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Differences in lean and obese skeletal muscle myoblasts of adult and post-natal LA/N (faf) rats: a confocal description of the skeletal muscle myoblast

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Differences in Lean and Obese Skeletal Muscle Myoblasts of Adult and Post-natal LA/N^{faf} Rats.

A Confocal Description of the Skeletal Muscle Myoblast

A Thesis

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TABLE OF CONTENTS

ABSTRACT

Mutant strains of obese rats are currently being studied to reveal the mechanisms of increased lipid synthesis and decreased lipid oxidation in these rats. It was originally thought that much of the fat production that leads to obesity is the result of metabolic processes in the liver and adipose tissue. Studies by Kahle et al.[3] showed that the liver and adipose tissue account for only 22% and 7% respectively, of total fatty acid synthesis. It was also shown that 21% of total *de novo* fatty acid was localized in the postural skeletal muscle.

The dry weight of the body is 40% skeletal muscle, thus it is likely that this tissue type has some role in controlling the body's metabolism.[1] Previous work has gone into characterizing differences present within the skeletal muscle of lean and obese phenotypes that may have an influence on metabolism. Electron microscopy was used to determine structural differences in skeletal muscle tissue as well as in myoblasts. It was shown that the myoblasts of the obese phenotype have a higher capacity metabolic synthesis. The work of this thesis was done in order to further investigate some of these previously studied parameters. The focus of this work was to characterize differences in metabolic potential in skeletal muscle myoblasts of the obese and lean phenotypes of the rodent model, the LA/N^{faf} rat.

One important parameter in question is the amount of intracellular lipid deposits present within the individual myoblasts. To determine differences between the two phenotypes, confocal microcopy was used to visually observe,

with a lipid specific probe nile red, both intracellular lipid concentration and lipid distribution within the cell. After digitally capturing micrographs into specifically designed computer software, it was possible to determine the amount of fluorescence present within a single cell in a culture. Post-natal (14 days of age) animals were also studied in the same manner to determine if pre-obese pups (Figure 1b) showed the same situation as their adult counterparts.

The results of the experiment indicated a significant difference ($p < 0.50$) present within the lipid content between obese and lean adult rats. The same situation was not shown in post-natal animals. The size of the cells studied however, differed significantly ($p < 0.05$) between the two age groups studied, but not within them. This difference may be directly attributable to the increased rate of mitosis of the post-natal myoblasts. This increased rate of mitosis may also explain the similarity in lipid content of the obese and lean pups. Another parameter briefly examined was the role of the mitochondria in the metabolism of the cell. No results were attained from the mitochondrial studies.

Skeletal muscle has recently been proven to be an important tool in the delivery of proteins and drugs throughout the body. It has been shown that hybrid skeletal muscle myotubes can be used to deliver such necessary proteins as dystrophin and human growth hormone.[33-37] Using this type of myoblast-mediated gene therapy it may be possible with continued study to determine regulatory hormones in lipid metabolism. These hormones when delivered to the body in the correct manner may be used as an effective means to not only study

the effect of certain drugs on metabolism but perhaps as an effective treatment to obesity.

INTRODUCTION

The use of genetic mutants has become an important tool in the study of biology and its related sciences. These mutants can be used for a wide variety of applications including the characterization of genes and their gene products. By studying genetic mutants, much information can be learned about an organism. Results may range from revealing details about the evolution of an organism on the population level to localizing mRNA involved in protein synthesis. One interesting example of a genetic mutant that extensive research has been conducted on is the obese rat.

Mutant strains of obese rodents are currently being studied to reveal the mechanisms of increased lipid synthesis and decreased lipid oxidation in the obese phenotypes.[2,3,4] Determination of these mechanisms will undoubtedly lead to a more thorough understanding of overall metabolism, and eventually may lead to new generation of genetic medicine and gene therapy. What is learned from rats, mice and other obese mutant species will lead to a more thorough understanding of the condition of human obesity.

There is a certain amount of body fat that is necessary physiological functions to be properly carried out.[5] When an individual exceeds this value (25% of total body weight for men and 30% for women), they are considered to be obese.[5] Many obesity studies rely on measures of relative weight, such as body mass index (BMI), an index of body weight that is normalized for height, to determine the prevalence of obesity in an individual. For example, the National

Center for Health Statistics currently uses the 85th percentile sex-specific values of BMI for persons aged 20-29 as a cutoff to define obesity in adults.[5]

Obesity pertains to an excess of body weight in relation to height. This includes all tissues, including intracellular lipid deposits, and therefore may reflect varying degrees of total body fat.[5] Based on data from The Third National Health and Nutrition Examination Survey (NHANES III), it has been estimated that approximately thirty percent of adult Americans aged 20 and older are estimated to be obese.[5] Using figures from the 1990 census, this corresponds to roughly 58 million Americans. The prevalence of obesity in the United States has increased dramatically during the past 15 years in men and women of all age and ethnic groups. Obesity is disproportionately high among African American and Hispanic women, being near 50% in the two groups. Obesity has also dramatically increased in juveniles and younger children as well. Based on NHANES III data, presently about one fifth of adolescents aged 12-19 are obese.[5] This number is up from only 5% in the 1960s and 1970s. The relationship of adult obesity to juvenile obesity will be one of the underlying themes of this thesis.

Morbid obesity is related to numerous health problems including increased mortality in adults. A person is considered to be morbidly obese if their weight is at least twice the desired weight.[5] Severe obesity has also been shown to be associated with increased mortality and other health problems as well.[5] Although some studies have reported a greater rate of mortality among the thinnest individuals,[6] a 1993 study that carefully controlled for smoking and illness-related

weight loss showed a close relationship between BMI and mortality.[6] Two studies have suggested that overweight children and adolescents may have increased early mortality.[6] Childhood obesity may be a significant risk factor for adult obesity. Also, according to studies obesity at younger than adolescent ages has yet to be determined to be an adequate predictor of obesity in adults.[5] Relationships between post-natal and adult obesity will be questioned in this thesis.

Obesity has been associated with several degenerative diseases or conditions that change the productivity and lifespan of the individual. Observational studies have established a clear association between excess weight and hypercholesterolemia and suggest an independent relationship between being overweight and development of coronary artery disease.[6] Being overweight has also been associated with several cardiovascular risk factors in children and adolescents, including hypercholesterolemia and hypertension.[7] Obesity has also been associated with an increased risk of certain cancers (including those of the colon, rectum, prostate, gallbladder, biliary tract, breast, cervix, endometrium, and ovary), and with other disorders such as cholelithiasis, obstructive sleep apnea, venous thromboembolism, and osteoarthritis.[5]

Finally, obesity can negatively affect the quality of life by limiting mobility, physical endurance, and other functional measures, as well as through social, academic, and job discrimination.[6] Because of the large numbers of obese individuals in the United States and throughout the world, there is much research

being conducted in this field. One of the most important discoveries made is the a single mutant gene in rodents that can lead to extreme obesity. [3]

The fatty (*fa*) mutation in the rat arose spontaneously in the Merck strain in 1961 and shows strong phenotypic similarities to obesity in humans.[8] The rats that now continue to carry this mutation are referred to as Zucker rats. The *fa* obese mutation is due to a single nucleotide substitution, Gln269Pro, in the *fa* allele of the leptin receptor ($Lepr^{fa}$).[9,10,11] Another mutation, the *f* mutant, arose spontaneously in the Koletsky's hypertensive rat strain and the mutation was introgressed into the LA/N and SHR/N strains developed at the NIH.[12] Recent studies have shown that *fa* and *f* are mutations on the same gene, the leptin receptor (*Lepr*)[13] This was accomplished in part by producing a *fa/f* compound mutant (Figure 1a). It has now been shown that the *f* mutation is a Tyr763Stop mutation in *Lepr* on chromosome 5.[2] The homologous region in humans for *Lepr* is 1p31-p22.[14] The leptin receptor is thought to be directly responsible for directing lipid metabolism is certain cell types. There are several forms of the leptin receptor that are present in varying tissues throughout the body.

It was originally thought that much of the fat production that led to obesity was the result of metabolic processes in the liver and adipose tissue.[15-18] As a result of this thinking, volumes of work have been written on the role of the liver in lipid biosynthesis. In 1981 Hollands and Cawthorne were able to demonstrate that the liver and adipose tissue account for only 22% and 7% respectively, of total fatty acid synthesis.[1] Their findings were based on whole body distribution of tritium-labeled water and its synthesis into fatty acids. Surprisingly 21% of

total de novo fatty acid was localized in the postural skeletal muscle, which include the predominantly red muscles, the psoas major and the iliacus.[1] These are very interesting results when coupled to the fact that the mutant LA/N^{faf} rat has decreased metabolism and increased capacity for energy storage (fat production). A recent study has shown that leptin plays a direct role in the depletion of fat content of both adipocytes and nonadipocytes.[19]

Skeletal muscle is one tissue examined by Shimabukuro et al.[19] Here, leptin was shown to deplete triglycerides in cells with intact (non-mutated) leptin receptors via a direct mechanism that involves both an increase in free fatty acid (FFA) oxidation and a decrease in esterification.[19] The study used Zucker^{fatty} rats to determine the role that leptin plays in lipid regulation of the body. Effects of leptin were recorded in lean rats while obese animals showed little or no response to the administered leptin.

The growing evidence supporting the hypothesis that skeletal muscle is a major contributor of fatty acid synthesis has prompted research into finding possible differences in the ultrastructure of the muscle tissue itself. Some of the initial work in this field was prompted by the finding of different rates of fatty acid synthesis as shown by skeletal muscles versus the liver and adipose tissue.[20] Other studies with light microscopy have shown a reduction in the diameter of glycolytic fast white and intermediate fibers with no significant difference in the fast red fibers.[21] It has also been shown that the obese red fiber contained three times as much fat as that of lean mice.[21] Current investigations of several skeletal muscles, including the soleus, gastrocnemius, and psoas major show

distinctive differences in undifferentiated myoblasts or satellite cells between obese and lean muscle.[22]

Skeletal muscle satellite cells are single cells associated with myotubes.[23] Mature muscle in postnatal animals is a syncytia of fused myoblats or satellite cells. Myofibers are the linear multinucleated fused cells functioning together and sharing a common cytoplasm. Satellite cells exist fused to the membrane of the myotube but do not share the cytoplasm of the myotube.[24,25] Distinct differences have previously been shown to exist between the satellite cells of lean and obese rats.[3] Satellite cells of lean phenotypes appear to lack organelles, have a very condensed nucleus, and posses few vesicles.[3] This is in contrast to the obese phenotypes which display a well developed endoplasmic reticulum and Golgi apparatus, greater numbers of mitochondria, a less condensed nucleus and greater numbers of lipid deposits than their lean counterparts.[3] These observations indicate that it is possible that the obese skeletal muscle cells have the capacity for increased metabolic activity. Based on these observations, two questions can be asked. The first is to focus on one of the differences noted specifically the amount of lipid droplets present in the lean and obese satellite cells. The second will focus on determining what relationship, if any, exists between obese adult individuals, and pre-obese (phenotype) postnatal individuals. Specifically, are the differences seen in the myoblasts of the obese and lean phenotypes (especially the amount of lipid deposits) actually direct differences caused by the mutation in the leptin receptor, or can the differences be attributed to some other mechanism, suggesting that

the lipid deposits are more of a secondary condition promoted by the obese condition itself. In order to answer these questions, it will first be necessary to isolate the myoblasts from the skeletal muscle of the rats to be studied. The isolated myoblasts were grown in culture and were examined using confocal microscopy in order to visually, as well as quantitatively determine differences between obese and lean cells. Findings will be compared to previous light and electron microscopy studies of the same cells. The confocal microscope was chosen as the investigative tool for this thesis because it has the ability to produce three dimensional images on a submicrometer level[26] and it can make time-lapse images of live cells. These images allow investigators to capture events such as lipid partitioning prior to and during events such as mitosis. The principle feature of confocal imaging is that only what is in focus is detected.[26] Because of this, it is possible to focus on very thin sections without fluorescence from regions around the section in focus to spill over into the region in focus. Out-of-focus regions of a sample appear black, thus allowing for more precise quantification of fluorescence.

MATERIALS AND METHODS

The LA/N^{faf} rat (formerly LA/N-fa^{cp}) was developed at The National Institutes of Health by Carl Hansen.[26] This was done by mating a Koletsky rat heterozygous for the corpulent mutation with an LA/N (Lister Albany/NIH) rat. The obese LA/N^{faf} rat unfortunately does not reproduce and must be maintained by heterozygote matings. The lean heterozygotes are phenotypically indistinguishable from the lean homozygous wild-type rats.

Breeding experiments took place in the Marshall University Animal Facilities with routine husbandry guidelines recommended by the Marshall University Animal Care Committee. The colony was maintained specific pathogen free on a 12h/12h (L:D) cycle. Adult animals were maintained ad libitum on laboratory chow (PMI Feeds, St. Louis, MO). The dietary analysis of the rodent diet is outlined in Table 1. Two week old animals were experimented on prior to being weaned.

Two age groups of animals were chosen for myoblast analysis. Those involved in the active development of obesity (10 to 13 weeks of age) and 14 day old pre-obese pups. Eight animals in the active obesity development stage were used, four lean (heterozygous and homozygous; distinction was not recognized) and four obese. Pups were genotyped prior to myoblast culturing. The genotyping procedure will be outlined below. After selection of an animal, hindlimb muscles were dissected, enzymatically treated to release myoblasts, and grown in culture. The procedure used for culturing the cells is outlined

below. Lastly, the cultures were stained with nile red and examined under the confocal microscope for lipid content. Culturing and staining procedures will be described first.

Procedure for Culturing of Myoblasts

The subsequent steps were conducted in a laminar flow hood. Animals were anesthetized using 2-bromo-2-chloro-1,1,1-trifluoroethane (Sigma, St. Louis, MO Cat # B-4388). The carcass was submerged in 70% ethanol for 5 minutes. Then, an initial incision was made at the juncture of the hindlimbs and trunk. The epidermis and facia were removed, exposing the skeletal muscle. The calf and thigh muscles were excised and placed into a petri dish containing Hank's Balanced Salt Solution (HBSS). The muscles were quickly diced with sterile surgical scissors then placed into a jacketed cell stirrer (Fisher Scientific, Pittsburgh, PA Cat # 06 416 3B) with a magnetic stirrer and an externally, separately sealed water flow-through channel which was kept at 37°C. HBSS/trypsin solution (50ml) was added to the muscle suspension and incubated for 15 minutes. Following sedimentation, the solution was discarded. The digestion procedure was repeated 3 times for 20 minutes each, with the liquid layer being saved in 50ml sterile centrifuge tubes. The solution was then split evenly into two tubes and 5 ml of skeletal muscle media (13.5g Dulbecco's Modified Eagle Medium, 900ml ddH_20, 4.00g Sodium Bicarbonate ($NaHCO_3$),

3.10g dextrose, and 3.75ml penicillin/streptonycin) with 20% horse serum was added to each tube. The tubes were spun for 10 minutes at 1000 RPM. At the end of this protocol, the supernatant was poured off and discarded. 2.5ml of skeletal muscle media (SKM) were added to each pellet. This procedure was repeated three times in addition to the initial 15-minute incubation that was discarded. At this time, the muscle-enzyme suspension was allowed to sediment and the liquid was discarded. Pellets were placed in a new sterile centrifuge tube with 10ml of SKM/20% horse serum and fungizone (Gibco, Grand Island, NY Cat # 15295-017). The solution was passed through a cell strainer (Fisher Scientific Cat # 08-771-2) into a new sterile centrifuge tube. The final strained solution was evenly distributed into culture plate containing six separate 13mm wells (Becton Dickinson, Franklin Lakes, NJ Cat # 3046) and allowed to incubate overnight in a 37°C incubator. Inside each well was a confocal microscope coverslip coated with matrigel (Collaborative Biomedical Products, Bedford, MA Cat # 40234). Matrigel allowed for the cells to adhere to the coverslip for later investigation by the confocal microscope. After 24h, media was removed from the dishes, discarded and 2ml of SKM with 20% horse serum and fungizone was added to each well.

Procedure for Staining Cells and Viewing with Confocal Microscope

Skeletal muscle myoblasts have no natural auto-fluorescence, thus it is necessary to stain the cells in order to visualize them using confocal microscopy. Nile red was chosen for this procedure. Nile red is a phenozazone dye that fluoresces intensely and in varying color, in organic solvents and hydrophobic lipids. However, the fluorescence is fully quenched in water. The dye acts, therefore, as a fluorescent hydrophobic probe.[28] A stock solution of nile red (100μg/ml) was prepared in acetone and stored protected from light. Staining was carried out on live cultures. The staining procedure began by washing the culture two times with 1ml of HBSS. This was followed by the addition of a dilution (1:100) of the stock solution of nile red in HBSS for 5 minutes. At the end of the period, the culture was washed two times with HBSS. The coverslip was removed and placed cell culture side down on a glass slide. The slide was then transferred to the confocal microscope for data collection and processing.

The confocal microscope used for the experiment was BioRad model number MRC 1024. The software used was developed specifically for the microscope by BioRad and was used under the operating system OS/2 Warp. For digital imaging of the cell cultures, or acquisition, specific settings were used in order to visualize the stained intracellular lipid droplets. These settings are given in Table 5. The same settings were used every time for consistency in acquisition.

After cell images were acquired, it was necessary to process the images to determine relative amounts of intracellular lipid per cell. This was accomplished by drawing a random line over the cell stretching from cell process to cell process. An example is shown in Figure 14. The line was then measured for distance in micrometers using the equation:

$$d(P,Q) = [(x_2 - x_1)^2 + (y_2 - y_1)^2]^{.5}$$

Where d is the distance between the points P and Q, and points P and Q are determined on a cartesian plane with x and y values determined in quadrant I by the computer based on the total size of the image. To determine differences in relative fluorescence between cells, sum fluorescence was calculated along the length of the line. Fluorescence was calculated for each pixel and was given a value ranging from 0 to 255 where 0 is total blackness (no fluorescence). After calculating sum fluorescence for all cells, and determining an average for each group, age and phenotype, differences in fluorescence between phenotypes and adults versus pups were also determined using a Student's t-test to compare average sum fluorescence and cell size for a particular group.

Procedure for Genotyping LA/N^{faf} Rat Pups

Four steps were followed to genotype post natal rats: 1. An initial DNA extraction, 2. Gene amplification using the polymerase chain reaction (PCR), 3. Restriction enzyme digestion followed by 4. Electrophoretic migration/separation of PCR fragments/digestion products on an agarose gel.

Extraction of DNA

The first step in the genotyping procedure is the extraction and subsequent purification of the test animal DNA. Earclips were taken from 10 - 12 day old LA/N rats using an animal ear punch (Fisher Scientific 01 - 337B). Earclips were placed into a 1.5 ml Eppendorf tube containing 900µl of lysis buffer (1% SDS, 10mM EDTA, and 10mM Tris Buffer) and 45µl of Proteinase K. Tubes were incubated overnight (10 - 12 hours) in a 65°C waterbath. Next, 100µl of 8M Potassium Acetate (KAc) was added to the tube. Tubes were cooled on ice for 15 minutes, then centrifuged at 14,000 RPM for 15 minutes. After centrifugation, the supernatant was transferred to a new tube and the pellet was discarded. Into the new tube, 100µl of phenol/chloroform was added. Tubes were vortexed, then spun at 14K for 5 minutes. The aqueous layer was removed and transferred to new tubes and 500µl of chloroform was added. Tubes were again vortexed and spun at 14K for 5 minutes. The aqueous layers were removed and transferred to new microfuge tubes. To the tubes 1/9 of the total volume of 3M NaAc and 1/2

that volume of isopropanol was added. Tubes were chilled for one hour at -20°C, then spun at 14K for 30 minutes. Supernatants were decanted and pellets were washed two times with chilled 70% EtOH. After the second washing, 300µl of ddH_2O was added to tubes and DNA was allowed to resuspend itself for 3 - 4 hours at 27°C.

PCR Amplification

After the DNA extraction and purification, it was necessary to amplify the *Lepr^{fa}* gene by means of the polymerase chain reaction (PCR). For the reaction, a master mix was made that contained 376µl of double distilled H_2O, 60µl of standard 10X PCR buffer, 60µl of dNTP mix, 72µl $MgCl_2$, and 2µl of Taq polymerase enzyme. This was enough to allow 6 PCR reactions. For an individual reaction, 50µl sample sizes were used. Of the 50µl, 47µl were master mix, 1µl was the purified DNA extract and 1µl each of the two specific primers for the *Lepr^{fa}* gene. The primers used were designated rLepr 14A and rLepr 14B. (Specific sequences for the two primers are listed in Table2.) The primers were made by the Marshall University DNA Core Facility. The 50µl PCR mix was injected by micropipette into a thin walled PCR tube. The tube was then placed into the PCR thermocycler (Perkin Elmer Gene Amp PCR System 2400 Thermal Cycler) and amplification was as follows. The temperature was brought to 94° for 5 minutes, then cycled 35 times from 94°C for 30 seconds to 55°C for 30

seconds to 72°C for 30 seconds. At the end of the 35 cycles, the temperature was left at 72°C for 7 minutes then lowered to 4°C.

Determination of Genotype by Electrophoretic Separation

The final step in determination of the genotype of the postnatal rat was electrophoretic separation of the PCR product after a restriction enzyme digestion. For this the restriction endonuclease DRA I was used. The primers used in the PCR reaction introduce a DRA I cut site in the obese allele, which does not exist in the wild type (lean) allele. This cut site is shown in Table 4. For the digestion, 22.5μl of PCR product, 1μl of DRA I, and 2.5μl of enzyme buffer were added to 1.5ml reaction tubes. The mix was incubated 24h in a 37°C waterbath. Next, an 8μl aliquot of the incubated mix and 2μl of generic electrophoretic gel dye were added to a new reaction tube and were loaded into a 3% agarose gel made of 2.1g agarose and 70ml of TAE and ethidium bromide. The sample was run for 25 minutes at 100V. The results indicated three possibilities: the first was one 125 base pair (bp) band, indicating a lean homozygote; the second result was two bands one being 102bp and the other being 23bp, thus indicating a obese homozygote; and finally three bands 125, 102, and 23bp indicated a lean heterozygote. Examples of all three genotypes are shown in Figure 16.

RESULTS

Obese rat skeletal muscle satellite cells have structural features that indicate the capacity for greater metabolic activity when compared to their lean littermates.[29-31] One of these features is the appearance of higher levels of lipid deposits in the obese cells. To date, it has not been determined quantitatively if this difference exists on a significant basis. The results of this series of experiments indicate that, indeed, adult rat myoblasts show a significant difference in lipid content when comparing cells of lean and obese phenotypes. However, when compared to cells taken from two-week-old animals not yet expressing obesity, myoblasts from the two phenotypes did not display the same results. Young rats show no significant difference in lipid content between phenotypes.

Adult lean cells, as a group, were found to have an average sum fluorescence of 3305.13+/-447 units of fluorescence (Figures 2 - 4). Adult obese myoblasts were to found to have a value of 5033.31+/-728 units of fluorescence (Figures 5 - 7). Table 6 shows the statistical analysis of this data. The lengths of the cells did not significantly differ between phenotypes with the lean phenotype having a mean length of 70.62+/-3.66μm and the obese cells having a mean length of 63.27+/-3.36μm. Statistical analysis of the data for the length of the cells is shown in Table 7. Data is further presented by histogram in Figure 14. These results indicate that when comparing adult cells not significantly different

in size, the amount of lipid present in the obese phenotype is significantly higher than that of the lean phenotype ($p < 0.05$).

When looking at post-natal animals however, a different situation was shown to exist. It was first necessary to genotype the baby animals because the animals have no easily detectable physical differences between phenotypes. For the experiments 4 obese and 4 lean animals (2 heterozygotes and 2 homozygotes) were used. Figure 16 shows an example of a gel separation of the different genotypes. From the genotyped post-natal animals, it was determined that a significant difference does not exist between phenotypes with respect to the amount of intracellular lipid deposits. The lean pups had a value of 3650.09+/-411 units of fluorescence and the obese pups had a value of 3781.90+/-329 units of fluorescence. Again the cells compared between phenotypes did not significantly differ in size from each other (lean cells = 39.83+/-1.46μm; obese cells = 36.20+/-1.57μm). Post-natal lean cells are shown in Figures 8 - 10 and post-natal obese cells are shown in Figures 11 - 13. Statistical analysis of collected data is shown in Tables 6 and 7. The data is presented by histogram in figure 17. The results of the post-natal animals indicate that the amount of intracellular lipid deposits do not significantly differ from each other ($p > 0.05$).

The results of the adult and post-natal animals were not compared statistically beyond the size comparison. When average length of adult to baby cell was compared, a significant difference was shown to exist between adult cells and post-natal pre-obese cells ($p < 0.001$). Adult cells had a length of 67.23

+/-2.52μm and post-natal cells had a value of 38.15+/-1.08μm. This data is presented in Table 7 and Figure 18. Lipid content was not compared between adult and baby animals due to the significant difference in sizes of the cells.

TABLES AND CHARTS

TABLE 1. Dietary Information.

LABORATORY RODENT DIET 5001

Crude protein:	not less than 23.0%
Crude fat:	not less than 4.5%
Crude fiber:	not more than 6.0%
Ash:	not more than 8.0%
Added minerals:	not more than 2.5%

Table 2. Sequences of *Lepr*^{fa} Gene Primers rLepr 14A and rLepr 14B.

rLepr 14A:

CTG GAC ACT GTC ACC TAA TGT TT

rLepr 14B:

CAT GGA TAT AAT ACT TGT TAA CAT

Table 4. Recognition Sequence of DRA I.

5'...TTT^AAA...3'

3'...AAA^TTT...5'

Table 5. Confocal Microscope Settings.

Lens Magnification	60X
Software Magnification	1X
Laser	3%
Filter 1	
Wavelength	585
Iris	4.7
Gain	1053
Blevel	0
Filter 2	
Wavelength	522
Iris	7.4
Gain	1500
Blevel	0
Filter 3	closed

Table 6. Statistical Analysis of Sum Fluorescence.

Animals	Number (N)	Average Fluorescence	Standard Error (SE)	t-value
Adult Obese	77	5033.31	728.0149	2.02
Adult Lean	66	3305.136	447.0347	
Baby Obese	80	3650.09	410.98	0.25
Baby Lean	68	3781.90	328.56	

Table 7. Statistical Analysis of Cell Size.

Animals	Number (N)	Average Length (μm)	Standard Error (SE)	t-value
Adult Obese	77	70.62	3.66	1.47
Adult Lean	66	63.27	3.36	
Baby Obese	80	39.81	1.46	1.69
Baby Lean	68	36.20	1.57	
Adult (all)	143	67.23	2.52	10.61
Baby (all)	148	38.15	1.08	

Figure 1a shows an adult obese animal while the animal in Figure 1b is an ungenotyped 14 day old rat. Both types of animals as well as lean LA/N rats were used in this study.

Figure 1a. LA/N$^{fa/f}$ Compound Mutant.

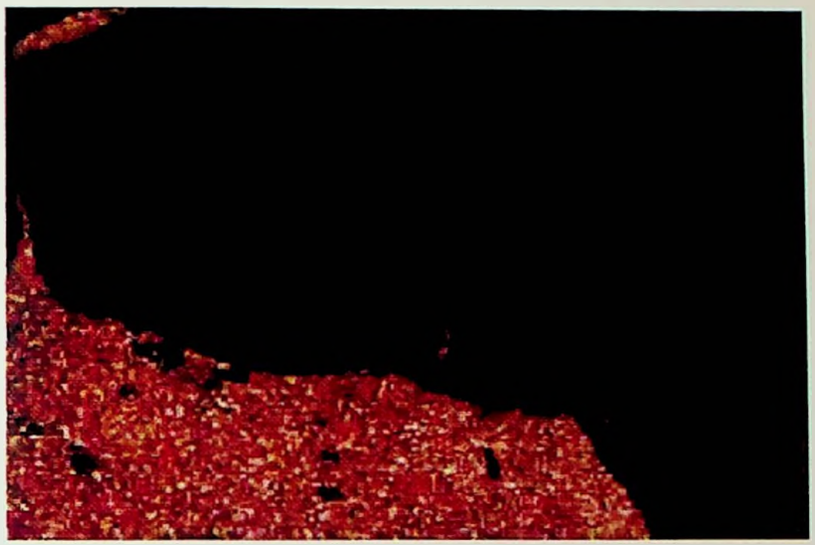


Figure 1b. 14 day old LA/N$^{fa/f}$ Rat pup

Figures 2 - 4 represent cells of adult lean animal cultures. The cells shown here clearly display a supply of lipid present throughout the cell. The cell membranes can be seen as a faint orange color while the lipid within the cell can be seen as much brighter globules located throughout the periphery of the cell. Significant differences can be seen by the cells in these figures versus those shown in Figures 5 - 7.

Figure 2. Adult Lean Animal Myoblasts.

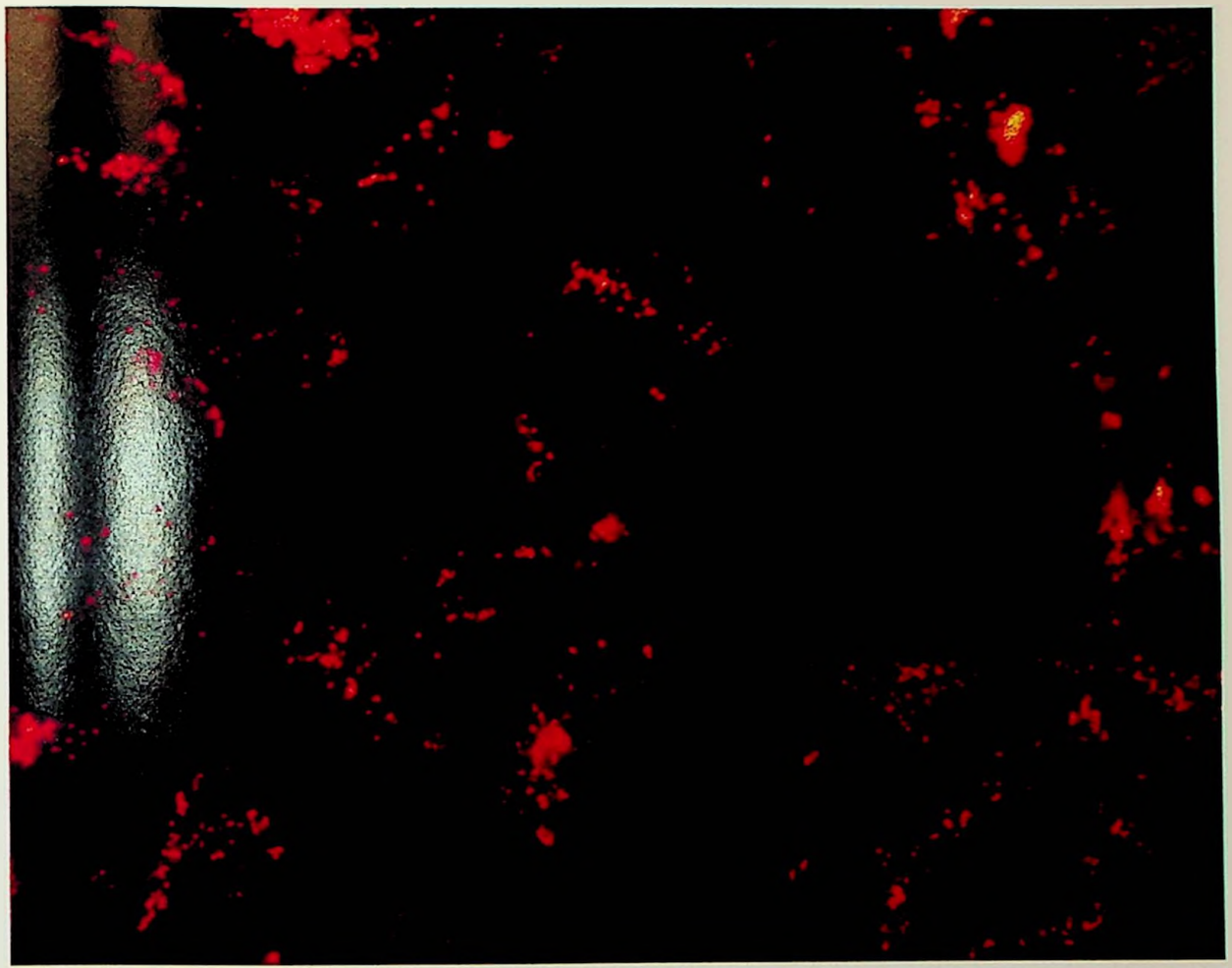


Figure 3. Adult Lean Animal Myoblasts.

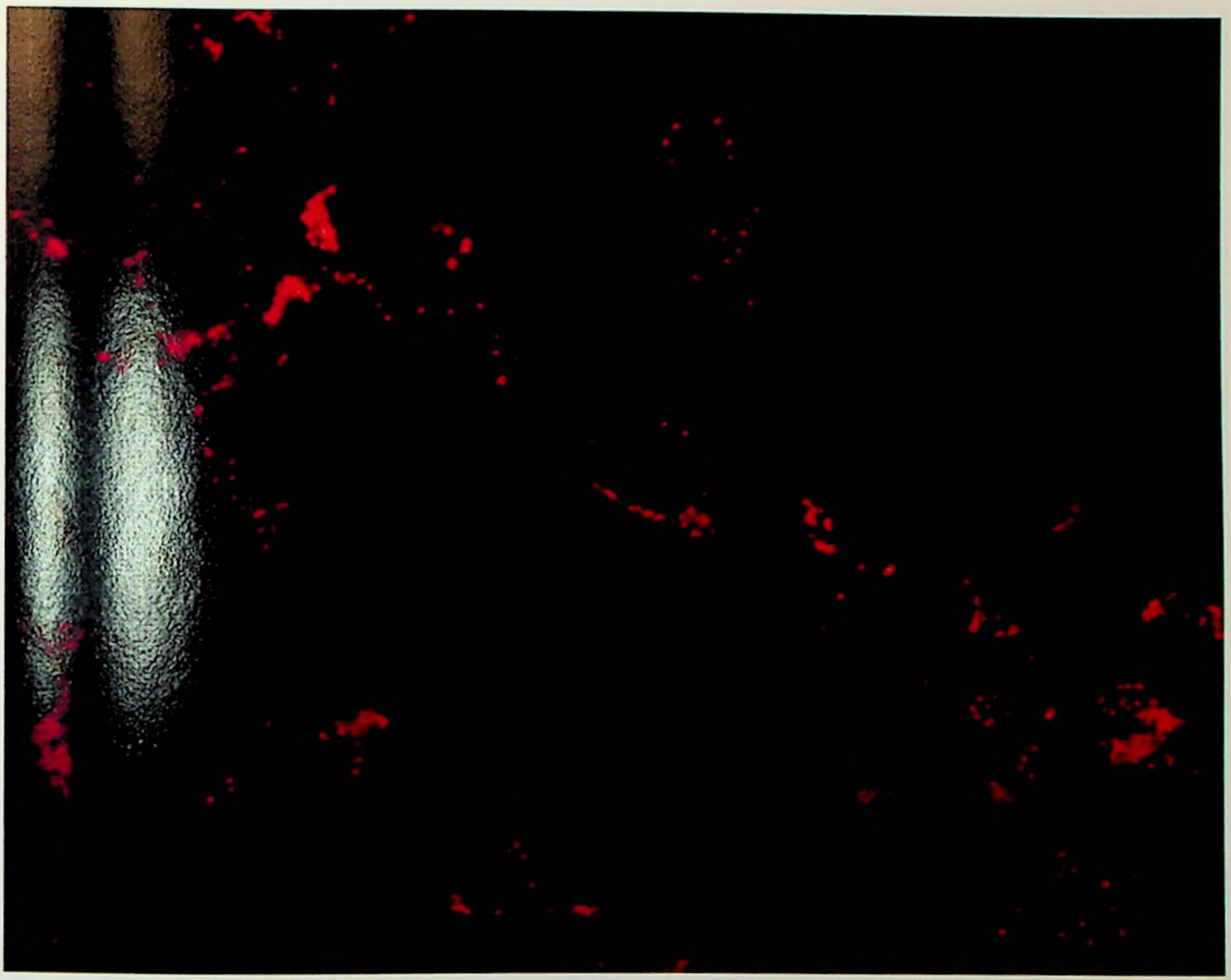
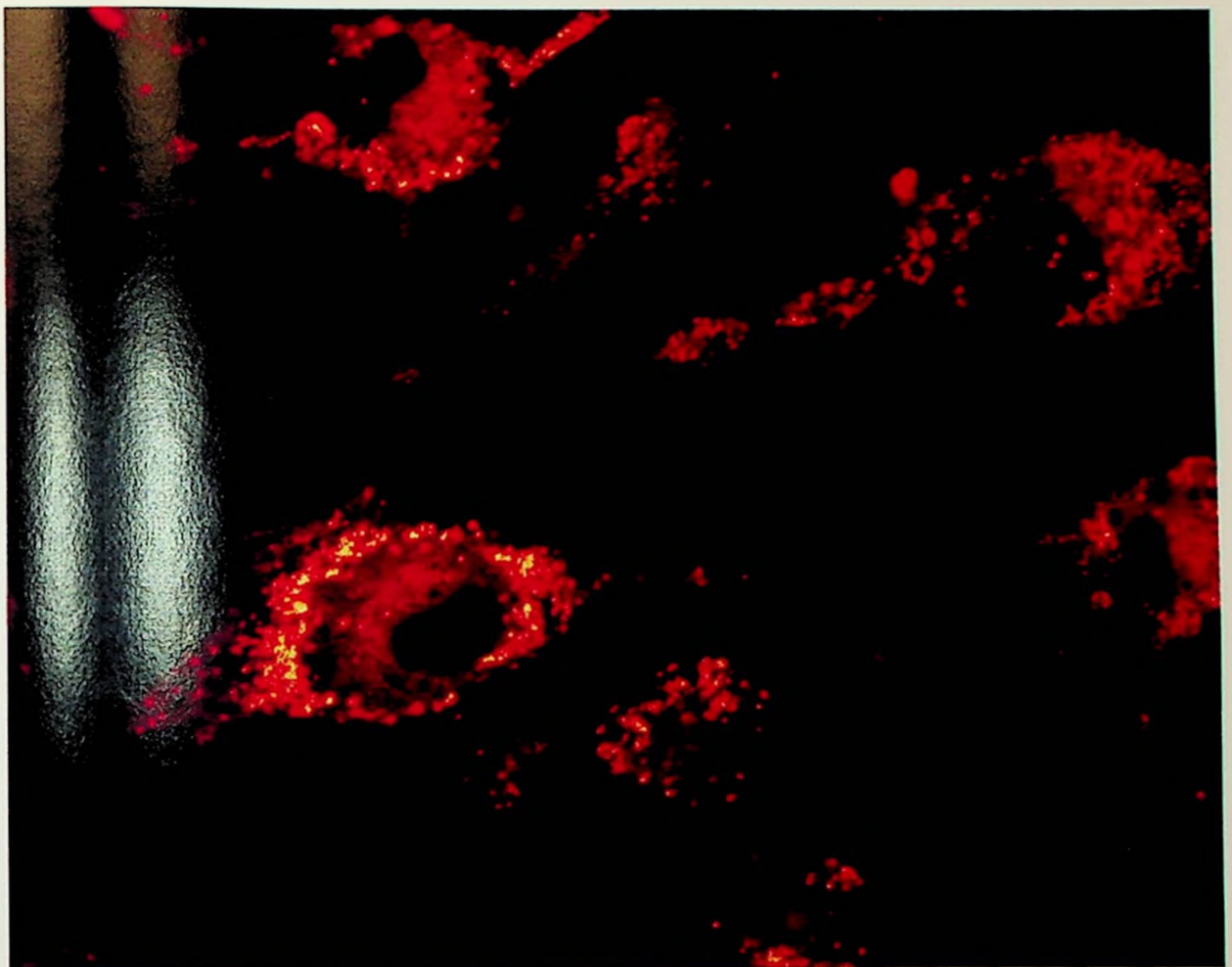


Figure 4. Adult Lean Animal Myoblasts.



Figures 5 – 7 represent cells of obese adult animal cultures. The cells shown here clearly display a supply of lipid present throughout the cell. The cell membranes can be seen as a faint orange color while the lipid within the cell can be seen as much brighter globules located throughout the periphery of the cell. Differences within phenotypes are best displayed by Figures 6 and 7. Three cells in these cultures show extreme levels of lipid within the cells. Lean cultures only show lipid droplets, while the concentration of lipids in these cells clearly show the significant differences within phenotypes.

Figure 5. Adult Obese Animal Myoblasts.

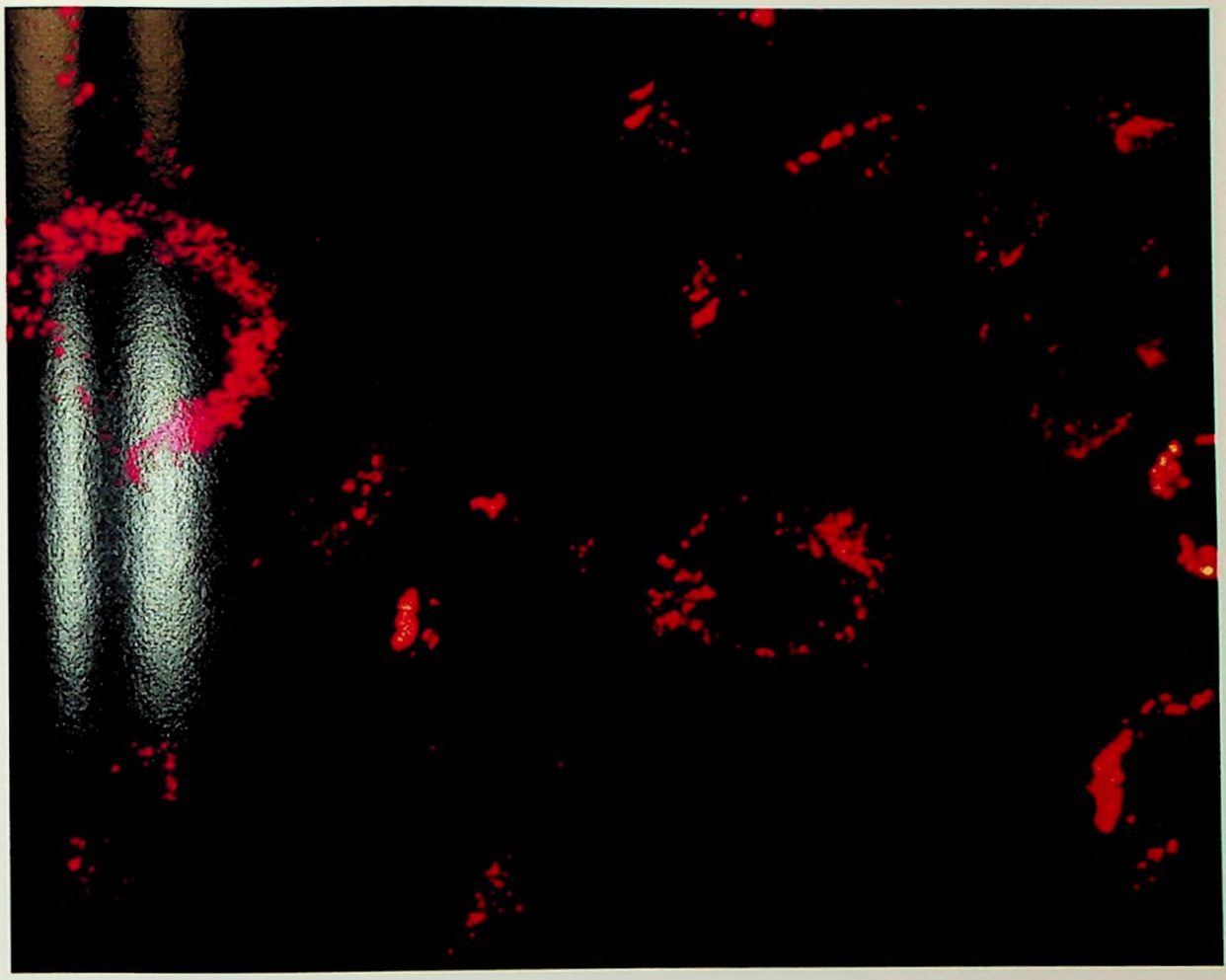


Figure 6. Adult Obese Animal Myoblasts.

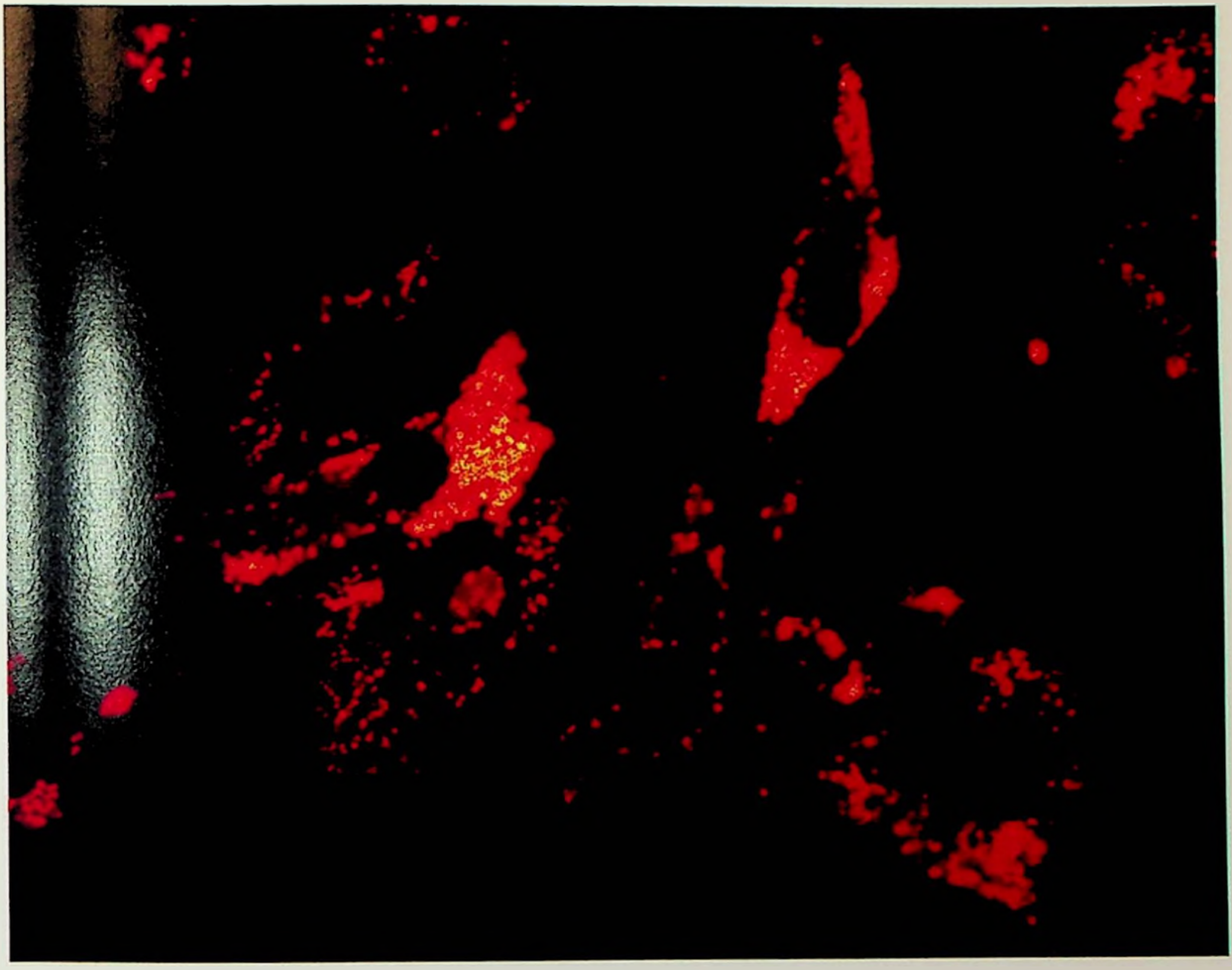


Figure 7. Adult Obese Animal Myoblasts.

Figures 8 – 10 represent cells of lean two-week old animal cultures. The cells shown here clearly display a supply of lipid present throughout the cell. The cell membranes can be seen as a faint orange color while the lipid within the cell can be seen as much brighter globules concentrated near the peripheries of the cell.

Figure 8. Post-natal Lean Animal Myoblasts.

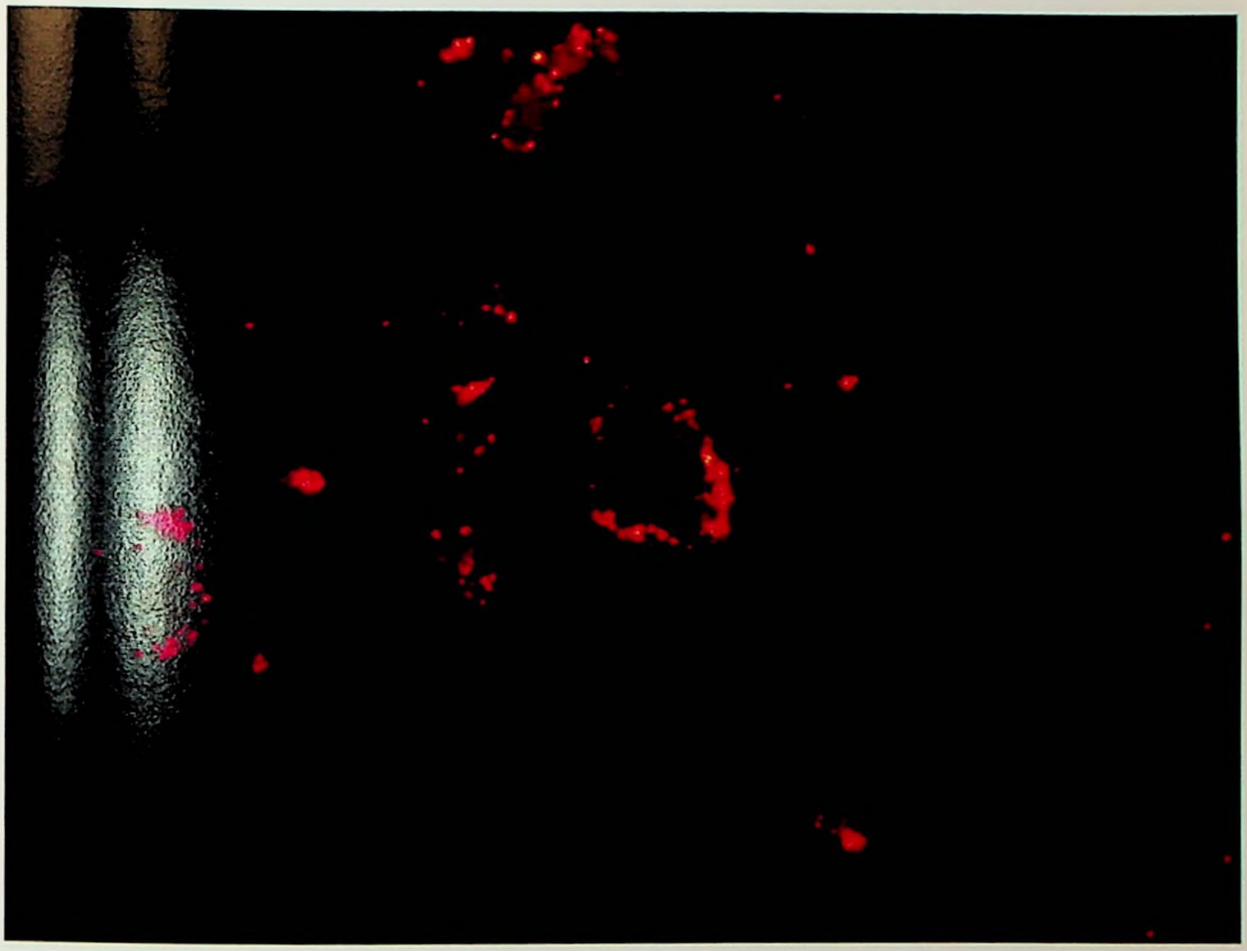


Figure 9. Post-natal Lean Animal Myoblasts.

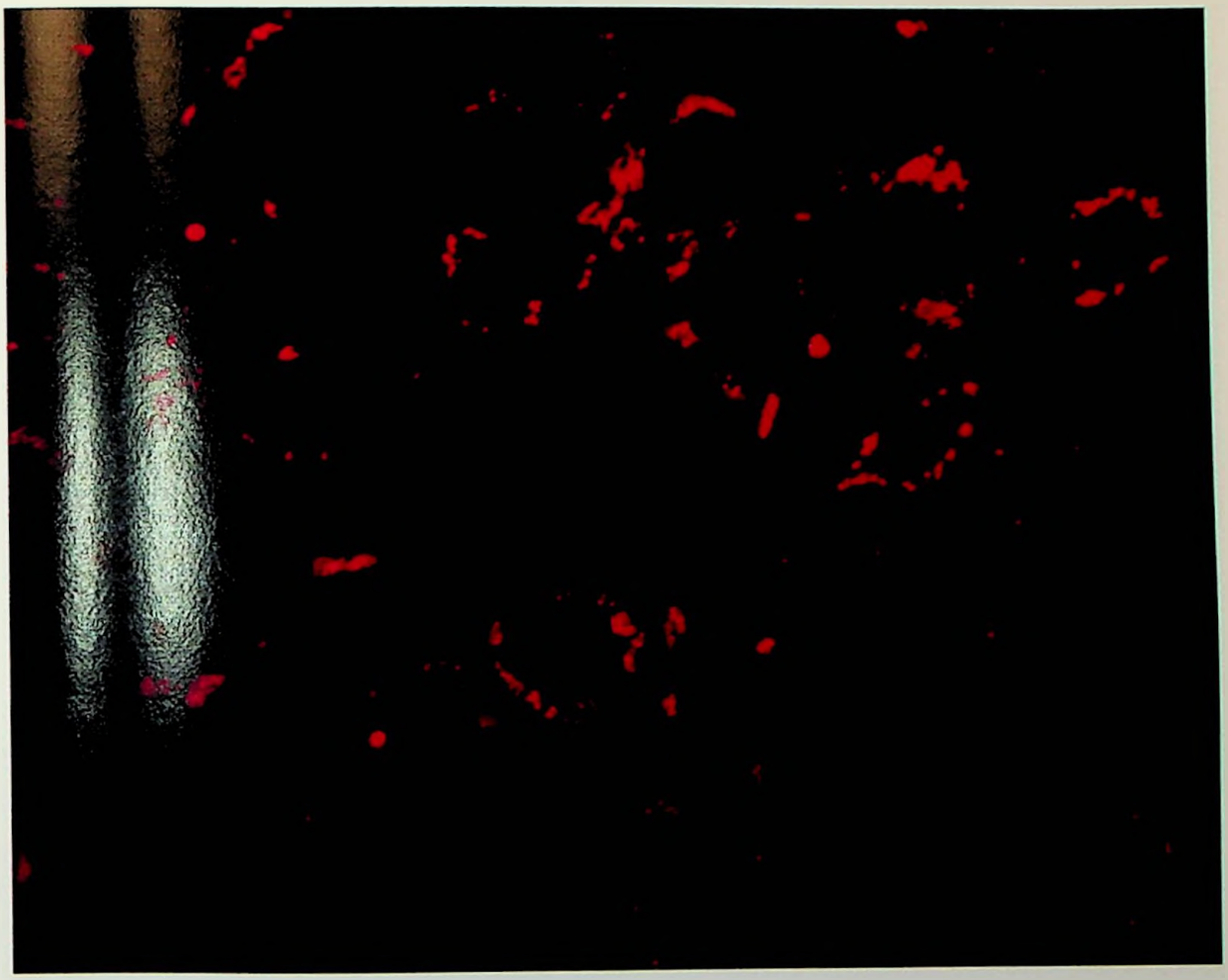
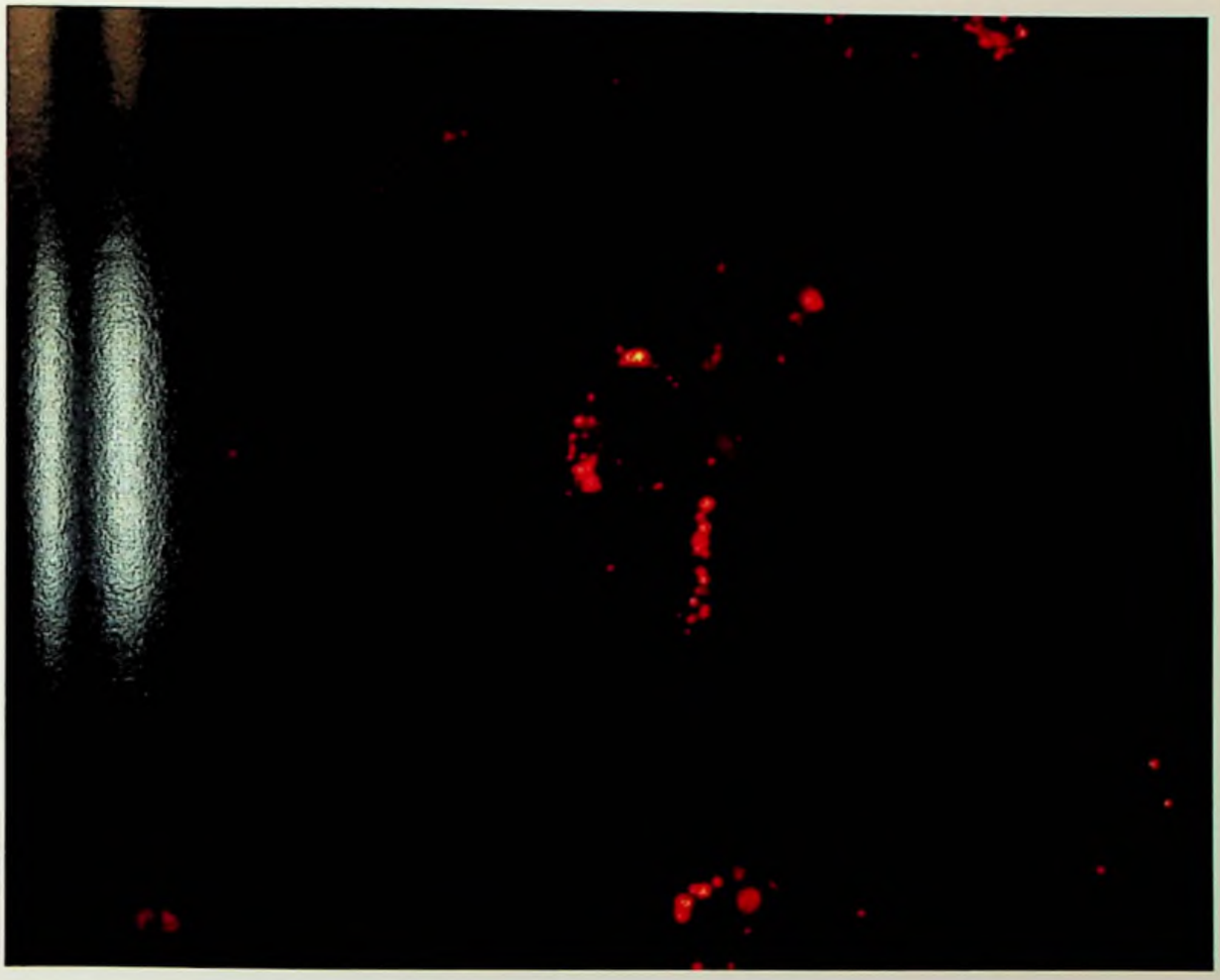


Figure 10. Post-natal Lean Animal Myoblasts.



Figures 11 – 13 represent cells of obese two-week old animal cultures. The cells shown here clearly display a supply of lipid present throughout the cell. The cell membranes can be seen as a faint orange color while the lipid within the cell can be seen as much brighter globules located throughout the periphery of the cell.

Figure 11. Post-natal Obese Animal Myoblasts.

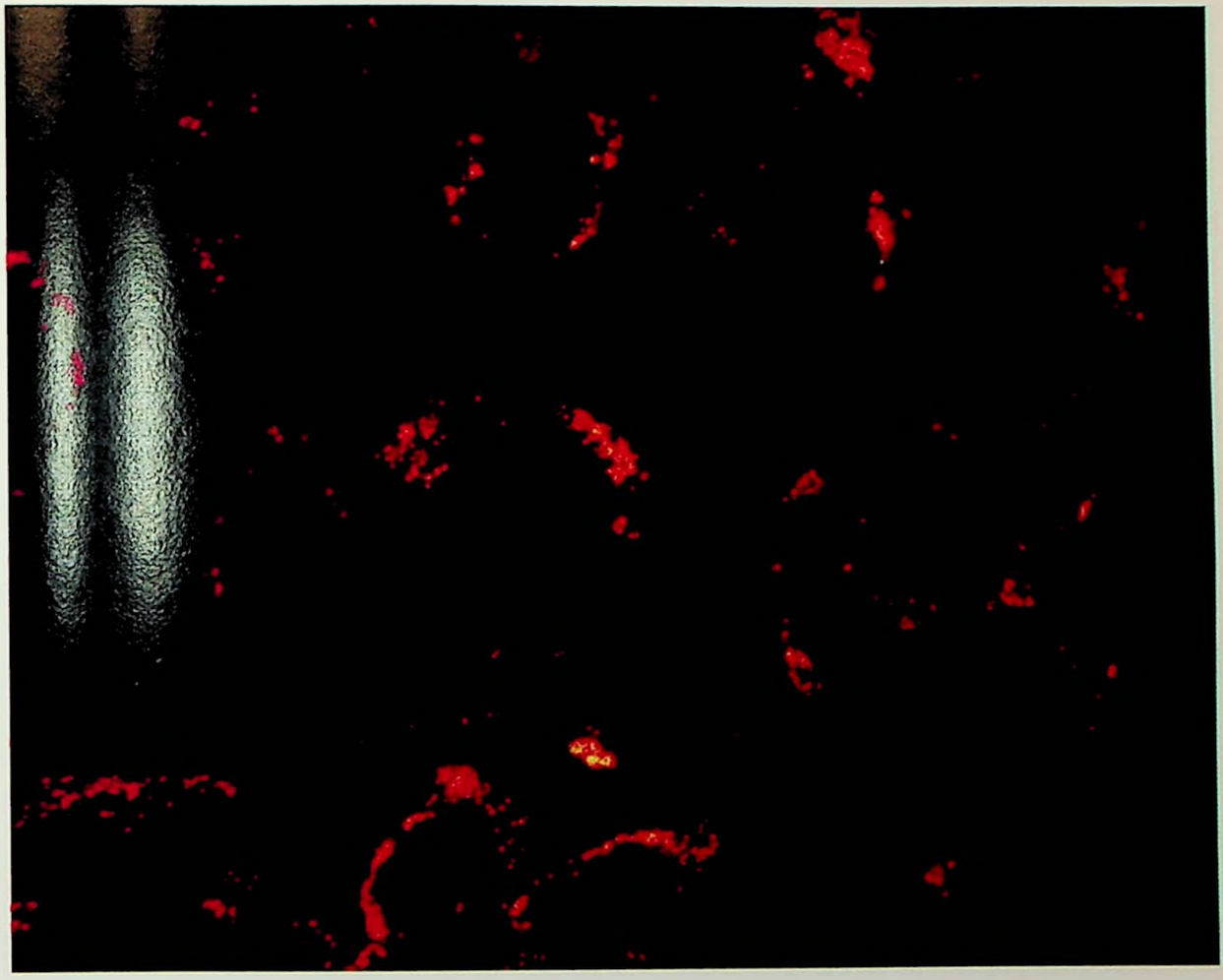


Figure 12. Post-natal Obese Animal Myoblasts.

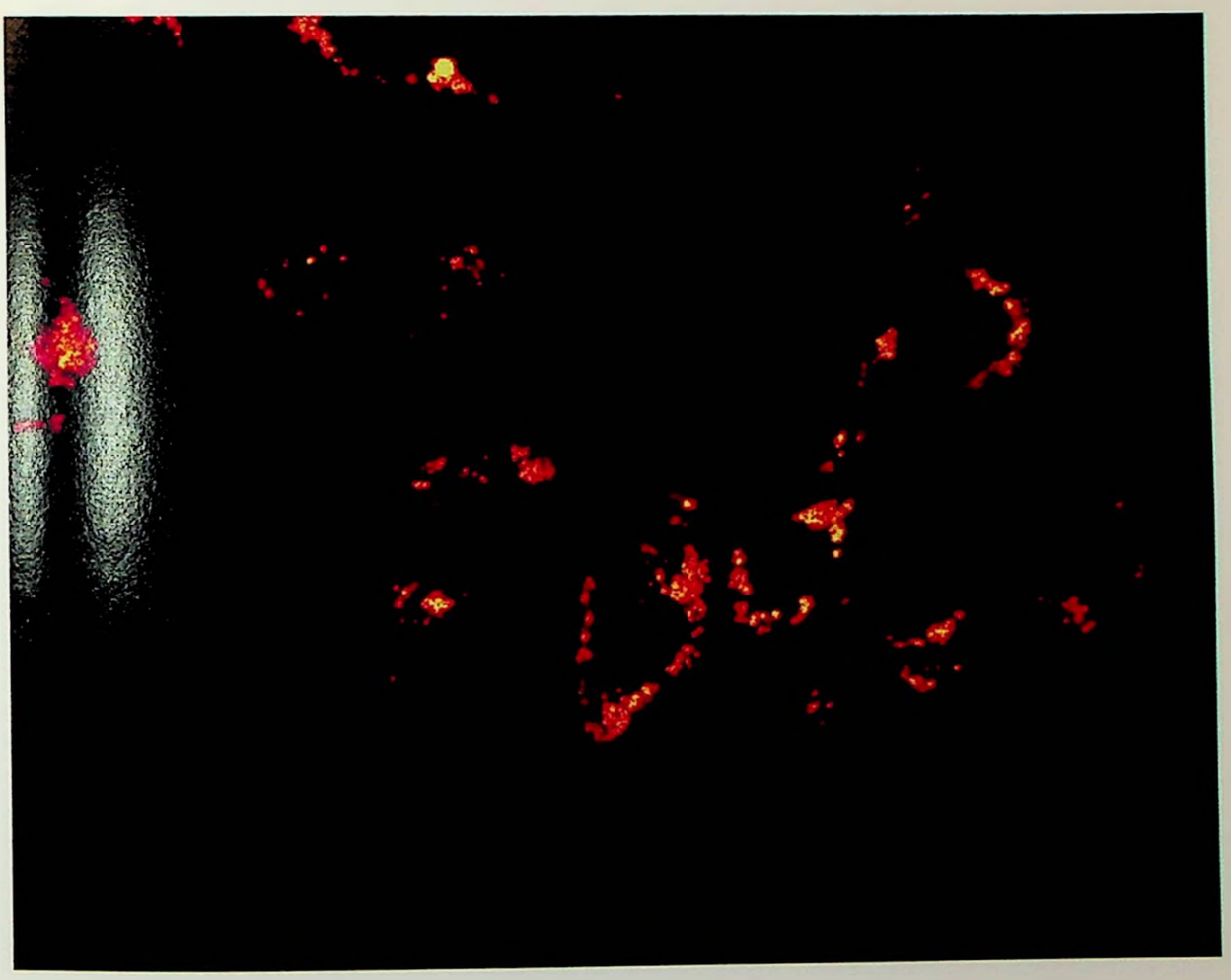
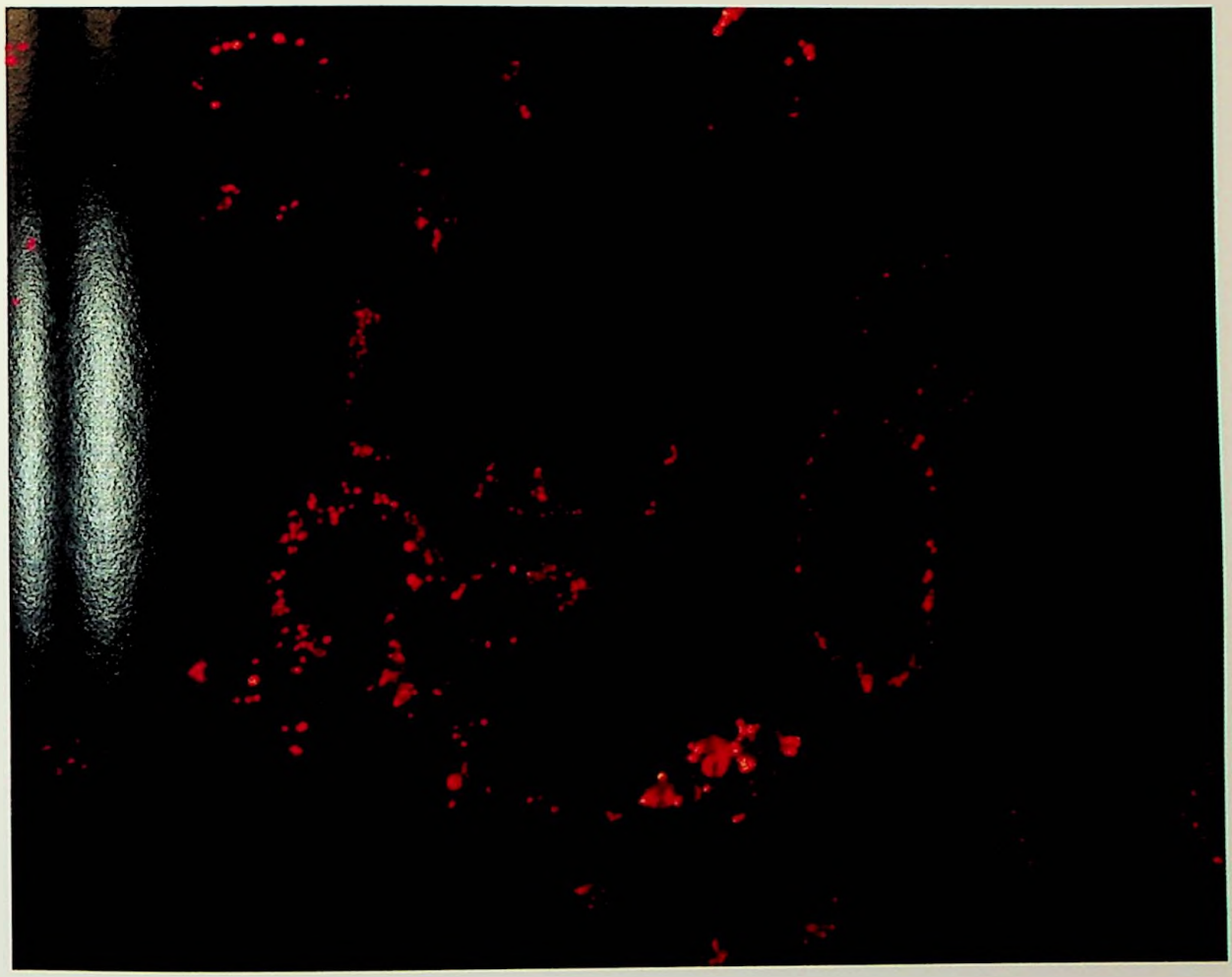


Figure 13. Post-natal Obese Animal Myoblasts.



The double-arrowed line above is an example of the random line used to calculate the amount of fluorescence present within cells. A value of 0 to 255 (0 = no fluorescence) was assigned to each pixel on the line and the sum of all pixel values was averaged to calculate the relative fluorescence within the cell.

Figure 14. Example of Random Line Used to Quantify Fluorescence.

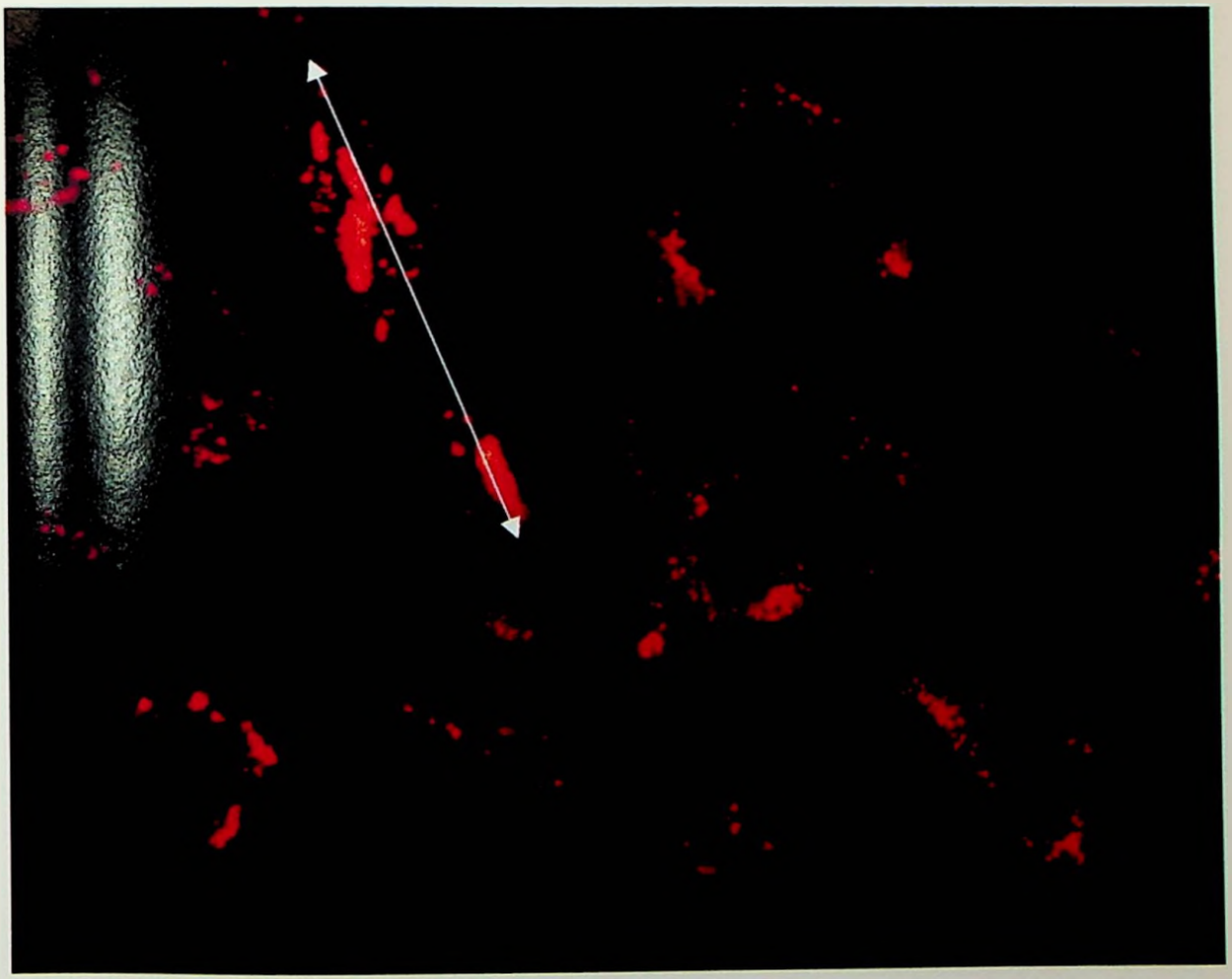
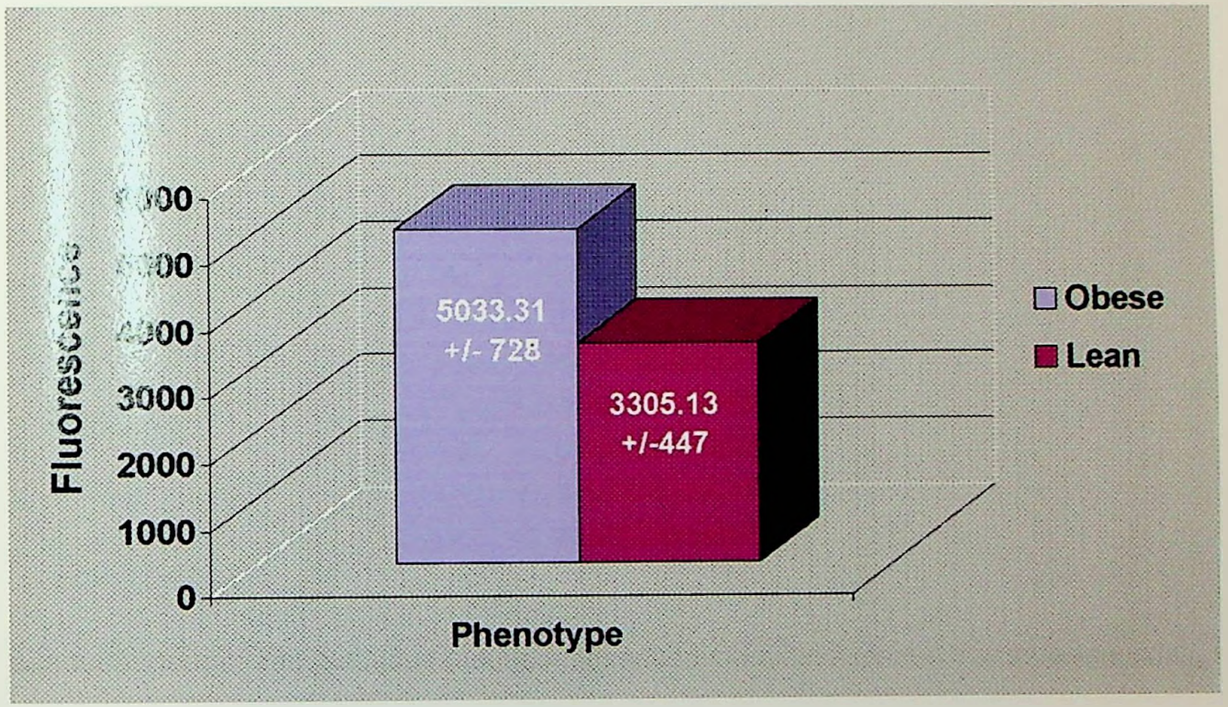


Figure 15. Histogram Showing Comparison of Fluorescence of Adult Cells.



Of the 6 lanes present in the gel, only 4 show PCR products. (The smallest DRA I digestion fragment, 23bp, is not visible.) The first lane (left) shows an obese animal (102bp). The forth and fifth lanes show heterozygotes (120bp and 120bp) and the last lane shows a lean animal (120bp).

Figure 16. Examples of Three Genotypes of Post-natal Animals on Agarose Gel.

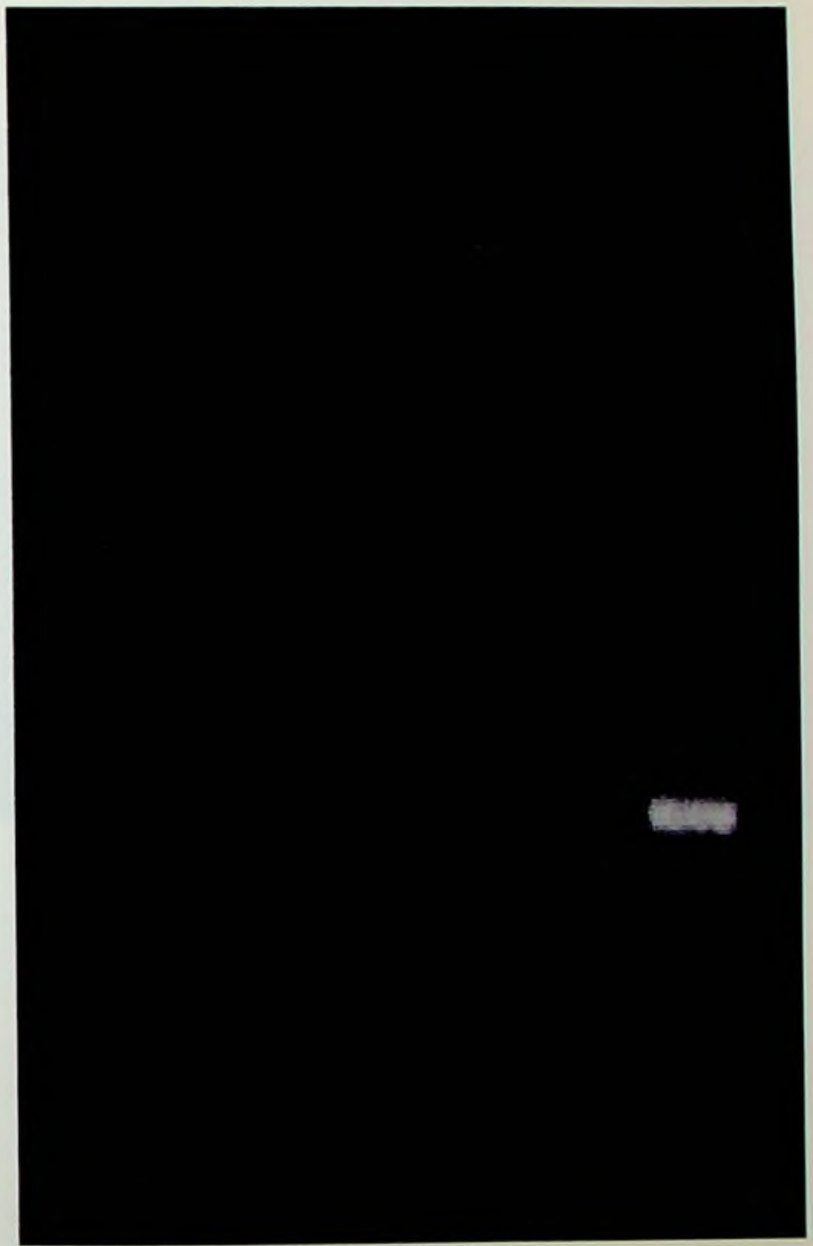


Figure 17. Histogram Comparing Fluorescence of Post-natal Cells.

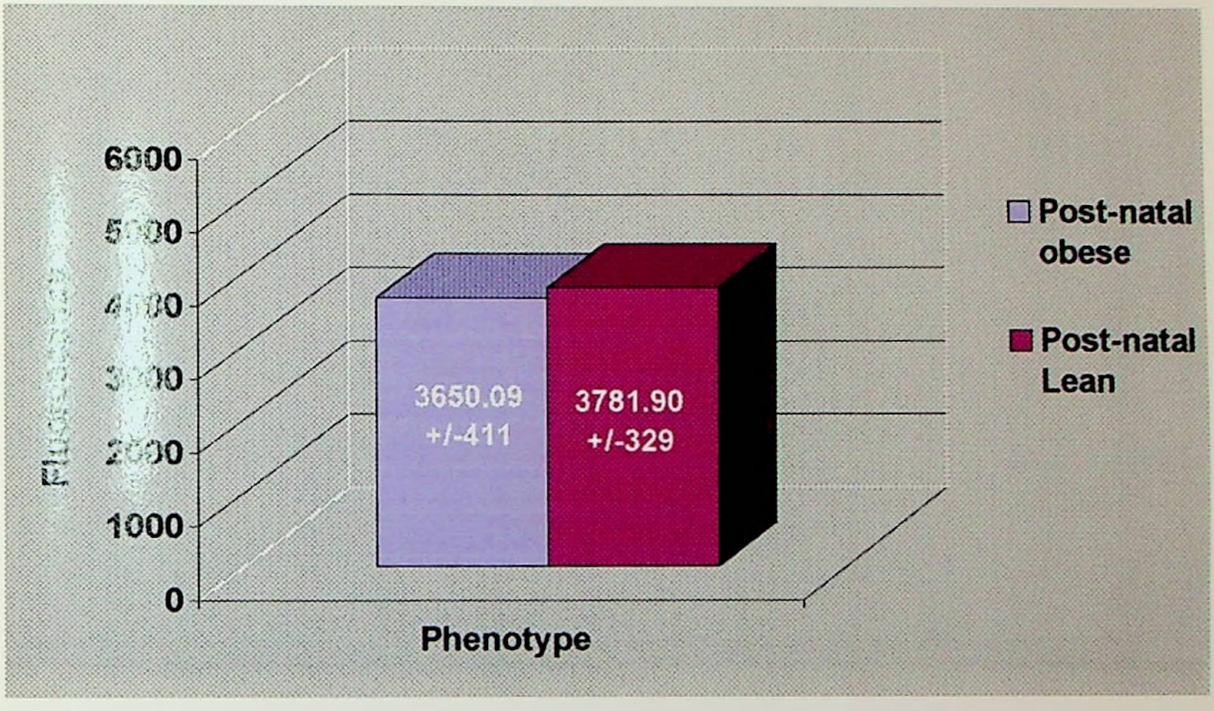
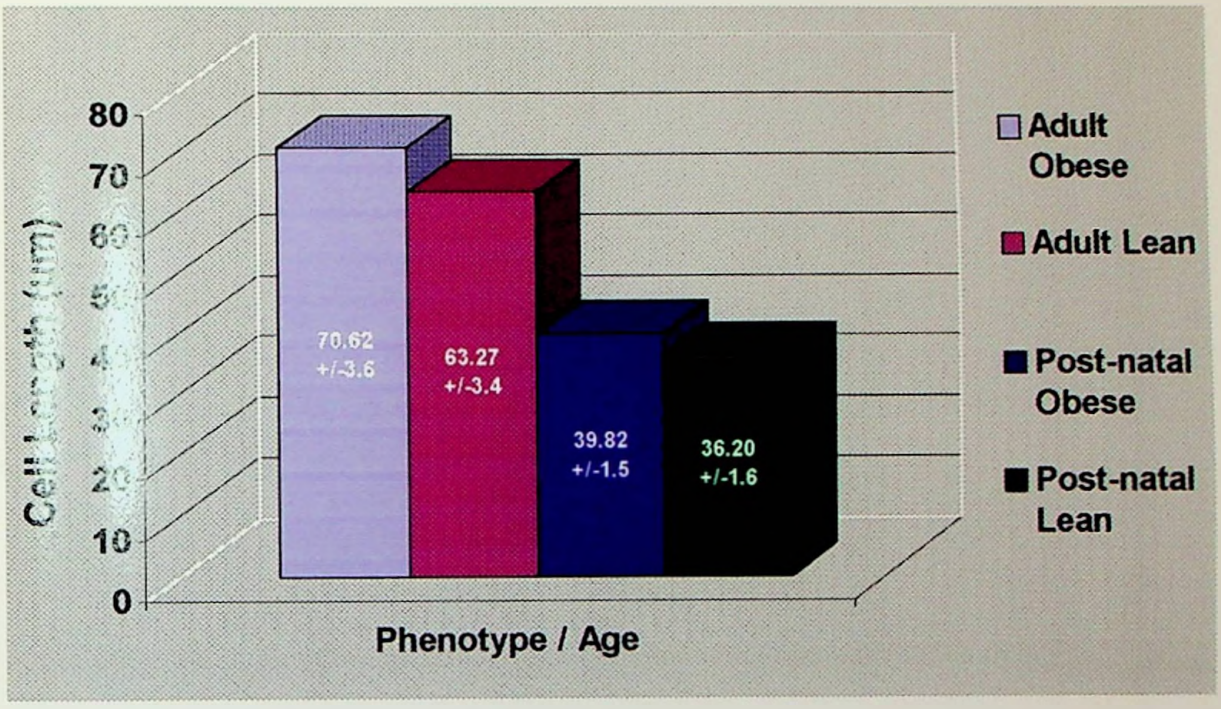
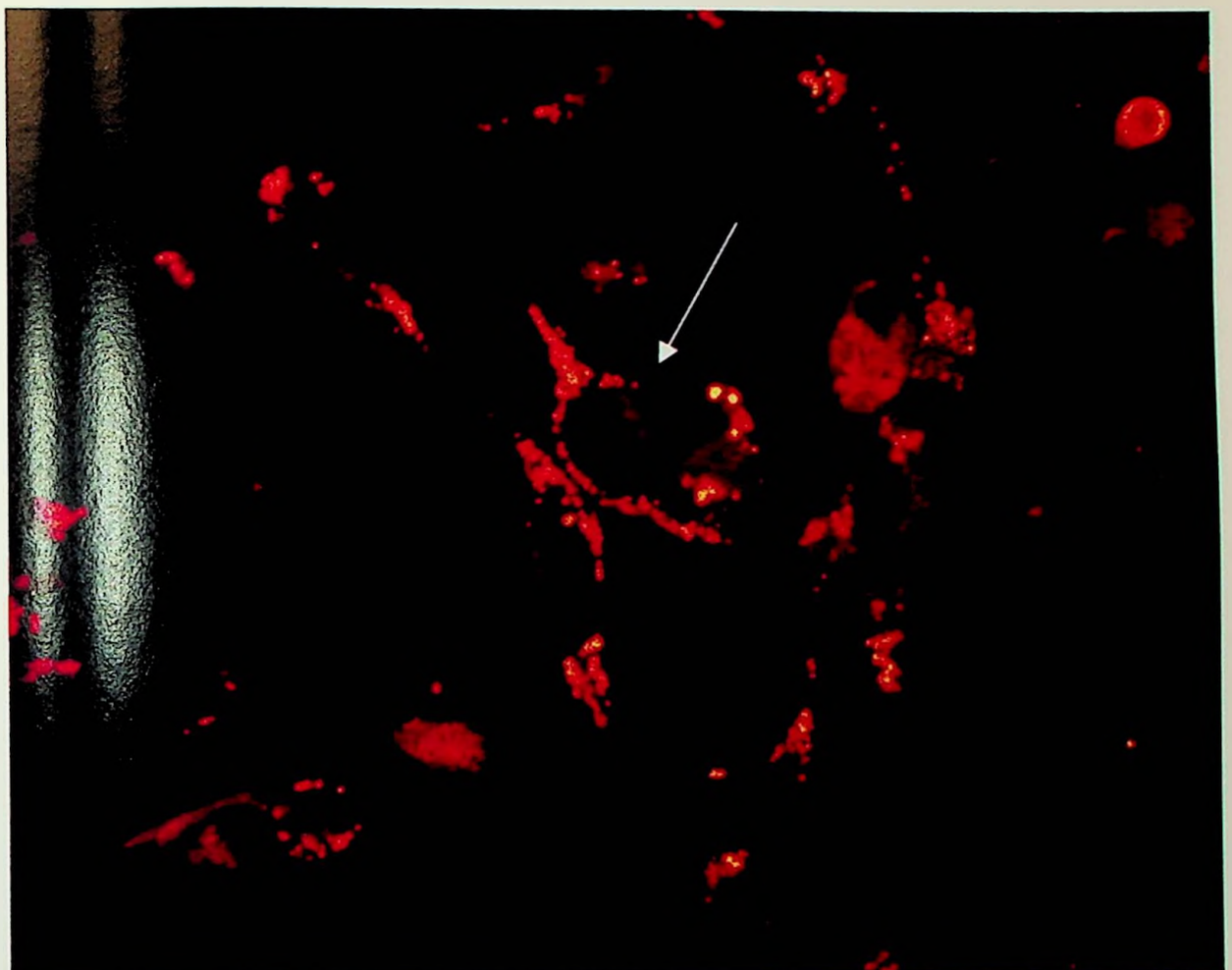


Figure 18. Histogram Comparing Sizes of Adult Animals and Baby Animals.



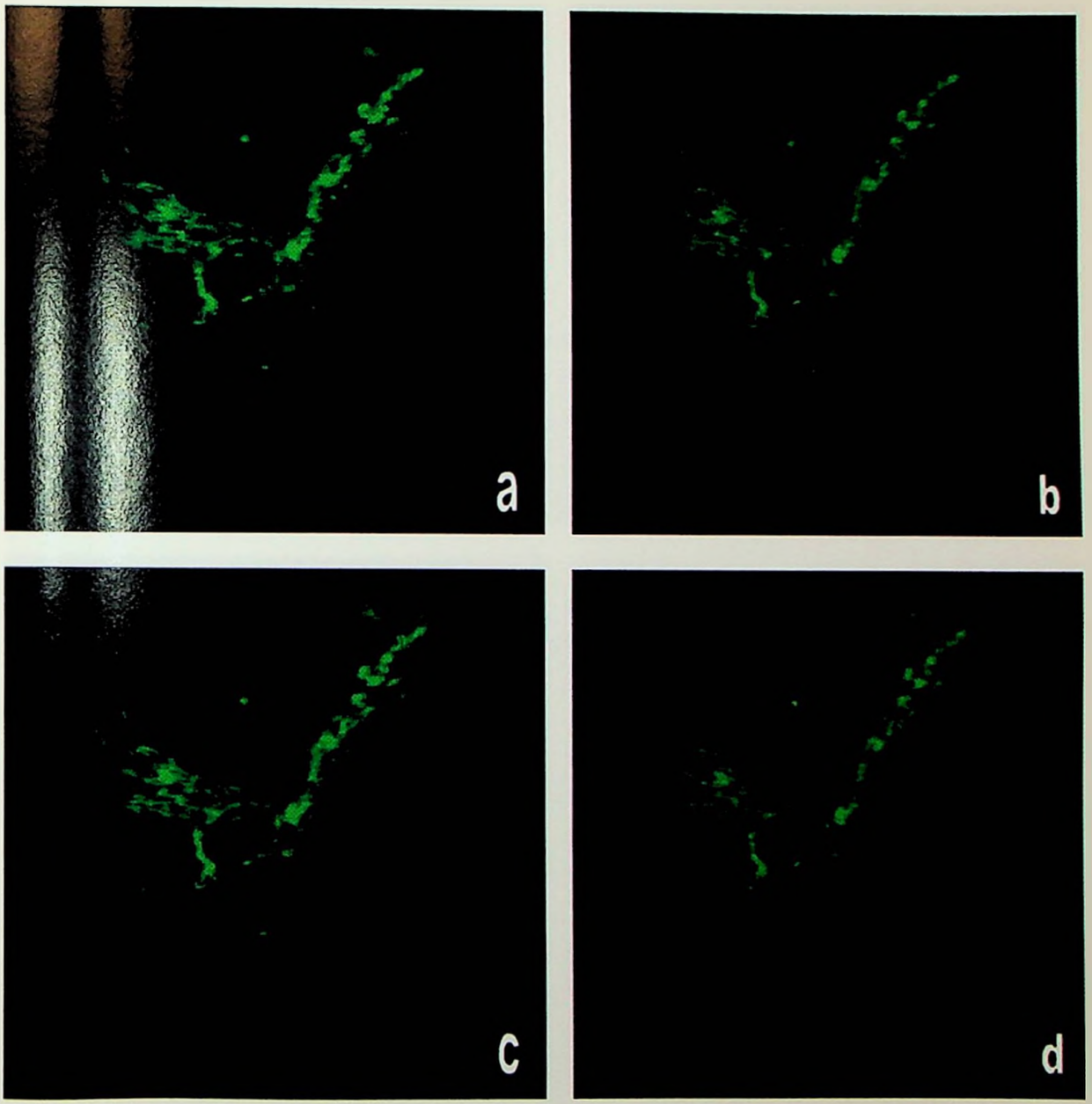
The arrow in the following micrograph indicates the cells involved in the process of mitosis. The cells shown in this micrograph display an example of lipid partitioning during mitosis.

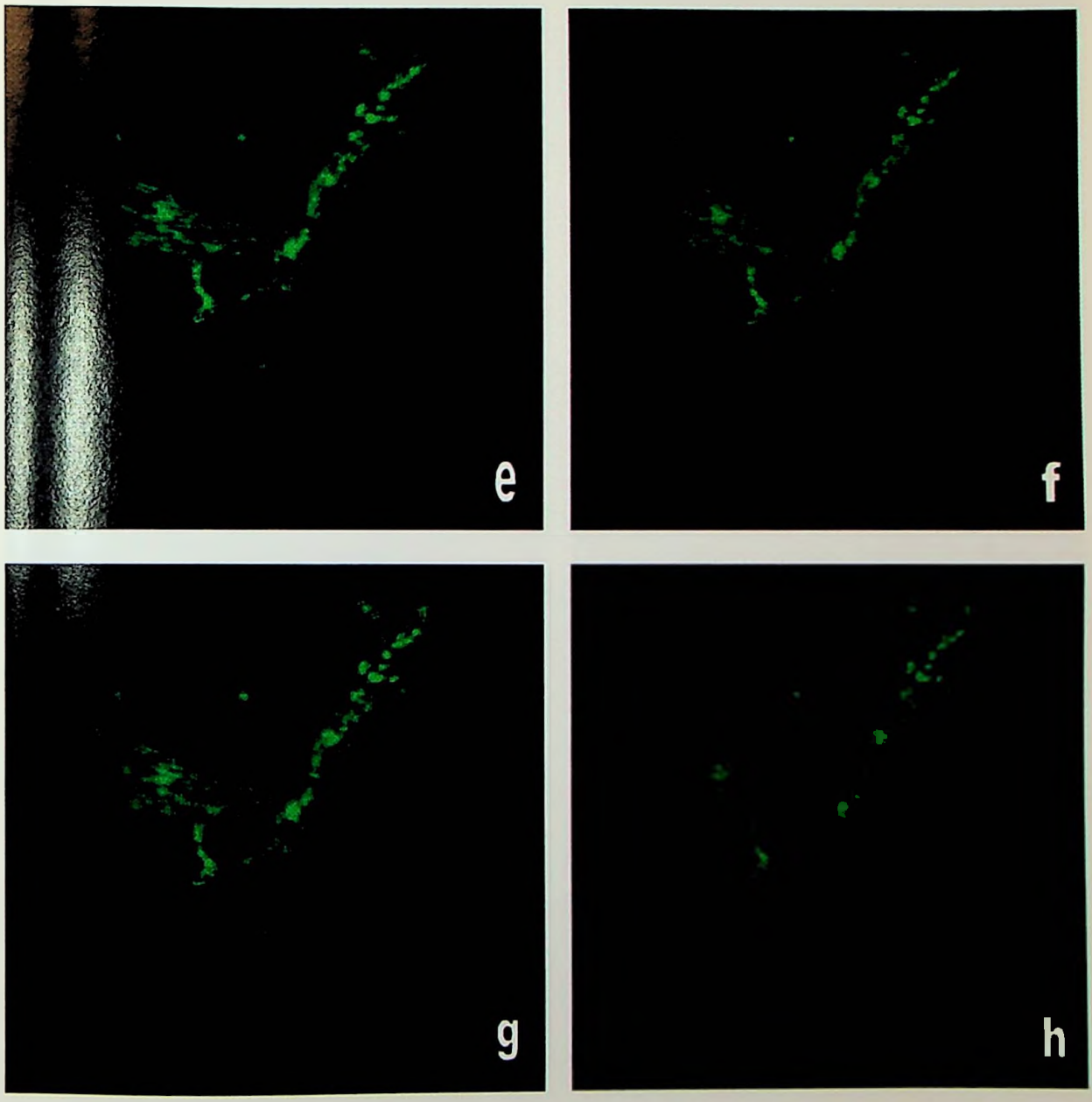
Figure 19. Cell Going through Mitosis.



The series of 12 images (a-l) represent a skeletal muscle myoblast stained with matrix-specific green fluorescent protein (GFP). The images represent the movement of the cells mitochondria over one hour. Starting at t = 0 each image is a 5 minute interval. Most movement seen in the images is displayed by individual mitochondria within the right cell process.

Figure 20. Movement of Mitochondria.

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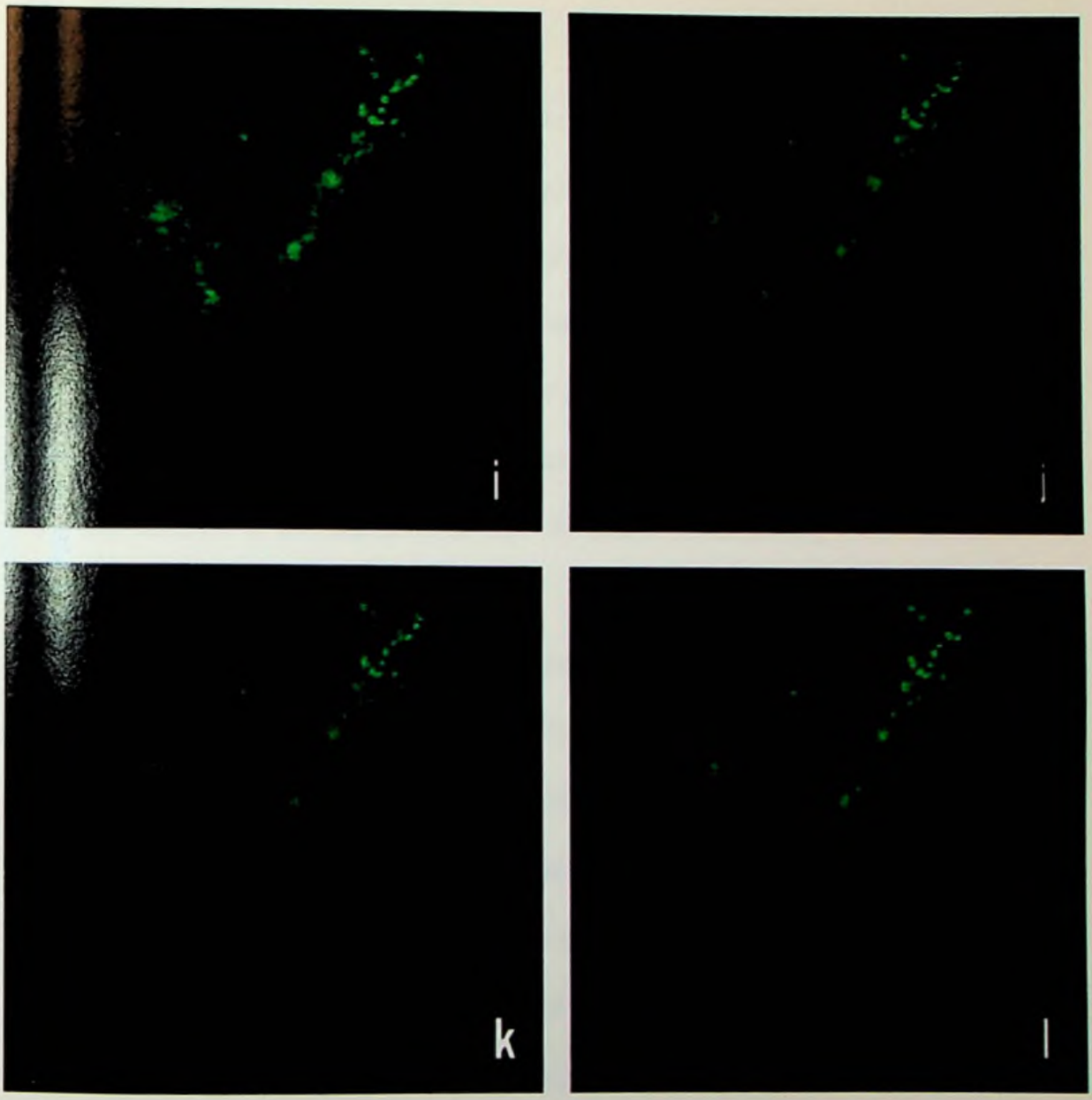
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DISCUSSION

The results of this experiment indicate that adult LA/N$^{fa/}$ rats have a significantly higher concentration of intracellular lipid deposits in obese versus lean skeletal muscle myoblasts. Although the same situation is not shown in post-natal animals, it has been hypothesized that the rate of lipid synthesis / oxidation in these cells may indeed differ from one phenotype to the next in both adults and babies. Measuring rates of lipid synthesis and oxidation will be one of the next focuses of the work in our lab. A reason for studying lipid metabolism is promoted by these experiments.

Cell cultures grown from adult animals showed very different characteristics than those of two week old animals. One difference was in the time necessary for the cells to differentiate into myotubes in culture. Adult cell cultures differentiated after 2 or 3 days of incubation. This is in stark contrast to the less than 24 hour differentiation time necessary for the post natal animal cells. Another major difference observed between cell types is the rate of mitosis. After 3 days, adult cells only displayed a small increase in the number of cells present that were adhered to the matrigel. The baby rat cells, however, multiplied very rapidly, so rapidly in fact, that if the cells were not imaged within 24 hours of culturing, imaging proved almost impossible due to the large amount of cells present on the coverslip. These differences suggest the presence of an internal mechanism(s) controlling the behavior of the cells.

The increased rate of mitosis in baby rat cells may explain the differences in the lipid concentrations of the two age groups. It may be necessary for the cells to persist in the growth, or "S" phases of the cell cycle for a longer period of time in order for the lipid deposits to show significant differences from phenotype to phenotype. One reason for this thinking was seen in numerous micrographs imaged during work on this experiment. An example of a cell going through mitosis is shown in Figure 19. As shown by the figure, the lipid source of the cell is partitioned to either side of the cytokinesis plate prior to cell division. No comparisons were made between ages or phenotypes with respect to this parameter. This partitioning of the lipid source shows the importance of lipid regulation within a cell.

Another parameter we looked at with respect to metabolism was the role of mitochondria in the cell. It was questioned as to whether the mitochondria were also partitioned prior to mitosis. Unfortunately, due to difficulties in staining procedures and locating cells in different stages of mitosis, this question remains unanswered. However, it was shown that the mitochondria are not static in position within the cell, but are quite motile, especially at the peripheries of the cell processes. An example of this movement is shown in Figure 20(a-l). Recent work[32] has yielded conflicting hypotheses as to what may control this mitochondrial movement, but there is compelling evidence that mitochondrial inheritance is an actin-mediated process.[32] The study of the myoblast is not limited to individual cells. Recent work using transplanted myoblasts to

regenerate hybrid muscle tissue may also give valuable insight to the role of skeletal muscle in the development of obesity.

Our reason for studying skeletal muscle is to determine the role 40% of the body's dry mass has in the development of obesity. Another reason to study skeletal muscle is to find out what role skeletal muscle myoblasts may have in the treatment of obesity.

It has been shown that transplantation of cultured myoblasts into mature skeletal muscle can be used for a new therapeutic approach to treating muscle and non-muscle diseases.[33-37] This type of therapy has been referred to as myoblast-mediated gene therapy. The general idea behind the technique is to implant into a host non-host myoblasts containing gene products absent from the host's muscle tissue, thus creating a hybrid myotube that is capable of producing proteins that the non-hybrid mutant is unable to transcribe.

Mature muscle is a syncytia of fused precursor cells called myoblasts or satellite cells as they exist in post-natal animals. Myofibers are the linear multinucleated fused cells functioning together and sharing a common cytoplasm. Myoblasts exist fused to the membrane of the myotube but do not share the cyotoplasm of the myotube. The formation of muscle or myogenesis is made up of three stages. The first is myoblast determination.

Several myogenic regulatory factors (MRF) are responsible for the determination of myoblasts from the somites they are derived from. In mouse embryos, myf5 appears in the dorsal part of the epithelial somite at the 4-somite stage. Shortly after MRF4 and myogenin are expressed in the myotomal part of

the somite. As the somite continues to develop, MyoD, another MRF, can be detected. These MRFs play an important role in the determination of myoblasts, however, it is still unknown what the exact functions of the proteins are. The next step in muscle development after the myoblasts have differentiated is the migration of the myoblasts.[38]

It has been shown that another protein, Pax-3, is involved in the process of myoblast migration in limb tissues. The cells in the lateral half of the somite significantly contribute to the migratory cells that form the limbs. Pax-3 has been shown to play a role in the process of directing cells to an area where they are able to further develop. As with the MRFs, the exact mechanism involved has yet to be determined. After the cells have been directed to a specific area, the final process in muscle development is set to begin.[38]

Differentiation is the final process in myogenesis. Differentiation can be viewed as the fusion of individual myoblasts. The fusion is a complicated process that involves a precise alignment of myoblasts involving Ca^{2+} mediated recognition, adhesion, and union of the plasma membranes.[39] Shortly after the myoblasts fuse, they actively engage in the synthesis of muscle contractile proteins. These newly synthesized proteins undergo self assembly into thick and thin filaments and are assembled into sarcomeres around the peripheral regions of the myotube.[38] The contractile proteins begin taking a larger volume of the cytoplasm and the nuclei migrate to an area just below the plasma membrane. By this stage, in order for the skeletal muscle fiber to continue to grow, the fusion of cytoplasmic myoblasts must occur.[38] There are some myoblasts that do not

fuse with the muscle fiber during the initial differentiation, but remain in the surrounding basal lamina as satellite cells. It is the presence of these satellite cells that first gave scientists the idea to use myoblast transfusion as a means of gene therapy.

By fusing with mature or regenerating fibers of the host, implanted myoblasts can form hybrid myofibers and contribute to the syncytium by providing missing or absent proteins to the host muscle.[33] The most well studied medical treatment using this type of therapy deals with the treatment of muscular dystrophy.

In mdx mice, the X chromosome carries a mutation that results in the absence of dystrophin in all skeletal muscle fibers.[33] This lack of dystrophin appears to be the cause of the segmental necrosis of muscle fibers that starts at age 15 days.[34] By culturing myoblasts biopsied from human biceps muscles that showed no signs of abnormalities and injecting the cultured cells into 6-10 day old mdx mice, it was possible to grow hybrid myotubes that showed no signs of necrosis. Similar experiments have been carried out in humans. It was shown that normal dystrophin transcripts could be detected in Duchenne muscular dystrophy patients after myoblast transplantation.[35] Dystrophies are not the only abnormality being treated with this form of therapy, Parkinson's disease is another candidate for this type for method of gene therapy.

The implantation of cells genetically modified to express tyrosine hydroxylase has been proposed for the treatment of Parkinson's disease.[36] Tyrosine hydroxylase converts tyrosine to L-DOPA and endogenous

decarboxylase activity then converts L-DOPA to the neurotransmitter dopamine, which alleviates the symptoms of Parkinson's disease. Researchers have shown that muscle can serve as an exocrine organ and secrete dopamine as well as L-DOPA. These secretions have been shown to be used as effective treatments of Parkinson's disease. [36]

There are many other uses for this type of gene therapy. One of these is to utilize myoblasts to systemically deliver human growth hormone. [37] These three examples are just the beginning of a whole new type of medicine that scientists are just now beginning to understand. Using this type of therapy, and what is known about the obese condition in humans and rodent models, myoblast transfection could prove to be a very effective means of treating a disease that afflicts 1 in 5 Americans.

By transplanting myoblasts from lean animals into obese animals it may prove possible to create a heterozygous lean individual from a genetically obese (homozygous double recessive) individual. In order to test this hypothesis, it will be necessary to take muscle tissue from a broad range of individuals, including several age categories. The reason for this is that it is not presently known if the myoblasts that persist in adult muscle are identical to their embryonic counterparts in function and in their ability to be determined into adult muscle tissue. Thus, ideally myoblasts in the early stages of determination in the embryo are the most likely candidates for this type of therapy. One technique that could be employed in this type of experiment would be to take one embryo, genotype it for homozygous lean dominance, remove somites or other tissue

closely involved in the formation of muscle and implant the tissue into a homozygous recessive obese embryo. The somites from the lean animal could play a role in forming muscle that is capable of supplying the leptin receptor and other vital lipid metabolizing enzymes in an obese animal.

The experiments reported in this thesis consist of some very new ideas. It is difficult to locate information, both new and background, with which to tie the details of these concepts together. In closing, it should be known that these new ideas and the work involved in determining the data needed to present them as a hypothesis are very long and can be very tedious in nature. What may seem to be the simplest non-informative detail may in actuality be a long and tedious process that could take anywhere from weeks to years to complete. The ideas presented here are only a small fraction of the world of research that is currently being undertaken in the growing field of genetic obesity.

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EPILOGUE

It can be argued that DNA is nothing more than a program designed to preserve itself. Life has become more complex in the overwhelming sea of information. Life, when organized into species, relies upon genes to be its memory system. So man is an individual only because of his intangible memory, and a memory cannot be defined, but it defines mankind. The advent of computers and the subsequent accumulation of incalculable data has given rise to a new system of memory, and thought paralleled to our own. Humanity has underestimated the consequence of computerization.

Ghost in the Shell, 1995