

Thesis Proposal:

**Transgenic Mice with partial lipodystrophy due to  
expression of Lamin A or C cDNA containing a  
Dunnigan-specific mutation.**

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## i. Background

Lamin A and Lamin C are intermediate filament (IF) proteins made from the two major splice variants of the same transcript. The two proteins share 566 identical amino acids (through exon 10) while Lamin C has 6 unique amino acids coded for at the end of exon 10 and Lamin A goes on to include 98 additional amino acids from exons 11 and 12 [1-3]. These IF proteins have a globular head domain, a central alpha-helical rod domain, and a C-terminal globular tail domain. They interact to form head to head (hetero or homo) dimmers and coiled-coil oligomers that assemble into the nuclear lamina in a Lamin B-dependent manner [4] by interacting with Emerin [5-7] and other integral nuclear membrane proteins. This meshwork of structural proteins lines the inner surface of the nuclear membrane, giving support to the nucleus, anchoring nuclear pore complexes, and contributing to chromatin organization [8]. The A-type lamins arise during embryogenesis and are present in nearly all differentiated cell types [9-11].

Lamin A is mutated in at least nine different human diseases: Dunnigan's-type Familial Partial Lipodystrophy (FPLD) [12], autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD) [13], autosomal recessive (AR-) EDMD [14], dilated cardiomyopathy and conduction system defect (DCM-CD) [15], limb-girdle muscular dystrophy (LGMD1B) [16], Mandibuloacral dysplasia (MAD) [17], autosomal recessive Charcot-Marie-Tooth disorder type-2 (CMT2B1) [18], and Hutchinson-Gilford Progeria Syndrome [19], finally a new spontaneous mutation has been found in a case of generalized acquired lipodystrophy with type-2 diabetes, skin papules and cardiomyopathy [20]. These mutations occur throughout the gene's length and are largely disease-specific and non-overlapping. There are even cases where different mutations in the same amino acid cause different diseases. R527P causes AD-EDMD while R527H results in MAD [17]. H222P results in AD-EDMD, but H222Y causes AR-EDMD [14]. (See table 1)

Dunnigan's-type Familial Partial Lipodystrophy (FPLD) is an autosomal dominant disorder in which patients begin to lose subcutaneous fat from the limbs and trunk around the age of puberty. The loss of fat in these areas is practically complete leaving patients with well-defined muscles in the arms and legs. Coordinate with the described fat loss, patients experience an increase in fat deposition around the face, neck, back, and labia majora [21-24]. They are also at an increased risk for hypertriglyceridemia, hyperinsulinemia, type-2 diabetes, hypertension, atherosclerotic heart disease, acute pancreatitis, hyperandrogenism, hirsutism, and polycystic ovarian disease [24-27]. Aside

from the secondary aspects of FPLD that affect only women, female patients also seem to be at greater risk for the remainder of secondary complications as well [27, 28].

Originally FPLD was believed to be inherited in a sex-dependent manner [22] as the visible manifestations of FPLD are more obvious in female patients than in males. The thicker face and neck that are due to the increased deposition of subcutaneous fat in these areas, the well-defined limb musculature, as well as the hyperandrogenism/hirsutism are all more conspicuous in females.

Later work defined transmission through pedigrees that demonstrated the syndrome to be autosomal dominant [21, 29, 30]. In January 2000 Cao and Hegele published the first report linking FPLD to a specific mutation [12]. They found 22 FPLD patients in five families to have an R482Q mutation in the LMNA/C gene.

Subsequent work has demonstrated several other LMNA/C mutations that have been linked to FPLD. Most of these mutations are located in the same region of the gene, coded for by exon 8 of LMNA/C. In fact there are at least three different mutations affecting the Arginine at position 482 (R482Q, R482W, R482L). 90% of the FPLD-associated LMNA patients and 12% of all laminopathy patients have a mutation in this highly conserved arginine [28, 31]. Nearby there are two more mutations changing the Lysine at position 486 (K486N, K486T) [32] and another that replaces the glycine at position 465 with an aspartic acid [33]. These three residues are located in the same region of the 3-dimensional protein structure. This portion of the C-terminal region is a modified Beta-sandwich [31, 34]. The six listed mutations in these three amino acid residues have a similar effect. They all result in either the loss of a positive charge or the introduction of a negative charge at the same face of this Ig-like domain.

A small number of mutations in other regions of the protein have also been associated with FPLD, though not exclusively to typical FPLD. An R28W mutation in the N-terminal head domain and an R62G in the alpha-helical rod domain have both been associated with a combination of FPLD and cardiomyopathy-related conduction system defects [35]. Also, two mutations exclusive to Lamin A in exon 11 are associated with an atypical-FPLD [33, 36]. The atypical state associated with the R582H and R584H mutations is generally less severe. Patients have a less severe loss of subcutaneous fat from all affected regions, particularly from gluteal region and thighs; lower serum triglyceride and decreased fat deposition in the labia majora [37]. Since these mutations are located in exon 11 the corresponding amino acid change would



protein) (figure 1). The mouse has a two base pair insertion (counted as 2 differences) and a one base pair deletion compared to the human. Of the other amino acid changes about half result in replacements with similar amino acids. Of the twenty-two amino acid residues that are different only three changes (both human to mouse and mouse to human changes were considered) are represented in a list of known disease causing mutations (table 1).

First, the valine at human position 549 is a methionine in mouse. V440M was reported as an FPLD-causing mutation by Hegele and colleagues [39]. However, the V440M change was found only twice and in one family. It was present in one patient who was a compound heterozygote for the R482Q mutation. The change was also present in a 71-year-old woman reported as being of “uncertain clinical phenotype” even though she had no apparent lipodystrophy, no dislipidemia and no diabetes. It should be obvious that while it is possible that this valine to methionine change could have a modifying affect it is not a “disease-causing” mutation in the traditional sense.

And second, there are two serines in the C-terminal end of lamin A that are glycines in the mouse. In an upcoming paper from Eriksson et al there is a G608S mutation that results in Hutchinson-Gilford Progeria Syndrome [19]. This amino acid change is not the main mutation, however. Two different nucleotide changes were found within this codon (one is “silent”, G608G) that increase the splice consensus fit to the point that a small quantity of aberrant proteins are made that act in a dominant negative manner to cause the progeria phenotype.

Finally, there is a histidine at the human position 566 (the last shared by lamin A and lamin C) that is an arginine in the mouse. Several similar mutations result in human disease. I have already mentioned the R582H and R584H mutations that contribute to the atypical-FPLD. Also, R377H causes LBMD-1B, R541H causes EDMD, and R527H causes AR-MAD. With these cases in mind this evolutionary change may seem like a conspicuous difference between the two homologues. However, this region is not part of a known domain and at least some of the region (AA 548-553) is unstructured[31, 40]. There is no data available on the portions of the protein C-terminal to position 553. The Ig-like  $\beta$  sandwich ends after amino acid 544. The short stretch of 20 amino acids between there and the last residue shared by lamins A and C contains 8 of the 25 differences between the human and mouse proteins. This region may be under little evolutionary control based on evolutionary changes, its location with respect to the Ig-like fold, and its proximity to the end of LMNC.

## ii. Specific Aims

The proposed research project will help elucidate some of the mechanisms that could be contributing to laminopathies and the pathophysiology behind FPLD.

Aim 1: To develop a partial lipodystrophic mouse model based on transgenic expression of mutated human LMNA or LMNC cDNAs. The transgenic mouse will allow us to study how mutated Lamin proteins can result in specific cell and tissue loss.

Aim 2: To determine how diet and obesity may affect the progression and pathophysiology of the disease (lipodystrophy) and its metabolic complications.

Aim 3: To determine if the lipodystrophy can be attributed to either loss of mature fat cells or a failure of adipocyte precursor cells to properly and efficiently differentiate.

### iii. Experimental Design

#### Aim 1: Development of a LMNA/C-dependent lipodystrophic mouse model.

We planned to develop a lipodystrophic mouse model based on the mutated human cDNAs of LMNA and LMNC. By using the first published FPLD-specific mutation we hoped to have a lipodystrophic mouse with some similar characteristics to FPLD. By expressing a mutated transgene selectively in adipose tissue we would be able to characterize any phenotype as primarily dependant on the adipocyte defect, thereby excluding the possibility that this was a multisystem disorder. Finally by expressing the lamin A and lamin C separately we would be able to determine whether either could cause a lipodystrophic phenotype on its own, only mutated lamin A resulted in lipodystrophy (as was previously suggested [39]), or both proteins were required to both be mutated together.

We received copies of the LMNA and LMNC cDNAs from Rob Moir. The genes had been cloned into a homemade e coli expression vector that was not useable for us. In order to begin the process of producing the planned transgenic insert we used hi-fidelity PCR to produce copies of the genes that are bordered by restriction enzyme recognition sequences. These products were then digested with the applicable enzymes and ligated into pBluescript II KS+ from Stratagene. We then used the QuickChange Site-Directed Mutagenesis Kit (Stratagene) to induce a specific 2 base pair change (figure 2).

Wild-type	ACT	TAC	CGG	TTC	CCA
R482Q	ACT	TAC	CAG	TTC	CCA
Induced Mutation	ACT	TAC	CAA	TTC	CCA

Figure 2: nucleotide sequence for LMNA/C amino acids 480-484 with mutations indicated in red.

The mutation was designed to have the following effects: an amino acid change to match the newly (at the time) published R482Q FPLD-associated mutation [12], a Tsp509 I restriction site is created, and an Age I site is destroyed. The restriction site changes were used to screen for mutation incorporation. Dideoxy sequencing was used to check the LMNA/C clones for unwanted mutations. The gene sequences were then removed from the pBluescript backbone and ligated into another pBluescript vector that had been previously modified [41]. The lamin cDNAs were now located 3' to the adipocyte-specific aP2 promoter and enhancer. 3' to the cDNA was the SV40 intron and

polyadenylation signal. Finally, the vector backbone was removed. The mutant LMNA/C transgene was purified, and injected into single-cell mouse eggs. After potential founders were transferred to our control they were screened for insertion of the transgene by PCR and Southern blotting.

When using the transgene strategy of genetic manipulation, incorporation of the construct can vary widely in copy number and insertion site. Expression levels can then vary not only due to the number of copies of the transgene but also because of the local chromosomal architecture. The chromatin arrangement can have a tremendous effect in silencing a transgene. In a gene rich region boundary elements and cis-repressors can also have a large effect on transcription levels. When studying transgenic mice it is never a good idea to trust the phenotype of one line whether the results are positive or negative, whether they agree with your hypothesis or not. It is not wise to assume that any effect (or lack of one) is due to the change you meant to cause and not the accidental one of which you are unaware. For example, had a transgenic mouse been produced that expressed non-mutated human LMNA or C what would it have meant for there to be no discernable phenotype? After screening for the transgene itself it may have been located in a region that suppressed its transcription. Perhaps the protein was not being incorporated into the nuclear lamina. Several lines of mice would have had to be followed congruently for many months to ever confirm that an observed negative result was actually the true negative result looked for and not just chance. Eight lines of mutant mice were kept and bred through the initial periods of the experiment in case they would be needed to confirm or rebut a result. Producing, housing, breeding, and studying several more lines as a negative control would have meant wasted time and resources for questionable results. For these reasons, as well as the extremely high amino acid identity and similarity mentioned in the introduction, the battery of non-mutated human LMNA and LMNC transgenic negative control mice were not produced.

Four founders were confirmed to be positive for the LMNA transgene. There were also four positive LMNC founders. From this pool one line was selected for each gene (LMNA and LMNC) based roughly on the number of copies of the transgene as judged by Southern blot (data not shown). These two lines (hereafter referred to as “11A” for the mutant LMNA transgenic mice and “17C” for the mutant LMNC transgenic mice) are those that have been and will continue to be used to determine the effect of the incorporation and expression of the mutant transgene.



Northern blots confirmed that the 11A and 17C lines had the highest expression levels. Northern blots were also used to demonstrate the tissue specificity of the transgene (figure 3). As expected, there was abundant, relatively consistent expression white adipose tissues (WAT) and in brown adipose tissue (BAT). There was very low expression in skeletal muscle, cardiac muscle, and spleen. The cardiac and skeletal muscle expression has been previously reported for this expression construct and for aP2 itself [41]. The expression seen in spleen is likely due to previously reported aP2 activity in monocytes.

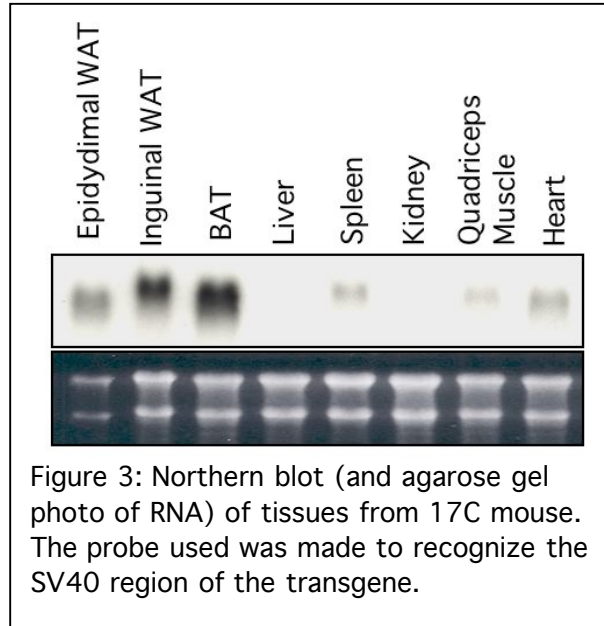


Figure 3: Northern blot (and agarose gel photo of RNA) of tissues from 17C mouse. The probe used was made to recognize the SV40 region of the transgene.

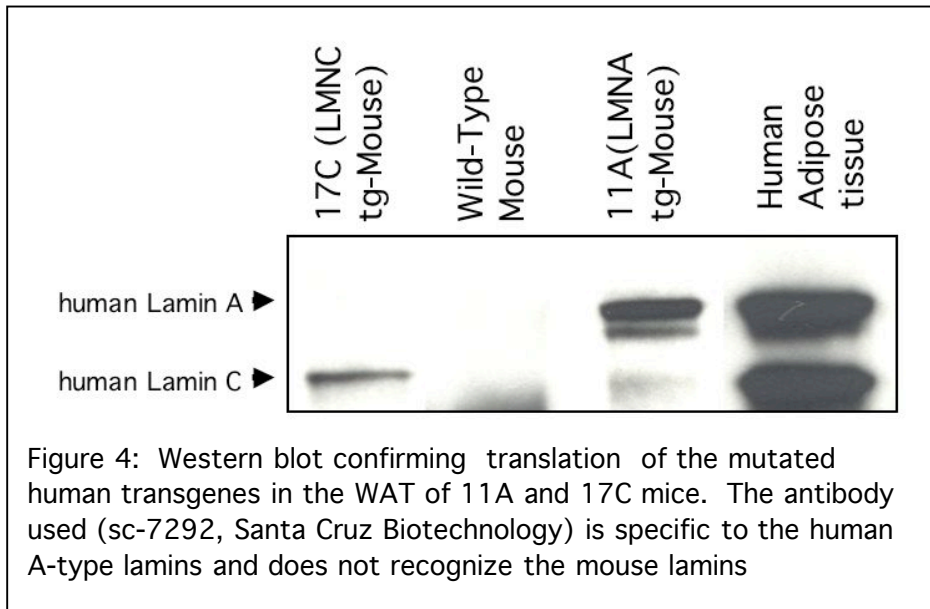


Figure 4: Western blot confirming translation of the mutated human transgenes in the WAT of 11A and 17C mice. The antibody used (sc-7292, Santa Cruz Biotechnology) is specific to the human A-type lamins and does not recognize the mouse lamins

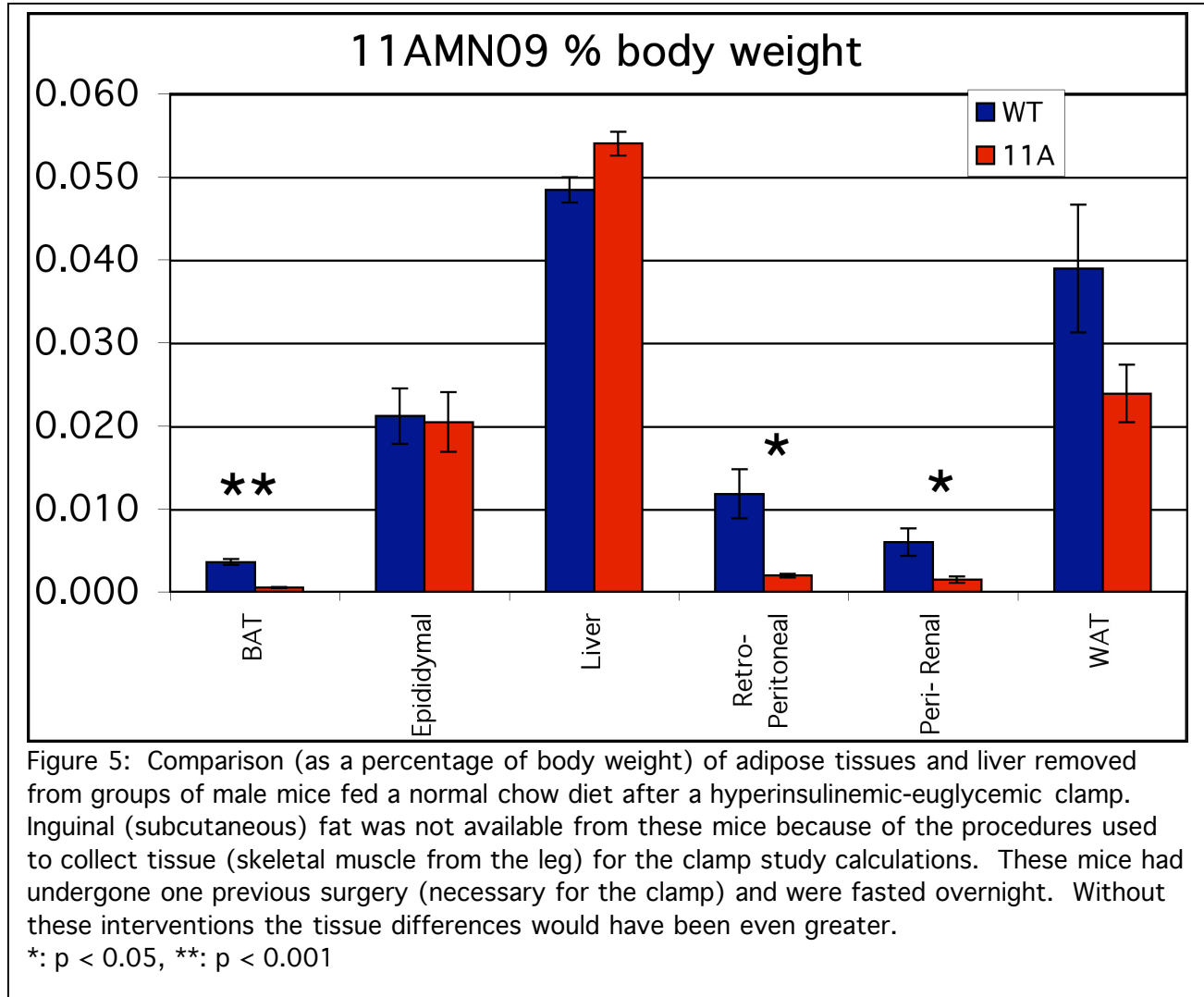
Western blots confirmed that the mutated human LMNA/C were translated into lamin A and lamin C in the mouse WAT (figure 4). Despite the >96% amino acid identity between the human and mouse A-type lamins, antibody

sc-7292 (Santa Cruz Biotechnology) is reactive with lamin A and C from humans but not from mice.

In order to determine the progression of the disease, mice were weighed weekly and blood samples were taken periodically in order to compare blood glucose, serum triglyceride, and serum insulin levels. In future studies these blood samples will continue to be taken to follow the progression of insulin resistance and the potential onset of diabetes.

Work to date has required that animals be euthanized in order to compare the size of the fat pads. To do this individual fat pads have been removed and

weighed. This data was compared between the transgenic mice and their wild type littermates to assess whether any lipodystrophy is present. Study groups to date have all indicated that the 11A mice do have a progressive lipodystrophy that also leads to profound insulin resistance (figure 5).



To determine the extent of insulin resistance one study group was put through a hyperinsulinemic-euglycemic clamp. The clamps demonstrated that both skeletal muscle and liver were insulin resistant in the 11A mice.

Long-term studies show that the 17C are also lipodystrophic and insulin resistant. This is the first demonstration that the mutated lamin C protein can cause lipodystrophy in the absence of lamin A mutations.

Future studies in this area will involve longitudinal investigation of 11A and 17C mice by the methods previously described and using a Bruker Minispec. This tabletop NMR will allow us to determine the percent body fat in live, non-anesthetized animals. By taking periodic measurements (i.e. bi-weekly) in a number of animals over a period of months we will be able to watch the accumulation and diminution of body fat in individual mice. This should help us to determine at what point the mice begin to lose their fat stores. The Minispec measures total lipids, however and not just the lipids in particular regions. Depot-specific changes will not be available and any excess fat deposited in the liver will contribute the value of total % body fat. Therefore, groups of mice will still have to be euthanized at different time points in order to collect data on any site-specific changes.

**Aim 2: Investigation of how diet and obesity can affect the progression and pathophysiology of the disease.**

While the primary phenotype of subcutaneous adipose tissue loss is consistent in FPLD patients, there is quite a bit of variation among the secondary metabolic complications. We plan to use dietary changes and a well-studied genetically obese mouse to investigate how these factors may affect some of the secondary disease states associated with FPLD.

We have already performed one set of experiments in which the diet of 11A mice was modified. Beginning at 9-11 weeks of age, groups of 11A mice and their wild-type littermates were fed (ad libitum) either a high-fat or a low-fat control diet throughout the experiment. The food was purchased from Research Diets Inc. The high-fat group received pelleted mouse chow in which 45% of the kCal were derived from fat (cat # D12451). From the control diet (also purchased from Research Diets in pellet form, cat # D012450B), only 10% of the kCal came from fat. While both the high-fat and low-fat groups of males exhibited similar effects on adipose tissue mass (55% and 53% decrease, respectively, in WAT collected as a % of body mass) there were differences in the liver weights. These lipodystrophic mice generally have enlarged fatty livers, but those on the control diet had a 1.2 fold increase in liver size over the wild type mice and those on the high-fat diet had >1.5 fold increase (calculated from liver weight as a % of body mass). The high-fat group also had more severe insulin resistance and higher glycated hemoglobin A1 (an indicator of long term blood glucose levels).

In order to further ascertain the effects of obesity we have acquired a number of mice with the long-studied ob mutation in the leptin gene that had been backcrossed onto the FVB/N inbred strain for greater than 10 generations [42]. This particular strain of ob mice will be used to decrease any strain-specific genetic effects when breeding them with the 11A mice that are already on the FVB/N background.

In the homozygous state this naturally occurring ob mutation [43] destroys the activity of leptin, a long-term satiety signal produced by adipose tissue. Without leptin the mice feel a constant urge to eat. As a result they grow to be extremely obese and develop insulin resistant type-2 diabetes. We predict that the 11Aob/ob mice would be leaner but sicker. They should have less fat due to the lipodystrophic effect of the 11A transgene but have more severe diabetes due to the potentially additive effects of the obesity inherent in the ob/ob mice and the insulin resistance of the 11A transgene.

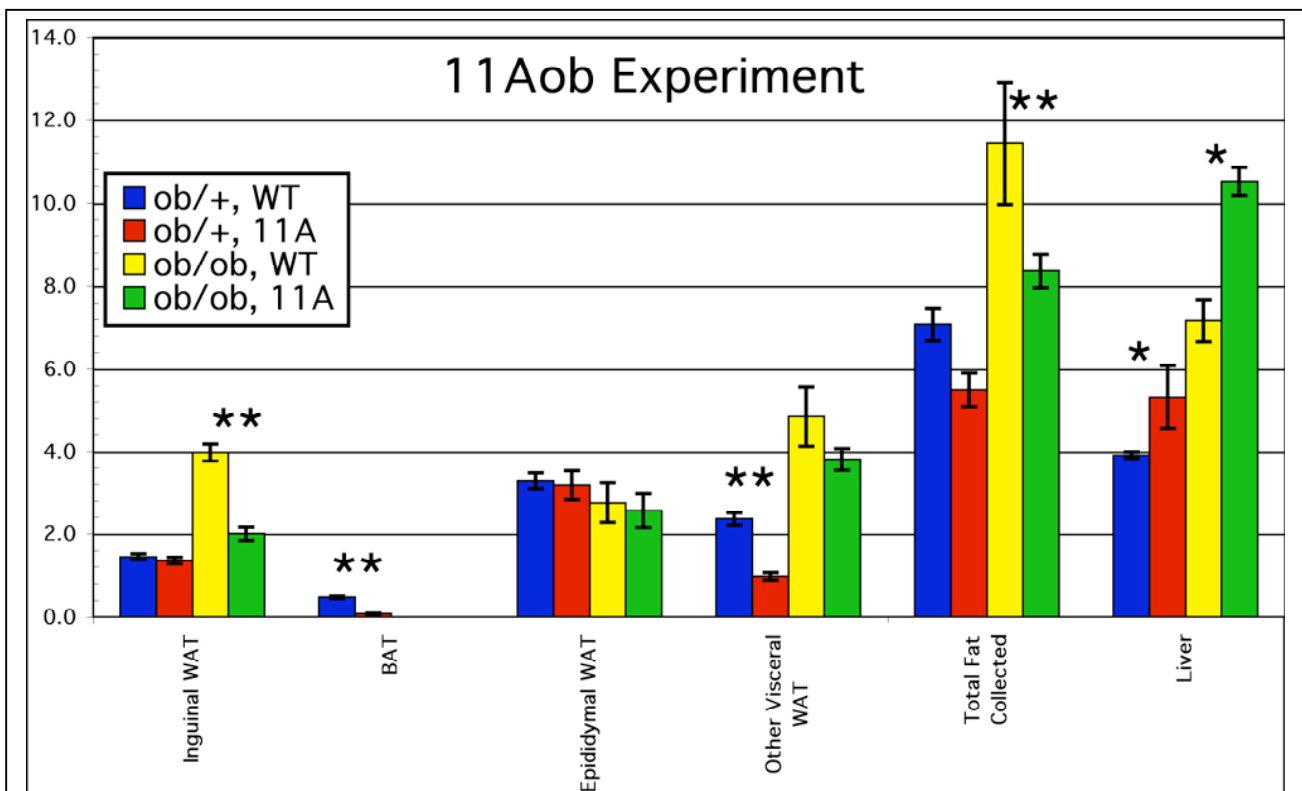


Figure 6: Comparison (as a percentage of body weight) of adipose tissues and liver removed from groups of male mice fed a normal chow diet until approximately 32 weeks of age. The indication of statistically significant differences refers only to the differences between transgenic and wild type mice within the same ob genotype.

\*:  $p < 0.05$ , \*\*:  $p < 0.001$

In the first 11Aob experiment (figure 6), the expected fat loss was seen in the leptin deficient mice. However, while there were obvious differences in glycated hemoglobin levels between obese and non-obese mice, this test did not show a statistically significant difference in the long-term blood glucose levels of transgenic and non-transgenic ob/ob mice. This test was done as an initial and quick inquiry. It will be followed up by determination of serum insulin levels.

Future experiments will include other longitudinal studies of 11Aob/ob mice (and the control mice produced as littermates) to determine the animals' depot-specific fat content and the onset of diabetes. From the future groups of mice periodic blood samples will be taken to search for any differences in the onset of insulin resistance. These mice may also be studied with the new Bruker minispec to investigate total body lipid levels.

**Aim 3: Efforts to determine if the lipodystrophic phenotype can be attributed to apoptosis or failure of adipocyte precursors to properly differentiate.**

There are several possibilities for what may be causing the lipodystrophic phenotype at the tissue level. First the adipocytes could be unable to properly maintain their fat stores. In this case they would be much smaller than normal adipocytes. This does not seem to be the mechanism in FPLD, but it is possible that the transgenic model is acting differently. The second possibility is that mature cells are being lost, perhaps through apoptosis. Finally, the FPLD mutation could be caused by a failure of differentiation into adipocytes. The following sets of experiments have been planned to give us some insight into whether any of these mechanisms are at work.

To determine whether there is a size difference in the adipocytes from the fat pads of wild type and transgenic mice these tissues have been digested and fixed with osmium tetroxide. Small samples of WAT (inguinal and epididymal) were weighed and exposed to osmium tetroxide in sealed vials. During an incubation at 37° C the osmium will fix the fat droplets by cross-linking the triglycerides into small black pellets. These pellets can be counted by a Coulter counter [44]. By comparing the results (# of cells/mg of tissue) to the results of a lipid extraction (mg of lipid/mg of tissue) the average size of the lipid droplet can be calculated. There was no statistically significant difference in the cell size data obtained in this manner. However, the test may have failed to give meaningful results due to the experimental design. For this test WAT was collected and fixed from individual mice. This would retain any differences in cell size between the individuals due to feeding habits, number of animals per cage, and dominance (fighting) issues. It was suggested that more reliable results might be maintained by pooling WAT from a small number of mice (3-4) and then testing the cells obtained after a collagenase digestion [45]. This would decrease the variability seen between individuals. Then statistical tests could be performed on results gathered from multiple pools of tissue.

In addition to cell size we will collect data to determine whether the adipocytes are functioning normally as well. Lypolysis and lypogenesis assays will help to determine whether the cells are functioning as proper adipocytes. We will isolate adipocytes from adult mice for these cell culture experiments or use cells isolated for the osmium tetroxide experiment mentioned above.

To address the second and third possibilities, epididymal WAT from 3 mice of each genotype was collected, fixed, and stained to detect either apoptosis (TdT end-labeling) or cell proliferation (Ki67 immunohistochemistry). These test efforts were not sufficient to see any obvious differences. The phenotype seems to act over the lifetime of the animals so it may not be vigorous enough to observe in tissue sections at a single time point in adult WAT. Instead a number of cell culture and Northern blotting experiments may be more useful.

One method of visualizing any differences in both apoptosis and differentiation would be to isolate the precursors and observe induced differentiation in culture. For the first experiment, embryonic mice will be harvested at about 15-16 days. After removing the internal organs the remaining tissue can be digested allowing for the isolation of the stromovascular fraction. These cells will then be induced to differentiate into adipocytes. Significant cell loss in the 11A mice could be appreciated simply by counting the mature adipocytes over time. Further, a failure of the precursors to differentiate could be detected by a lack of mature adipocytes and an abundance of remaining stromovascular cells.

In FPLD patients recognition of the phenotype is delayed until approximately the age of puberty. If there is a component of the disease that is dependent on reaching a certain level of maturity (such as hormone levels) then apoptotic or differentiation differences may not be detectable or even present in cells taken from embryonic tissue. In this case tissue for the same cell culture experiments will be taken from animals that have passed the age of sexual maturity (approximately 8 weeks).

Another way to detect changes in the levels of differentiation is to look for changes in levels of differentiation markers. For adipocytes those would include SREBP-1, PPAR $\alpha$  and aP2. Northern blots will be done to detect the expression levels of these adipocyte marker genes. A phosphorimager will then be used to look for any changes. Pref1 is a marker of preadipocytes. A Northern blot for Pref1 would indicate any increase in the population of preadipocytes caused by a defect in differentiation.

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