Project Code: TCC "PRMT4-5 Histone Modifiers"

An Assessment of Research that Answers the Question: Does Adipose Differentiation require either PRMT4 or PRMT5 Histone Modifiers?

A Major Qualifying Project Report
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ABSTRACT

This project involved an assessment of research carried out at an off-campus site that addressed a problem related to adipose differentiation PRMTs are a class of histone modifers, which can help "open" or "close" DNA by methylating histone tails at arginine residues. PRMTs are especially important during differentiation, when turning genes "on" and "off" is crucial. Recent findings show that PRMT4 & PRMT5 are required for muscle differentiation. The project's purpose was to determine if PRMT4 & PRMT5 are also required for adipose differentiation. This was accomplished by comparing the efficiency of differentiation of a fibroblast into an adipocyte, using wild-type, PRMT4-, and PRMT5- fibroblast cell lines. Fibroblasts are capable of transdifferentiating into adipocytes after being given a nuclear hormone receptor, PPAR-gamma, and the ligand, troglitazone. mRNA expression results show adipose genes (Leptin, Resistin, and FAS) are transcribed at significantly lower rates in cells lacking PRMT5. Expression of adipose mRNAs was significantly higher in wild-type cells: 159,900% for resistin, 3,256% for FAS, and 1,750% higher for leptin. Lipid stains show that cells lacking PRMT5 did not produce nearly as much lipid as wild-type cells. These results suggest that PRMT5 is required for adipose differentiation. The PRMT4 experiment never produced a complete and reproducible set of mRNA expression data or consistent lipid stains. In the PRMT4 experiment, Resistin mRNA was found to be expressed 2,264% more in PRMT4- cells. And, AP2 mRNA

expression was found to be 57% higher in PRMT4+ cells. This incomplete and conflicting data might have been due to difficulty with bacterial contamination and inefficient MEF 13 retroviral infections. Modifications to the protocol could provide the opportunity retrieve reproducible data from the PRMT4 experiment. And, adding protein data to both the PRMT4 and PRMT5 experiments would make the results more complete. Knowing the role of PRMT4 and PRMT5 in adipose differentiation would help clarify the role PRMTs (histone modifiers) play in the differentiation process.

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Chromatin & Histones

Almost two meters of DNA is compacted to fit into the eukaryotic nucleus through the use of proteins, especially histones. The complex of proteins and DNA is called chromatin. The histone packaging proteins (H1, H2A, H2B, H3, and H4) are small, positively charged (basic) proteins that can bind to negatively charged DNA. Histones contain many basic amino acids. These include lysine and arginine. In some histones, the percentage of lysine is as high as 27% of the amino acids, and the percentage of arginine can be as high as 15% (See Figure 1). No histone has more than 265 amino acids. H1, also known as linker histone, is the largest of all the histones.

Figure 1 shows data on histones including amino acid size and % arginine. (Waterborg, 1998)

Histone	Amino Acids	% Arginine
H1	200-265	2
H2A	129-165	9
H2B	121-148	6
H3	135	15
H4	102	4

There are 4 core histones and 1 linker histone. Core histones (H2A, H2B, H3, and H4) have the same basic structure. Core histones contain a long hydrophobic alpha helix surrounded by two shorter hydrophobic alpha helices. These

structures enable core histones to interact such that an octamer can form. Histone subunit H2A interacts with H2B, and H3 interacts with H4. To form the histone core octamer, three steps must occur. First an H3-H4 tetramer must form. Then, two H2A-H2B dimers must form. Then, these 3 subunits must combine to form an octamer. (Peterson, 2004)

Nucleosomes consist of DNA wound around a group of eight histones, called an octamer. Two of H2A, H2B, H3, and H4 subunits make up the octamer, while a single H1 subunit exists outside the octamer. 145 base pairs of DNA wrap around the histone octamer approximately 1 and ¾ turns. Between 10 and 60 base pairs exist between nucleosomes as "linker" DNA. "Linker" DNA can easily associate with proteins while DNA wound around the histone octamer cannot. In this way, chromatin structure can regulate gene transcription.

Two forms of chromatin can be isolated from cells. At low ion concentration, chromatin is isolated in a form known as "beads on a string" (10nm thick). This form of chromatin contains single nucleosomes separated by "linker" DNA. H1 is usually absent with this form of chromatin. Gene transcription commonly occurs in this form of chromatin. At physiological ion concentration, chromatin is isolated in a 30nm fiber form that is much denser than the "beads on a string" form. This form exists as a solenoid of nucleosomes. This type of chromatin is not genetically transcribed. Also, this form contains a much higher level of H1 linker

histone than the 10nm form. (Of note: Histone H1 stabilizes interactions between nucleosomes). Denser conformations of chromatin can exist during mitosis, especially metaphase. Conformations of chromatin can reach 100-400nm thick. However, the 300nm looped domain conformation is best documented. This consists of loops of 30nm chromatin fibers looped and attached to a protein scaffold. 300nm chromatin forms chromosomes, which are 700nm in width or 1400nm during mitosis.

Chromatin remodeling enzymes have the ability to alter the structure of chromatin so that proteins can bind to certain regions and are blocked from binding to others. One class of chromatin remodeling enzymes is the histone modifiers (See Figure 2). These enzymes can add a variety of functional groups to histones. And, the functional groups are most frequently added to the tails of the histones. Histone tails are 20-35 amino acid residues long on the amino terminal. These extensions from the nucleosome contain amino acids that are basic (positively charged). Histone tails function to bind to DNA, fold chromatin, and to interact with other histones.

Another common type of chromatin remodeling enzyme is related to 2MDa yeast SWI/SNF complex. These enzymes use energy, ATP, to break histone-DNA bonds (Salma, 2004). Another common type of chromatin remodeling enzyme is a class of nuclear histone acetyltransferases (HATs). These enzymes modify

histones by adding acetyl groups to lysines in the histone proteins. HATs belong to the histone modifers, but because they have been studied for so long, many scientists consider them to be their own category of chromatin remodeling enzymes.

SWI/SNF complexes (in vitro) have been shown to remodel chromatin. In vitro results show (1) greater accessibility of DNA to protein factors, (2) loss of coiling in the chromatin, and (3) shifting of position of histone octamers. Researchers believe that ATP is used to "drive a change in the twist of nucleosomal DNA" to remodel the chromatin (de la Serna, 2006). Two SWI/SNF-like complexes are found in mammalian cells and are known as hSWI/SNF or BAF. These enzymes have an ATPase subunit, which is either BRG1 or BRM. These enzymes are needed to make activators function. Some of these activators include the estrogen receptor, heat shock factor, and glucocorticoid receptor. SWI/SNF enzymes mostly activate genes.

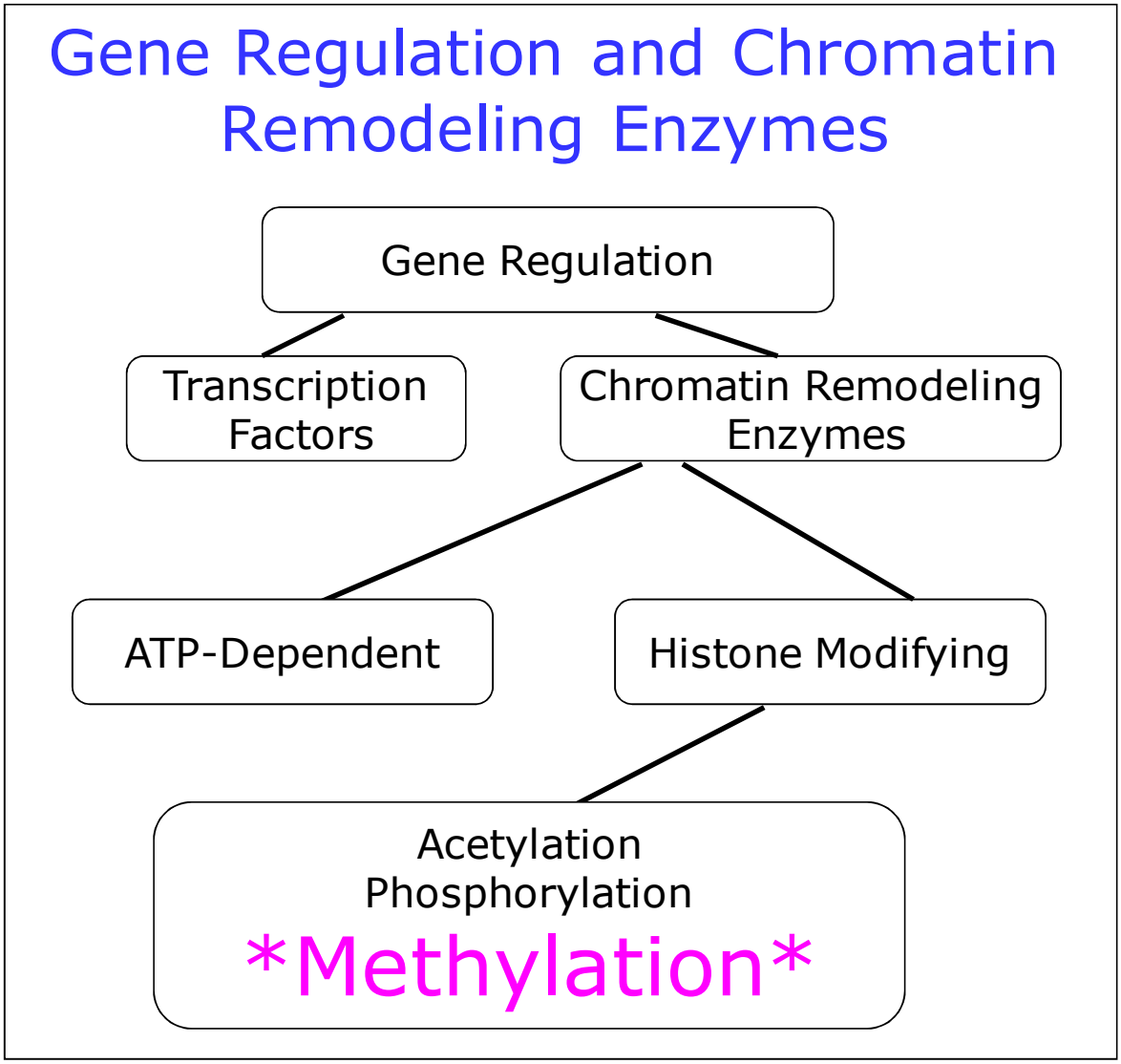


Figure 2 shows different mechanisms that regulate genes: transcription factors and chromatin remodeling enzymes. The project focused on histone methylation enzymes.

Post-translational modifications to histone tails are very common. Acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, and ribosylation are some of the common modifications. However, histone acetylation is the best known. Acetylation involves the reversible addition of acetyl groups to N-terminal core histones. Acetylation is associated with (1) reduced binding of histones to DNA and (2) less stable forms of chromatin. Acetylation occurs on lysines, which are highly conserved in H3 and H4 histones. Histone Acetyl Transferases (HAT) enzymes and histone deacetylase enzymes control these reactions.

Each post-translational modification also has a level of complexity. For example, there is more than one way to methylate an arginine. In fact PRMTs can modify arginines in at least four different ways. In addition, multiple arginines can be modified. And modifications can occur at different histones. Recent studies suggest that histone modifications can even occur deep within the histone core, not just on histone tails.

PRMTs (Protein Arginine Methyl Transferases) & Arginine

There are 20 standard amino acids. However, posttranslational modifications to amino acids can extend this number. Post-translational modifications are useful

for changing protein structures, which can activate or turn off that protein's function. Methylation is a common post-translational modification. According to one study published in 2003, it is estimated that 1% of all mammalian genes encode methyltransferase enzymes (Fackelmayer, 2005). PRMTs are a type of enzyme that methylates arginine amino acids (See Figure 3) in both cytoplasmic and nuclear proteins, using the conserved methyltransferase (MTase) domain. PRMTs are expressed ubiquitously in human cells but can have specificity based on alternative splicing. PRMT1 and PRMT4 are vital for existence.

PRMTs are classified based on how they methylate arginine residues. Arginine consists of an "R-group" (CH2)3-NH-CNH2NH2. Because arginine contains 3 nitrogen groups, methylation can occur at 3 different sites (see Figure 3 below). PRMTs transfer methyl groups from S-adenosylmethionine (AdoMet) to nitrogen groups on arginine. The top nitrogen circled in red in figure 3 is known as the delta nitrogen. The other two nitrogens on the end of the arginine are known as omega nitrogens. At most, the delta nitrogen can receive one methyl group (see Figure 4 below). The omega nitrogens could each receive two methyl groups. If arginine receives one methyl group it is known as mono-methylated arginine (MMA). If arginine receives two methyl groups it is known as di-methylated arginine (DMA). Di-methylated arginines can be asymmetrical, which means they have methyl groups added to the same omega nitrogen (notated as aDMA). However, di-methylated arginines can also be symmetrical,

which means they have methyl groups on both omega nitrogens (notated as sDMA).

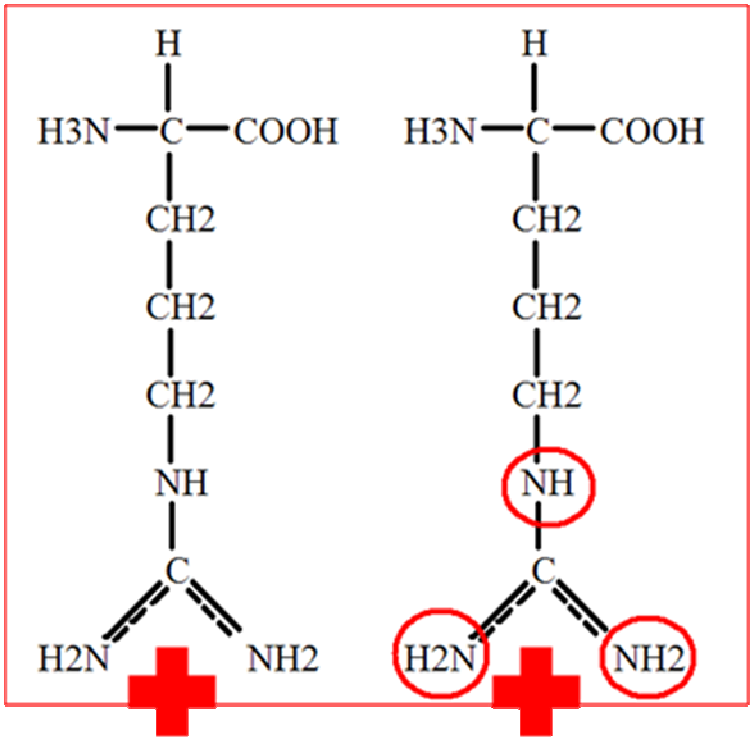


Figure 3 The figure on the left shows arginine, and the figure on the right shows the sites where potential methylation can occur. A positive charge is distributed over the two outermost nitrogen groups.

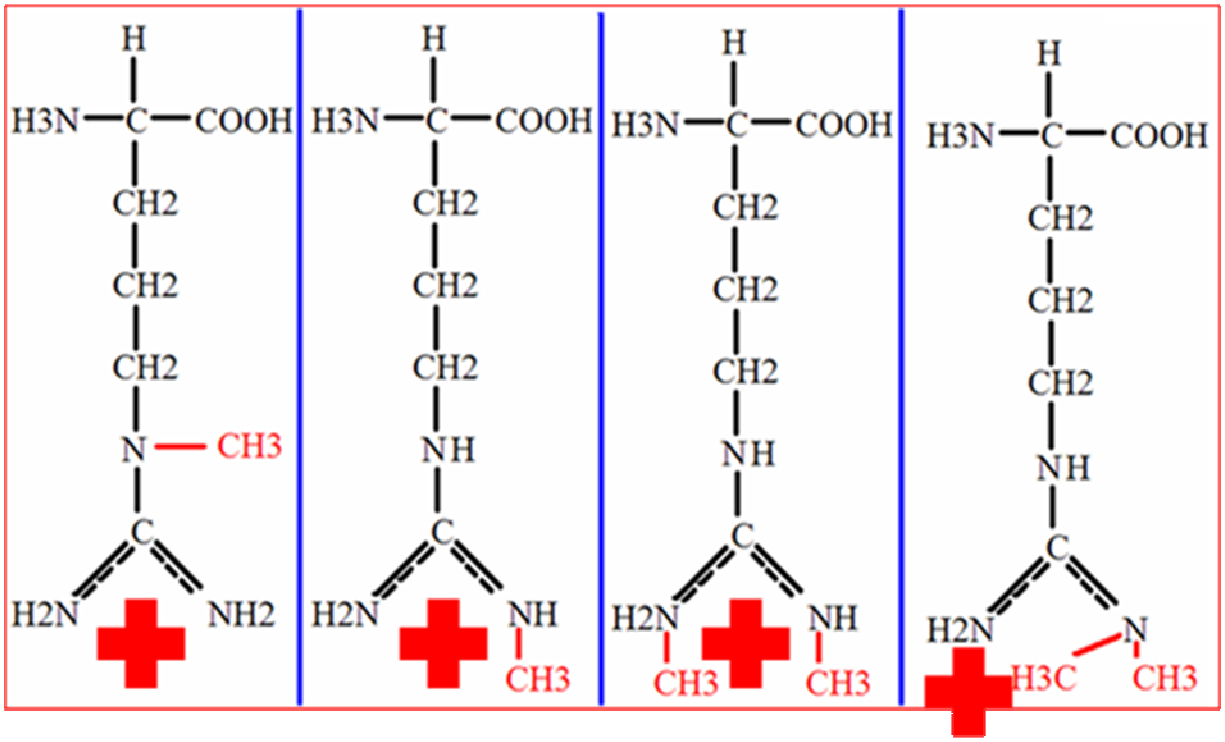


Figure 4 shows the possible methylation products of the four classes of PRMTs. A positive charge is distributed over the two outermost nitrogen groups.

PRMTs are classified as either Type 1, 2, 3, or 4. Type 1, 2, and 3 methylate omega nitrogens. Type 1 form asymmetrical di-methylated arginines (aDMA). Type 2 form symmetrical di-methylated arginines (sDMA). Both Type 1 and Type 2 PRMTs form di-methylated arginines from mono-methylated arginine intermediates. Type 3 enzymes form a variety of mono-methylated arginines (MMA). Type 4 PRMTs only methylate the internal or delta nitrogen.

PRMT 4 = Type 1 = Asymmetric Di-Methylation

PRMT 5 = Type 2 = Symmetric Di-Methylation

PRMT#	Classification Type
PRMT1	1
PRMT2	4
PRMT3	1
PRMT4	1
PRMT5	2
PRMT6	1
PRMT7	2,3
PRMT8	1
PRMT9	2

Classification Type	Description
1	(aDMA) = asymmetrical di-methylated arginine
2	(sDMA)= symmetrical di-methylated arginine
3	(MMA) = mono methylated arginine
4	(omega MMA) = mono methylated arginine

Figure 5 shows classifications of PRMTs and definitions of each classification.

Several types of methyltransferases exist and are categorized by class. Class 1 has a seven-stranded Beta-sheet structure. Class 2 are SET lysine methyltransferases. A third class exists and is involved with membrane-bound methylation. PRMTs belong to Class 1 methyltransferases. As of December 2007, 10 mammalian PRMTs have been identified. However, PRMT2 and PRMT9 have not demonstrated methylation activity (Bedford, 2008).

PRMTs contain 5 "signature" motifs. These include VLD/EVGxGxG, V/IxG/AxD/E, F/VVDI/L/K and LR/KxxG and a THW loop. Each PRMT contains these 5 motifs (see Figure 6). PRMT2 also carries a SH3 domain and PRMT3 contains a Zn++

finger domain which aid in recognition of the substrate. PRMT7 also contains a duplication of the 5 motifs. PRMT4 contains one of each of these motifs in the center of the protein. PRMT5 also contains each of these motfis. However, these motifs are found on the ends of the protein. Each of these motifs in PRMT4 and PRMT5 both have good sequence similarity to PRMT consensus motif sequences.

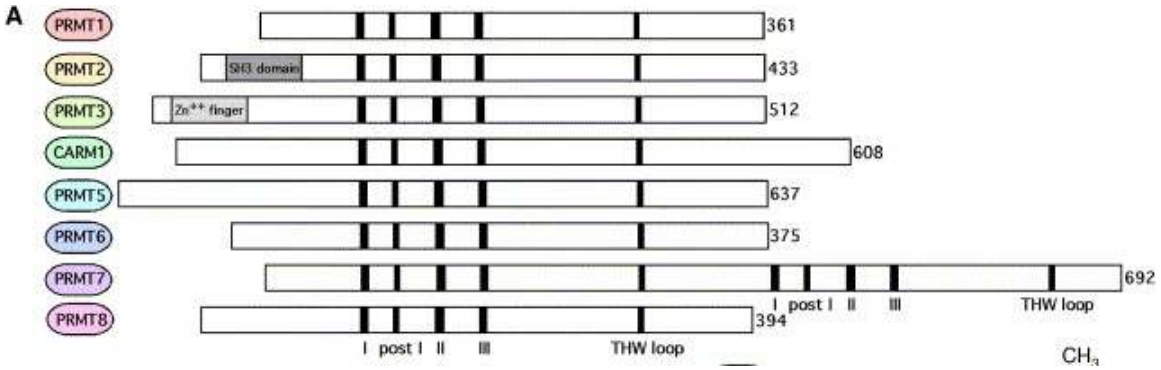


Figure 6 shows the 5 conserved motifs of PRMTS. This image was obtained from Arginine Methylation an Emerging Regulator of Protein Function by Mark T. Bedford and Stephane Richard. (Bedord, 2005)

Proteins with methylated arginines are involved with (1) DNA repair, (2) RNA metabolism, and (3) transcriptional regulation. PRMTs mostly methylate within GAR motifs (Glycine and Arginine), which are found on the substrate. Arginine methylation can be carried out in multiple ways. Methylated arginines have a variety of functions including regulation of transcription, nuclear trafficking, mRNA splicing, and DNA repair. PRMTs are expressed in all cells and may have a "housekeeping" function. Experimental evidence shows that PRMT-deficient mouse embryos die at or before birth (Yadav, 2003). However, stem cells from

the embryo are viable and do not have noticeable defects. **These results suggest that PRMTs are needed for differentiation.**

When arginine is methylated, the net "+" charge does not change. As a result, the properties of arginine should remain fairly similar to arginine that is not methylated. It is believed that methylated arginine does not act like an on/off switch, but either slightly dims or increases its activity. Experiments have shown that cells without PRMTs usually still have some level of activity associated with methylated arginines.

Until recently, arginine methylation was thought to be a permanent or very long lived modification. However, in 2004 demethylases were discovered. One of first of these proteins discovered was peptidyl arginine deiminase (PAD-4). In 2007, an arginine demethylase was discovered and named JMJD6. It contains a Jumonji domain. In total, two families of arginine demethylases have been discovered. Still, the difference in prevalence of PRMTs and demethylases is substantial. Some PRMTs are seen as "housekeeping" proteins found in all cells. Arginine demethylases have only been found in a few cell types with Ca2+ concentrations high enough for activation. Most cells do not have arginine demethylases.

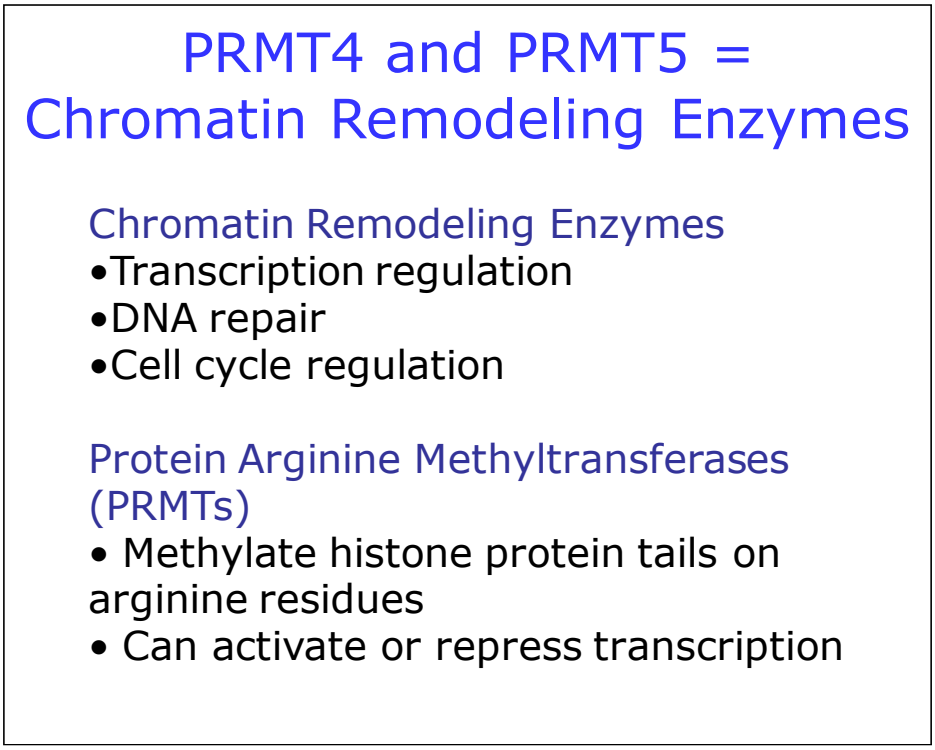


Figure 7 shows a summary of information about PRMT chromatin remodeling enzymes.

PRMT4 (CARM1)

PRMT4 (608 amino acids) is also known as CARM1, Coactivator-associated arginine methyltransferase, has been shown to increase transcription (Wyatt, 2007). PRMT4 is a secondary coactivator. It is recruited to the promoter site of hormone response genes via glucocorticoid receptor-interacting protein-1 (GRIP1, SRC2/TIF2). Once there PRMT4 interacts with histone acetyltransferase p300/CBP and AIB1, while methylating Arg17 and Arg26 on histone 3, resulting in gene activation. CARM 1 is also able to methylate p300/CBP, RNA-binding proteins, and splicing factors. NUMAC is a protein discovered to regulate PRMT4. Kinases phosphorylation is associated with decreased activity of PRMT4. Of note, PRMT4 overexpression is associated with breast and prostate tumors.

It should be noted that p300/CBP modifies histones by adding acetyl groups to lysine amino acids. It is believed that there is "interplay" between lysine acetylation and arginine methylation. This is not the only example of cooperative histone modification.

Proteins Methylated by PRMT4	Substrate Function
Histone H3 Histone H2A CBP P300 A1B1	Chromatin-Associated
SmB CA150 U1C SAP49	hnRNPs and splicing
PABP1 HuR HuD	mRNA-binding
FTARPP	Signalling

Figure 8 shows a list of proteins methylated by PRMT4.

PRMT5

Less is known about PRMT5 (637 amino acids) compared to PRMT4. PRMT5 modifies arginines on H2A, H3, and H4 histone subunits and can be found in both the cytoplasm and nucleus. Cytoplasmic PRMT5 is involved with the methylsome, which methylates Sm proteins. Nuclear PRMT5 is involved with SPT4

and SPT5 transcription regulators. Nuclear PRMT5 also complexes with human SWI/SNF ATPase proteins BRG and BRM, which increases PRMT activity. Recent findings suggest higher PRMT5 methylation levels of H3R8 in lymphoid cancer (Pal, 2007). Experiments show that this methylation can result in either increased or decreased transcription. PRMT5 is believed to operate with chromatin remodeling complexes (hSWI/SNF, BRG1/BRM, and MBD2-based NURD). These chromatin remodeling complexes enable PRMT5 to reach histone tails where it can modify arginine amino acids.

Proteins Methylated by PRMT5	Substrate Function
Histone H4 Histone H3 Histone H2A SPT5 FCP1	Chromatin-Associated
SmB CA150 SmD1 and D3	hnRNPs and splicing
EBNA1 EBNA2	Viral Proteins

Figure 9 shows a list of proteins methylated by PRMT5.

More about PRMT Substrates

While Glycine and Arginine (GAR) motifs are common methylation sites for PRMTs, Proline, Glycine, Methionine, and Arginine (PGM) motifs are also common sites for methylation. As an exception, PRMT4 cannot methylate GAR motifs. As a result, it mostly methylates PGM motifs. PRMT5 methylates both GAR and PGM motifs. PRMT4 and PRMT5 are the only two PRMTs that can methylate PGM motifs.

Adipose Differentiation Pathway & Peroxisome Proliferator-Activated Receptor Gamma (PPAR Gamma)

Peroxisome Proliferator –Activated Receptors (PPARs) exhibit 3 isoforms - alpha, delta, and gamma (Mukherjee, 2002). These molecules increase production of peroxisomes, organelles involved in fatty acid metabolism. Peroxisomes mainly work through beta-oxidation, the breakdown of fatty acids into Acetyl-CoA molecules (Tontonoz, 1994).

PPARs are part of the Nuclear Receptor (NR) family. Nuclear Receptors are proteins activated by hormones that regulate gene expression. NRs have 5 domains: N-terminal, DNA-binding (DBD), hinge, Ligand-binding (LBD), and C-terminal domains. The hinge domain connects the DBD to the LBD (Salma, 2006).

PPARs function as Type II NRs. Type II receptors bind to corepressors when the ligand is absent. After biding the ligand, they dissociate from the corepressor and hetero-dimer with RxR (retinoid X receptor). (See Figure 10) Then, they recruit coregulatory proteins and RNA polymerase to regulate gene transcription. These regulatory proteins can be involved in chromatin remodeling or recruitment of other proteins to DNA. Two common coactivator proteins associated by PPAR-gamma are Src-1 and Tif2 (Marathe, 2007).

The general mechanism of action for PPAR is to bind a ligand, dimerize with retinoid x receptor (RXR), and bind Peroxisome Proliferator Response Elements (PPREs) with coregulators. The PPRE is found in the promoter and has the consensus sequence AGGTCA**X**AGGTCA. Genes are then either activated or repressed. **It is important to note that PPARs regulate gene expression related to lipid metabolism.** Because of this, PPARs are clinically important because it is associated with diseases such as obesity, cancer, atherosclerosis, and diabetes.

The ligands for PPARs are either fatty acid derivatives or derived from Thiazolidinedione. Derivates of Thiazolidinedione include Troglitazone (Rezulin), Piogliazone (Actos), and Rosiglitazone (Avandia). Troglitazone was used as the ligand for this experiment.

Adipose differentiation begins when fatty acids or fatty acid derivatives bind to the PPAR-gamma receptor (See Figure 5). The receptor then binds to RXR (Retinoid x Receptor) and forms a heterodimer. The complex then binds to DNA and binds to other transcription factors. This leads to gene activation and adipose differentiation as well as an increase in insulin sensitivity.

Of note, drugs called thiazolidinediones (TZDs) treat type II Diabetes by binding to PPAR-gamma receptors. Activation of the PPAR-gamma receptor leads to an increase in insulin sensitivity. TZDs are sold under names such as Avandia, Actos, and Rezulin. An unwanted side effect of these drugs is an increase in weight!

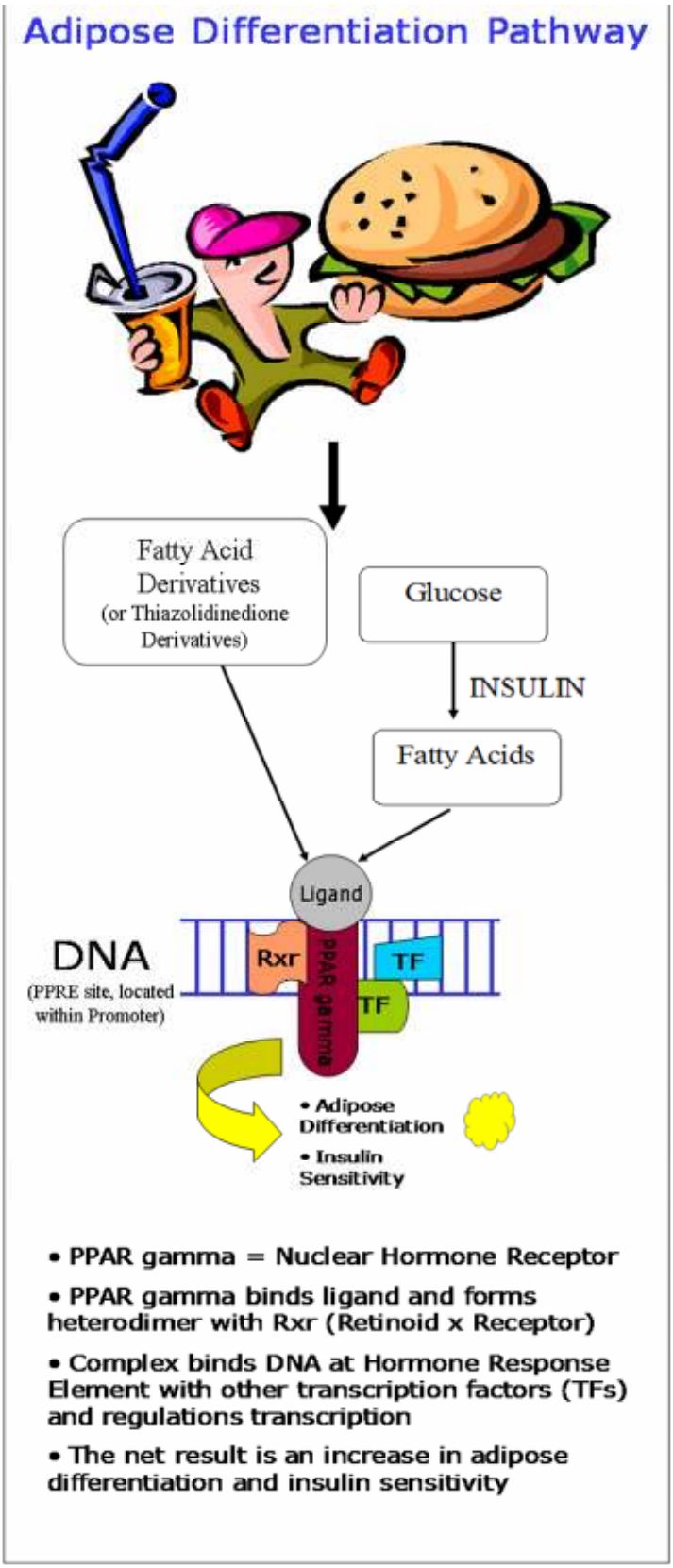


Figure 10 shows the adipose differentiation pathway.

Adipose mRNA Markers

- Leptin – This 16-kDa hormone (167 amino acid protein) is produced by adipocytes. The amount of leptin in the blood stream is directly proportional to the amount of body fat. It binds to LepR(x) receptors, where x is a, b, c, d, e, or f. Leptin binds to several regions of the brain, including the hypothalamic nuclei and hypothalamus. Binding of leptin to its receptor tells the brain to produce a signal of fullness or satiety.
- Resistin – Resistin is a protein hormone found in mice that acts on the liver. It is 108 amino acids in length and causes insulin resistance effects in the liver. Because the liver stores sugar deposits as glycogen, it plays an important role in regulating blood sugar. Insulin resistance of the liver causes blood sugar levels to increase. This is because insulin tells the body to reduce blood sugar levels. Of note, macrophages in mice also produce large amounts of resistin.
- Fatty Acid Synthase – FAS (272kDa) is responsible for converting carbohydrates to fat. It is the only enzyme with the ability to create fat, however fat can still be obtained through diet. Inhibitors of FAS have been developed by several companies to help fight obesity. Recently, however, FAS has been implicated in tumor growth. And, FAS inhibitors have been found to help prevent tumors.
- AP2 – Adipocyte Protein 2 is also known as Fatty Acid Binding Protein 4 (FABP4). FABP4 is a cytoplasmic protein that attaches to hydrophobic

substrates, such as fatty acids. Proteins in the class of FABP are involved with fatty acid metabolism and transport.

Recent Research Findings

PRMT4

Recent research into PRMT4 has revealed a link between the enzyme and **activation** of estrogen-mediated genes. It is believed that this activation is caused by methylation of H3 and H4 histones. It is also believed that PRMT4 acts at "estrogen-induced promoters" as part of a chromatin remodeling complex. PRMT4 has also been shown to be involved in myogenesis.

PRMT5

Recent finding about PRMT5 have revealed that it may be involved with **repression** of "growth control" and tumor suppression. Also, PRMT5 has been shown to decrease elongation factor Spt5's affinity for RNA polymerase – thus repressing gene expression. Generally, PRMT5 is able to repress genes by methylating histones and transcriptional subunits.

Dr. Imbalzano's Lab Findings

In 2007, my research mentor Caroline Dacwag published an article in the journal Molecular and Cellular Biology showing evidence that suggests PRMT5 is needed for myogenesis (muscle cell differentiation).

Muscle cell differentiation requires several transcription factors, including MyoD, Myf5, Mrf4, myogenin, and Mef2 members. These transcription factors are needed for gene activation during differentiation. The role of chromatin remodeling enzymes in myogenic gene activation had not been thoroughly studied. As a result, my mentor Caroline Dacwag set out to determine the role of PRMT5 in muscle differentiation.

Prior to her experiments, research on chromatin remodeling enzymes and myogenic gene expression revealed both positive and negative myogenic gene expression effects. At the time, a recent study linked PRMT4 to myogenesis (Chen, 2001). Because PRMT5 is a close relative of PRMT4 and because PRMT5 and PRMT4 had both been linked to another chromatin remodeling subunit, BRG1 (ATPase subunit of SWI/SNF), the role of PRMT5 in myogenesis was explored.

Dacwag used 3T3 fibroblasts to determine the role of PRMT5 in myogenesis. Dacwag used the knowledge that 3T3 fibroblasts expressing MyoD initiate myogenesis differentiation (Dacwag, 2007). Dacwag also used fibroblasts deficient in PRMT5 from an antisense vector. Fibroblasts deficient in PRMT5 did

not activate myogenic genes. Dacwag was able to show that the lack of arginine methylation on Histone 3 resulted in a loss of interaction with SWI/SNF subunit Brg1 and no ATP driven chromatin remodeling. Of note, PRMT5 was also shown to interact with transcription factor myogenin's promoter. (Myogenin is one of the earliest genes activated in the muscle cell differentiation pathway.)

Because the lab had previously sponsored adipose research and still had adipose cells culturing, the logical question was to ask if either PRMT4 or PRMT5 is needed for adipose differentiation. This MQP utilized the results of experiments performed during the past summer as the basis for this final report.

Hypothesis

It is predicted that because PRMT4 and PRMT5 are needed for muscle differentiation, they should also be required for adipose differentiation. It is expected that PRMT4- and PRMT5- cell lines should not transdifferentiate into adipose as efficiently as wild-type fibroblasts. As a result, there should be fewer lipids, fewer adipose mRNAs (leptin, fatty acid synthase, resistin, AP2), and fewer adipose proteins in PRMT4- and PRMT5- cells, compared to wild-type cells.

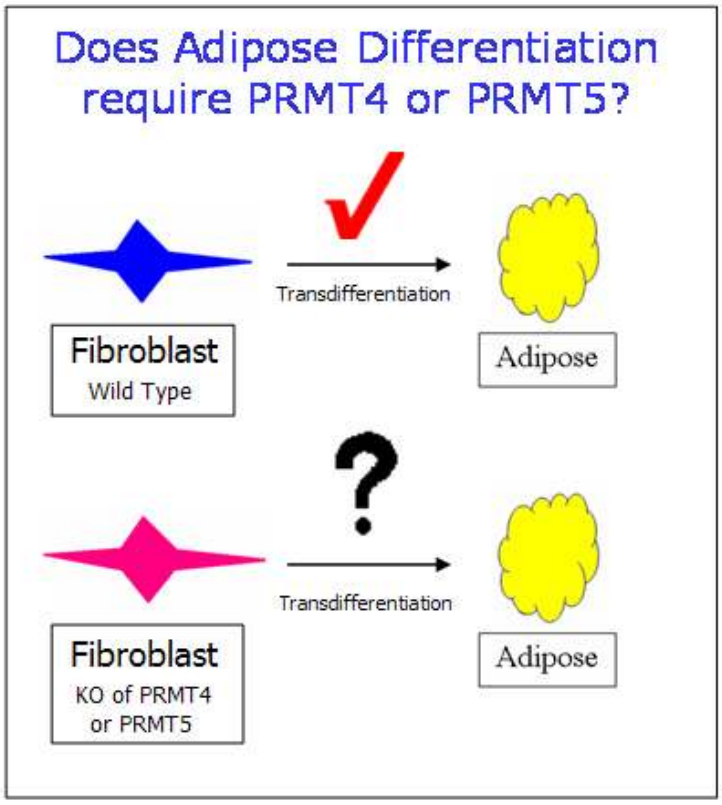


Figure 11 shows the experimental objective. It is known that fibroblasts can be transdifferentiatied into adipocytes when given PPAR-gamma. It is unknown if either PRMT4 or PRMT5 chromatin remodeling enzymes is needed during this process. If adipose differentiation is not as efficient when either PRMT4 or PRMT5 is removed, then adipose differentiation requires these chromatin remodeling enzymes.

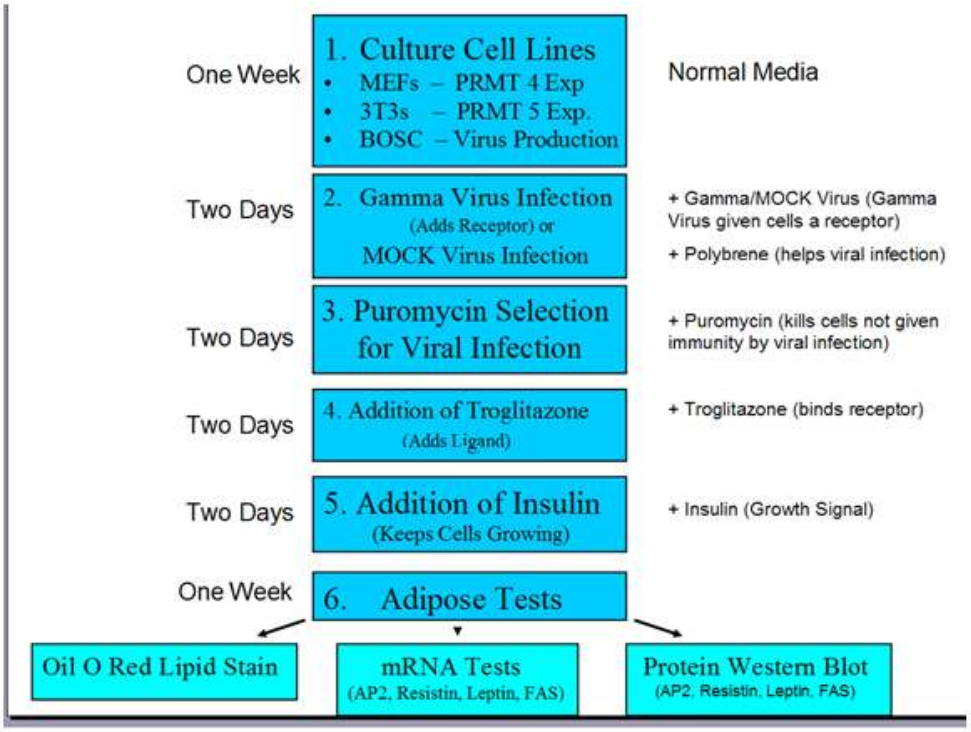


Figure 12 shows a brief experimental summary.

Cell Culture Materials

- Incubator (capable of operating at 37°C)
- 60 and 100mm Cell Culture Plates
- 95% Ethanol Spray
- Pasteur Pipets
- 1, 5, 10, 25 mL Pipets
- 15 and 50 mL conical tubes
- PBS (Phosphate Buffered Saline)
- Trypsin (10X stock diluted 1:10 with 1XPBS)
- DMEM (Dulbecco's modified Eagle's medium)

- 10% Calf Serum/ 10% Fetal Calf Serum
- Puromycin (diluted 1:1000 from stock to 2ug/mL)
- Glutamine (essential for cell culture but added separately to media because of stability issues)
- Penicillin-Streptomycin (bacterial antibiotics) - Penicillin blocks cell wall formation in Gram- bacteria, while streptomycin blocks protein synthesis in Gram+ bacteria.
- Normocin Antibiotic (stock = 50mg/mL, used at 5mg/mL) - Normocin is commonly used, alone or with Pen/Strep, to prevent growth of mycoplasma (bacteria without cell walls), fungus, and bacteria. Normocin is bactericidal, killing bacteria, not just preventing their growth. Normocin prevents Gram+ and Gram- bacterial growth. One active ingredient binds to the 50s ribosome subunit, blocking protein synthesis. The other component interferes with DNA synthesis. Mammalian cells are not affected by Normocin. Of note, Normocin also blocks flungal growth by interfering with ion exchange. A third component of Normocin acts on yeast and fungus but not bacteria.
- *IBMX* (.5mM), *Insulin* (.5ug/mL), and *Dexamethasone* (.5ug/mL) – IsoButylMethylXanthine is used in combination with insulin and dexamethasone for differentiation into adipocytes. IBMX activates CEBP-alpha, CEBP-beta, CEBP-delta, and PPARy transcription factors. This results in adipose differentiation.

- Polybrene (1ug/mL) is added to media to help viral infection. Polybrene is a positive molecule that binds to the surface of the cell and neutralizes the charge so that viral glycoprotiens can bind to receptors more efficiently.
- Troglitazone is a member of the thiazolidinediones and is the ligand for PPAR-gamma.
- TRIzol Reagent – This chemical (from Invitrogen) was used to isolate RNA. TRIzol disrupts cells and dissolves cellular components without damaging RNA.
- Inverted Light Microscope and Digital Camera

Cell Lines

- Mouse Embryonic Fibroblast Cells (MEF 13, 20, 20.3) –– Three MEF cell lines were cultured. MEF 13 cells were wild-type. MEF 20 cells (PRMT4-) were made with "transfection of Carm1-/- lines with PCAGGS-FLPe-puro". Of note, these cells were donated by Dr.Mark Bedford. He obtained these cells via S. Dymecki of Harvard Medical School. MEF 20.3 cells were created via PRMT4- cells that had PRMT4 function restored. This was done using a Flp/puro construct. MEF 20.3 are essentially wild-type cells. (The MEF cells required a 10% DMEM and Calf Serum mixture and were difficult to split.)
- 3T3 and C15 Fibroblast Cells – Two 3T3 derived cell lines were cultured. The wild-type cells are called 3T3. The PRMT5- cells were made from 3T3,

but are called C15 (Clone 15). PRMT5- cells were made by using an anti-sense PRMT5 vector, which reduced both protein and mRNA expression. These cells are also known as NIH 3T3/AS-PRMT5 (AS = Antisense). (3T3-derived cells required a 10% DMEM and Calf Serum mixture.)

- BOSC Packaging Cell Lines (BOSC-alpha, BOSC-beta, BOSC-gamma viruses) — The BOSC cells produce a retrovirus that carries a gene needed for adipose differentiation (PPAR-gamma). Three BOSC cell lines were cultured during the experiment BOSC-CEBP-alpha, BOSC-CEBP-beta, and BOSC-PPAR-gamma. **However, only BOSC-PPAR-gamma was used to infect cells.** Each BOSC produces a retrovirus that carries a master adipose differentiation gene. The gene encodes a receptor that can bind a ligand, troglitazone. When the ligand troglitazone binds to the receptor, it activates adipose genes and induces differentiation. Each BOSC line carries a gene that acts as a master adipose differentiator. (These cells required 10% DMEM and Fetal Calf Serum mixture and were easy to split.)

It should be noted that retroviruses are enveloped viruses and contain RNA. Once inside the host cell, the RNA is converted to DNA using reverse transcriptase. Then, the enzyme integrase is used to integrate the viral DNA into the host DNA.

- NOTE: [The BOSC cell line, C15 cells, and 20.3 cells have innate Puromycin (PURO) resistance. Puromycin is an antibiotic that kills both prokaryotes and eukaryotes by blocking translation of mRNA. Puromycin looks like an aminoacylated tRNA. So, it attaches to the growing polypeptide chain, but then causes the chain to release before completion of translation.]

Lipid Stain Materials

- Oil O Red
- Isopropanol
- ddH2O
- Buffered Formalde Fresh (10% Formalin) – (Formalin is another name for Formaldehyde. It is commonly used to preserve tissues.)

Making Media

500 mL DMEM (Gibco #11965-092)

5 mL L-Glutamine (200 milimolar)

5 mL Pen/Strep

50 Calf Serum or Fetal Calf Serum

Normocin Antibiotic (stock = 50mg/mL, used at 5mg/mL)

Puromycin (diluted 1:1000)

Step #1 Thawing Cells from N_2 (l) Freezer (MEFs, 3T3s, BOSCs)

1. Add 9mL of appropriate media into 15mL conical tube. Do this for each vial of frozen cells that will be retrieved
2. Remove vial from freezer
3. Thaw vial in 37° C warm water bath
4. Douse vial with 95% ethanol spray and dry
5. Remove 1mL of vial contents and add to conical containing 9mL of media
6. Centrifuge at 1.0-1.2 x 10^3 rpm for 2.5 minutes to pellet cells
7. Aspirate media
8. Resuspend pellet in 10mL of fresh media
9. Add to plate
10. Shake plate to distribute cells
11. Incubate at 37°C

Step #2 Splitting/Passaging Cells on 10cm Plate

1. Spray hood with 95% and wipe away with paper towel
2. Aspirate media from plates using Pasteur pipets
3. Add enough PBS to cover cells (5-6 mLs). Do not pipet directly on cells. Instead, pipet on side of plate.
4. Aspirate PBS using clean Pastuer pipet
5. Add .5-1mL of Trypsin and tilt plate to spread all around

6. Wait 3 minutes while cells detach. 7. Hit the side of the plate against the table to help cells detach from plate.

7. Use microscope to verify detachment. Cells should appear rounded when they have detached.

8. Add desired amount of media +10% serum to cells and resuspend. (Serum inactivates the trypsin.)

9. Distribute the resuspended cells to new plates which already contain media, and split to a desired ratio. (Add cells to media to ensure the cells attach to all surfaces of the plate surface.) It is important to note that the total volume on a 10cm plate is 10mLs. The total volume on a 6cm plate is 5mLs. These volumes can be used to calculate how much media + serum to add to the cell suspension.

10. Evenly distribute cells by rotating and shaking plate back and forth. (All cell plates can be shaken at once if they are placed on a cell tray.)

11. Incubate at 37^{o}C on a flat cell tray. If the tray is not flat, cells may not cover the surface evenly.

Step #3 Retroviral Infection (Gamma Virus and Mock Virus)

(Jain, 2007)

Gamma Virus Media

15 mL PPAR-gamma Virus

1 mL Calf Serum

5 mL 10% CS + DMEM

21 uL polybrene

add 7mL/plate (enough for 3 plates)

Mock Virus Media

9 mL PBabe Virus

1 mL Calf Serum

11 mL 10% CS + DMEM

21 uL polybrene

add 7mL/plate (enough for 3 plates)

1. Cells should be between 50-60% confluent. This is to ensure that the cells are in the cell cycle dividing and will incorporate the viral DNA.
2. When cells reach 50% confluence, aspirate old media and add 7mLs viral media to plates.
3. Incubate at 37^{o}C for two days
4. After two days, check confluency. If cells are 100% confluent,change media to back to original media, but add puromycin to all media. This will select for cells infected with virus. The virus gave the cells puromycin resistance. Adding PURO will kill cells without resistance. (Puromycin selects for cells that incorporated the virus)

5. After 2 days, check the confluency.
6. When the confluence reaches 100%, add media containing ligand. It is optional whether puromycin is added to the media beyond this step.

Troglitazone Ligand Media

10 mL of 10% FCS media

1 uM Dexamethosone

5ug/mL insulin

10uM Troglitazone

2ug/mL Puromycin

7. After 2 days, change media to insulin media.

Insulin Media

10 mL 10% FCS media

5 ug/mL insulin

8. The insulin media keeps the cell growing. Tests can be done at anytime after the insulin media has been added. This media should be changed every two days.

Step #4 Oil O Red Lipid Staining Test

1. Add .50 grams of Oil O Red to 100 mLs of isopropanol and mix for 3 hours
2. Add 40 mLs of ddH20 to 60mLs of the Oil O Red solution and incubate at RT for 1 hour
3. Filter stain through vacuum suction filter (.2 micron)
4. Aspirate cell media and wash with 1X PBS.
5. Add Buffered Formalde Fresh (10% formalin, Fisher Catalog # SF93-4) to cover cells
6. Incubate at room temperature for half an hour
7. Aspirate and add Oil O Red to cover cells
8. Leave for 1 hour at room temperature
9. Dump Oil O Red off cells and wash with PBS 2-3 times. Leave cells in PBS.
10. Take images under microscope.

Step #5 mRNA Testing

mRNA isolation

1. Lyse cells by adding 1mL TRIzol Reagent. Add 1mL/10cm^2 plate.
2. Incubate for 5 minutes at room temperature.
3. Add .2mL chloroform per 1mL of TRIzol. Scrape cells into eppendorf tube. Cap tube and shake for 15 seconds.

4. Centrifuge at 12000x g for 15 minutes at 2°C.
5. Transfer the upper aqueous phase to a fresh tube. Add .5mL of isopropyl alcohol per 1mL of TRIzol reagent.
6. Incubate for 10 minutes at room temperature.
7. Centrifuge at 12000x g for 10 minutes at 2°C.
8. Dry RNA pellet under vacuum centrifugation.
9. Redissolve RNA in 100% formamide and store at -70°C

TRIzol disrupts cellular components without harming RNA. Adding chloroform and then centrifuging will cause the formation of two layers, one aqueous and one organic. RNA stays in the aqueous phase. The isopropyl alcohol acts to precipitate the RNA.

Making cDNA from mRNA

1. Resuspend 1ug RNA into DEPC water for a total volume of 10.5uL
2. Place on ice for 5 minutes
3. Add 19.5 uL of RT mix

RT Mix (enough for 1 sample)

10uL (1:100) 200ng random hexamer

5x Buffer

1uL .1M DTT

1.5uL 10mM dNTPs

1uL MoMulV Reverese Transciptase

4. Run PCR

Composition of Media when Passaging Cells

BOSC-PPAR-gamma	C15 and MEF 20.3	MEF 13 and 20 and 3T3
10% DMEM	10% DMEM	10% DMEM
Fetal Calf Serum	Calf Serum	Calf Serum
Penicillin-Streptomycin (Pen-Strep)	Penicillin-Streptomycin (Pen-Strep)	Penicillin-Streptomycin (Pen-Strep)
Normocin Antibiotic	Normocin Antibiotic	Normocin Antibiotic
PUROMYCIN	PUROMYCIN	No PUROMYCIN

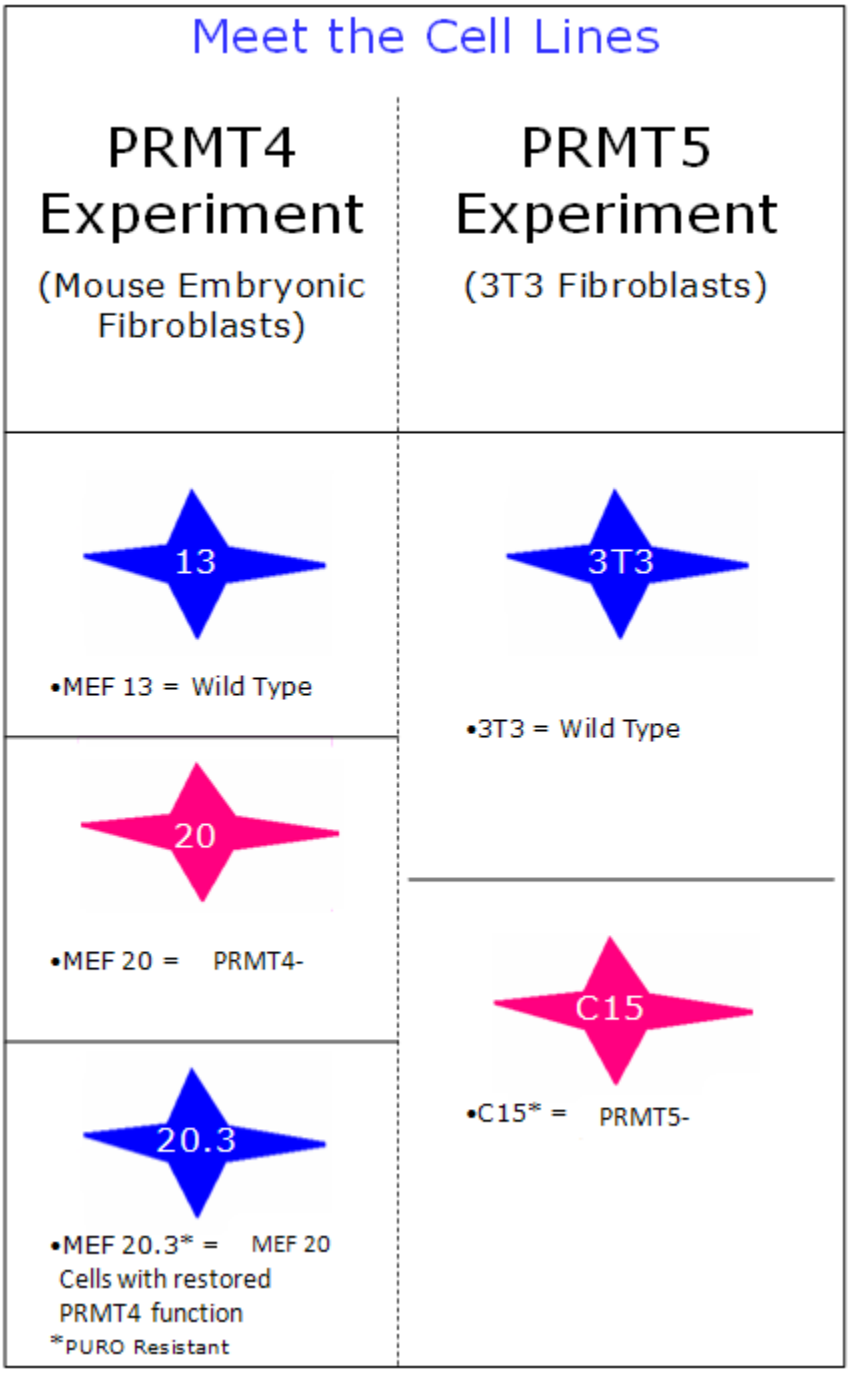


Figure 13 shows the cell lines used in the experiment.

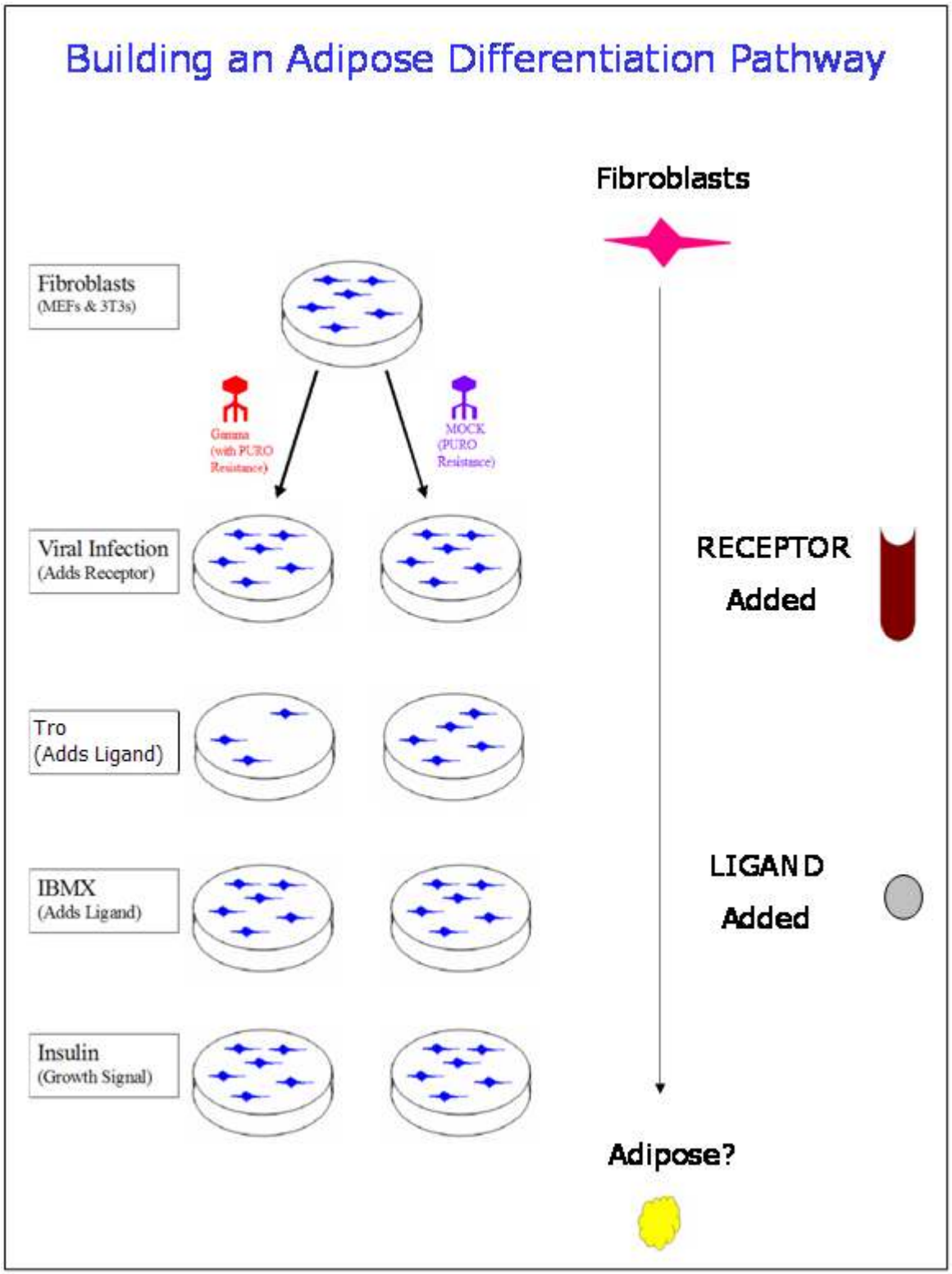
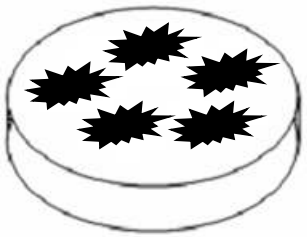


Figure 14 shows how the adipose differentiation pathway was built in a fibroblast. The gene for the PPARγ receptor was transferred to the fibroblast using a viral vector. The ligand for the recptor was then added to the growth media given to the fibroblasts.

In order to transdifferentiate a fibroblast into an adipocyte, the fibroblast must be given the master adipose differentiator. A virus can be used to introduce the genetic sequence for the receptor, PPAR-gamma. In the experiment, MEF and 3T3 cells were given either of two viruses. One virus contained the PPAR-gamma gene and a puromycin resistance selection marker. A second virus containing just a puromycin resistance selection marker was used as a control. After infection, the virus-infected cells were selected for by adding puromycin to the growth media. Cells not infected with the virus died. After the cells became confluent, the ligand for the adipose receptor, IBMX , was added to the growth media. Insulin was used as a growth signal to keep the cells growing. After the cell plates became confluent tests for adipose differentiation were run.



8 weeks of Bacterial Contamination!!!

Figure 15 shows a tribute to the 8 weeks of bacterial contamination that plagued the project on and off.

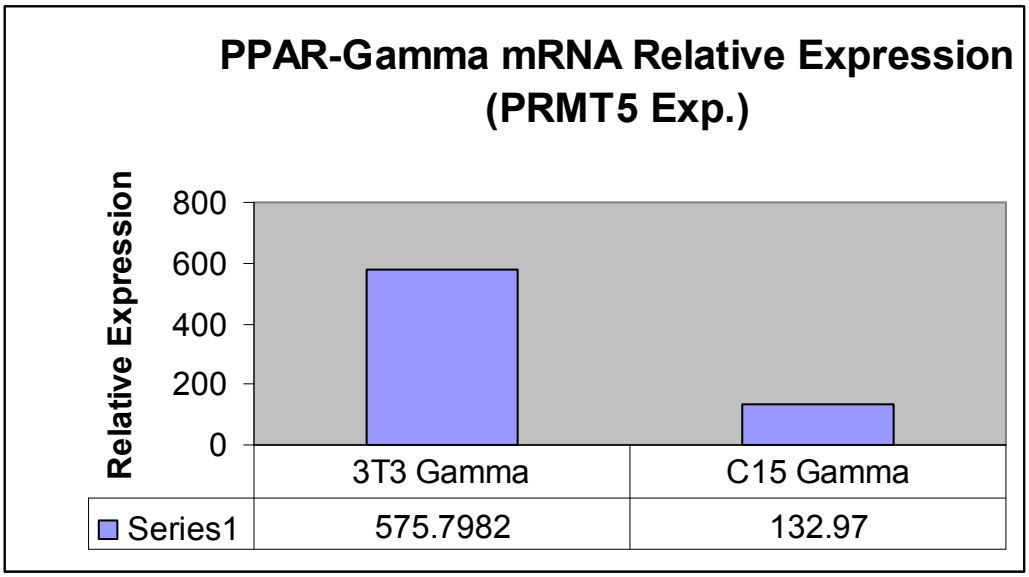


Figure 16 shows the raw PPAR-gamma mRNA expression in the PRMT5 experiment. The PRMT5 mRNA data were adjusted such that the level of PPAR-gamma expression in the cells was equal.

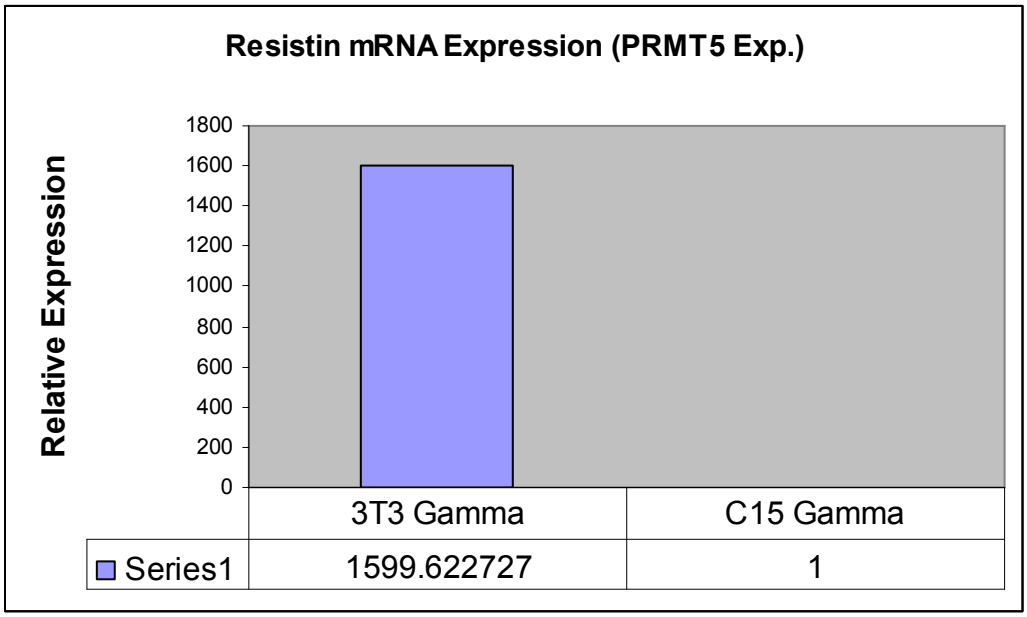


Figure 17 shows the Resistin mRNA expression for the PRMT5 experiment.

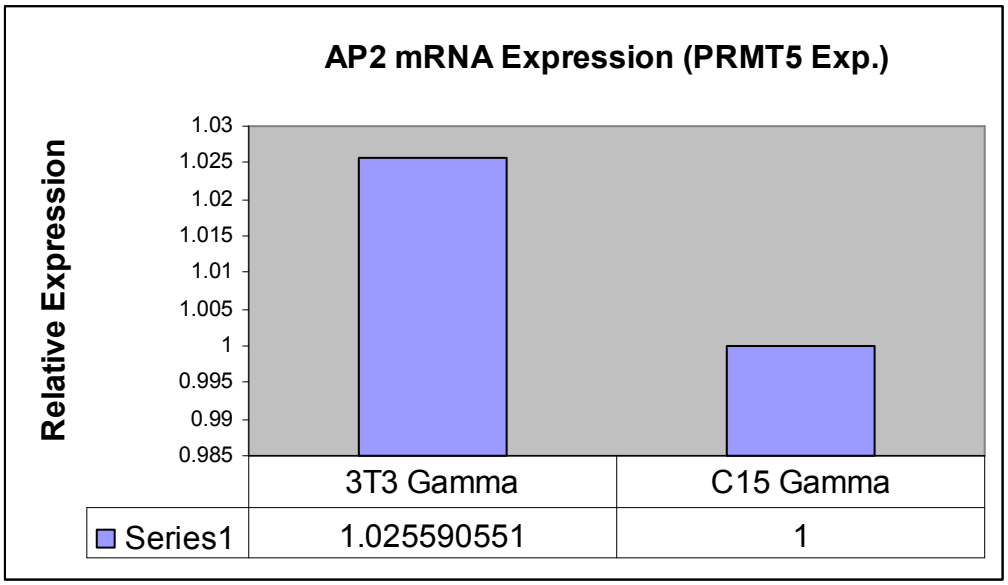


Figure 18 shows the AP2 mRNA expression for the PRMT5 experiment

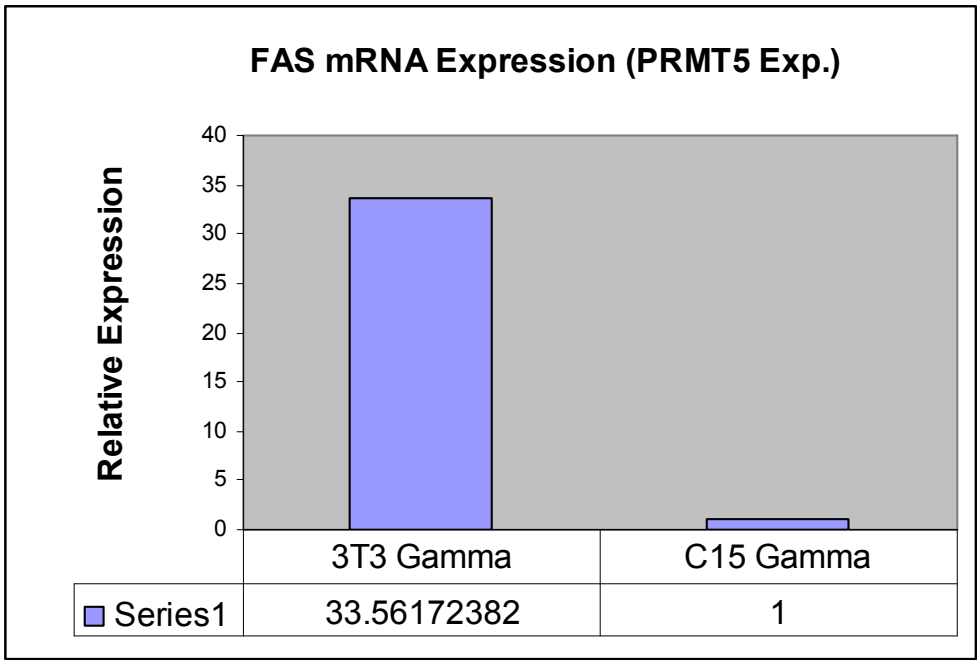


Figure 19 shows FAS mRNA expression for the PRMT5 experiment

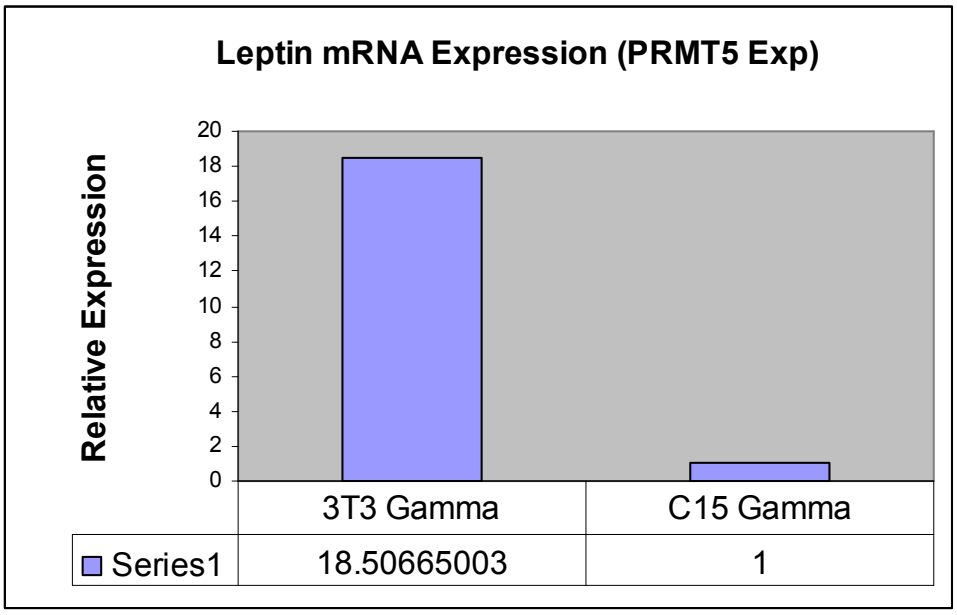


Figure 20 shows Leptin mRNA expression for the PRMT5 experiment

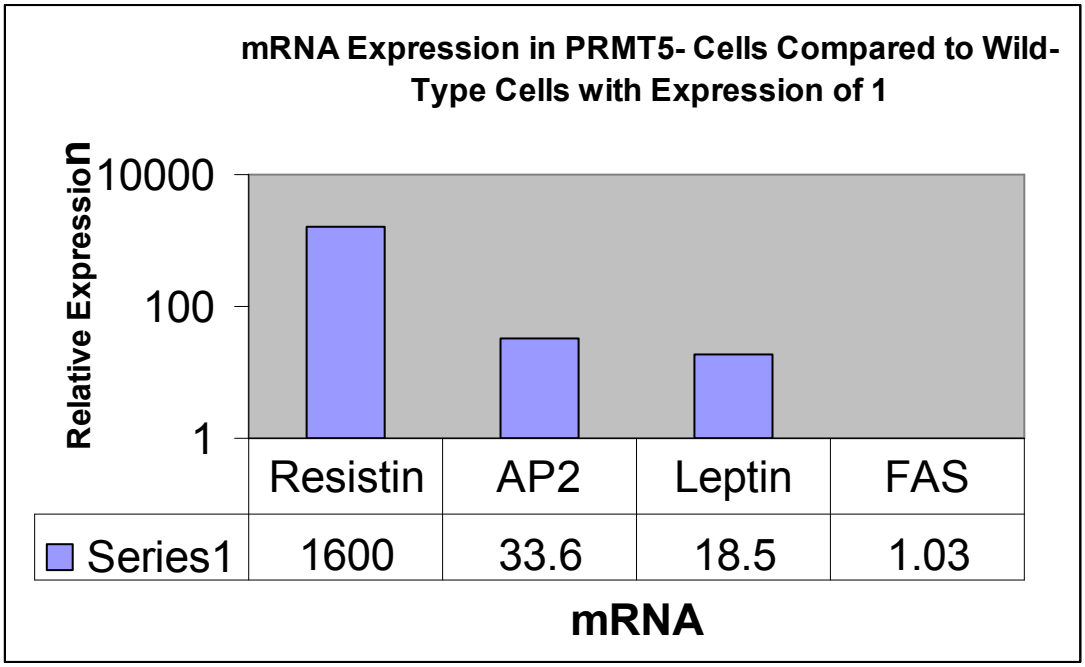


Figure 21 shows a summary of the mRNA expression results in the PRMT5- experiment.

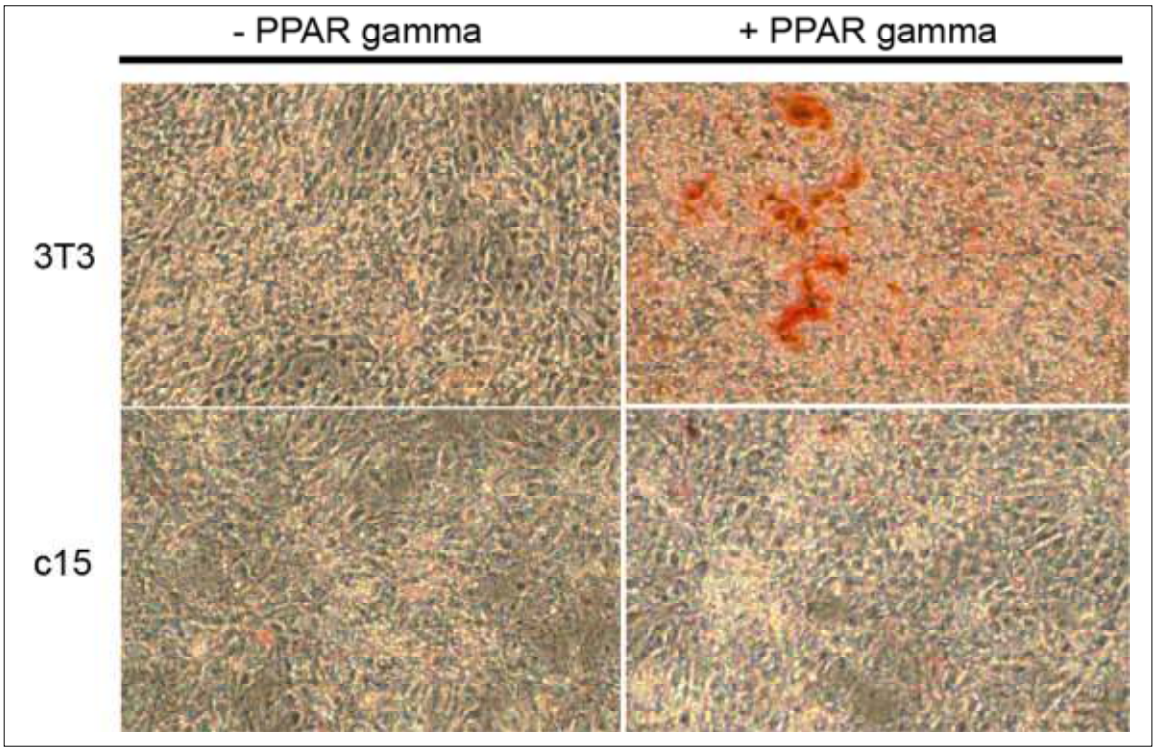


Figure 22 shows the Oil O Red (lipid stain) for the PRMT5 experiment. Lipid stains RED. 3T3 cells represent wild-type fibroblasts and C15 represent PRMT5 deficient cells.

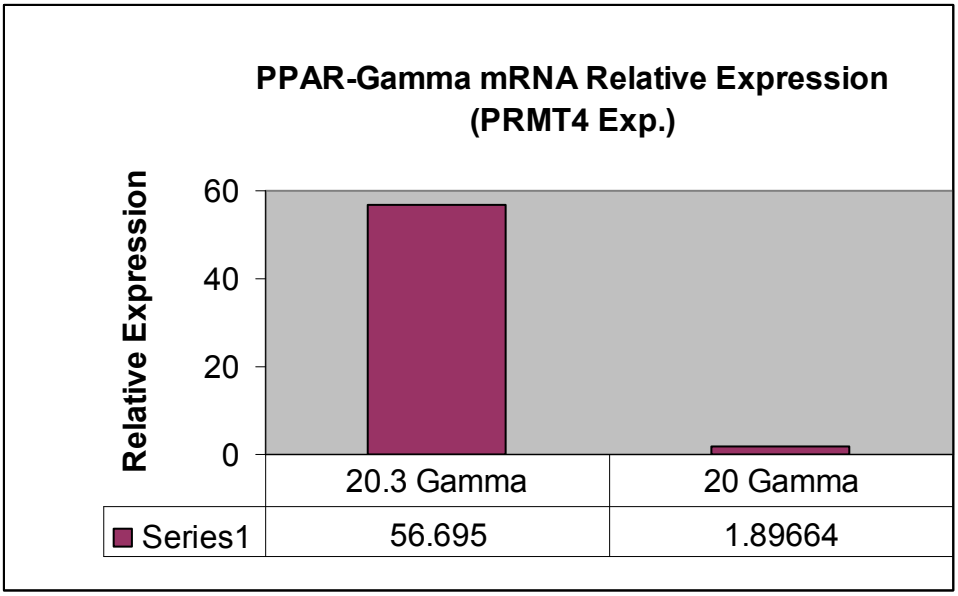


Figure 23 shows mRNA expression of PPAR-gamma for the PRMT4 experiment. The data were adjusted such that the relative expression of PPAR-gamma in the 20.3 and 20 were equal.

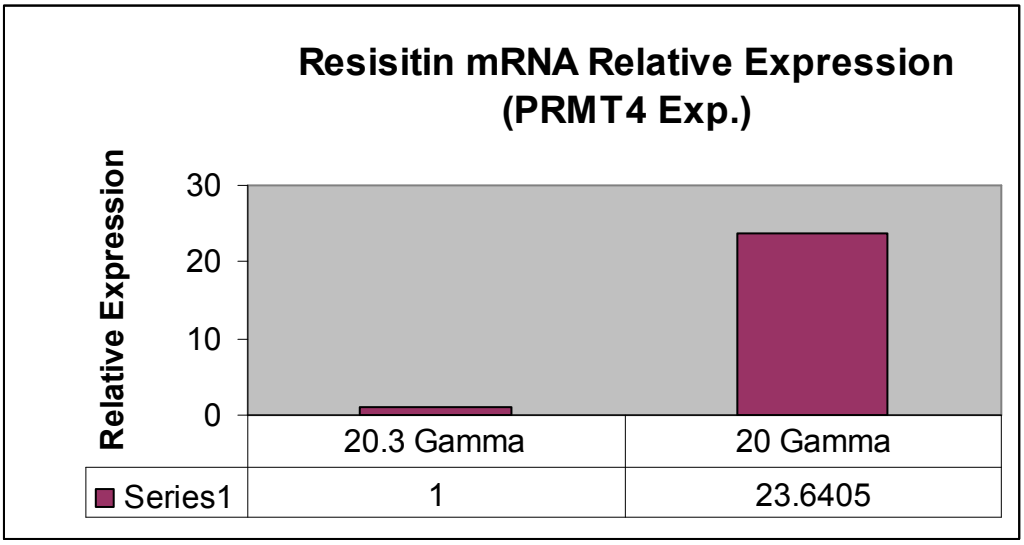


Figure 24 shows mRNA expression of Resistin in the PRMT4 experiment.

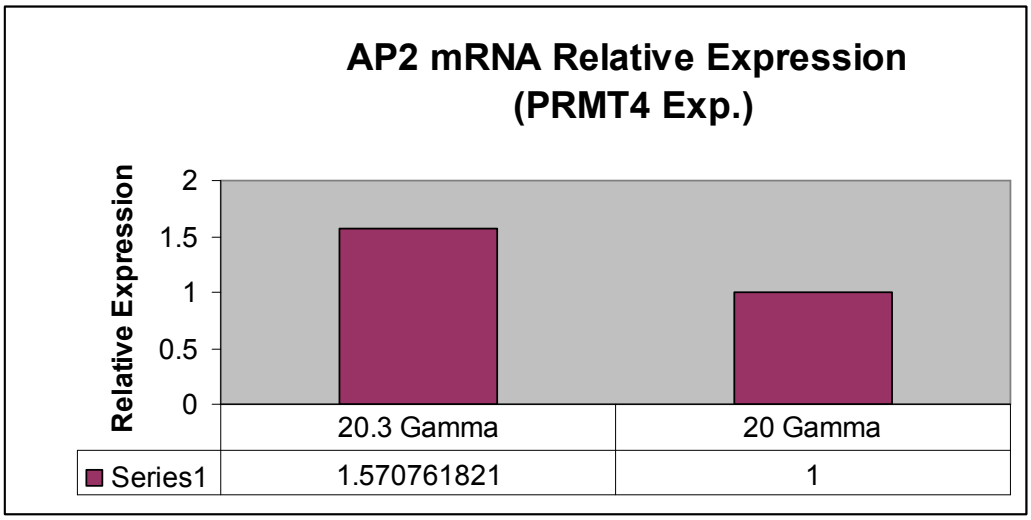


Figure 25 shows mRNA expression of AP2 in the PRMT4 experiment.

DISCUSSION

Obstacles affecting data collection

Contamination in the MEF cell lines set the project back several weeks. Randomly, during the course of the experiments, free-floating bacteria would appear in the cell cultures (See Figure 15). The media in the cell culture disks would appear cloudy, with particulate matter floating in the media. The incubation time before the appearance of the bacteria could last as long as 2 weeks. The penicillin/streptomycin that was added to the media did not kill the bacteria. Although Pen/Strep has the ability to kill both Gram+ and Gram- bacteria, resistance to these chemicals is becoming more common. Eventually, Normocin antibiotic was added to the media to block growth. The MEF data could only be obtained through the use of Normocin antibiotic.

Inefficient PPAR-gamma and MOCK virus infections of the MEF13 cells left few cells alive after puromycin selection. Without successful viral infection of the cells, no puromycin resistance could be obtained by the cells. Therefore, when virus-infected cells were selected for by adding puromycin, most cells died. Waiting for the few cells left occupying the plate to become confluent slowed the experiment down. In most cases, the MEFT13 cells were never able to grow to high enough confluence levels for tests to be performed. In other words, there were not enough cells on the plate to run tests of mRNA, lipid, and protein.

Innate puromycin-resistance in the MEF 20.3 and C15 cell lines prevented selection for virus-infected cells. Because of this, cells not infected with virus continued through the experiment. If the data were not normalized to account for PPAR-gamma expression, this would affect the results. If the data were not normalized, the degree of lipid formation and adipose mRNA transcription would be reduced by the cells not infected, and therefore not differentiating. Normalizing PPAR-gamma expression ensures that the total production of PPAR-gamma is the same on the wild-type plate and the experimental plate. This is necessary because this method helps to "exclude" the cells not infected with PPAR-gamma and not producing adipose mRNAs.

mRNA expression levels of Leptin and FAS were not reproduced in the PRMT4 experiment. The first trial revealed (1) mRNA expression of Leptin as 324.3% higher in Wild-Type cells compared to PRMT4- cells and (2) expression of FAS as 48.9% higher in Wild-Type cells compared to PRMT4- cells. However, during the second trial, this information was not successfully collected. And, a mistake caused resisitin and FAS mRNA levels to not be taken. Contamination was found in the second trial and data on mRNA expression was not collected. As a result of the lack of reproducibility, this information was not included in the results section in proper graph format.

The 10-week experimental time frame prevented additional experiments to tweak the protocol for a more complete set of data. Several plans were made to alter the protocols to achieve better results, however, research mentors insisted on continuing with the established protocols. Specifically, protein data was not collected in either experiment. And, mRNA data was either not reproduced or not completely collected in the PRMT4 experiment.

Normalized mRNA Expression Data

The data sets were normalized such that PPAR-gamma mRNA expression was the same in 3T3 and C15 cells (See Figure 16) and the same for MEF 20 and MEF 20.3 cells (See Figure 23). The reasons for differences in PPAR-gamma mRNA expression come from (1) different efficiencies of viral infection and (2) different efficiencies of gene transcription. The location of viral gene incorporation (into the genome) can affect relative transcription into mRNA. However, these discrepancies were eliminated when the data was normalized.

PRMT5 mRNA Expression

The adjusted mRNA data for the PRMT5 experiment shows that wild-type (3T3 cells) produce more adipose mRNAs than PRMT5 knock-downs (C15 cells). Resistin mRNA production was 159,900% greater in 3T3 cells compared with C15 cells (See Figure 17). FAS mRNA production was 3,256% greater (See Figure 19), 1,750% greater for leptin (See Figure 20) mRNA expression, and 2.559% greater

for AP2 expression (See Figure 18). This data suggests that adipose differentiation is more efficient when PRMT5 is present.

3T3 (WT) Cells Produce More Adipose mRNA than C15 (PRMT5-) Cells.

Adipose mRNA	% increased expression in Wild-Type 3T3 cells Compared to PRMT5- Deficient Cells
Resistin	159,900%
FAS	3256%
Leptin	1750%
AP2	2.559%

Figure 26 shows the adjusted mRNA data for the PRMT4 experiment.

The adjusted mRNA data for the PRMT4 experiment shows that the cells with restored PRMT4 function (MEF 20.3) produce 57.20% more AP2 than PRMT4- cells (See Figure 25). However, PRMT4- cells produced 2,264% more resistin mRNA than wild-type cells (See Figure 24). As noted earlier, Leptin and FAS data were omitted because they were never reproduced. Leptin expression was 324.3% higher in wild-type cells. FAS expression was 48.9% higher in PRMT4- cells.

This set of data brings to light one concern. The problem is that two pieces of data are not valid because they were not reproduced. When the data from the two valid trials is examined, it reveals that resistin mRNA expression is much higher in PRMT4- cells compared to wild-type cells. This suggests that PRMT4 is not needed for adipose differentiation. However, AP2 expression was higher in wild-type cells compared to PRMT4- cells. This means that when PRMT4 function

was removed, AP2 expression decreased. This suggests PRMT4 is needed for AP2 expression. No conclusion about whether PRMT4 is needed for adipose differentiation can be made.

Adipose mRNA Transcription does NOT seem to depend on PRMT4.

Adipose mRNA	mRNA Expression (written in relative terms)
Resistin	2,364% higher in MEF 20
AP2	57.20% higher in MEF 20.3

Figure 27 shows the adjusted mRNA data for the PRMT4 experiment.

Oil O Red (Lipid Stain)

The Oil O Red lipid stain clearly shows less lipid staining red in the C15 PRMT5-PPAR-gamma+ cells compared to the 3T3 wild-type cells infected with PPAR-gamma (See Figure 22). In other words, cells without PRMT5 produce fewer lipids than cells with PRMT5. This suggests that PRMT5 is somehow needed for adipose differentiation. Neither of the mock (control) infections produced anything more than background lipid staining. The PRMT4 experiment did not have lipid data because neither the MEF13 nor MEF20 designated for lipid staining survived the infection. The mock-infections (controls) do not show lipid staining because these cells were not given the master adipose differentiator PPAR-gamma.

CONCLUSIONS

Results suggest that PRMT5 is needed for Adipose Differentiation

Three of the four adipose mRNA markers tested (Resistin, FAS, Leptin) show at least 1750% increased mRNA expression in wild-type cells compared to cells deficient in PRMT5. Leptin mRNA expression was 1750% higher in wild-type cells. Fatty acid synthase (FAS) mRNA expression was 3256% higher in wild-type cells. And, Resistin mRNA expression was 159,900% higher in wild-type cells. This suggests that when cells become deficient in PRMT5, adipose mRNA production dramatically decreases. From this, support is provided for the idea that PRMT5 is needed to enable these adipose genes to be expressed.

It should be noted that AP2 expression was only slightly higher (2.55%) in wild-type cells, compared to cells deficient in PRMT5. This means that there is approximately the same level of AP2 mRNA expression in wild-type and PRMT5- cell lines. However, this does not mean that PRMT5 is not involved in adipose differentiation. It was assumed that AP2 would have a higher level of mRNA expression in adipose cells compared non-adipose cells. However, this was never shown in the project. The relative levels of AP2 mRNA expression in fully differentiated adipose and non-adipose cells should have been determined before the experiment as a control. This way, it could be verified that AP2 (and

Resistin, FAS, and Leptin) mRNA expression is truly higher in adipose cells compared to non-adipose cells.

(mRNA expression of adipose markers in adipocytes and fibroblasts could also be useful in measuring the extent of adipose differentiation. In other words, adipose mRNA markers should have been measured in (1) fibroblasts and (2) adipocytes. Then, a scale could be created for each adipose mRNA marker. Then, when fibroblasts are transdifferentiated into adipocytes, the mRNA expression levels could be used to determine the extent of adipocyte differentiation.)

There is another possible reason why the AP2 mRNA expression was approximately equal in both wild-type and PRMT5- cell lines. If the mRNA test had been done before the differentiation process was fully complete, the level of wild-type expression of AP2 might have increased as the differentiation process proceeded. To address the issue of how far the differentiation process had proceeded, lipid and mRNA tests should have been done at different time intervals during the differentiation process. This way, it could be ensured that the adipose cells were fully differentiated when the mRNA levels were determined.

There is still another possible explanation for why the AP2 mRNA expression was approximately equal in both wild-type and PRMT5- cell lines. PRMT5 may not be involved in allowing this gene to be accessible for expression. PRMT5 could still be involved in "opening" up many adipose genes for expression, but not AP2. Maybe, a different PRMT is involved in allowing PRMT5 to become accessible to protein factors. Or, maybe experimental error caused a skewed result. More tests confirming the AP2 data in the PRMT5 experiment would help clarify this topic. And, collecting this data at different time intervals after differentiation would ensure that these mRNA levels are from fully differentiated adipose cells.

From the Oil O Red lipid stain, it can be shown that only wild-type cells infected with the master adipose differentiator PPAR-gamma can produce lipid. The Oil O Red stain reveals a strong presence of red-stained lipid in the environment, with localized red dots. Red color was only lightly found in the background of the images of cells not infected with PPAR-gamma. This is evidence that the cell plate was stained with a red dye. However, the lack of a strong red color in the background and the lack of red color localized within the cells is evidence that the cells did not produce substantial amounts of lipids. However, it is important to consider that all cells do produce lipids for (1) membranes, (2) energy storage, and (3) signaling. These lipids are responsible for retaining a small amount of Oil Red O dye.

Cells deficient in PRMT5 did not produce lipid anywhere near the extent that wild-type cells did. Only a faint dot of red is found in the image of cells infected with PPAR-gamma, but deficient in PRMT5. This visual observation provides support for the idea that more lipid forms when PRMT5 is present. And, this result was visually supported by viewing individual cells under a microscope at high magnification. Wild-type cells infected with PPAR-gamma were found to contain several large granules, which resembled lipid granules. An expert in the field of adipose tissue confirmed this observation. These granules were not found in PRMT5- cells or any cells not infected with PPAR-gamma.

This combination of Oil O Red lipid stain images, mRNA data, and the observation of numerous large granules in wild-type cells containing PPAR-gamma supports the hypothesis that PRMT5 is involved in adipose differentiation. PRMT5- cells have fewer adipose mRNAs being expressed, less lipid staining red, and few if any granules resembling lipid droplets within the cells. This supports the expected results of the hypothesis. This outcome that PRMT5 seems to be needed for adipose differentiation follows nicely in the footsteps of my research mentor's recent finding that PRMT5 is needed for muscle cell differentiation.

Results from PRMT4 Experiment are too incomplete to make Correlation

The PRMT4 experiment did not produce any valid Oil O Red stains. This was due to the fact that the MEF 13 (Wild-Type) cells infected with PPAR-gamma could not be produced in significant quantity to stain. This was a caused by inefficient MEF13 viral infections. Without viral infection and delivery of puromycin resistance to the MEF13 cells, they were killed when puromycin was added during the viral selection phase. Without a wild-type to compare the PRMT4- cells to, staining the MEF20s was useless and therefore not done. (Suggestions for improving the efficiency of the MEF 13 viral infection can be found later in this section.)

The mRNA results for the PRMT4 experiment were also incomplete. Although two pieces of data were retrieved, these pieces of information conflicted with each other. Resistin expression was found to be over 2,264% higher in PRMT4- cells compared to cells with partially restored PRMT4 function. And, AP2 expression was found to be 57.203% higher in cells with partially restored PRMT4 function (MEF 20.3) compared to PRMT4- cells. It should be restated that MEF20.3 cells are not wild-type but are rescued versions of PRMT4- deficient cells (MEF20s). In this way, MEF20.3 cells can act like a wild-type, with active PRMT4 expression. mRNA data about FAS and Leptin was only retrieved during one trial. A second attempt to retrieve this data could not be made in the experimental time frame. Because this data was not reproduced (confirmed) it was omitted. No

conclusion can be made from this information as to if PRMT4 is needed for adipose differentiation.

Suggestions to Improve Viral Infection of MEF 13 cells

Incomplete mRNA and lipid data in the PRMT4 experiment was caused by a lack of cells that were infected with the PPAR-gamma viruses. Specifically, MEF13 cells infected by either the MOCK or PPAR-gamma viruses were difficult to obtain from the few cells that survived viral selection. Few MEF13 cells were successfully infected by the viruses and given Puromycin resistance. As a result, when puromycin was added to the MEF13 plates to select for viral infected cells , most of the MEF13 cells died. To improve the efficiency of viral infection in the future, MEF13 cells should be infected with virus at 75% confluence (instead of 50%). In this case, the cells are still dividing and would incorporate the viral genes into their DNA. And, there would be more cells infected on the plate, increasing the numbers of surviving cells. This would enable the surviving cells to more quickly repopulate the plate.

In addition to infecting the MEF 13 plates at 75% confluence, the PPAR-gamma and MOCK viruses should be harvested from the supernatant of the BOSC cells the moment before infection. This way, the virus does not have to be frozen and preserved. Freezing a virus can reduce the number of virus particles that can

successfully infect cells. In fact, using this technique near the end of the experiment did increase the number of MEF 13 cells infected by the viruses.

Another way to improve the MEF 13 viral infection is to infect a substantial number of MEF 13 plates. This way, cells that survive puromycin selection can be "pooled" together and can more quickly become confluent.

Suggestions to address Innate Puromycin Resistance in C15 and MEF 20.3 cells

One issue associated with this experiment was puromycin resistance. Both the PPAR-gamma and mock viruses conferred puromycin resistance to cells so that they could be selected for. However, two of the cell lines used, MEF 20.3 and C15, had innate puromycin resistance. Because of this, there was no way to kill these cells that were not infected with the virus. To address this issue, new cell lines that do not contain PURO resistance should be used. This way, cells can be selected for after viral infection. After selection, only the cells infected with the virus would survive. Then, these cells would divide and fill the plate. It should be noted, that this issue was addressed by adjusting data such that the expression of PPAR-gamma mRNA was equal for all cell-lines.

Outlook

While the PRMT5 experiment shows substantial data to suggest PRMT5 is needed for adipose differentiation, protein data would make the argument more convincing. Data showing adipocyte protein marker production decreasing in PRMT5- cell lines would improve the argument for PRMT5 playing a role in adipose differentiation. This is an important idea because mRNA expression levels do not always correspond to protein expression levels. Protein expression data corresponding to mRNA expression data would provide strong evidence confirming the mRNA results.

In addition, redoing the PRMT4 experiment with the inclusion of protein data would help make PRMT4's role in adipose differentiation known. Using the suggestions made in this project, achieving PRMT4 data on adipose mRNA expression and lipid formation is likely.

Knowing the role of PRMT4 and PRMT5 in adipose differentiation would help clarify the role of PRMTs in the differentiation process. Recent findings about the role of PRMTs in muscle differentiation led to the creation of this project. A new finding that PRMTs are involved in adipose differentiation could stimulate more research about PRMTs and their role in differentiation of other cell types, such as neurons, bone cells, or blood cells.

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