

**THE IMPACT OF POLARIZED ACTIVATED MACROPHAGES ON
ADIPOGENESIS**

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Adipogenesis Affected by Adipose Tissue Macrophage-Secreted microRNA. (May 2015)

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Adipose tissue plays a major role in energy storage and Adipokine secretion to maintain systemic balance. However, adipocytes that experience hypertrophy or hyperplasia have negative effects on the processes of obesity-associated metabolic syndromes. In addition, adipose tissue macrophages (ATMs) have been reported to regulate adjacent adipocytes via various cell-cell communications including, microRNAs, which have been discovered to exist in extracellular space. In my research, I studied the effects polarized macrophages have on adipogenesis. By obtaining quantitative real time-PCR (qRT-PCR) results, I monitored the growth of adipocytes through measuring their levels of adipogenesis genes such as Adiponectin, and FAS, chronologically. Moreover, oil red O staining assay was applied to record lipid formation in adipocytes as evidence of adipocyte maturation. By comparing adipocytes response to macrophage-conditioned medium (CM) it was shown that macrophages suppress adipogenesis. The various types of macrophages had varying degrees of suppression on adipogenesis, which leads to the need for further investigation of the mechanism of how this happens.

DEDICATION

This research project is dedicated to my family for their love and support in my research and academic career. Without their support I would not be as successful in my undergraduate career.

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I would also like to take the time out to acknowledge my research laboratory professor, Dr. Beiyan Zhou, who has allowed me to use her funding to do my project as well as allowed me to be apart of this research laboratory. A special thank you goes to my graduate student mentor, Richard Chang, who has taught me many laboratory techniques and supported me in order to help me complete this project. Lastly, I acknowledge the laboratory members under Dr. Beiyan Zhou's lab, for their help and support through this project.

CHAPTER I

INTRODUCTION

Importance of adipose tissue and ATMs

Adipose tissue is comprised of infiltrated immune cells, the precursor cells, and adipocytes, which is the main cellular component¹. Adipocytes are considered to originate from fibroblast-like precursor cells known as pre-adipocytes undergoing adipogenesis where the maturation of the adipocyte experiences growth arrest². It has been observed that the phenotype of the adipocyte during adipogenesis changes in gene expression, which occurs chronologically^{3,4}. In mammals where obesity is seen, there is an increase in dead adipocytes and ATMs form crown-like structures in adipose tissue⁵.

ATMs are neighboring cells to adipocytes, so it is important to study ATMs-regulated adipogenesis. In a study conducted in 2006, when culturing pre-adipocytes in macrophage-CM, adipogenesis was down-regulated but it wasn't specified as to what inhibited it⁶. Also, it is assumed that classically activated (M₁) ATMs are the type that inhibits adipogenesis by secreting pro-inflammatory factors⁷. However, no studies have been conducted to show the effect alternatively activated (M₂) ATMs have on adipogenesis.

Objective

The objective of this research was to determine how ATMs affect adipogenesis via cell-cell communications. I hypothesized that ATMs polarized activation (M1 and M2) have an opposite impact on adipocyte formation. To test this hypothesis, I have completed two specific aims: (1)

to establish the *ex vivo* adipogenesis system and confirmation differentiation by monitoring gene expression, adipocyte lipid accumulation, and adipokine secretion, and (2) to study the change of adipogenesis under the treatment of various macrophages CM after polarized activation as well as various macrophages co-culture after polarized activation.

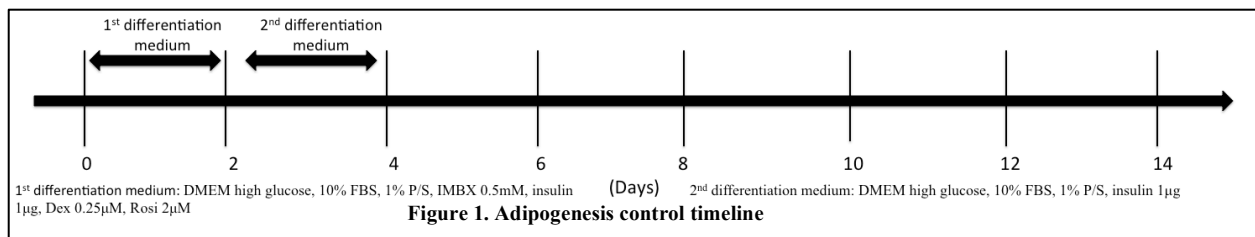
CHAPTER II

METHODS

Monitoring adipogenesis

3T3-L1-cell line was used to monitor adipogenesis. Adipogenesis gene expression, adipocyte lipid accumulation, and adipokine secretion, were monitored. The cell line was cultured in a basal medium of Eagle's minimal essential medium (DMEM) with 10% fetal bovine serum, and 1% penicillin/Streptomycin. The mother plates for the 3T3-L1 cells were cultured and allowed to grow up to the 18th passage. The mother plates were allowed to grow to 80-90% confluence before splitting. 2 12-well plates at 1.5×10^6 cells/plate were seeded in order to begin the cell culture for adipogenesis.

Once reaching confluence, the cells were exposed to a differentiating medium consisting of 0.5mM 1-methyl-3- (2-methylpropyl)-7H-purine-2,6- dione, 850 μ M insulin, 0.25 μ M dexamethasone, and 2 μ M rosiglitazone for 48 hours. A second differentiating medium consisting of basal medium plus 850 μ M insulin is required to maintain adipogenesis for another 48 hours. After this day (day 4) the cells medium is renewed every 48 hours with basal medium until day 14 (Figure 1).



The adipogenesis genes that were monitored were, adiponectin, fatty acid synthesis (FAS), CCAAT- enhancer binding protein alpha (C/EBP-alpha), and Beta- Actin. These genes were used to create a control timeline as well as collect data to observe changes in adipogenesis when under the treatment of various macrophages CM after polarized activation as well as miR-223 enriched or depleted medium. The total RNA was extracted every 48 hours from day 0-14 from cells. From there the RNA was analyzed for gene expression of the previously mentioned genes through qRT-PCR. The next step in indicating adipogenesis occurred as well as creating a control for it, was Oil Red O staining assay. This was done in order to provide evidence of lipid accumulation in the cells.

Analyzing macrophage conditioned medium affect on adipogenesis

To test the effect of macrophages on adipogenesis, my mentor, Richard Chang, prepared bone marrow-derived macrophages (BMDMs) and supplement BMDMs-CM, after activation, to culture the 3T3-L1 cells. Mouse bone marrow cells were isolated from the femur and tibia bones and were then cultured in basal medium plus macrophage colony stimulating factor. After 6 days of differentiation, the BMDMs were ready to undergo activation to either M1 or M2. The 3T3-L1 cells were treated with control, M1, or M2 BMDMs-CM. The effects of each of these BMDMs on adipogenesis were monitored through RNA extraction and then qRT-PCR, using adiponectin and FAS genes were measured on day 4 and day 6.

Analyzing macrophage co-culture affect on adipogenesis

Macrophages were co-cultured with 3T3-L1 cells in order to validate the results from CM effects on adipogenesis. Mouse bone marrow cells were isolated from the femur and tibia bones and were then cultured in basal medium plus macrophage colony stimulating factor. After 6 days of differentiation, the BMDMs were ready to undergo activation to either M1 or M2. After 24 hours, the BMDMs were transferred to a 12-well culturing plate that had 3T3-L1 cells on day 0.

CHAPTER III

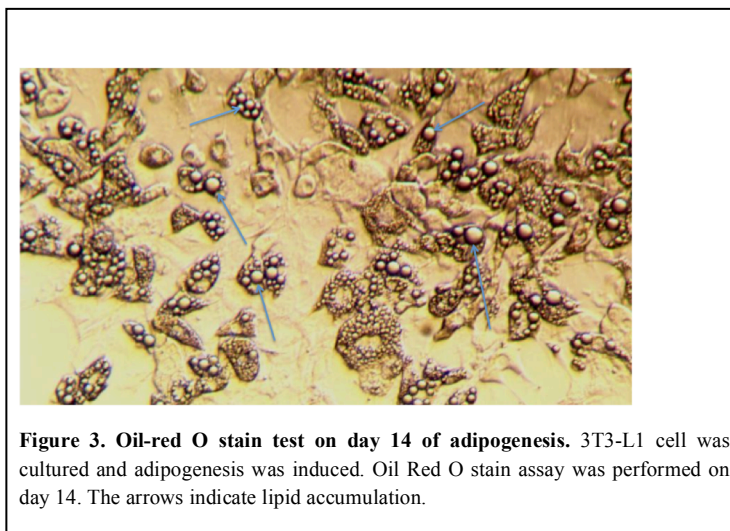
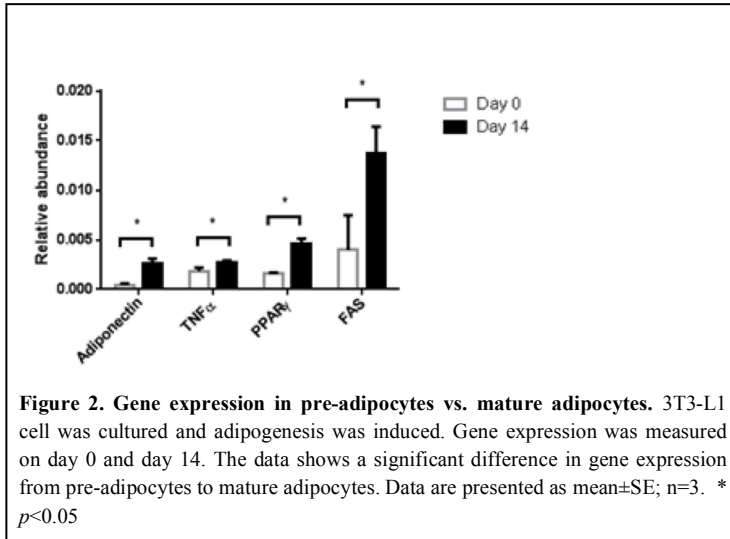
RESULTS

Regulation of gene expression in adipogenesis

3T3-L1 cells were used for the *ex vivo* induction of adipogenesis. The cells were seeded in a 12-well plate at 1.5×10^6 cells/well. 48 hours after seeding, 3T3-L1 cells grew to 100% confluence, which was deemed day 0. On day 0 the cells are exposed to a differentiating medium consisting of 0.5mM 1-methyl-3- (2-methylpropyl)-7H-purine-2,6- dione (IBMX), 850 μ M insulin, 0.25 μ M dexamethasone, and 2 μ M rosiglitazone for 48 hours. Rosiglitazone binds to the PPAR γ receptor. IBMX increases intracellular cAMP, dexamethasone binds to the glucocorticoid receptor and insulin binds to the insulin receptor. These combinations of these three pathways are important for the activation of the PPAR γ and C/EBP family genes. Adipocytes contain PPAR γ and C/EBP α , which activate adipocyte-specific genes encoding secreted factors, insulin receptor, and proteins involved in the synthesis and binding of fatty acids that compose lipid droplets.

A second differentiating medium consisting of basal medium plus 850 μ M insulin is required to maintain adipogenesis for another 48 hours. After this day (day 4) the cells medium is renewed every 48 hours with basal medium until day 14. Total RNA was extracted every 48 hours from day 0 to day 14. Adipogenesis genes were expressed and measured by methods of total RNA extraction and qRT-PCR. Adiponectin, TNF- α , PPAR- γ , and FAS were all measured quantitatively to prove that adipogenesis was successfully induced. These genes were chosen based upon at what stages of adipogenesis they are expressed. By using one that is expressed at the beginning, middle, and end of adipogenesis, we were able to prove that the pre-adipocyte

cells successfully underwent adipogenesis. The levels of gene expression were measured and compared from day 0 and day 14 (Figure 2). The increased levels of each gene from day 0 to day 14 show that the cells were maturing and secreting Adipokines. Oil red-O staining assay shows that lipid accumulation was present and adipocytes underwent maturation (Figure 3).



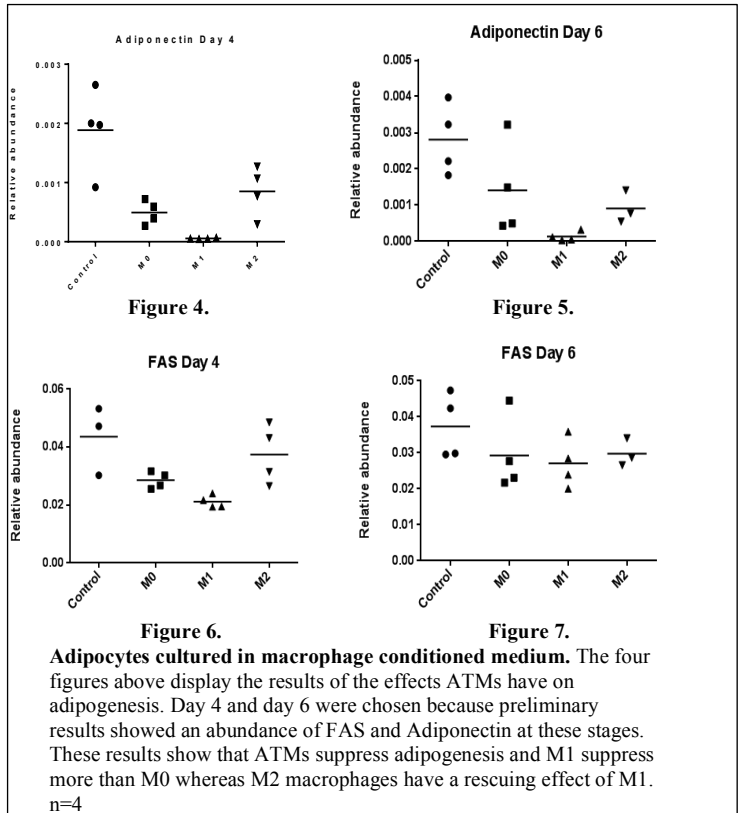
BMDMs conditioned medium affects on adipogenesis

After inducing adipogenesis, the cell line was treated with macrophage-conditioned medium. Mouse bone marrow cells are harvested from the mouse femur and tibia bones. Ammonium chloride is applied for erythrocyte lysis. Cells were then seeded in plates with GM-CSF (15% L929 supernatant is used as the source). In order to test the maturation of BMDM flow cytometry after day 7 was used. The cells must have a purity minimum of 90% of CD11b+F4/80+ to be used in the experiment. BMDMs are treated with lipopolysaccharide (100ng/mL)/interferon- γ (20ng/mL) or IL-4 (20ng/mL)/IL-13 (5ng/mL) for M1 or M2 activation respectively. Surface markers were examined by flow cytometry and gene expression in polarized activation macrophages was measured via qRT-PCR.

Also, adipose tissue was collected from lean and obese mice after 10-week diet-feeding regimen. ATMs were isolated using magnetic beads-conjugated antibody against macrophage surface marker. The purity of ATMs was evaluated by flow cytometry. Richard saved the macrophage-conditioned medium (CM) after 24-hour culture and gave to me on day 0, day 2, and day 4 of adipogenesis.

Adiponectin and FAS adipogenesis genes from were measured through qRT-PCR. The gene levels were compared to 3t3-L1 cells that were cultured in basal medium. It was shown that classically activated macrophages significantly depress the expression levels of the genes.

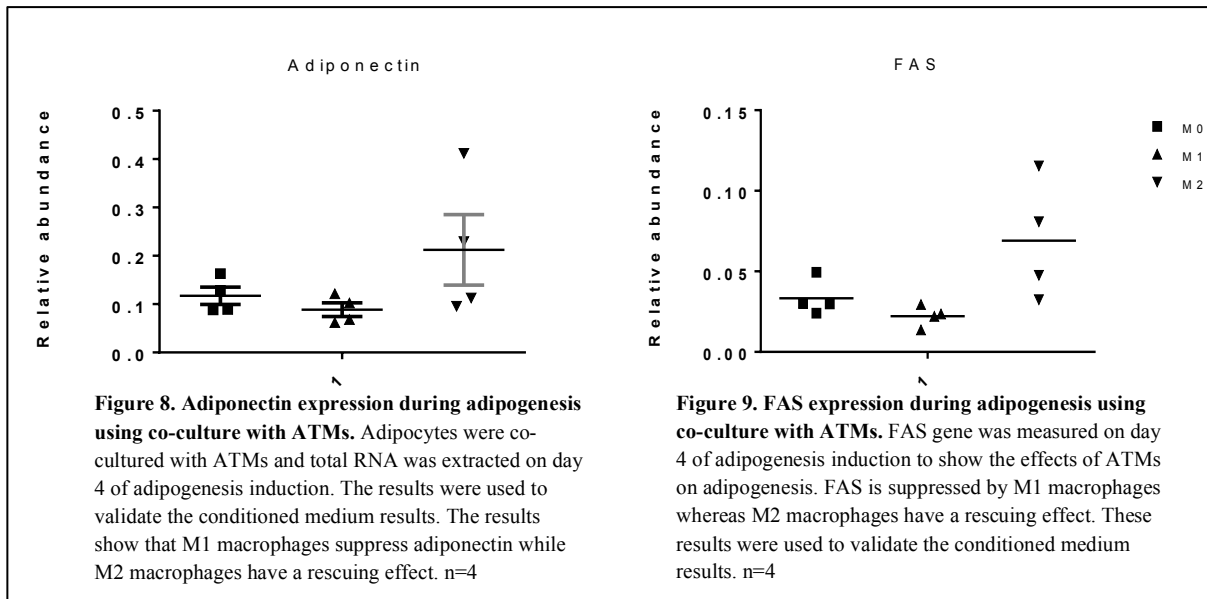
Whereas, alternatively activated macrophages have a rescuing function of the gene levels. M2 gene expression levels were higher than the M1 levels (Figure 4, 5, 6, and 7).



Polarized activated macrophages co-culture with pre-adipocytes

The next step for comparing control adipogenesis to the regulation of macrophages was using co-culture techniques. Macrophages were activated the same way they were with the conditioned medium experiment. Polarized activated macrophages were examined for cell receptors through flow cytometry before co-culturing. The polarized activated macrophages were placed in 12-well co-culturing dish. They were then placed in treated 12-well with 3T3-L1 cells at day 0 and were co-cultured to day 4. Co-culture procedure was used to confirm the results from BMDMs CM.

This procedure was also used to test the effects of direct cell-cell communication between the macrophages and adipocytes. The results confirmed that macrophages suppress adipogenesis but M2 have the opposite effect that M1 have. M2 have a rescuing function of M1. Total RNA was extracted on day 0, 2, and 4 and then qRT-PCR was performed, adipogenesis genes of co-culture were measured (Figure 8 and 9).



CHAPTER IV

CONCLUSION

Adipogenesis is an important metabolic process that occurs in the adipose tissue niche. ATMs are observed to experience hyperplasia and hypertrophy in an inflamed state of adipose tissue. Due to macrophages being adjacent cells to pre-adipocytes and adipocytes in the adipose tissue niche it is important to study the cell-cell communication between them. Macrophages are immune cells and in the adipose tissue niche they surround dying adipocytes and ingest them to protect the tissue from harmful substances the adipocytes may release. M1 and M2 macrophages may secrete pro-inflammatory and anti-inflammatory cytokines, respectively.

This study was able to successfully induce adipogenesis *ex vivo* by using the techniques mentioned in the methods and results section. In this study it was shown that polarized activated macrophages suppress adipogenesis *ex vivo*. M1 macrophages suppressed adipogenesis the most where M2 suppressed the least. It is suspected that M2 have a rescuing function on adipogenesis after it has been suppressed by M1 macrophages. This study can help in future studies pertaining to adipogenesis. Adipogenesis can be useful in studies pertaining to metabolic associated disorders.

CHAPTER V

FUTURE WORK

This study shows the relevance of ATMs in the adipose tissue niche pertaining to adipogenesis but it does not show how ATMs communicate with adipocytes. To my knowledge, there is a lack of data on how ATMs directly communicate and effect adipogenesis. In my future work I will be focusing on adipose tissue macrophage secreted microRNA, more specifically miR-223 and its effects on adipogenesis.

MicroRNAs are single stranded RNAs in length of 21-24 nucleotides that regulate gene expression in the post-transcriptional level. MicroRNAs can either silence mRNAs translation by binding to the 3'UTR of the targeted mRNA or enhance mRNAs degradation like siRNAs⁸.

Recent studies have reported the existence of extracellular microRNAs (exRNAs) as the biomarkers of certain diseases; however, the function and the mechanism exRNAs perform are not clear. Our group has proved M2 macrophages highly express intracellular microRNA-223 (miR-223) (Figure10)⁹. In addition, our preliminary data show the abundance of extracellular miR-223 in macrophage-CM (Figure 11). Taken together, macrophages-secreted extracellular miR-223 could be a potential regulator on adipogenesis.

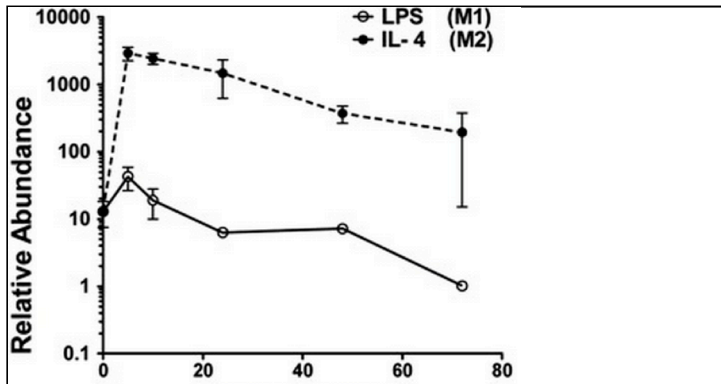


Figure 10. Expression of intracellular miR-223 in M1 and M2 macrophages
Differentially expressed miR-223 in bone marrow-derived macrophages (BMDMs) on lipopolysaccharide (LPS; M1) or interleukin (IL)-4 (M2) was measured at various time points after stimulation. Data are presented as mean±SE; n=4

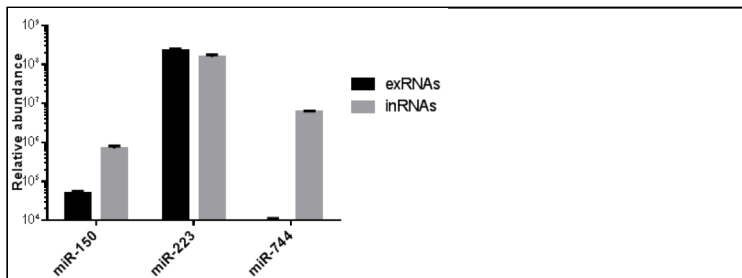


Figure 11. MicroRNA-223 is relatively abundant in and outside of macrophages. Intracellular RNAs (inRNAs) were extracted from collected BMDMs, whereas exRNAs were extracted from BMDM-CM. qRT-PCR confirmed that miR-150 and miR-744 were abundant in BMDMs but were significantly low in exRNAs samples. miR-223, however, was abundant in inRNAs and exRNAs samples collected from macrophages. Data are presented as mean±SE; n=4.

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