and regulates thermogenesis in the adipose tissue. FASN deletion in white adipocytes induces browning and ameliorates glucose**

tolerance. FASNKO in white adipocytes stimulate neuronal signaling to control their thermogenic program through a mechanism that remains unexplained to a great extent. **[35, modified]**

CRISPR/Cas9 technology for gene editing

In the recent decades, a new method of genomic editing has been developed. Clustered regularly interspaced short palindromic repeats (CRISPR) – associated protein 9 (Cas9) exploits the ability of DNA endonuclease Cas9 associated with guide RNA oligos (sgRNAs) to recognize protospacer – adjacent motif (PAM) on a target gene. Three base pairs upstream that gene, double – stranded breaks (DSB) are generated. These mutations are repaired by non-homologous end joining (NHEJ) and by homology directed repair (HDR) with the use of a guide template DNA and, as a result, random insertions and deletions (indels) are caused that lead to permanent gene deletion (Fig. III).[40] Due to its promising perspectives, this system has been intensively studied mainly *in vitro* and *ex vivo* in addition to several ongoing *in vivo* clinical trials.[36, 37, 38, 39] In order to transfer the CRISPR/Cas9 gene therapy to clinical practice, it is essential to achieve efficient and safe delivery of the required macromolecules for genome editing.[41, 42, 43, 44] For that purpose, adeno-associated virus vectors have been used[45, 46, 47, 48], however, they are also associated with a limited packaging capacity and significant off-target effects.[49, 50, 51] In addition to the off-target effects, long-term expression of bacterial Cas9 protein and the AAV

capsids can trigger immune reactions that eliminate the perspectives for applications in humans. [46] Various CRISPR/Cas9 delivery approaches have been used efficiently including electroporation [52, 53, 54], micro-injection[55] and hydrodynamic injection[56, 57]. However, there have been concerns with these methods due to poor cell viability, toxicity and technical difficulty for applications *in vivo* or *ex vivo*.

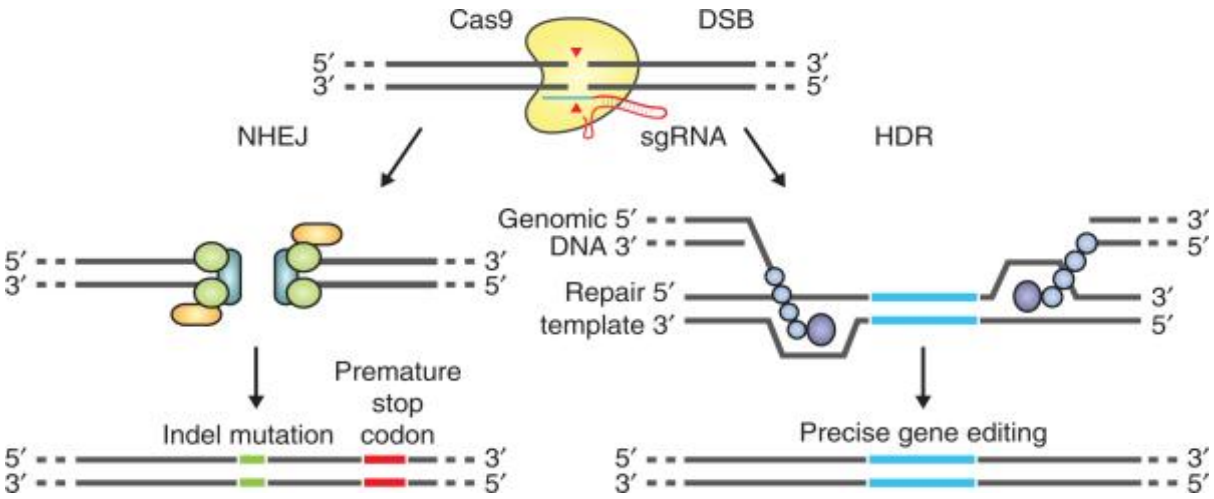

Figure III. **In CRISPR/Cas9 systems genome editing is promoted through DSB repair. Cas9–sgRNA complex recognizes protospacer–adjacent motives (PAM) on a target gene. Three base pairs upstream that gene, double–stranded breaks (DSB) are generated. DBS are repaired by NHEJ and by HDR with the use of a guide DNA template. As a result, random insertions and deletions are caused that lead to permanent gene deletion.** **[40]**

Purpose of project

The purpose of this project is to develop a novel *ex vivo* therapeutic approach for type 2 diabetes that would utilize the positive effects of DNL pathway disruption by

using CRISPR/Cas9 systems to engineer adipocytes. The ultimate goal is to obtain adipocytes from an individual with impaired glucose homeostasis and genetically modify them by targeting DNL. Once the DNL pathway is disrupted, the adipocytes will be reintroduced to the individual, and ultimately improve glucose tolerance. We believe this approach to implant genetically modified adipocytes will be a viable method for a therapeutic approach due to the recently published work that demonstrates human adipocyte xenografts implanted into the subcutaneous area of mice form a viable fat pad and positively impact glucose metabolism. [58] Thus, it is reasonable to expect that heterologous transplantations of engineered mouse cells into mice, and ultimately autologous transplantations of human engineered adipocytes back into the donor will have the potential to induce a positive phenotype. Therefore, this project has two main directions that are being worked in parallel.

A major primary goal of this project is to improve the efficiency of CRISPR/Cas9 systems to edit mature primary adipocytes *ex vivo* in order to achieve a remarkable FASN knock down. The adipocyte is a challenging cell type to transfect and is known for its fragility and low transfection efficiency without the use of viral vectors expressing Cas9 and sgRNA that would risk the safety of the technique for clinical applications. One caveat to depleting FASN is that it must be targeted after the differentiation of pre-adipocytes into mature adipocytes, because it is required for their differentiation with a mechanism that has not been explained. This obstacle

cannot be bypassed by simply enriching the differentiation media with fatty acids upon FASN depletion to achieve normal adipogenic differentiation.[59, 60, 61] In order to achieve that, a promising RNP-modified approach was recently developed by our group with CRISPR/Cas9 delivery particles (CriPs) were the Cas9-sgRNAs are physically inserted in the cells and 24 hours following the uptake, Cas9 is degraded and thus the chances for off-target effects and immune reactions to bacterial Cas9 are reduced.[62] The advantage of this approach is that, unlike with interfering RNA, the genome editing with CriPs is permanent and, in combination with the long lifespan of adipocytes, will potentially have long-lasting effects on glucose homeostasis.

The other major goal of this project is to determine if adipocyte implants in which DNL pathway is disrupted, likewise adipose tissue transplantations, can induce similarly improved glucose tolerance. Unpublished data produced by our group show that visceral space transplantation of FASNKO WAT even in chow-fed mice causes browning and improves systemic glucose metabolism. This project also aims to evaluate the hypothesis that FASNKO white adipocyte implants in the visceral or subcutaneous space are capable of inducing a similar phenotype. If this is correct, it could possibly provide a novel approach to exploit DNL down-regulation effects in adipocytes to improve glucose tolerance in patients with diabetes. Progress in these two directions will be analyzed in the Results section, in parts A and B, respectively.

MATERIALS AND METHODS

Materials

All chemicals are purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified and are used as received. Cas9 protein is purchased from PNA BIO, INC. (Newbury Park, CA, USA). DNA oligonucleotides are purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA). MEGAshortscript T7 Transcription Kit, Lipofectamine® RNAiMAX, Platinum™ Taq DNA Polymerase High Fidelity kit and Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F12 1X) are purchased from ThermoFisher Scientific (Waltham, MA, USA). BsaI, DraI, T7E1 and NEBuffer 3 are obtained from New England Biolabs Inc. (Ipswich, MA, USA). pUC57-sgRNA expression vector was purchased from Addgene (plasmid # 51132) (Cambridge, MA, USA). Endo-Porter (EP) is purchased from Gene Tools (Philomath, OR, USA). QIAquick PCR Purification Kit is purchased from Qiagen Inc. (Valencia, CA, USA). 4-20% Mini-Protean TBE Gel is purchased from Bio-Rad Laboratories (Hercules, CA, USA). Insulin is purchased from Cell Application (San Diego, CA, USA) Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) and Indomethacin are purchased from Sigma (St. Louis, MO, USA) and Rosiglitazone is purchased from Cayman Chemical (Ann Arbor, MI, USA). For cellular implants, Matrigel extracellular matrix is purchased from Corning Life Sciences (NY, USA).

Preparation of sgRNA template and synthesis of sgRNA

sgRNA sequences targeting *Fasn* and *Gfp* were designed using sgRNA Designer and CHOPCHOP online platform developed by Broad Institute and Harvard University, respectively. Templates for sgRNAs are generated by inserting annealed complementary oligonucleotides with the sgRNA sequences to the pUC57-sgRNA expression vector encoding a T7 promoter. [63] The sgRNA templates are linearized by DraI and transcribed *in vitro* using the MEGAshortscript T7 Transcription Kit according to manufacturer's instructions. Transcribed sgRNA is resolved on a 10% denaturing urea-Polyacrylamide gel electrophoresis to check the size and purity.

Preparation of the CriPs

Purified bacterial Cas9 protein is processed to remove endotoxin and other contaminants and is used for loading of sgRNA. The lyophylized Cas9 protein is suspended in water containing 20% glycerol. Cas9 protein and sgRNA are mixed in NEBuffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT, pH 7.9) and incubated at 37°C for 10 minutes to form nano-size complexes. These loaded Cas9-sgRNA complexes are then complexed with EP in PBS at room temperature for 15 minutes to form the final CriPs.

T7E1 assay

Two days after treatment, the cells are lysed in cell lysis buffer (1M KCl, 1M $MgCl_2$, 1M Tris-Base pH 8.3, 0.45% NP40, 0.45% Tween20, 0.1 mg/ml proteinase K) and the lysates are used as templates in PCR reactions to amplify the targeted genomic loci of *Fasn* using Platinum™ Taq DNA Polymerase High Fidelity kit according to the manufacturer's instructions. PCR products are purified using QIAquick PCR Purification Kit and quantified by Nanodrop. 200ng of purified PCR products are mixed with 2µl of 10x NEBuffer 2 (New England Biolabs, Inc) up to a total volume of 19µl and denatured then re-annealed with a thermal cycler protocol as follows: 95°C for 5 minutes, 95 to 85°C at 2°C /second, 85 to 20°C at 0.2 °C /second. The reannealed DNA is incubated with 1µl of T7Endonuclease 1 at 37°C for 15 minutes and the reaction is stopped with the addition of 1.5µl of 0.25 M EDTA, and analyzed on a 4-20% gradient Mini-Protean TBE Gel electrophoresed for 1.5 hours at 100 Volts, then stained with ethidium bromide. The frequency of indels is calculated based on the band intensities quantified using Image Lab (Bio-Rad Laboratories). The intensities of the cleaved bands are divided by the total intensities of all bands (uncleaved and cleaved) to determine the frequency of indels and thus provide an estimate of gene modification levels.

Primary pre-adipocyte cultures and differentiation

C57BL6/J mice male or female mice, gender-matched from 3 to 4 weeks old are used for iWAT collection. Subcutaneous inguinal white adipose tissue is obtained from 4 or 5 mice to start each culture of primary pre-adipocytes. The iWAT is minced in HBSS with 3% BSA and 2mg/ml collagenase and incubated in 37^{o}C while shaking, then filtered and centrifuged to remove the cells deriving from stromal vascular fraction. Erythrocytes are lysed with the use of lysis buffer and the cells are plated on a 100mm dish with DMEM/F12 (1X) enriched with 10% Fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA), 100 μg/mL streptomycin and 100 units/mL penicillin (Thermo Fisher Scientific, Waltham, MA) and 0.5% Normocin (InvivoGen, San Diego, CA, USA), from now on referred as regular media. After one passage, the primary cells can be passaged up to P2 before CriPs treatment for which they are plated in 12-well-plates to grow to full confluency before differentiation. Primary differentiation media is added 1 day following full confluence that consists of regular media enriched with insulin (1:400), dexamethasone (1:4000), indomethacin (1:1000), IBMX (1:500) and rosiglitazone (1:10000). This media is replaced 48 hours later with secondary media containing regular media enriched with insulin and rosiglitazone. On Day 4, secondary media was replaced with regular media containing insulin only. On day 6, the cells are considered mature primary white adipocytes.

Cell line culture and differentiation

An immortalized brown pre-adipocyte cell line had been previously established and frozen at passage 5 by our group derived from C57BL6/WT mice. The cells are thawed and maintained in regular media. Cells are maintained in a water-jacket 37°C/5% CO_2 incubator. Before implantation, they are plated in 145mm culture dishes (Corning) each of them destined to be implanted in one recipient. When the pre-adipocytes are 100% confluent, differentiation is started with the primary mix containing regular media enriched with insulin, dexamethasone, indomethacin, IBMX, rosiglitazone and T3 which at 48 and 96 hours after differentiation was replaced exactly as mentioned above for primary white pre-adipocytes.

Western blots

Protein expression analyses are performed to confirm *Fasn* knock-down in cells treated with CriPs targeting *Fasn* and in idFASNKO primary adipocytes in order to confirm the depletion of the protein before transplantation. Cells are lysed for protein extraction with radioimmunoprecipitation assay (RIPA) buffer containing 20mM HEPES [pH 7.4], 150mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS, 10% glycerol, 0.5% sodium deoxycholate that has been supplemented with Halt protease and phosphatase inhibitors (Thermo Pierce). 20μg of sample, diluted to 20mg/mL in 4x SDS sample buffer from cell lysates are then resolved by SDS-PAGE. After

samples are resolved on gels the proteins are transferred to nitrocellulose member by electroporative transfer. Membranes are blocked in 5% non-fat milk and blotted with antibodies against FASN (Rabbit, BD Biosciences) and Tubulin (Mouse, Sigma Aldrich) in 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween20 (TBS-T).

Animals

C57BL6/J mice were purchased from Jackson Laboratory and are used for primary white pre-adipocytes isolation and for the transplantation experiments as BAT donors and recipients. For tamoxifen-inducible deletion of *Fasn* (idFANSKO) in cultured primary adipocytes, idFASNKO mice are generated as previously described. [34, 35] All mice are housed on a 12-hour light/dark schedule and have free access to water and food (normal chow), unless otherwise specified. All procedures involving animals are approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

Glucose tolerance tests and serum collection

Glucose tolerance tests are performed on WT recipient mice for transplantation studies, as indicated, after overnight fasting. 1g/kg of glucose is intraperitoneally injected. Blood samples are drawn from the tail vein at the indicated times and blood glucose levels are determined using a Breeze 2 glucose meter (Bayer and Alpha -

Trak) or a Contour Next One glucose meter, due to Breeze 2 strips discontinuation. Blood collected whenever indicated from the submandibular vein of the mice that runs across the lower jaw with the technique of cheek bleeding in anesthetized individuals. At the end of the transplantation studies follow up, when the mice are euthanized blood is collected with cardiac puncture. Blood (100-200μL per bleed) is placed in EDTA-treated tubes and placed on ice. To collect sera, the blood samples where centrifuged at 4^0C, 2,000xG for 10 minutes and the sera (50-100μL per sample) are placed into sterile Eppendorf tubes and stored at -80^0C.

Transplantation procedures

Both for transplantations in the subcutaneous dorsal adipose tissue area and in the perigonadal visceral fat pad, donor mice were anesthetized. Anesthesia was delivered in a heated chamber that provides stable flow 500cc per minute of O_2 mixed with isoflurane 3% (v/v) for induction and then a continuous administration of established isoflurane 2% (v/v) concentration in O_2 during the procedure. Immortalized brown adipocytes, idFASNKO primary adipocytes and their control cells were trypsinized on the day of transplantation with Trypsin – EDTA (1x) (Thermo Fisher Scientific, Waltham, MA), washed and suspended in PBS 1X and placed on ice for transfer to the procedure room. At the animal facility procedure room, cell suspension in PBS up to a volume of 500μl were mixed with 500μl of matrigel on ice. Matrigel is in liquid form and injected in the subscapular area or

inside the visceral fat pad with an 18 ½ G and 20 ½ G needle for the subcutaneous and visceral injection, respectively. Constant pressure is applied at the injection site until the implant solidified at 37^{o}C to avoid any leakage. BAT transplantation procedures are described in the results section.

RESULTS

PART A: CriPs EFFICIENCY FOR PRIMARY WHITE ADIPOCYTES

The primary aim of this project was to optimize a simple delivery vehicle for highly efficient gene deletion in mature adipocytes via CRISPR/Cas9-based genome engineering. The ultimate goal is to achieve a permanent FASN depletion in primary white adipocytes using safe techniques with the possibility to be transferred to clinical practice in the future. For that purpose, two distinct steps should be accomplished. Firstly, a major step is to develop an optimized CRISPR/Cas9 technique for mature adipocytes with high efficiency and consistency and secondly, to optimize a method as accurate and invariable as possible, to detect and quantify the gene deletion. Both these aspects will be analyzed below.

CriPs technology development for gene deletion

A major goal of this project was to optimize a simple delivery vehicle for specific gene deletion via the CRISPR/Cas9-based genome targeting mechanism. For therapeutic applications, Cas9 protein direct intracellular delivery, as opposed to continuous expression by vectors in cells, seems promising because it could bypass uncontrolled integration of plasmid DNA into the host genome, immune responses and off-target effects. For that purpose, CriPs that can deliver Cas9 protein bound to sgRNA to mediate gene deletion *in vitro* and *in vivo* were prepared by our group. For CriPs formation purified bacterial Cas9 protein is used for loading sgRNA. The

sgRNA sequence is designed to target the gene of preference at a site adjacent to a PAM sequence. The loaded Cas9-sgRNA complexes are then covered with an amphipathic Endo-Porter (EP) peptide which mediates the uptake of the Cas9-sgRNA by live cells with minimum toxicity or detectable damage. Thus, the CriPs contain only the three molecular components: Cas9 protein, sgRNA an EP (Fig. 1). (*Shen, Y., et al., 2018,* posted online on biorxiv.org) [77] CriPs with different ratios of EP:Cas9-sgRNA complexes were measured for their sizes and charges (Table 1). Dynamic light scattering (DLS) measurements showed that the average hydrodynamic sizes of EP and Cas9 alone, as well as Cas9-sgRNA (1:1) were 0.83±0.05 nm, 7.8±0.9 nm and 14.4±1.0 nm, respectively.

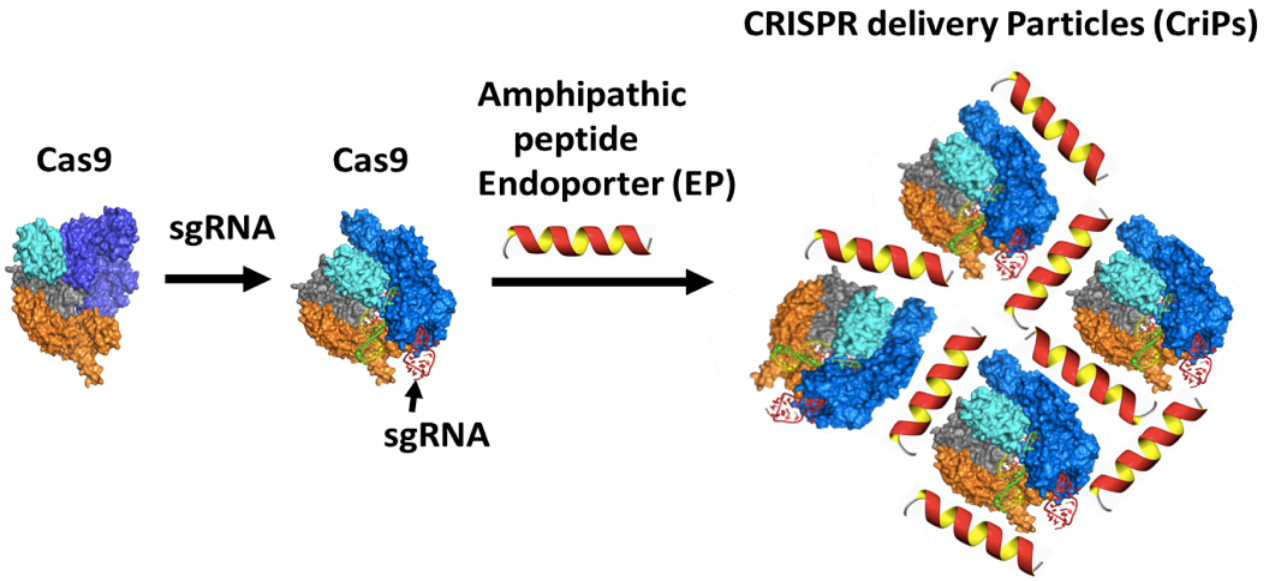

Figure 1. **Preparation of CRISPR delivery particles (CriPs). Purified bacterial Cas9 protein that is carefully processed to remove endotoxin and other contaminants is used for loading of sgRNA. The sgRNA sequence is designed to**

target a selected gene at a site adjacent to a PAM sequence in the DNA of that target gene. The loaded Cas9-sgRNA nanocomplexes are then coated with an amphipathic peptide, Endo-Porter (EP), which is required to mediate uptake of the Cas9-sgRNA complexes into live cells without toxicity or detectable damage. (*Shen, Y., et al., 2018,* posted online on biorxiv.org) [77]

The hydrodynamic size of the CriPs consisting of Cas9-sgRNA-EP (1:1:20) was 376.8±20.2 nm. The sizes of the CriPs were further increased when coated with more EPs, suggesting that each particle contains multiple Cas9-sgRNA complexes associated with EP peptides. The sizes of CriPs remained unchanged with the dilution in the DMEM which mimics the *in vitro* cell culture conditions, suggesting the stability of the particles in the culture media (Table 1).

Zeta potentials of the particles were also measured. Positive charges of +6.0 ± 4.4 mV, +14.6 ± 6.3 mV and +20.7± 6.0 mV were observed for the CriPs loaded with Cas9-sgRNA-EP with a molar ratio at 1:1:20, 1:1:150 and 1:1:250, respectively. The overall positive charge on the surface of the particles could facilitate the cellular uptake by interacting with the negatively charged cell membranes (Table 1).

Table 1. **Size and charge measurements of particles. *DMEM media (*Shen, Y., et al., 2018,* posted online on biorxiv.org)** [77]

	Size (nm)	Charge (mV)
EP	0.83 ± 0.05	18.0 ± 5.5
Cas9	7.8 ± 0.9	13.9 ± 6.8
Cas9-sgRNA (1:1)	14.4 ± 1.0	-2.4 ± 3.8
Cas9-sgRNA-EP (1:1:20)	376.8 ± 20.2	6.0 ± 4.4
Cas9-sgRNA-EP (1:1:150)	1240.9 ± 243.8	14.6 ± 6.3
Cas9-sgRNA-EP (1:1:250)	1238.7 ± 34.8	20.7 ± 6.0
Cas9-sgRNA-EP (1:1:20) in DMEM*	371 ± 30.5	N/A
Cas9-sgRNA-EP (1:1:150) in DMEM*	1155.6 ± 85.3	N/A
Cas9-sgRNA-EP (1:1:250) in DMEM*	1543.3 ± 56.3	N/A

Synthesis of sgRNA targeting *Fasn*

The first step to achieve efficient FASN knock down in primary white adipocytes with CriPs is to design and assess sgRNAs for *Fasn* as far as their efficiency and then *in vitro* transcribe the most efficient for the CriPs experiments. Firstly, the most efficient sgRNA sequence targeting *Fasn* was determined between four different candidate sgRNA sequences that were designed:

1) GGGTGGACACCGGATTCGGT,

2) GGCTCTATGGATTACCCAAG,

3) GGCAGGGTCGATATAGATGG,

4) GGTTGGCCCAGAACTCCTGT

All candidate sgRNAs were purchased in small quantities and each of them was used to treat mature primary adipocytes using the CriPs delivery system described above. Two days after treatment, the cells were lysed and a T7E1 assay was used to measure the efficiency by quantifying the intensities of the main band and indel bands and calculating the indel frequency that corresponded to each sgRNA sequence. The fourth sequence was shown to be the most efficient one with an indel frequency of approximately 20%, which is considered low, yet the highest among the candidate sgRNAs (Fig. 2). Thus, all following experiments were performed with the FASN sgRNA sequence 4, from now on referred as FASN sgRNA.

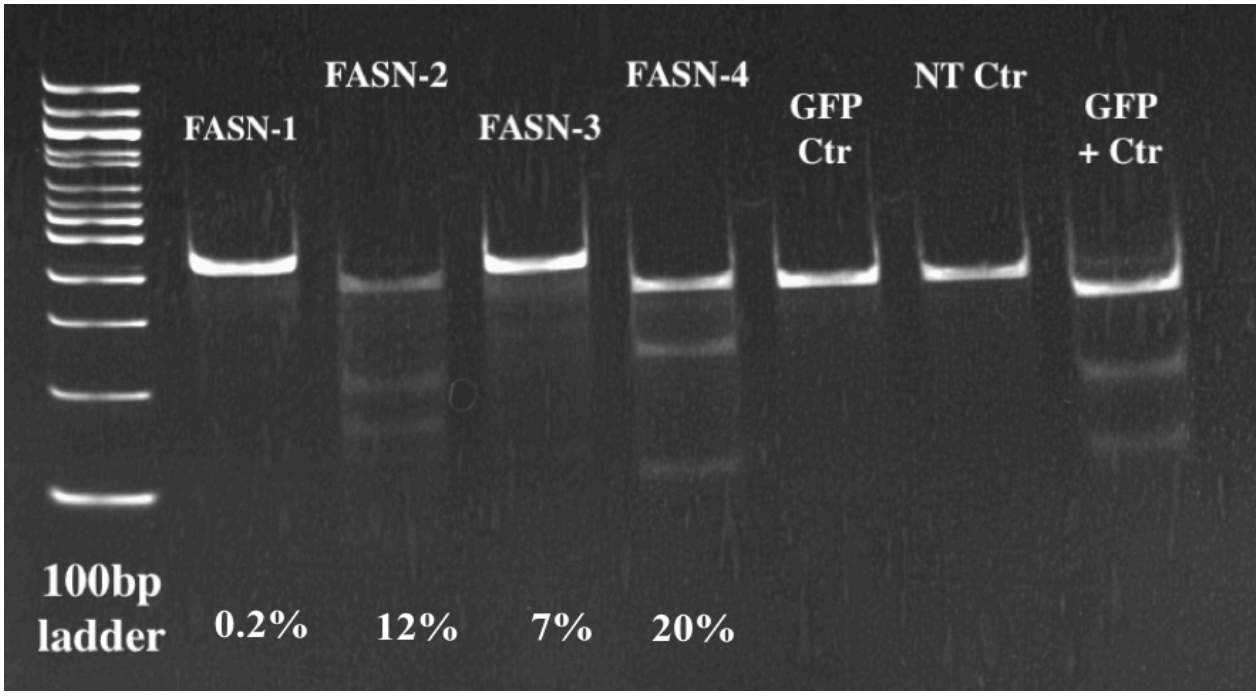

Figure 2. **T7E1 for the selection of sgRNA targeting *Fasn* with the highest efficiency among the candidate sgRNA sequences. *Fasn* sgRNA 4 is shown in this figure to be the most efficient with an indel percentage of approximately 20% when mature primary adipocytes were treated. In this figure, *Fasn* was amplified in the initial PCR with primers specific for the gene modified by sequence 4. Main product and expected indel sizes in base pairs: Sequence 1 [399=305+94], Sequence 2 [355=153+202], Sequence 3 [393=127+266], Sequence 4 [331=225+106].**

For the *in vitro* transcription (IVT) of FASN sgRNA, linearized plasmid DNA was used as a template. pUC57-sgRNA expression vectors [63] with sequence No4 incorporated was used to produce the template according to the MEGAshortscript™

Kit instructions and the product ran in a 10% denaturing gel in order to confirm the sgRNA integrity. Additionally, *in vitro* cleavage assay was performed to confirm the ability of the sgRNA to target *Fasn* gene as designed when applied with Cas9 endonuclease in purified PCR product with *Fasn* gene amplified (Fig. 3).

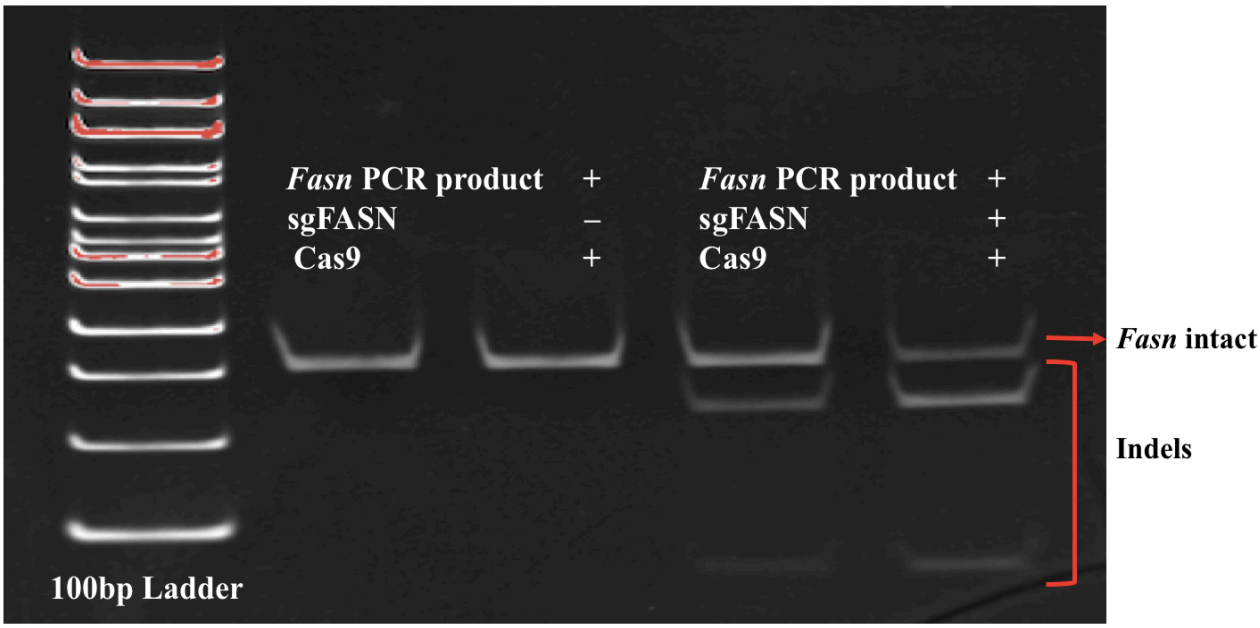

***Figure 3. In vitro* cleavage assay for IVT FASN sgRNA with a 100bp ladder (1st lane), and samples with purified *Fasn* PCR product were treated with Cas9 without or with FASN sgRNA (2nd, 3rd and 4th 5th lanes, respectively). The samples ran in a polyacrylamide 4-20% gradient TBE gel. In 4th and 5th lanes, indels are clearly visible and thus, the proper functionality of the IVT sgRNA synthesized was confirmed.**

Treatment of primary white adipocytes with CriPs targeting *Fasn*

The next step was to evaluate if a consistent knock-down of *Fasn* in murine primary differentiated adipocytes can be achieved with the use of CriPs. Gender – matched mice, aging 3 to 4 weeks old, are used for the isolation of primary white pre-adipocytes. These cells are plated in 12well-plates up to 100% confluency. Two days after having reached 100% confluency, differentiation began according to the lab's standard protocol described on materials and methods section. After six days post differentiation, when primary cells are considered fully mature adipocytes, treatment with CriPs targeting *Fasn* or *Gfp* is applied.

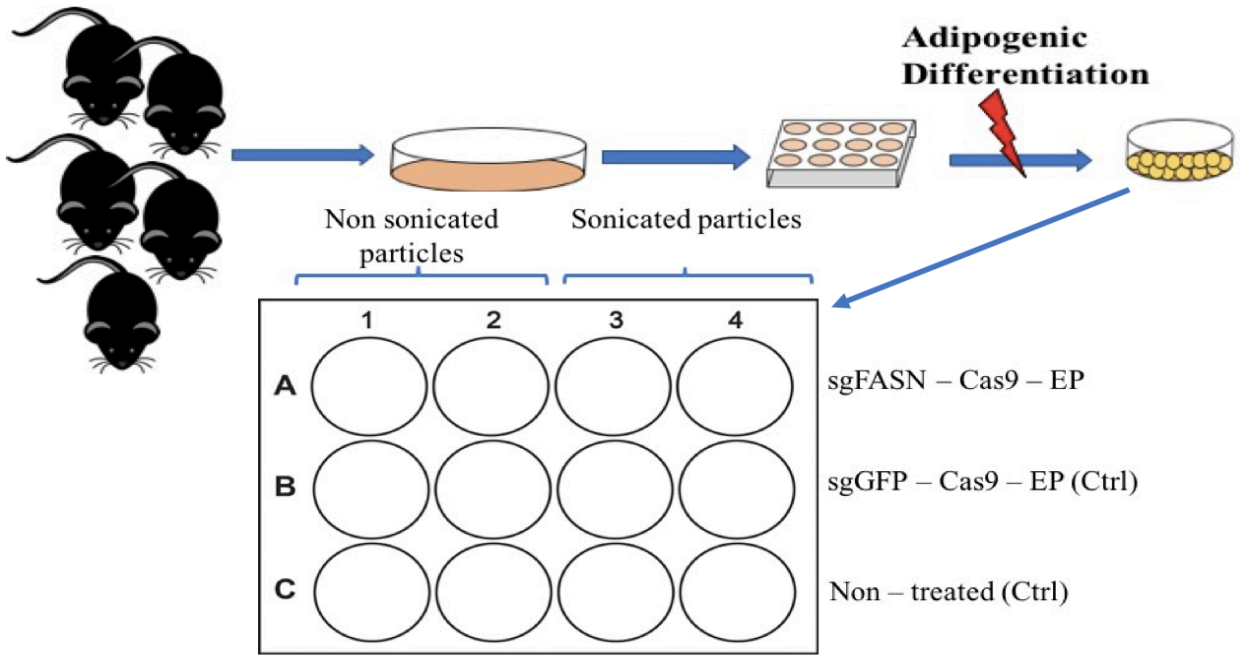

Figure 4. **Design and setting of a typical treatment experiment of murine primary mature white adipocytes deriving from the inguinal adipose tissue, with CriPs to knock-down FASN.**

CriPs targeting *Gfp* is used as negative control since the mice used do not express *Gfp*. In the context of these preliminary efforts, both sonicated and non-sonicated CriPs particles have been used to evaluate sonication efficiency. Sonication might facilitate the dilution of the particles in the treatment media. Three days after treatment, the adipocytes are harvested for protein and T7E1 assay (Fig. 4).

A challenging aspect of this project is to establish an accurate and consistent method to detect the gene editing we have achieved. So far, two approaches have been used, T7E1 assay that indicates the insertions into the gene loci and western blotting that shows the knock down at the protein level. Previously, our group used flow cytometry to assess GFP knock down with CriPs in J774A.1 cells and peritoneal exudate cells from transgenic mice expressing GFP. T7E1 assay has been used to assess pre-adipocytes knocked down with CriPs targeting Nrip1 because there is no available antibody for Nrip1 protein for western blotting. Moreover, deep sequencing which is the gold standard method to detect the gene editing has been used for the same purpose by our group. (*Shen, Y., et al., 2018,* posted online on biorxiv.org) [77]

In order to detect FASN knock down in primary white adipocytes, three approaches (T7E1 assay, western blotting and deep sequencing) are available. Surprisingly, in a series of repetitive experiments with CriPs targeting FASN treatments, a remarkable discrepancy between T7E1 assay and western blotting has been continuously

observed. T7E1 assay showed inconsistent, from extremely weak (<10%) to completely absent, indel bands sometimes visible in the control cells apart from the engineered cells, while western blotting revealed a highly consistent and repetitive knock down of FASN in the CriPs targeting FASN cells. Indeed, western blot analysis for FASN showed a remarkable 51% down-regulation of FASN levels in the treated cells compared to control, when cells were treated with non-sonicated particles (Fig. 5). A less significant down-regulation of FASN was achieved with sonicated CriPs (Fig. 6). These results are encouraging towards a highly efficient and safe *ex vivo* gene editing of cells with CriPs and the gene editing going to be confirmed with deep sequencing shortly.

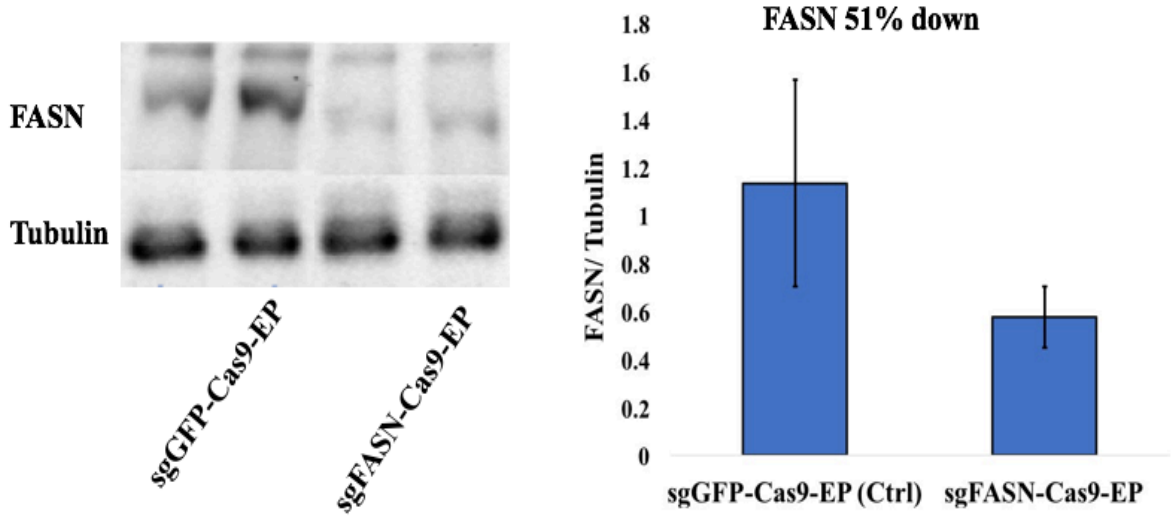

Figure 5. **Western blotting in mature white adipocytes 3 days after treatment with non-sonicated CriPs targeting *Fasn* and *Gfp* (negative control). FASN protein levels were decreased by 50.998% in the cells treated with CriPs**

targeting *Fasn*. This is a remarkable down-regulation achieved with a CRISPR/Cas9 technology without the use of viral or plasmid Cas9 expression.

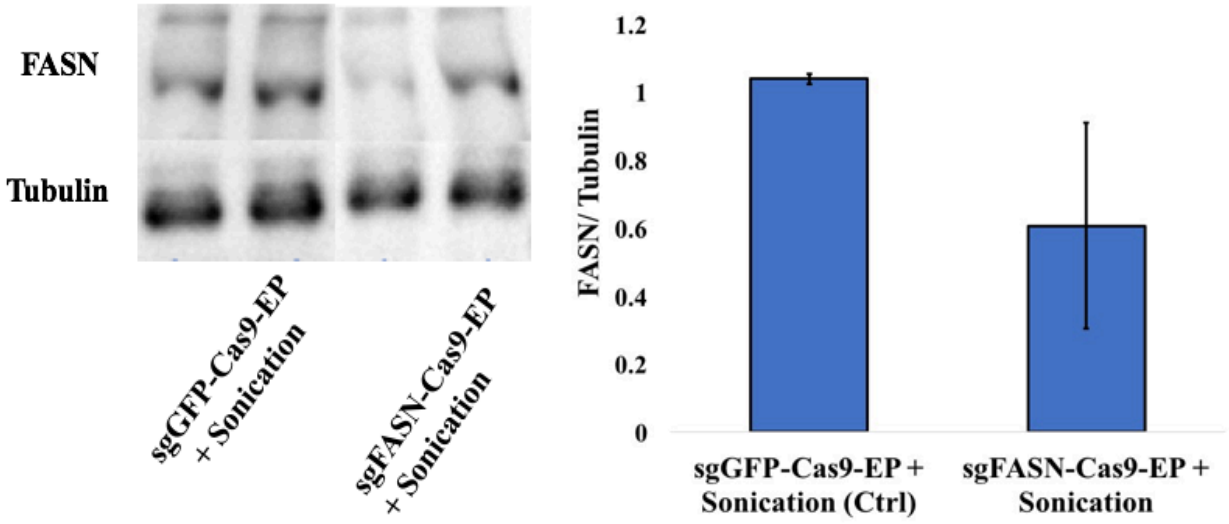

Figure 6. **Western blot analysis in mature white adipocytes three days after treatment with sonicated CriPs targeting *Fasn* and *Gfp* (negative control). FASN protein levels were decreased in the cells treated with CriPs targeting *Fasn* but without the consistent remarkable knock-down achieved with the non-sonicated particles.**

PART B: HETEROLOGOUS TRANSPLANTATION STUDIES TO EVALUATE CELL IMPLANT EFFICIENCY

In parallel with the experiments to optimize CriPs to genome edit primary adipocytes, transplantation studies were performed. The ultimate goal of these studies is to evaluate the potential of CriPs engineered–cells for *Fasn* knock-down to form a fat pad when transplanted and induce a metabolically healthier phenotype as previously shown with FASNKO fat tissue transplantations. The first step to reach this goal was to assess our positive controls and for that purpose immortalized brown adipocytes (IBA) and BAT were initially transplanted. The major hypothesis to examine is that IBA implants into the subcutaneous or visceral space can be transformed into a vascularized and innervated fat pad. Similarly, the BAT engraftment needs to be evaluated. Then, if engraftment is successful, the impact of these interventions on glucose homeostasis will be assessed in order to confirm whether they can function as positive controls for the FASNKO adipocyte implants. For these experiments, cultured cells were mixed with matrigel which is liquid at $4^{o}C$, becomes solid at $37^{o}C$ and provides a supportive environment for cells after implantation. It has been previously demonstrated by our group that when it is mixed with primary adipocytes and injected in the subcutaneous area, it can form a fat pad. We followed up the recipients for 12 weeks with weight and wherever possible, food

uptake measurements, glucose tolerance tests and morning sera collection for future appetite hormones levels panel. Both subcutaneous and visceral transplantations were studied to assess the site with the most effective phenotype, taking into account that subcutaneous space access is less interventional. An outline of the transplantation studies that have started in the context of this project, is provided below on table 2.

Table 2. **Outline of the transplantation experiments. Cohort A has been completed, Cohorts B and C are in progress.**

Cohort	Transplantation Groups (mice per group)	Mice/ Group	Sex	Recipients' Age	Donors' Age	BAT quantity	IBA Differentiation Day
A	1. SC IBA + M/g 2. SC BAT 3. SC PBS + M/g	4	F	6-7 weeks	10 weeks	± 0.25g/ mouse	16
B	1. Vsc IBA + M/g 2. Vsc BAT 3. Vsc PBS + M/g	5	M	8-9 weeks	11 weeks	± 0.45g/ mouse	9
C	1. SC IBA + M/g (4) 2. SC BAT (5) 3. SC PBS + M/g (5) 4. SC idFASNKO Adipocytes +M/g (4) 5. SC non-idFASNKO Adipocytes +M/g (4)	4-5	F	7 weeks	16 weeks	± 0.24 g/ mouse	19

SC=Subcutaneous; Vsc=Visceral; M/g=Matrigel; IBA=Immortalized Brown Adipocytes; BAT=Brown Adipose tissue; F=Female; M=Male
***All donors – recipients were sex - matched**

BAT transplantation procedures

BAT was collected from the donor WT chow-fed mice on the day of transplantation in a sterile environment (Fig. 7A). Contaminated cells, tissue or matrigel and the consequent host immune reactions could jeopardize the engraftment of the implants or interfere with the outcome of the studies. Donor mice were euthanized with CO_2 and BAT was removed from their subscapular space and kept in saline preheated at 37°C. BAT collected was weighted and split into as many portions as the recipient mice, in sterile normal saline and transferred on ice to the animal facility procedure room. Recipient mice were anesthetized with isoflurane as previously described (Fig. 7B). The skin in sites of subcutaneous and visceral transplantations and visceral injections was shaved to avoid any contamination with fur that could cause inflammation and abscesses. BAT was surgically inserted into the subscapular or visceral space (Fig. 8, 9).

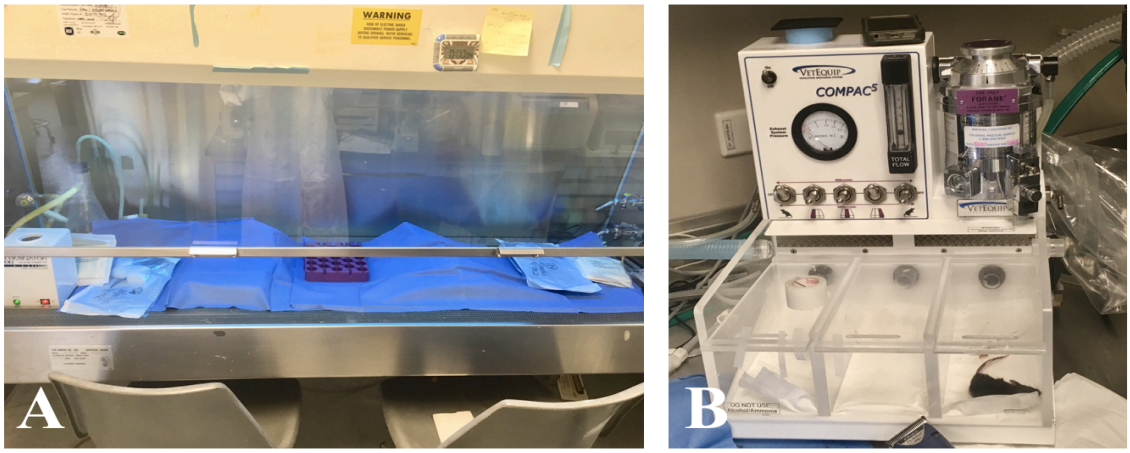

Figure 7. **A. BAT allografts were collected in the tissue culture room under laminal flow. All tools were autoclaved on the previous day and were sterilized**

with a beads-sterilizer among the different samples. Sterile gauzes and surgical gloves were used to handle the donor mice. B. Anesthesia device with three chambers and breathing tube. O_2 and isoflurane administration is controlled at desirable concentrations at a constant flow of 500cc per minute. For this project, it is used to anesthetize recipient mice for transplantation procedures and cheek bleeds.

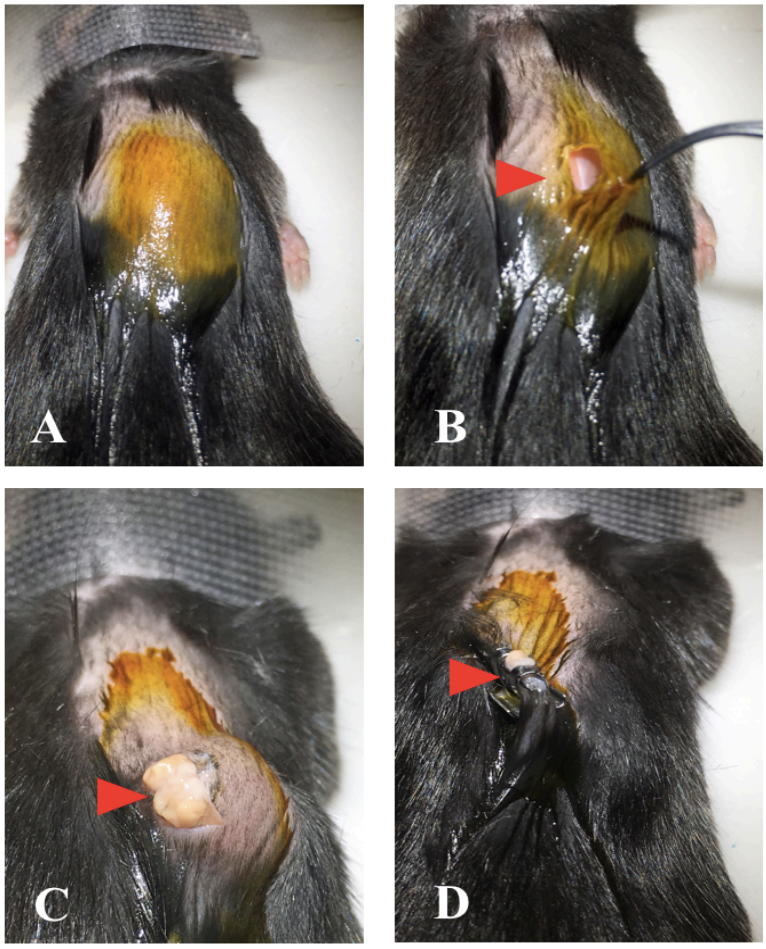

Figure 8. **Transplantation process of BAT in the subcutaneous space. A. The dorsal area of recipient mice was shaved and prepared with application of povidone iodine and ethanol for three times. B. An incision was performed**

in the dorsal area to access the subscapular space and create the "pocket" were fat pads were transplanted. C. BAT was inserted in the "pocket" created. The arrow shows the transplant while it is being inserted. D. The skin was closed with a metal clip which was removed 14 days after the procedure.

For the visceral transplantations, BAT was surgically inserted into the perigonadal fat with a novel technique that we developed for this study.

Previous attempts to transplant tissue into the perigonadal fat have been challenged because the host tissue was severely damaged due to ruptures when the donor implants were inserted. As a result, the implants could not be sustained and engrafted into the target host organ, the visceral fat space. Additionally, excessive inflammation and abscesses formation into the intraperitoneal space risked the success of these studies and interfered with the reliability of the data.

For these series of studies, we developed an approach that has not been previously described. We determined through a trial-and-error process that for 20-30g mice, 700µL was the maximum saline volume an average size visceral fat pad could sustain without leakage or ruptures. After the exposure of the visceral fat, 700µL of sterile saline were slowly injected. Saline makes the tissue expand in a similar way to an edema, without any damage. After the incision, saline leaks out but the tissue remains expanded and thus its capacity to host implants is increased without any severe complications (Fig. 9).

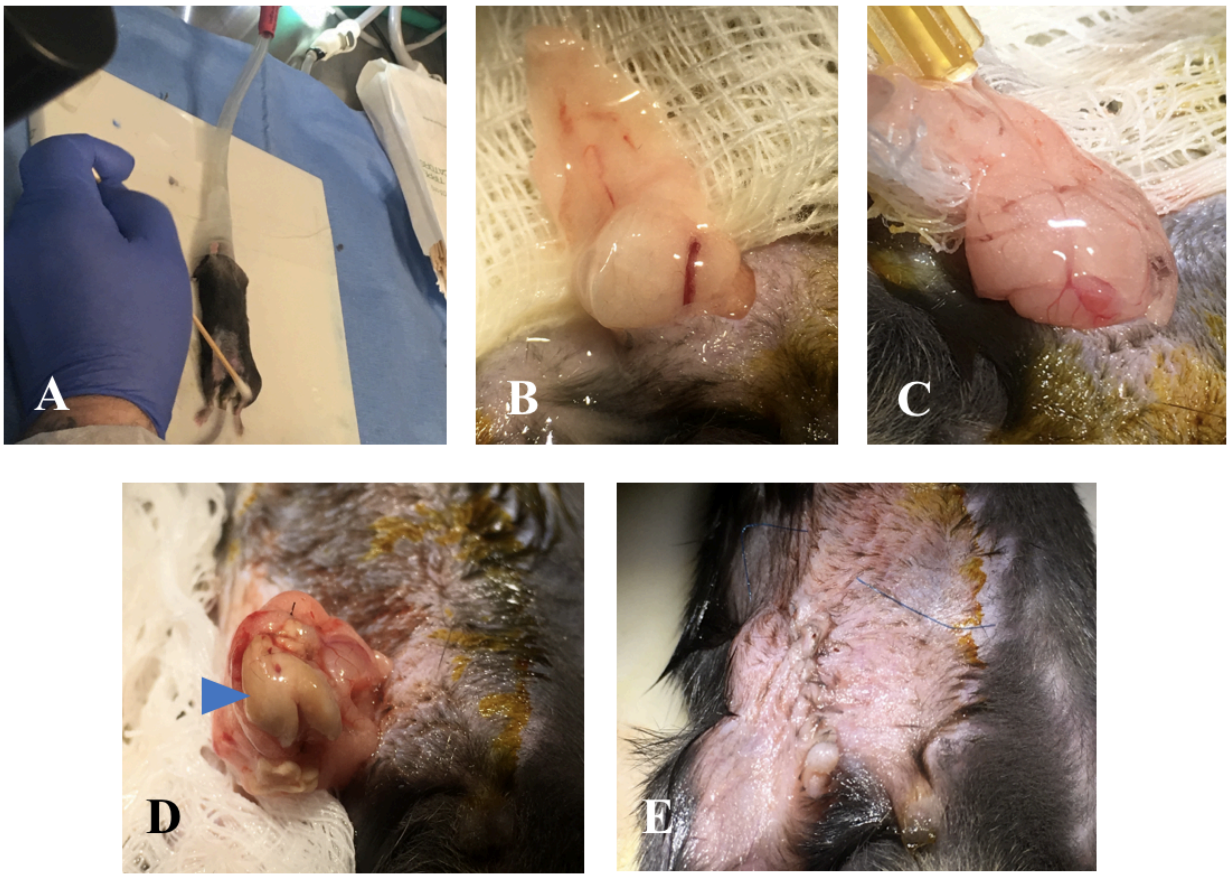

Figure 9. **Transplantation process of BAT in the visceral space. A. The inguinal area of recipient mice was shaved and prepared with application of povidone iodine and ethanol for three times. B. The perigonadal adipose tissue is exposed and pulled out. C. Visceral adipose tissue expanded after the injection of normal saline (700μL) without any macroscopically visible tissue damage. D. BAT (less red in color due to disruption of blood supply) in the visceral fat "pocket". E. Three-layer closure (visceral fat, peritoneum, skin) after the transplantation with absorbable sutures.**

Subcutaneous transplantation study (Cohort A) - Implant fates

Twelve weeks following the transplantation, Cohort A recipient mice were dissected and the implants were macroscopically observed. Matrigel mixed with IBA cell grafts in the dorsal space had transformed into a whitish soft solid formation surrounded by solidified matrigel macroscopically (Fig. 10A,10B). Negative control implants consisting of matrigel mixed with PBS had no macroscopic signs of cell involvement (Fig. 10C, 10D). BAT grafts had signs of inflammation and abscess formation (Fig. 10E). Thus, even in these small groups, it is clear that IBA implants could not be engrafted into the dorsal area and form a fat pad.

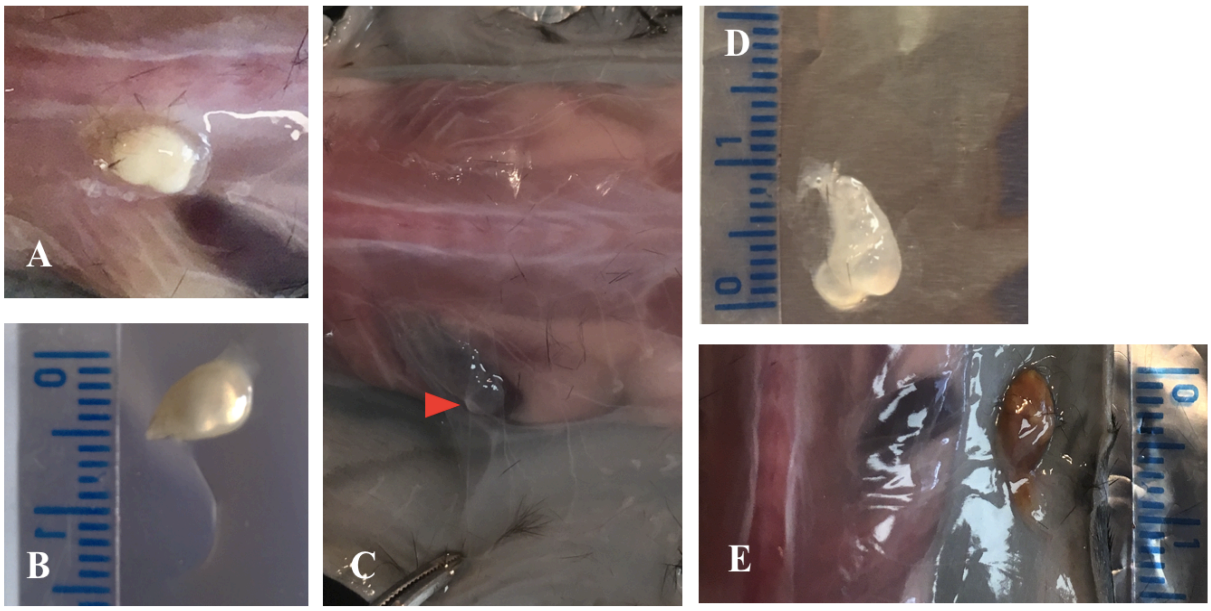

Figure 10. **Implanted matrigel and BAT macroscopic pictures on dissected Cohort A mice at the end of follow up. A-B: IBA implant. C-D. Matrigel mixed with PBS implant. E. BAT allograft.**

IBA and BAT subcutaneous space transplantations impact on glucose homeostasis (Cohorts A and C)

The first part of the transplantation studies was primarily designed to evaluate the positive and negative control groups for subcutaneous space transplantations. As *Fasn* deletion of adipose tissue causes browning, the optimal positive control for the transplantation experiments would be BAT grafts. If a similar phenotype could be achieved with IBA implants in the subcutaneous space, these cells could be used as a positive control which would facilitate the procedure. In these experiments the primary aim is firstly to assess the ability of IBA implants to form a fat pad after transplantation and secondly to improve glucose homeostasis. The secondary aims of these preliminary studies were to optimize our transplantation techniques and procedures including cell manipulations, interventional procedures in donor and recipient mice, data analysis and produce preliminary data.

As each cohort had a small number of mice, in order to evaluate the effect of the IBA implants and BAT transplantations on glucose tolerance, data from Cohort A and the corresponding groups (1,2,3) of Cohort C were combined. For these preliminary data, statistical significance was assessed with student two-tailed T-tests between each experimental group with the negative control (PBS implants). All recipients were C57BL6 WT female mice, 6-7 weeks old and chow-fed. They were all weighted and a baseline GTT was performed on the day before transplantations

(Fig. 11C). In cohort A, two mice have been removed from the analysis, one from the IBA group and one from the PBS group in order to have baseline AUC GTTs without statistically significant differences. Overall the recruited mice are divided into three groups: the first group received IBA cells mixed with matrigel (n=7), the second group received BAT (n=9) and the third group received injection with PBS mixed with matrigel (n=8) as a negative control for the procedure.

Because of the clear differences observed in weight gain in both cohorts, with BAT and IBA mice gaining less weight overtime compared to the controls (Fig. 11A), food consumption was measured from week 8 till the end of the study in cohort A, as well as in cohort C since the transplantation day and then every two weeks. So far, the average food uptake per group seems to match the weight graphs at least in cohort A. Spillage of 0.1g/mouse/day was taken into account in the measurements [64] (Fig.11B). Due to the observation of the differences in food consumption, serum was collected on weeks 9, 11 and 12 in cohort A, and before transplantations and on week 2 so far (week 4, 8, 12 in the future) in Cohort C to evaluate appetite-related hormones at the end of the study. Up to 2 weeks of follow up, no differences hav been displayed in glucose tolerance of the IBA (1) or BAT (2) transplanted mice (Fig.11C, 11D).

At the end of follow up of Cohort C, like Cohort A, the grafts will be macroscopically and histologically evaluated for the formation of functional adipose

depots. Thermogenic and lipogenic genes expression will be evaluated in the grafts, in the endogenous fat tissues (BAT, visceral and subcutaneous WAT) and in the liver. Additionally, the grafts and endogenous fat tissues will be assessed for sympathetic innervation with TH immunohistochemistry.

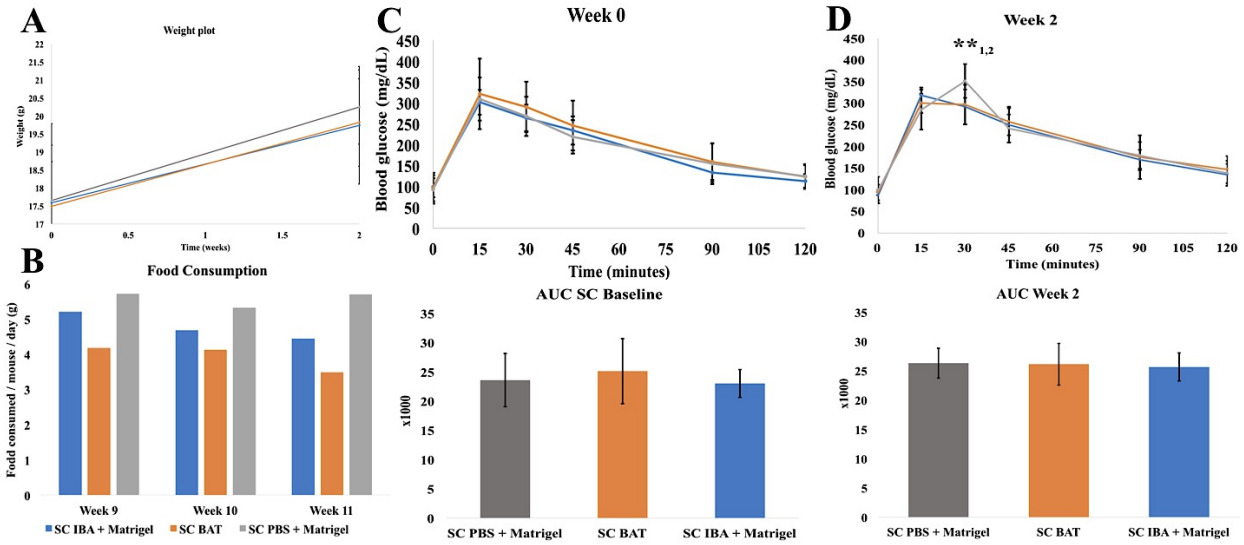

Figure 11. **A. Weight gain overtime, up to week 2 following transplantations. IBA (1) implanted and BAT (2) transplanted mice gained less weight compared to controls although their baseline weights were not significantly different. B. Food consumption on weeks 9, 10, 11 (Cohort A data only) C. Baseline GTT before the transplantations. D. GTT 2 weeks following the transplantations. (* $p < 0.05$, ** $p < 0.01$)**

IBA and BAT visceral space transplantations impact on glucose homeostasis (Cohort B)

The next transplantation study was designed, similar to Cohorts A and B, to evaluate the control groups, BAT and IBA transplantations into the visceral space. The beneficial effect of BAT transplanted into the visceral space has been previously described. [65] Cohort B recipients were C57BL6 WT chow-fed male mice at 8-9 weeks of age. Before transplantations, they were randomly divided into three groups, they were all weighted and a baseline GTT was performed. Three mice were removed from the study in order to achieve baseline average GTT AUCs and weights without any statistically significant differences between the three groups. The first group received IBAs mixed with matrigel (n=4), the second group received BAT (n=4) and the third group received PBS mixed with matrigel in the visceral space (n=4) as negative controls. For these preliminary data, statistical significance was assessed with student two-tailed T-tests between each experimental group with the negative control (PBS implants). After the transplantation procedures, the IBA transplanted mice started gaining significantly less weight compared to BAT transplanted and to controls. This effect has been preserved until week 8 which is the current status of the study (Fig. 12A). Food consumption measurement is not able in this study because each cage contains mice treated differently. Up to 2 weeks following the transplantations, no differences in glucose tolerance were observed

(Fig. 12B, 12C). IBA and BAT transplanted mice displayed improved glucose tolerance compared to controls from week 4 until week 8 which is the current status of the study.

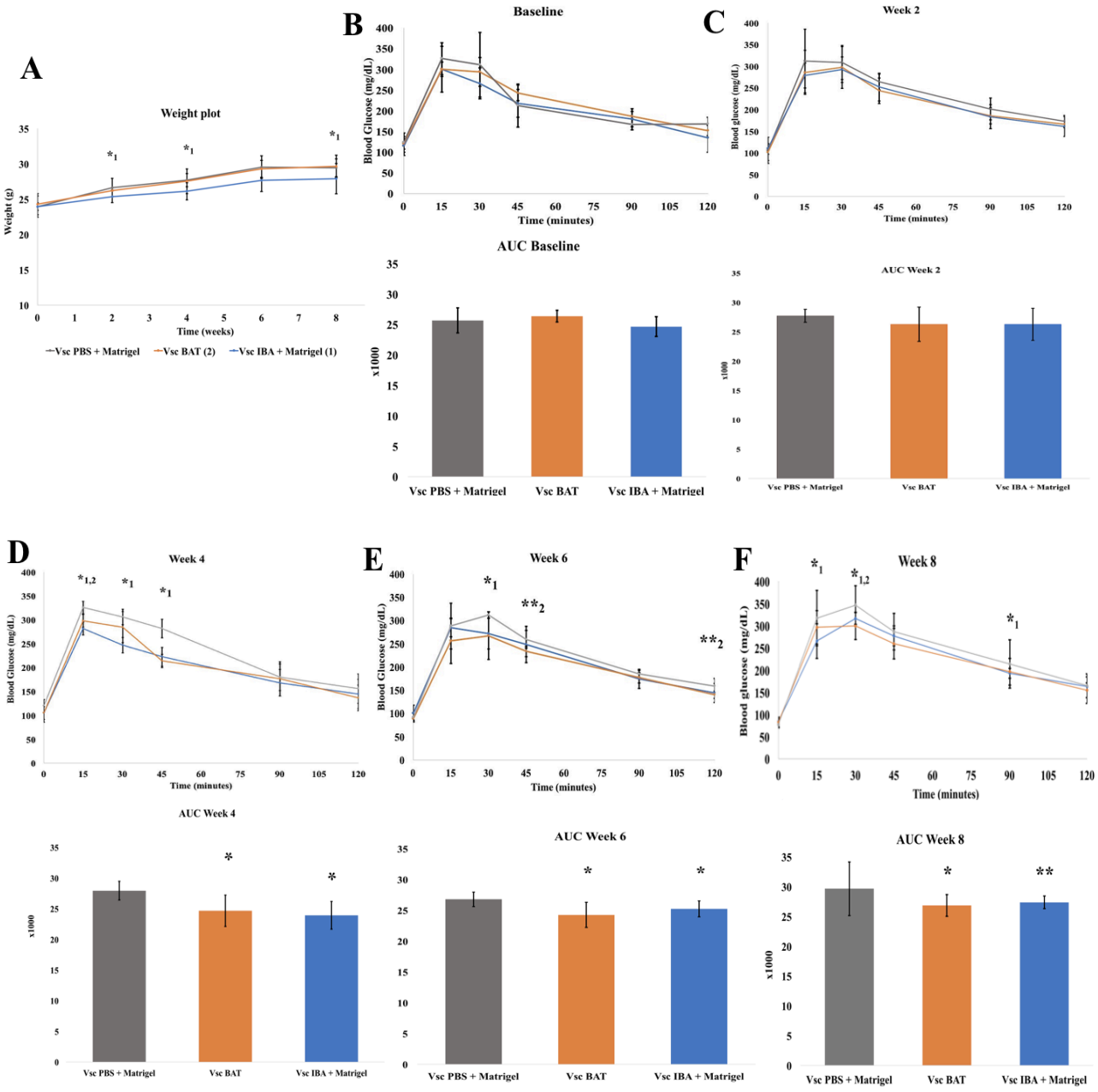

Figure 12. **A. Weight gain overtime, up to week 8 following transplantations. IBA (1) implanted mice gained less weight compared to BAT (2) transplanted**

and control mice B. Baseline GTT before the transplantations C. GTT 2 weeks following transplantations. D – F. GTTs on weeks 4, 6, 8 respectively, after the transplantations. (* $p < 0.05$, ** $p < 0.01$)

Although the study is still in progress and requires a bigger number of mice per group to gain statistical power, these data are encouraging towards the use of IBA implants as positive controls in the transplantation studies for FASNKO white adipocytes in the visceral adipose tissue. However, it is not safe to reach any conclusions before the examination of the grafts in the end of the follow up to assess if the IBA mixed with matrigel could form a fat pad in the visceral space, as opposed to the subcutaneous space.

Thus, after the follow up period (week 12), the visceral fat of these mice will be examined to assess the macroscopic and microscopic morphology of the matrigel pad. Surrounding adipose tissue will be harvested for histological analysis, lipogenic and thermogenic gene expression with RT-PCR and western blot analysis, as well as sympathetic innervation with TH immunohistochemistry.

Generation of FASNKO mice and FASNKO cellular implants

Data produced by our group demonstrate a metabolically healthier phenotype induced by FASNKO WAT transplantation experiments. In order to exploit this effect for therapeutic purposes though, cell implants and not tissue transplantations must be able to induce a similar phenotype. To assess this hypothesis that transplanted, FASN depleted adipocytes will promote glucose tolerance, a series of transplantation experiments were designed using FASNKO primary white adipocytes transplanted in the subcutaneous or visceral adipose tissue spaces of hosts. These cells were obtained from tamoxifen (TAM)-inducible FASN KO (idFASNKO) mouse models where *Fasn* was knocked-out after the differentiation of the primary cells into mature adipocytes, so that the FASN deletion will not interfere with normal differentiation process. For this purpose, idFASNKO and control mice were generated with breeding set-ups as shown on figure 13. All litters were homozygotes for the floxed *Fasn* and same gender Ubiquitin Cre+ and Cre- mice were selected for the experiments (Fig. 14).

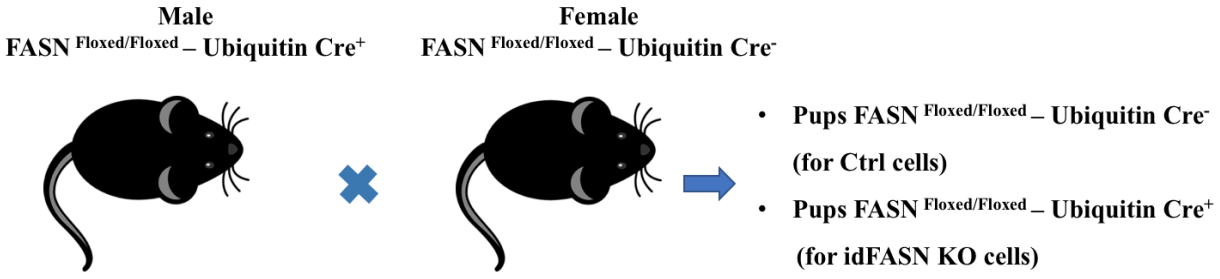

Figure 13. **In order to obtain FASNKO primary white adipocytes we crossed**

Fasn$^{fl/fl}$-Ubiquitin Cre+ male mice and Fasn$^{fl/fl}$-Ubiquitin-Cre– female mice. 4-5 pups aged 3-4 weeks old and gender and genotype matched (ubiquitin Cre positive or negative) were used for the isolation of the idFANSKO or control primary adipocytes.

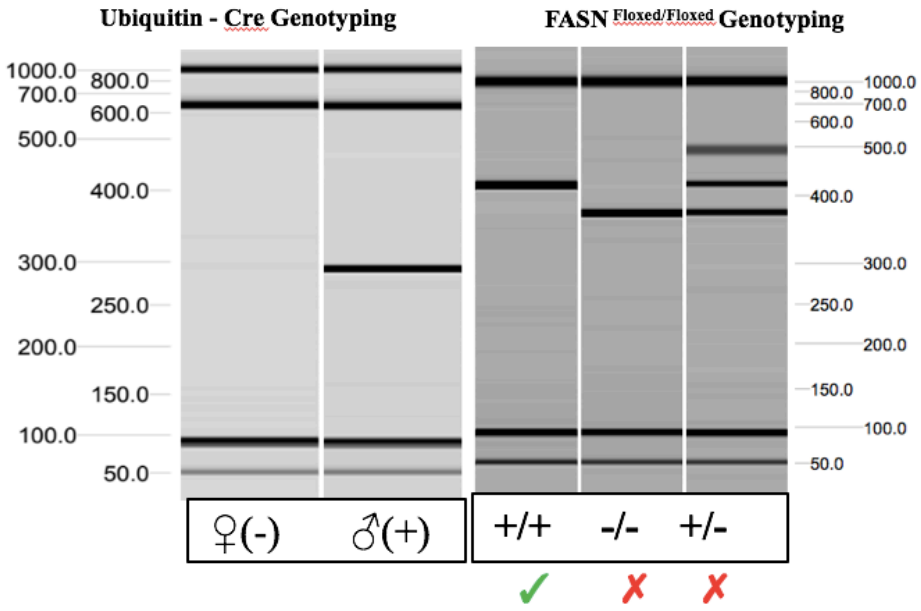

Figure 14. **Representative genotyping image for the breeding pairs. Male breeders, unlike females, express Cre recombinase and both males and females should be homozygotes for floxed *Fasn.* The same genotyping protocol is used to assess the pups that are used for the idFASNKO and control cell implants.**

The ubiquitin Cre + mice were used to obtain primary adipocytes where *Fasn* KO would be achieved with tamoxifen application. The ubiquitin Cre– cells that would continue expressing *Fasn* after tamoxifen application were used as negative controls. *Fasn* KO was validated at an mRNA level with RT-PCR and at a protein level with Western blot analysis after the application of tamoxifen or ethanol (control) in both

Fasn$^{flox/flox}$ – Ubiquitin Cre+ and *Fasn*$^{flox/flox}$ – Ubiquitin Cre– mature primary adipocytes. Additionally, mRNAs specific for adipocytes (eg. Adiponectin) and pre-adipocytes (eg. PREF1) expression was also measured to confirm that FASN KO is not a false effect due to selective poor differentiation of the Ubiquitin Cre+ cells, since FASN expression is low in pre-adipocytes. Tamoxifen or ethanol was applied on days 7 and 8 post differentiation and the cells were added in TriZol for RNA or harvested for protein after day 9 (Fig. 15-16).

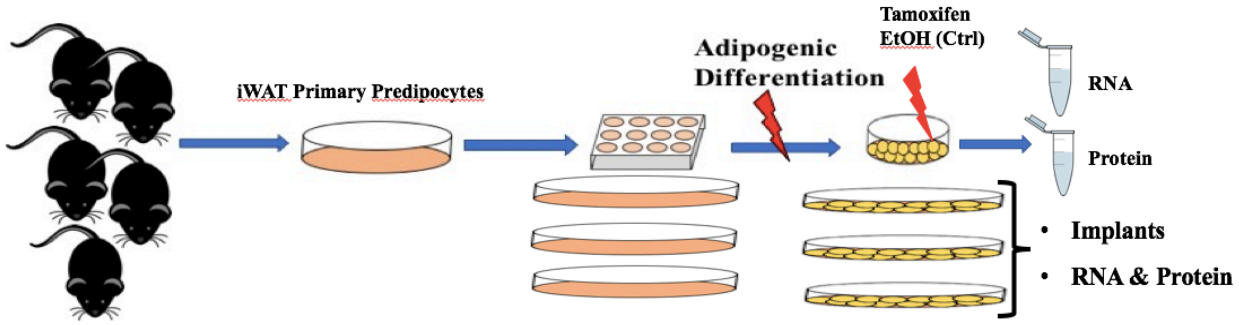

Figure 15. **The process of isolation white primary pre-adipocytes from the inguinal fat pads of either *Fasn*$^{flox/flox}$-Ubiquitin Cre+/– mice, differentiation to mature primary adipocytes and apply tamoxifen or ethanol (control) on days 7 and 8 post differentiation. TAM mediates Cre entrance in the nucleus and thus the deletion of *Fasn*. On day 9, triplicates are harvested for both RNA and protein validation of the FASN deletion. Pre-adipocytes are also plated in 150mm plates for differentiation and TAM application to be implanted into mice. On the transplantation day, a small sample of these cells is used for validation of the idFASNKO.**

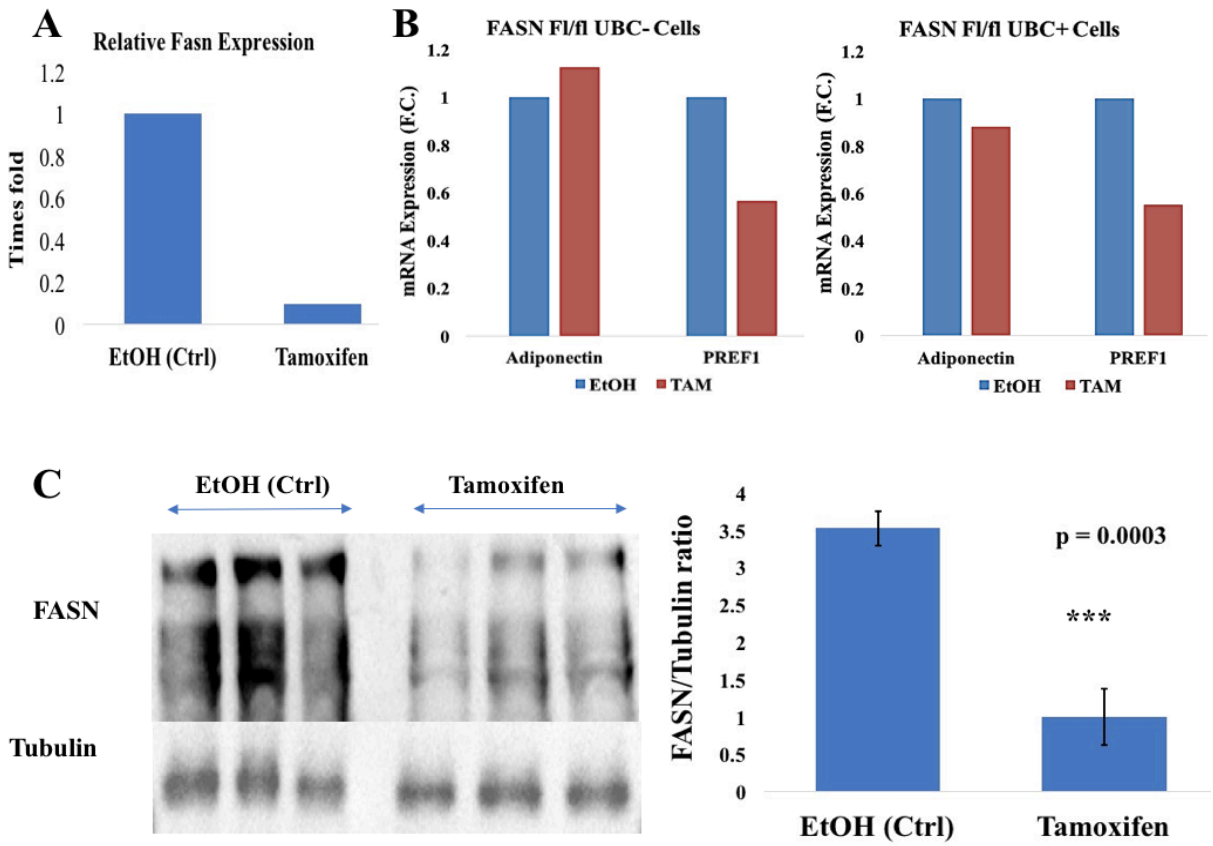

Figure 16. **A. mRNA expression of *Fasn* in *Fasn*$^{fl/fl}$*-Ubiquitin Cre+* treated with TAM or EtOH (Ctrl) for the validation of the idFASNKO. B. Confirmation of high expression of Adiponectin and low expression of PREF1 in the idFASNKO cells that indicates the deletion of FASN is due to TAM and not because of selective poor differentiation. C. FASN protein levels *Fasn* in *Fasn*$^{fl/fl}$*-Ubiquitin Cre+* treated with TAM or EtOH (Ctrl) for the validation of the idFASNKO confirming that these cells can be used as idFASNKO mature adipocytes in the transplantation studies.**

idFASNKO adipocyte implants in the subcutaneous space (Cohort C)

The purpose of the third transplantation study is to assess the original hypothesis that the FASNKO adipocyte implants in the subcutaneous space can improve glucose metabolism, as previously demonstrated with idFASNKO white adipose tissue transplantations in the visceral space.

C57BL6 WT, 7-week-old and chow-fed, female mice were recruited as recipients for this study. Before the transplantations, the recipient mice were weighted, a baseline GTT was performed and blood serum was collected. The recipients were divided into five groups: 4 mice to receive idFASNKO adipocytes with matrigel prepared as described in the previous paragraph, 4 mice to receive IBA implants as positive control, 4 mice to receive WT primary mature adipocytes TAM-treated as negative control for the TAM treatment, 5 mice to receive BAT transplantation as control and 5 mice to receive PBS mixed with matrigel as a negative control for the matrigel. C57BL6 WT, 4-month-old, chow-fed female mice were used as donors for BAT removed from the dorsal area to be transplanted. All recipient groups baseline average weights and glucose tolerance had no significant differences (Fig. 17). The follow up will take place for twelve weeks with weight measurements and GTTs every other week, morning serum collections on weeks 2, 4, 8 and 12 and average daily food consumption follow up.

This study is in progress and is expected to provide insight whether FASNKO

adipocyte implants can improve glucose homeostasis when transplanted into the subcutaneous space which has easier accessibility compared to the visceral fat space. Moreover, the data concerning IBA, BAT and PBS groups can be combined with the data generated from Cohort A to increase the statistical power of the study concerning the control groups.

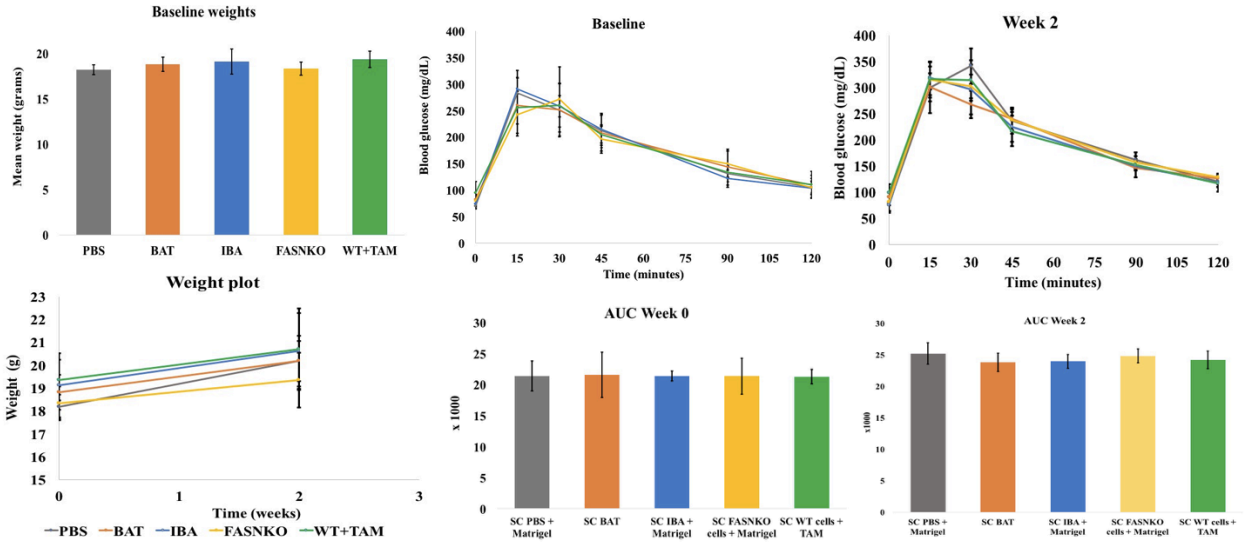

Figure 17. **Cohort C baseline average weight, weight plot and GTTs before and two weeks following transplantations. All groups of Cohort C displayed no significant differences of their mean weights and GTT AUCs two weeks after transplantations. (* $p < 0.05$, ** $p < 0.01$)**

DISCUSSION

Although still on a preliminary stage, this project provides a promising approach for advance a novel therapeutic strategy to target type 2 diabetes combining leading CRISPR/Cas9 tools and cutting-edge knowledge about the role of *de novo* lipogenesis in adipose tissue. CRISPR/Cas9 is a state-of-the-art, "clever" technique for permanent gene editing as it takes advantage of the endogenous natural response of bacterial to viral elements that provoke DNA damage. With the novel CriPs tool, recently developed by our group, new approaches for both *ex vivo* and *in vivo* applications to treat obesity and diabetes can now be designed and evaluated.

Concerning the first section of this project with CriPs targeting FASN in mature adipocytes, preliminary data are encouraging, though additional obstacles need to be overcome. The aims reached in this project so far could be summarized as follows:

1. CriPs targeting FASN in mature primary white adipocytes can achieve knock-down efficiency up to 50% of FASN protein three days following transfection.
2. Efficiency in these experiments is best measured with western blot, since measurement by the indel frequency in T7E1 assays appears to yield underestimates of gene deletion.

An approximate 50% knock down of FASN is very encouraging compared to published work, regardless of its ability to induce a phenotype in glucose homeostasis. However, efforts for further improvement of the efficiency are

required and several approaches will be discussed with the future directions. Additionally, a second validation method that would quantify the gene editing will be desirable. Since there have been developing concerns about the ability of T7E1 assay to detect gene editing in CRISPR/Cas9 engineered cells, deep sequencing seems a possible solution to an alternative validation procedure. [75]

Concerning the transplantation studies, although Cohorts B and C are in progress and Cohort A analysis being incomplete, a few goals have been already reached:

1. We developed safe and minimally tissue-damaging techniques for subcutaneous and visceral space transplantations.
2. After Cohort A completion, we have strong evidence that IBA mixed with matrigel are not able to form a functional fat pad when implanted in the subcutaneous space.
3. Similarly, BAT transplanted into the subcutaneous space did not seem engrafted and healthy.
4. Cohorts A and C data suggest that mice that receive IBA implants or BAT allografts in their subcutaneous space, gain less weight overtime.
5. Cohort B data indicate that mice that received IBA cells in their visceral space gain less weight overtime compared to mice that received BAT or PBS mixed with matrigel in their visceral space.
6. Mice that received BAT allografts or IBA implants into their visceral space have

consistently showed improved glucose tolerance from week 4 to week 8 after transplantation. This may be an excellent approach in future experiments to test the efficacy of transplanting FASN depleted white adipocytes.

7. We were able to generate idFASNKO mature primary white adipocytes and transplant them into the subcutaneous space of mice (Cohort C, group 4).

From cohort A data, although the number of mice transplanted was not adequate to reach significant conclusions concerning the effect on glucose tolerance, it is safe to argue that IBA mixed with matrigel implants in the dorsal area are not able to be transformed into a fat pad and thus be used as positive control in the future experiments with FASNKO adipocyte implants or CriPs targeting FASN engineered adipocyte implants. In macroscopic observation, the implants resembled sterile abscesses without any signs of vascularization or innervation. We could hypothesize that the viral component for immortalization or the fact that mixed gender cells were used to establish this cell-line might have triggered immune responses that prevented the engraftment. Furthermore, sterile abscesses were also macroscopically observed in almost all BAT grafts. Literature about BAT transplantations into the subcutaneous area has been controversial, so it is necessary to examine the combined data produced from both cohorts A and C to reach conclusions. [69, 76] From these cohorts, there have been no differences in glucose tolerance between the different groups yet. This is not surprising as it has been shown that at least 9 weeks are

required for matrigel with cells to form an innervated, vascularized and functional fat pad [58] and for the engraftment BAT allografts.

Many additional experiments are yet to be completed to enable further accurate conclusions. It needs to be clarified if IBA cells with matrigel in the visceral space can be transformed into a fat pad and ameliorate glucose homeostasis, so that they can be used as positive controls, instead of BAT allografts, for the idFASNKO adipocyte implantations to improve glucose homeostasis. If IBA grafts fail as positive controls, is primary brown adipocytes which will be gender-matched with the recipients could act as an alternative positive control.

As far as the cell implants are concerned, we need to assess if white primary adipocyte implants into the subcutaneous or visceral space can be transformed into a vascularized and innervated fat pad by the end of cohort C follow up. In addition, we need to evaluate if FASNKO mature adipocytes with matrigel implanted in the subcutaneous or visceral space in mice can form a functional fat pad and induce a beneficial phenotype in glucose tolerance. If FASNKO white adipocyte implants are capable to induce such a positive effect, the next step is to assess if that can be also induced with FASN knock-down white adipocytes achieved with CriPs. A 51% knock-down of FASN in mature adipocytes that has been already achieved with CriPs, remarkably suppresses DNL pathway. It is yet to be determined with transplantation studies in the visceral and subcutaneous space if these genetically

modified cells implanted into mice, could have a positive impact on glucose tolerance.

FUTURE DIRECTIONS

Alternative approaches can be engaged to increase CRISPR/Cas9 efficiency to genome edit of adipocytes. For example, combination of CriPs with electroporation which has already been effectively used for other cell types in combination with RNPs, could increase CriPs efficiency and can be a useful technique for *ex vivo* approach.[38, 50, 52, 53, 54, 62, 66, 67] Furthermore, advances in degradable vectors expressing Cas9 and sgRNA, even in non-dividing cells such as mature adipocytes, could be a potential way to increase CRISPR/Cas9 efficiency without the safety concerns associated with the continuous Cas9 expression by adeno-associated virus vectors. Such strategies might upgrade the *ex vivo* CRISPR/Cas9 applications to genetically modify as challenging cell types as the adipocytes. An efficient and safe technique to induce permanent and precise genome editing in adipocytes would be a breakthrough achievement with possibilities to be applied even with different target genes and in various disease contexts.

Transplantation studies have been a useful research method to evaluate the different types of adipose tissue effect on whole-body metabolism. [65, 68, 69, 70, 71] Such studies are the major method to evaluate the efficiency of any *ex vivo* or *in vivo* CRISPR/Cas9 applications we will use. In the ongoing studies for this project,

recipients are WT, young (6-9 weeks old at transplantation studies and 18-21 weeks old at the end of follow up), chow-fed mice at 25^{o}C. Future studies with metabolically challenged experimental models need to be designed to assess the FASNKO white adipocyte implants effect on glucose homeostasis. Such experimental models could be WT mice on HFD or under thermoneutral conditions (30^{o}C) [72] or transgenic mice with impaired glucose metabolism (eg. Ob/Ob, ApoE3*Leiden.CEPT). [73, 74] As positive controls for the cell implants, since the preliminary data are not in favor of IBA cell implants, we could evaluate gender-matched primary brown adipocytes. The latter cells have not undergone virus-induced immortalization that may trigger immune responses and jeopardize the engraftment procedure.

Type 2 diabetes is a disease with safe medications available that can efficiently decrease blood glucose levels for many years following initial diagnosis in most patients. Due to this fact, any novel therapeutic intervention aiming to a permanent cure has to guarantee safety upon application to humans, unlike in diseases with poor prognosis. Thus, an *ex vivo* therapeutic approach using a CRISPR/Cas9 tool associated with rapid Cas9 and sgRNA degradation and reduced immune responses, such as CriPs, is a promising method in translational research for type 2 diabetes.

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