Regulation of adipogenesis by microRNA-378/422b and RNAi associated proteins Dicer and Ago2

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Acknowledgments

Abstract

  Adipogenesis, or the differentiation of preadipocytes into fat cells, is a cellular process present in all higher organisms. Regulated by activation of specific transcription factors and signaling pathways, adipocyte differentiation is also affected by a recently discovered class of gene regulators termed microRNAs (miRNAs). Transcribed in the nucleus, miRNAs are moved into the cytoplasm and processed by dicer 1, ribonuclease type III (Dicer) and assembled onto the RNA-induced silencing complex (RISC), which includes the eukaryotic translation initiation factor 2C (Ago2). The mature miRNA then base pairs to mRNA to cause translational repression. This study attempts to discern target genes of miR-378/422b to better understand miRNA regulation of adipogenesis. miR-378/422b was upregulated when miRNAs were differentially screened for expression between 3T3-L1 preadipocytes and adipocytes via miRNA microarrays. Compared to wild-type cells, ST2 cells with miR-378/422b overexpression contained bigger lipid droplets on day 3 of differentiation. Because of the important functions Dicer and Ago2 have in RNA interference (RNAi) and miRNA action, their roles in adipocyte differentiation were also studied. Luciferase assay results from transfection of cells with vectors that contained the miR-378/422b seed and vectors with an insert of the predicted target gene sequence indicate that DIX domain containing 1 (Dixdc1), LIM domain containing preferred translocation partner in lipoma (LPP), Sp1 transcription factor (Sp1), nuclear receptor interacting protein 1 (Nrip1), and nuclear receptor co-repressor 2 (NCOR2) are not target genes of miR-378/422b. Western blot and Thin-Layer Chromatography analyses suggest that both Dicer and Ago2 are involved in lipid metabolism and that Ago2 is not involved in adipocyte differentiation. This study serves as a basis for further research on understanding the variety of ways miRNAs and associated processing proteins function during adipocyte differentiation.

Introduction

Adipogenesis and obesity

      Adipogenesis, or the differentiation of preadipocytes into fat cells, is a cellular process present in all higher organisms. Adipogenesis begins when a pluripotent stem cell commits to the adipocyte lineage, discarding its ability to differentiate into other cell types. Recent interest in adipose tissue properties and adipogenesis has increased in parallel with the rise in obesity rates and associated diseases worldwide [1].

      Prior to the 1980s, adipocytes were only considered to be sites of fat storage. Whether lipid was released from or added to adipocytes depended on what hormones were active [2]. Recently, advances have been made in understanding adipocytes as an active metabolic machine in energy homeostasis and as secretors of molecules important for regulation of bodily processes such as blood pressure and angiogenesis [1]. For example, leptin, a hormone secreted by adipocytes and coded by the *obese* gene, was discovered to be important for body-weight homeostasis and appetite. Mice with an *ob/ob* loss of function mutation weigh three times greater than wild-type mice even when both sets of animals are fed the same diet [3]. Because of the critical role adipocytes are now known to play in metabolism, understanding of how cells differentiate into adipocytes and how adipogenesis is regulated have become increasingly important in addressing illnesses related to excess adiposity.

Regulation of adipogenesis

      The activation of specific transcription factors causes the pluripotent stem cell to commit to a particular cell lineage. Presence of runt-related transcription factor 2 (Runx2) and Sp7 transcription factor (Osx), for example, causes stem cells to differentiate into osteoblasts. Other transcription factors can also cause the progenitor cell to eventually mature into myocytes or

chondrocytes (Figure 1) [4]. The activities of several transcription factors, including peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding proteins (C/EBPs) lead to adipocyte cell determination [4, 5].

PPARγ has two isoforms, PPARγ1 and PPARγ2; the latter is found predominantly in adipocytes [5]. During differentiation of preadipocytes into adipocytes, PPARγ is induced and found to be both necessary and sufficient for adipogenesis [6]. More than 5000 binding sites for PPARγ have been identified in 3T3-L1 adipocytes [7]. Along with PPARγ, C/EBPs (C/EBPα, -β and -δ) have also been recognized to play important roles in proper adipogenesis [5]. Deletion of the C/EBP genes results in abnormal accumulation of fat. In C/EBPα-knockout mice, white and brown adipocytes did not amass lipid; the mice died eight hours after birth from liver abnormalities and hypoglycemia [8]. Deletion of C/EBPβ or C/EBPδ did not result in as severe of phenotypes as in C/EBPα mice, although fat abnormalities were also observed [9].

In addition to PPARγ and C/EBPs, there are more than 100 other pro-adipogenic factors expressed in adipocytes. Other transcription factors exist to also repress adipogenesis, indicating a complex transcriptional cascade for the differentiation pathway. Many anti-adipogenic factors that show decreased expression during adipogenesis display higher expression during differentiation of cells into other cell fates. While adipogenesis is mediated by a chain of transcription factors, other factors contribute to adipocyte differentiation. Wnt signalling, when activated, results in an increase in osteoblastogenesis and a decrease in adipogenesis. Additionally, bone morphogenetic protein 4 (BMP4) causes stem cells to commit to adipogenesis, while DLK1/PREF1 signalling has been shown to repress it [1]. Finally, as the recently discovered field of gene silencing emerges as a prominent factor in gene regulation, its role in adipogenesis has also been identified [10]. A recently discovered class of regulators termed

microRNAs (miRNAs), with a function similar to gene interfering molecules, has been evidenced to affect adipocyte differentiation. miR-143 was the first miRNA discovered to be important for preadipocyte maturation [11].

Gene interference and microRNAs

Newly discovered in the 1990s, post transcriptional gene silencing plays important roles in cellular function and gene regulation. Rather than acting through post-transcriptional or post-translational modification of genes, gene silencing involves directly degrading messenger RNA [10]. Gene silencing was recognized to occur in *Caenorhabditis elegans* where overproduction of antisense RNA homologous to the endogenous gene of interest resulted in disruption of expression and function of the endogenous gene [12].

RNA interference (RNAi), one form of gene silencing, involves introduction of foreign strands of double-stranded RNA (dsRNA) into cells and the subsequent splicing of the dsRNA into 19-21 nucleotide long sequences called short interfering RNA (siRNA) by dicer 1, ribonuclease type III (Dicer). The double-stranded siRNA is unwound by ATP helicase and complexed to the RNA-induced silencing complex (RISC), composed of various proteins, including the proteins from the Argonaute (Ago) family. The RNA-protein complex then binds to the mRNA to induce cleavage and successive degradation of the mRNA [10].

miRNAs function as another form of gene silencing and differ from RNAi in that miRNAs are endogenously produced. Found in viruses, plants, and animals, the noncoding miRNA is the inherent biological form of gene silencing in organisms [10, 13]. miRNAs were first discovered while researching larval development in *C. elegans*. Lee at al. discovered that *lin-4* causes a decrease in Lin-14 protein expression. Additionally, *lin-4* does not encode a protein, and parts of its sequence are complementary to regions in the 3'-untranslated (3'-UTR)

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region of lin-14 mRNA [13]. The discovery of the unique function that *lin-4* possesses resulted in an increase in miRNA research, including better understanding of how miRNAs are processed and how they bring about mRNA translational repression.

microRNA processing

miRNAs are transcribed in the nucleus into imperfectly formed stem-loop hairpin structures termed pri-miRNAs (Figure 2). RNase III type endonuclease Drosha processes the pri-miRNA, and the resulting pre-miRNA is exported out of the nucleus with the help of exportin5. In the cytoplasm, the protein Dicer cleaves the pre-miRNA, resulting in a 19-22 nucleotide long miRNA:miRNA* duplex. The duplex is unwound and one strand is usually degraded. At times, both strands are utilized in gene silencing. Finally, the mature miRNA is assembled onto the RISC. The miRNA, along with the RISC, then docks at the mRNA. Either the miRNA base pairs with the mRNA with perfect complementarity, resulting in mRNA cleavage similar to that in siRNA function, or the miRNA base pairs imperfectly, usually in the 3'-UTR region of the mRNA, resulting in translational repression [14].

miRNA with the RISC can bind to the mRNA 3'-UTR to result in deadenylation of the poly(A) tail and successive degradation of the mRNA. The miRNA-induced repression can also occur by slowing elongation at the post-initiation of translation stage or initiation block by repressed cap recognition. Repressed mRNAs are moved into P-bodies, discrete cytoplasmic granules, for storage [14].

Proteins from the Argonaute family is a crucial component of the RISC, which includes the aforementioned Argonaute proteins and also the mature miRNA which binds to complementary mRNA sites [14]. Results from manipulation of the Argonaute protein genes give evidence to the family's critical role in cell development, differentiation, and maintenance.

Eukaryotic translation initiation factor 2C (Ago2) has been identified in two separate studies as the only member of the Argonauate subfamily in humans to possess endonuclease characteristics and to cleave substrate RNA [15, 16]. Some Ago proteins may also need to be posttranslationally modified or interact with other proteins to be functionally active [17].

In addition to the Argonaute proteins, Dicer is also an important molecule in miRNA processing. Dicer is an RNAse III enzyme that cleaves pre-miRNA into miRNA duplexes which eventually give rise to the mature single-stranded mature miRNA [14]. Its importance in cell differentiation is evidenced in inhibition of osteoclast differentiation in cells transfected with Dicer siRNA. Additionally, mice with mutant Dicer$^{f/f}$ suffer from mild osteoporosis [18]. Research on effects of Dicer knockdown on adipogenesis in human multipotent stromal cells (HMSCs) shows that adipocyte differentiation in the HMSCs are inhibited when Dicer expression is decreased [19].

Along with an increased understanding of miRNA processing and of proteins such as Dicer and Ago2 that act in RNAi and miRNA function, the number of miRNAs reported and predicted also continues to rise. Today, more than 5300 miRNAs are known [10]. miRNAs have been found to be involved in a number of roles in the cell. A study of miRNAs during cardiogenesis discovered that miR-1 is activated during differentiation and targets heart and neural crest derivatives expressed 2 (Hand2), a transcription factor involved in ventricular cardiomyocyte expansion [20]. In hematopoiesis, miR-181 is a positive regulator of B-lymphoid cell differentiation [21]. The effects of miRNAs have also been observed in adipogenesis [11].

microRNAs function in adipogenesis

Through the use of antisense oligonucleotides (ASOs), Esau et al. determined that miRNA-143 regulates adipocyte differentiation. In microarrays that profiled miRNA expression

in preadipocytes and developing cells, miRNA-143 is upregulated in the differentiating

adipocytes. An ASO directed at miRNA-143 causes inhibition of adipocyte-specific gene

expression and decreases triglyceride accumulation. Mitogen-activated protein kinase 7 (ERK5)

is found to be upregulated in ASO-targeted cells and hypothesized to be the target gene for

miRNA-143 [11].

In another more recent study, Sun et al. utilized microarray analysis to determine

miRNAs that are highly expressed during 3T3-L1 differentiation. miRNA *let-7* is upregulated

and was found in subsequent analyses to inhibit adipocyte differentiation and clonal expansion

when introduced ectopically into 3T3-L1 cells. High mobility group AT-hook 2 (HMGA2)

expression is notably affected by *let-7* expression in cells; high expression of *let-7* corresponds to

low expression of HMGA2 in 3T3-L1 cells. The researchers concluded from the research results

that *let-7* affects adipocyte differentiation and targets HMGA2 [22].

Further evidence of miRNA involvement in adipogenesis and linkage to obesity was

demonstrated from a study in which miR-103 was characterized. Xie et al. showed that miR-103

is upregulated during adipogenesis and downregulated during obesity. When expressed in 3T3-

L1 preadipocytes, many adipocyte-important genes are upregulated, and there is increased

expression of PPARγ2 and molecules associated with metabolism and homeostasis. Interestingly,

miR-103 exhibits decreased expression in cells from *ob/ob* mice, suggesting that obesity results

in loss of miRNAs necessary for proper development of adipocytes [23]. In Esau et al., Sun et al.,

and Xie et al., miR-378/422b is mentioned to be among the miRNAs that are found to be

expressed at significantly different amounts between the differentiated and undifferentiated 3T3-

L1 cells.

microRNA 378/422b functions in adipogenesis

miR-378/422b, termed so due to both miR-378 and miR-422b originating from a common stem loop precursor, was first identified in human leukemia cells (HL-60) cells. The hairpin precursor is found in both *Homo sapiens* and *Mus musculus* [24]. In unpublished research conducted in the MacDougald laboratory, miR-378/422b was found to be upregulated when miRNAs were differentially screened for expression between 3T3-L1 preadipocytes and adipocytes via miRNA microarrays. Northern blot analysis validated the upregulation of the specific miRNA. Via a retroviral system, miR-378/422b was then overexpressed in ST2 cells during adipocyte differentiation to determine if there were phenotypic changes in cells that had miR-378/422b overexpression as compared to wild-type cells. Compared to wild-type cells, ST2 cells with miR-378/422b overexpression contained bigger lipid droplets on day 3 of differentiation. The results suggest that miR-378/422b plays a role in adipogenesis and lipid metabolism.

While miRNAs have been evidenced to function through translational repression, this method of function may not be the only process through which miRNAs work. Peroxisome proliferator-activated receptor gamma, coactivator 1 beta (PGC1β) is a coactivator that helps to regulate oxidative metabolism. In one study, variation in the coding region of PGC1β was suggested to be related to the pathogenesis of obesity [25]. Interestingly, the sequence of miR-378/422b is found within the first intron of PGC1β (Figure 3). Although the exact significance of the miR-378/422b sequence within the PGC1β gene is unknown, it is possible that the miR-378/422b sequence possesses an important role. Perhaps the intron that contains the miR-378/422b sequence acts independently to regulate PGC1β protein expression in the cell after it is cleaved from the PGC1β gene.

Purpose of investigation

The purpose of my investigation is to better discern the role that miRNAs and proteins involved in miRNA processing possess in adipogenesis. Specifically, I looked for potential target genes of miR-378/422b to better understand the process and pathway the miRNAs take in causing changes in adipocyte characteristics. Additionally, because of the essential role Ago2 plays in miRNA and RISC association with mRNA, I investigated phenotypic changes in adipogenesis in 3T3-L1 Ago2 knockdown cells. Additionally, the expression of Ago2 and Dicer over time during 3T3-L1 and ST2 differentiation was studied. The important roles Ago2 and Dicer play in miRNA processing and function provided a compelling reason to investigate them in my research. I hypothesized that my results would indicate a target gene of miR-378/422b and/or lead to better understanding of how the miRNA functions in adipogenesis. I also expected to find knockdown of Ago2 to result in abnormal differentiation of cells into adipocytes.

3T3-L1 preadipocyte cells and ST2 cells were the cells of choice used in my research. 3T3-L1 cells are derived from the mouse fibroblast line 3T3 and are capable of becoming adipocytes, taking on the characteristics of real adipose cells [26]. ST2 cells are a stromal cell line derived from mouse bone marrow and possess the ability to differentiate into adipocytes when induced by insulin [27].

Methods

Cell culture

3T3-L1 preadipocytes were grown and maintained using Dulbecco's modified Eagle's medium (DMEM) (GIBCO catalog #11965) with 10% calf serum (FCS) (Atlanta Biologicals) at 37°C and 10% CO_2. Cells that had reached confluence for two days were treated with methylisobutylxanthine, dexamethasone, and insulin (MDI). After two days, the medium was replaced with FCS supplemented only with insulin.

ST2 cells were grown and maintained at 37°C and 5% CO_2 using alpha-minimal essential medium with 10% fetal bovine serum (FBS), ascorbate, L-glutamine, and 100 units/ml streptomycin. For adipogenesis, once cells had reached confluence for one day, they were fed 10% FBS, MDI, and troglitazone (5 mM; Pfizer Inc., Groton CT). After two days, the medium was replaced with FBS supplemented with insulin only.

Western blots

Equal quantities of protein from lysed 3T3-L1 cells from day 9 were separated via SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% powdered nonfat milk (Bio-Rad, Hercules, CA). Solute carrier family 2 (facilitated glucose transporter), member 4 (Glut4), fatty acid binding protein 4, adipocyte (FABP4), and C/EBPα were each detected on the membrane with an antibody specific for Glut4, FABP4, and C/EBPα, respectively. Primary antibodies were probed using bound horseradish peroxidase-coupled (HRP-coupled) goat-anti-rabbit secondary antibodies; protein bands were visualized using Pierce Super Signal or Super Signal Ultra enhanced chemiluminescence substrates (Thermo Fisher Scientific Inc., Rockford IL).

Immunoprecipitation

To immunoprecipitate NCOR2, protein was extracted from confluent Hela cells, 3T3-L1 cells, ST2 cells infected with pMSCV-puro, and ST2 cells infected with miR-378/422b pMSCV-puro. The protein extracts were incubated with antibody against NCOR2. Sepharose-linked Protein G was then added to the protein extract. The solution was centrifuged and the supernatant removed. A Western blot was performed and the membrane was probed with a HRP-coupled goat-anti-rabbit secondary antibody. Bands were visualized using chemiluminescence.

Quantitative Real-Time PCR

RNA was extracted from harvested 3T3-L1 or ST2 cells using chloroform and purified with QIAGEN's RNeasy Mini Kit (QIAGEN Inc., Valencia, CA). Total RNA concentration was measured using a spectrophotometer. Using Taqman reagents and protocol, 1 µg of total RNA was transcribed to cDNA (Applied Biosystems, Foster City, CA). Gene expression levels were analyzed by quantitative PCR (qPCR) using MyiQ real-time PCR detection system (Bio-Rad, U.K.). Amplified products were detected using SYBR Green fluorescent dye. TATA-box binding protein (TBP) was used to normalize gene expression. Fold change was calculated based on the levels from TBP normalized against the samples amplified by Dicer primers or Ago2 primers. The primer sequences for Ago2 were: 5'-CTGTTGGCCGTTCCTTCTTC-3' (F) and 5'-ACTTCTCTGCCCCCACCC-3' (R). The primer sequences for Dicer were: 5'-CCAAC CTGCTCATTGCAACA-3' (F) and 5'-TGCCCGTCCTTTAGACTGGA-3' (R). Primers used for TBP were designed as reported by St-Pierre et al. [28]: 5'-ACCCTTCACCAATGACTC CTATG-3' (F) and 5'- TGACTGCAGCAAATCGCTTGG-3' (R).

Transient Transfection

PCR was used to amplify 300-400 bp long sequences from the 3'-UTR of DIX domain containing 1 (Dixdc1), LIM domain containing preferred translocation partner in lipoma (LPP), Sp1 transcription factor (Sp1), nuclear receptor interacting protein 1 (Nrip1), and nuclear receptor co-repressor 2 (NCOR2) from mouse genomic DNA. All amplified sequences included the 7-bp mir-378/422b seed: 5'-UCAGGAA-3'. PCR products were inserted into the TOPO Cloning Vector (Invitrogen Corp., Carlsbad, California), containing a Kanamycin-resistant gene, and then transformed into DH5α cells. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit or the QIAGEN Plasmid Maxi Kit (QIAGEN Inc., Valencia, CA), depending on the amount of DNA needed. Colonies were grown on LB Kanamycin plates, and colonies were selected for sequencing. Clones with correct sequences were enzyme digested and ligated into the pGL3-Control Vector (Promega BioSciences, San Luis Obispo, CA) that contains a luciferase-based reporter gene. The FuGENE-6 Transfection Reagent (Roche, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen Corp., Carlsbad, California) was used to transfect pGL3 plasmids with and without the 3'UTR inserts from Dixdc1, LPP, NCOR2, Nrip1, or Sp1 into NIH/3T3 cells or 293T cells. For the luciferase assays conducted for Dixdc1 and LPP, pcDNA3.1 and pcDNA3.1+miR-378/422b plasmids were also transfected into the same cells. Because the pMSCV vector was more stable, for the luciferase assays conducted for NCOR2, Nrip1, and Sp1, pMSCV-puro and miR-378/422b pMSCV-puro were used in place of pcDNA3.1 and pcDNA3.1+miR-378/422b. pRL-SV40 Renilla plasmid (Promega BioSciences, San Luis Obispo, CA) was used to normalize the luciferase activity. After 24 hours, the cells were harvested and a luciferase/Renilla assay was conducted for all samples using a luminometer. There were three samples for each treatment. Analysis of variance (ANOVA) statistical analysis

was performed using GraphPad Prism 5.0 software with a Bonferroni post-hoc test to determine statistical significance for the measurements. The Dixdc1 sequence was amplified using: 5'-CACTCCCTGGCTGCTTCTC-3' (F) and 5'-GTCTGCACTTGGGCTTTCAT-3' (R). The LPP sequence was amplified using: 5'-AGACACCTCGCTTTGCAGTT-3' (F) and 5'-GCTGTGTGACAATGGCCTAA-3' (R). The NCOR2 sequence was amplified using: 5'-GAGCGCTCTGGCTTTGGT-3' (F) and 5'-AGAAGATTGATTTAAAGACATCATGG-3' (R). The Nrip1 sequence was amplified using: 5'-ATACATCTAGGGAAATCGGGTTCGGAATAC-3' (F) and 5'- TTACTTCTAGAGCT GTGCAGAGGACAGTTTG-3' (R). The Sp1 sequence was amplified using: 5'- ATACATCT AGTCCATTTGGTCCTTTCTATGC-3' (F) and 5'-TTACTTCTAGAGCCCTCTTCCTCCAG AGATG-3' (R).

Knockdown of Ago2and Dicer in 3T3-L1

21-nt long short hairpin RNA loops were designed to knockdown Ago2 mRNA and Dicer mRNA. The sequences were designed as reported by Schmitter et al. [29]. Ago2_sh2: 5'-GATCCCGCAGGACAAAGATGTATTATTCAAGAGATAATACATCTTTGTCCTGCTTTT TGGAAA-3' (F) and 5'AGCTTTTCCAAAAAGCAGGACAAAGATGTATATCTCTTGAAT AATACATCTTT GTCCTGCGG-3' (R). Dicer_sh2: 5'-GATCCCATTGGCTTCCTCCTGGTT ATGTTCAAGAGACATAACCAGGAGGAAGCCAATTTTTGGAAA-3' (F) and 5'-AGCTTT TCCAAAAAATTGGCTTCCTCCTGGTTATGTCTCTTGAACATAACCAGGAGGAAGCCA ATGG-3' (R). The forward and reverse oligo strands were annealed and cloned into the pSUPERIOR.retro.puro vector and linearized with BglII and HindIII. The vector was transformed into DH5α cells. The correct inserts were confirmed by sequencing. Retroviral plasmids were transfected into 293T cells by calcium phosphate coprecipitation. Media from the 293T cells that had been transfected with the virus was collected and filtered through a 0.44 µm

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sterile Millipore filter into tubes with polybrene. The media was then poured onto subconfluent

3T3-L1 preadipocytes. Cells were split after they had reached 80% confluence. 3T3-L1 cells

were transfected with pSUPERIOR control and pSUPERIOR+Ago2. 2 sets of 3T3-L1 cells were

transfected with pSUPERIOR control, pSUPERIOR with the short hairpin Ago2 (shAgo2), and

pSUPERIOR with the short hairpin Dicer (shDicer). On day 9, RNA was extracted from each set

of cells and reverse transcribed via RT-PCR to produce cDNA. Then, Real-Time Quantitative

PCR was conducted for each set of cells with TBP, Ago2, and Dicer primers to confirm that

Ago2 and Dicer were knocked down (data not shown).

Thin-Layer Chromatography

Cells were incubated for 2 hours in the presence of [^{14}C]acetic acid and were then lysed

with 0.5 mL of methanol. 1 mL of chloroform was then added and mixed. Finally, an additional

0.5 mL of 0.1 N HCl was added and mixed. The mixture was centrifuged at $500 \times g$ for 30 min.

The organic phase was washed with 0.5 mL of distilled water twice, and the dried pellet was

resuspended in chloroform. For separation of extracts, dichloroethane-acetic acid (100:1, vol/vol)

was used. Accumulation of extracts on the silica gel plates were exposed to film. A two-tailed

Student's t-test was used to analyze significance of the data.

Results

Identification of miR-378/422b target genes

To investigate whether predicted target genes were true targets for miR-378/422b, NIH/3T3 cells or 293T cells were transfected with either the pGL3 control vector or the pGL3 control vector containing the 3'UTR sequences of the respective target gene in the presence of pcDNA3.1 or pcDNA3.1+miR-378/422b plasmids, or pMSCV-puro or miR-378/422b pMSCV-puro. pRL-SV40 Renilla plasmid was used to normalize the luciferase activity. After 24 hours, the cells were harvested and a luciferase/Renilla assay was conducted for all samples. A significant ($p<0.05$) decrease in luciferase activity when cells are transfected with the pcDNA3.1/pMSCV-puro vector with the miR-378/422b insert in the presence of pGL3+3'UTR compared to cells transfected with only the pcDNA3.1/pMSCV-puro vector in the presence of pGL3+3'UTR is expected if the predicted target gene is actually a target of miR-378/422b. Alternatively, a significant decrease ($p<0.05$) in luciferase activity when cells are transfected with pcDNA3.1/pMSCV-puro vector with the miR-378/422b insert in the presence of pGL3+3'UTR compared to cells transfected with pcDNA3.1/pMSCV-puro with miR-378/422b would also suggest the gene to be a target of miR-378/422b.

In the luciferase assay conducted for Nrip1, no significant changes are observed in luciferase activity between any of the treatments, suggesting that Nrip1 is not a target gene of miR-378/422b (Figure 5*D*). For Dixdc1, LPP, and Sp1, there are significant increases in luciferase activity when both miR-378/422b and the 3'-UTR sequence are present compared to when only miR-378/422b is present, suggesting an interaction between the miRNA and the 3'-UTRs, although there is an increase observed rather than a decrease (Figure 5*A*, *B*, *C*). However, there are also significant increases in luciferase activity when cells were transfected with

pcDNA3.1/pMSCV-puro and the pGL3+3'-UTR sequence compared to cells transfected only

with pcDNA3.1/pMSCV-puro, suggesting that the presence of the 3'-UTR sequence alone may

have caused a change in luciferase activity. In addition, specifically for predicted target gene Sp1,

there is a significant increase in luciferase activity when both the 3'-UTR and the miRNA are

present compared to when only the 3'-UTR is present. However, there is also a significant

difference in luciferase activity between the two treatments in the control. Based on the data

available, Nrip1, Dixdc1, LPP, and Sp1 do not appear to be targets of miR-378/422b. The

luciferase assays should be repeated to determine the reason for the unexpected increases in

luciferase activity.

NCOR2 is the only predicted target gene in which a significant decrease in luciferase

activity is observed in cells transfected with pMSCV-puro vector containing the miR-378/422b

seed in the presence of pGL3+3'UTR compared to cells transfected with pMSCV-puro and the

miR-378/422b seed only (Figure 5*E*). However, there is also a significant increase in luciferase

activity in cells transfected with the pMSCV-puro vector with the miR-378/422b in the presence

of pGL3+3'UTR compared to cells that are transfected with only the pMSCV-puro vector in the

presence of pGL3+3'UTR. Additionally, there is a significant difference in activity between the

two treatments in the control. A significant decrease in luciferase activity is also observed when

cells are transfected with pMSCV-puro and the pGL3+3'-UTR sequence compared to cells

transfected only with pcDNA3.1. Because the luciferase assay results for NCOR2 are

inconclusive, an immunoprecipitation was done specifically for NCOR2.

Confirmation that NCOR2 is not a target gene for miR-378/422b

Because of the contradictory luciferase assay results of NCOR2, an immunoprecipitation

was conducted to confirm that NCOR2 is not a target gene for miR-378/422b. Protein was

extracted from 3T3-L1 cells, ST2 cells infected with pMSCV-puro, and ST2 cells infected with miR-378/422b pMSCV-puro. Bands blotted from samples before the immunoprecipitation served as controls. NCOR2 protein was expressed in ST2 cells infected with pMSCV-puro, ST2 cells infected with miR-378/422b pMSCV-puro, and 3T3-L1 cells in bands blotted from samples after the immunoprecipitation (Figure 6). However, no decrease in NCOR2 protein expression is seen in ST2 cells infected with miR-378/422b pMSCV-puro compared to ST2 cells infected with pMSCV-puro only. The results of this immunoprecipitation suggest that NCOR2 is not a target gene of miR-378/422b. However, because the data is from one sample, to definitively confirm that NCOR2 expression levels do not change with overexpression of miR-378/422b, more immunoprecipitations will need to be done with a greater number of samples.

Gene expression levels of Ago2 and Dicer in ST2 and 3T3-L1 cells

To determine Ago2 and Dicer gene expression levels in ST2 and 3T3-L1 cells, RNA was extracted from harvested 3T3-L1 or ST2 cells, and expression levels were analyzed by quantitative PCR. The data was based off of only one sample, so no error bars could be calculated (Figure 7). However, the preliminary results of this experiment show that Dicer expression stays relatively constant throughout the six days of differentiation in both ST2 and 3T3-L1 cells, with a slight increase on day 4 in both types of cells. Ago2 expression stays relatively constant in 3T3-L1 cells, but a large increase in expression is exhibited on day 4 in ST2 cells. This preliminary experiment suggests no substantial difference in Dicer expression during adipocyte differentiation. However, changes in expression of Ago2 during adipogenesis in ST2 cells indicate that 3T3-L1 and ST2 cells vary in how they differentiate. Furthermore, the data indicates that Ago2 expression levels do change during differentiation. Further experimentation must be conducted to give definitive conclusions, however.

Lipid metabolism in Ago2 knockdown and Dicer knockdown cells

To produce Ago2 knockdown and Dicer knockdown cells, 3T3-L1 cells were transfected with pSUPERIOR control and pSUPERIOR+Ago2. Two sets of 3T3-L1 cells were transfected with pSUPERIOR control, pSUPERIOR with the shAgo2, and pSUPERIOR with the shDicer. Thin-Layer Chromatography was used to separate extracts (Figure 8). A comparison between the control versus the shAgo2 samples shows that Ago2 knockdown results in a significant decrease in triacylglycerol ($p<0.05$). No differences in triacylglycerol accumulation are seen between the control and shDicer. Both shDicer extracts and shAgo2 extracts exhibit significant decreases in phospholipid ($p<0.05$). The decrease in lipid metabolism in Ago2 knockdown cells indicate that Ago2 plays a crucial role in lipid metabolism in differentiating adipocytes. Additionally, the observation that Dicer knockdown cells did not exhibit changes in triacylglycerol accumulation but did display a decrease in phospholipid content compared to control cells suggests that Dicer plays a role in lipid metabolism, but perhaps one that is not as over-encompassing as that of Ago2. Because the initial cells from the extract were incubated with $[^{14}C]$acetic acid, the decrease in triacylglycerol and phospholipid from shAgo2 knockdowns indicate that Ago2 plays a crucial role in lipogenesis, suggesting that there is an abnormality in the conversion of acetate to fatty acids when Ago2 is knocked down.

Adipocyte differentiation in Ago2 knockdown cells

Equal quantities of protein from lysed 3T3-L1 Ago2 knockdown cells from Day 9 were separated via SDS-PAGE and transferred onto nitrocellulose membranes. Glut4, FABP4, and C/EBPα were each detected on the membrane with use of antibodies, and protein bands were visualized. As three common adipocyte markers, changes in Glut4, FABP4, and C/EBPα expression in Ago2 knockdown cells indicate that Ago2 affects cell differentiation. No changes

are observed in protein expression in cells transfected with pSUPERIOR+Ago2 compared to

cells transfected with pSUPERIOR only in blots prepared for all three adipocyte markers (Figure

9). The results of this experiment show that Ago2 does not have a significant effect on adipocyte

differentiation in 3T3-L1 cells.

Discussion

The purpose of this research was to better elucidate the roles of miRNAs and the known miRNA-processing proteins in adipogenesis. A core component of my investigation was focused on locating the target gene(s) of miR-378/422b, termed so because it is unknown which strand of the stem in the stem-loop precursor eventually becomes the mature, functional miRNA. miR-378 and miR-422b were both upregulated when miRNAs were differentially screened for expression between 3T3-L1 preadipocytes and adipocytes via miRNA microarrays. Furthermore, overexpression of miR-378/422b in ST2 cells led to increased lipid droplets on day 3 of differentiation (Figure 4). Because these preliminary results indicate that miR-378/422b influences adipogenesis in some manner, it was chosen to be the main miRNA of focus in my research.

Using the method of transient transfection, cells were transfected with vectors containing the miR-378/422b seed and vectors containing a 300-400 3'-UTR base pair insert of the predicted target gene. Because miRNAs are understood to function by repressing translation, a decrease in luciferase activity is expected when both miR-378/422b and the 3'-UTR of the target gene with the miR-378/422b seed are transfected into cells. The luciferase assay results from the transfection did not show conclusively that Nrip1, NCOR2, Sp1, Dixdc1, or LPP to be target genes of miR-378/422b (Figure 5). Although my research did not pinpoint a target gene of miR-378/422b, this does not eliminate the possibility that a target gene for the specific miRNA does not exist. Based on TargetScan 4.2, there are 334 predicted targets of miR-378/422b. One or more of these predicted targets, which I have not yet explored, may be the target of miR-378/422b. In addition to the possibility that the target gene for miR-378/422b exists but has not been identified, there is the possibility that miR-378/422b functions in a manner that differs from

the conventional way that miRNAs have been known to act. The fact that the miR-378/422b

sequence is found to be located within the first intron of PGC1β provides a compelling reason to

investigate the possibility that the specific sequence gives PGC1β a unique function in its action

as a coactivator in metabolic pathways (Figure 3).

Another indication that miRNAs may act in ways still unknown comes from a

preliminary study conducted in the MacDougald laboratory suggesting that miR-378/422b may

be a transactivator of the leptin promoter in the presence of C/EBPα. Based on luciferase assay

results, introduction of the miR-378/422b seed into cells with a wild-type leptin promoter in the

presence of C/EBPα results in an increase in relative luciferase activity compared to the

introduction of the miR-378/422b seed into cells with a leptin promoter mutated in the C/EBP

binding site in the presence of C/EBPα. When miR-10a, a miRNA with a different sequence

from miR-378/422b, is used in place of miR-378/422b in the experiment, no changes are seen in

relative luciferase activity between the use of the wild-type leptin promoter and the use of a

leptin promoter mutated at the C/EBP binding site. The results of this experiment suggest that

miR-378/422b, unlike miR-10a, is specific for leptin and C/EBPα, although the exact mechanism

for how miR-378/422b can cause increased activity is unknown. Yet, the data suggests that miR-

378/422b may not function in the conventional method of translational repression.

Because Ago2 is an integral part of the miRNA processing procedure, I investigated the

effects Ago2 knockdown had on adipocyte differentiation and metabolism. After creating Ago2

knockdown cells, I conducted a Western blot of key adipocyte markers to assess any changes in

adipocyte differentiation. There are no differences seen in protein expression of Glut4, FABP4,

and C/EBPα between the control cells and the Ago2 knockdown cells (Figure 9). From these

results, I conclude that Ago2 does not visibly affect adipocyte differentiation. The data suggests

that Ago2 does not process any miRNAs that target Glut4, FABP4, and C/EBPα; if Ago2 does, I would have expected to see changes in protein expression of the adipocyte markers in the knockdown cells compared to the control cells. Another explanation for the results is that Ago2 does process the miRNAs that target the three adipocyte markers, but perhaps another processing protein, or another member of the Argonaute family yet unidentified, is capable of complementing the role of Ago2 in the cell. In a very recent publication by Iwasaki et al. studying translational repression in *Drosophila*, Ago1-RISC is able to induce translational repression through deadenylation of mRNA. Also, Ago1 is suggested to inhibit translation of mRNA after m^7G-cap recognition. Ago2, on the other hand, is believed to repress translation of mRNA by blocking the function of the m^7G-cap structure [30]. To fully determine the role of Ago2 in adipocyte differentiation and metabolism will require further research into the other members of the Argonaute family and their ability to translationally repress mRNA. Additionally, other adipocyte markers should be assessed via Western blot to determine if Ago2 knockdown affects the protein expression of adipocyte markers that I did not investigate in my research.

To better understand the role Ago2 and Dicer may play in adipocyte-related mechanisms, I studied lipid metabolism via Thin-Layer Chromatography. Comparisons were made of triacylglycerol and phospholipid content in control cells, Ago2 knockdown cells, and Dicer knockdown cells with incorporation of $[^{14}C]$acetic acid (Figure 8). While no changes are seen in triacylglycerol content in Dicer knockdown cells, changes are observed in the Ago2 knockdown cells. For both triacylglycerol and phospholipid content, shAgo2 samples have decreased quantities compared to the control. shDicer samples only show decreased quantities in phospholipid content compared to the control. Because the initial cells from the extract are incubated with $[^{14}C]$acetic acid, the decrease in triacylglycerol and phospholipid from shAgo2

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knockdowns indicate that Ago2 plays a crucial role in lipid metabolism, suggesting that Ago2 knockdown leads to an abnormality in the conversion of acetate to fatty acids. Perhaps Ago2 affects, indirectly through miRNAs or even directly in an unknown manner, genes involved in lipid metabolism. Dicer may also directly or indirectly affect miRNAs involved in lipid metabolism, but perhaps it affects those that are more specifically involved in phospholipid metabolism rather than triacylglycerol metabolism. Although not examined in my research, expression of key lipid metabolism genes such as PPARγ should be analyzed when Ago2 is knocked down to determine if shAgo2 affects such genes. Expression of common adipocyte differentiation markers and other lipid metabolism markers should also be examined in shDicer cells via Western blots and Thin-Layer Chromatography, respectively.

To gain a better understanding of Ago2 and Dicer characteristics during adipogenesis, qPCRs were conducted to study Ago2 and Dicer expression levels throughout differentiation in 3T3-L1 and ST2 cells. Both Ago2 and Dicer expression levels stay relatively constant throughout differentiation in 3T3-L1 cells, while Ago2 expression has a substantial increase in ST2 cells on day 4 (Figure 7). This preliminary experiment suggests no substantial difference in Dicer expression during adipocyte differentiation. However, changes in expression of Ago2 during ST2 cell differentiation suggest that 3T3-L1 and ST2 cells have distinct properties during adipogenesis. Furthermore, the data indicates that Ago2 expression levels do change during differentiation. The results from this experiment were compared to data retrieved from NCBI's GEO Datasets. Gene expression of Ago2 at various points in 3T3-L1 preadipocyte differentiation display an increase in expression from preconfluence to 12 hours, followed by a decrease in expression until day 3 and a subsequent increase in expression until day 7 [31]. Although expression of Ago2 was analyzed at different time points during adipogenesis of 3T3-L1 cells in

my experiment, the constant Ago2 expression observed throughout the days of differentiation analyzed (day 0, day 2, day 4, and day 6) in my data differs greatly from the rises and falls in expression in the GEO Dataset. Although a set of results on Dicer expression in 3T3-L1 cells were found in the GEO Dataset, the investigators who provided the data expressed no confidence in accuracy of the data. No GEO Dataset is present for Ago2 and Dicer expression in ST2 cells. While my initial experiment suggests differences in Ago2 and Dicer expression in ST2 cells and also differences in Ago2 expression between ST2 and 3T3-L1 cells, no definite conclusions can be made. Because the qPCR for Ago2 and Dicer expression levels only involved one sample, this experiment will have to be repeated with a greater number of samples to gain better data accuracy. Once more data has been gathered, this can be compared with the GEO Dataset results for Ago2 expression in 3T3-L1 cells again.

My research attempted to provide a better understanding of miRNA processing and to decipher specific miRNAs involved in adipogenesis. While miR-378/422b has been determined to play a role in proper adipocyte development, no target gene of miR-378/422b was found in my research. Preliminary studies also suggest miR-378/422b may function unconventionally and perhaps as a transactivator. The experiments conducted in this research together indicate that Dicer is involved in lipid metabolism and that Ago2 is involved in lipid metabolism rather than adipocyte differentiation. Perhaps Ago2 only processes a specific group of miRNAs involved in similar functions. However, with new insight into the other members of the Argonaute family, it may be possible that other Ago proteins complement Ago2's functions in the cell.

Because of the novelty of research on the role of miRNA in adipogenesis, this investigation served as a basis for further research on understanding the variety of ways miRNAs function in the cell during differentiation and development. Further studies to better elucidate the

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specific role of miR-378/422b include investigations of other potential target genes and further research into the possibility of miRNAs functioning in ways other than through translational repression. Again, repetitions of the experiments conducted in my research will be vital in gathering stronger data, leading to more conclusive results. Additionally, because of the intricate balance between osteogenesis and adipogenesis and the common set of proteins that affect both differentiation pathways, it is worthwhile to investigate changes in osteoblast markers when miRNAs, such as miR-378/422b, are manipulated.

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Figure 1. Commitment of mesenchymal stem cells to a cell lineage depends on various factors.

Various factors determine the outcome of mesenchymal stem cell (MSC) differentiation, including cytokines, transcription factors, and hormones. PPARγ and C/EBPs are important transcription factors for committing MSCs to the adipocyte lineage [4].

Figure 2. miRNA processing begins in the nucleus and the mature form exhibits its activity in the cytoplasm.

DNA coding for miRNA is transcribed in the nucleus by RNA Polymerase II and the resulting pri-miRNA is initially processed by RNase III enzyme Drosha. The pre-miRNA contains a hairpin loop and imperfect stem. Once it is exported out of the nucleus by exportin 5, it follows a processing pathway similar to that of siRNA. Dicer cleaves the hairpin loop off of the pre-miRNA, leaving a miRNA:miRNA* duplex. The duplex is unwound and assembled onto the RISC. Here, Ago2 of the Argonaute family of proteins, joins the assembly. The final assembled complex can follow one of two paths. (A) The miRNA binds with imperfect complementarity to the 3'-UTR region of the mRNA, resulting in translational repression, or (B) the miRNA binds with perfect complementarity to the mRNA, resulting in mRNA cleavage [14].

Figure 3. The miR-378/422b sequence is found within PGC1β.

miR-378 and miR-422b are derived from the same stem-loop precursor. (A) miR-378 is derived from the 5' end of the precursor while miR-422b is derived from the 3' end. (B) The miR-378/422b sequence is found within the first intron of PGC1β, shown in red. The bars displayed below the PGC1β gene show that the area in which the miR-378/422b sequence is present in PGC1β is highly conserved amongst organisms. This figure is used with permission from Dr. Isabelle Gerin.

Figure 4. Overexpression of miR-378/422b in ST2 cells leads to increased lipid accumulation at day 3.

To begin the study of possible miRNA roles in adipocyte differentiation and metabolism, miRNAs were differentially screened between 3T3-L1 preadipocytes and adipocytes using microarrays. Northern blot analysis confirmed the up- or down-regulation of several miRNAs during adipocyte differentiation. One specific miRNA, miR-378/422b, is upregulated during adipocyte differentiation. Thus, the miRNA was overexpressed in ST2 cells using a retroviral system. Compared to (A) control cells, (B) overexpression of miR-378/422b corresponds to increased lipid accumulation in the adipocytes at day 3. Approximately the same number of adipocytes can be found in any given field in both samples, but cells with an overexpression of miR-378/422b contain larger lipid droplets.

Figure 5. Dixdc1, LPP, Nrip1, Sp1, and NCOR2 are not targets of miR-378/422b.

293T cells were transiently transfected as described in *Methods.* Error bars were based on standard deviations from the mean of the relative luciferase activities of three samples. The controls for each luciferase assay were cells transfected with pGL3 and with pcDNA3.1/pMSCV-puro with or without miR-378/422b. The experimental samples were cells transfected with pGL3+3'UTR of the respective target gene and pcDNA3.1/pMSCV-puro with or without miR-378/422b. Analysis of variance (ANOVA) statistical analysis was performed using GraphPad Prism 5.0 software with a Bonferroni post-hoc test to determine statistical significance for each measurement, with $p < 0.05$ (*), $p < 0.01$ (#), and $p < 0.001$ (+). Relative luciferase activity is shown for (A) Dixdc1, (B) LPP, (C) Sp1, (D) Nrip1, and (E) NCOR2.

Figure 6. Immunoprecipitation of NCOR2 confirms that it is not downregulated when miR-378/422b is overexpressed.

An immunoprecipitation experiment was conducted to study NCOR2 protein expression levels. No considerable NCOR2 protein bands are visible in the samples before immunoprecipitation, and a sample from HeLa cells was used as a control. The amount of protein between ST2 cells infected with pMSCV-puro and cells infected with miR-378/422b pMSCV-puro are quantitatively similar. NCOR2 protein is also present in the 3T3-L1 cell sample, but in a lower amount.

Figure 7. Dicer mRNA expression differs from Ago2 mRNA expression during differentiation in ST2 and 3T3-L1 cells.

A qPCR was conducted to measure levels of Dicer and Ago2 expression in adipocyte differentiation of (A) ST2 cells and (B) 3T3-L1 cells. Dicer expression levels stay relatively constant throughout the six days of differentiation in both the ST2 and 3T3-L1 cells. Ago2 expression levels stay constant in the 3T3-L1 cells but there is a noticeable increase in expression on day 4 in ST2 cells.

Figure 8. Ago2 knockdown affects lipid metabolism.

Thin-Layer Chromatography of cells incubated with [^{14}C]acetic acid was conducted to study changes in lipid metabolism in Ago2 knockdown cells and Dicer knockdown cells. A two-tailed Student's t-test was used to analyze significance of the data ($p < 0.05$). The extracts of triacylglycerol from shAgo2 compared to the control show that there is a significant decrease in triacylglycerol when Ago2 is knocked down. No significant difference is observed in triacylglycerol quantities between the control and shDicer. Both shAgo2 and shDicer samples exhibit a significant decrease in phospholipid concentration when compared to the control.

Figure 9. Ago2 knockdown does not affect adipocyte differentiation.

Western blots were conducted to investigate any quantitative changes in adipocyte markers expressed in Ago2 knockdown cells compared to control cells. The Western blots conducted of three different adipocyte markers, (A) Glut4, (B) FABP4, and (C) C/EBPα, show no significant changes in expression in each of the proteins between the pSUPERIOR control and the Ago2 knockdowns. This suggests that Ago2 does not directly affect differentiation in adipocytes.

Figure 1.
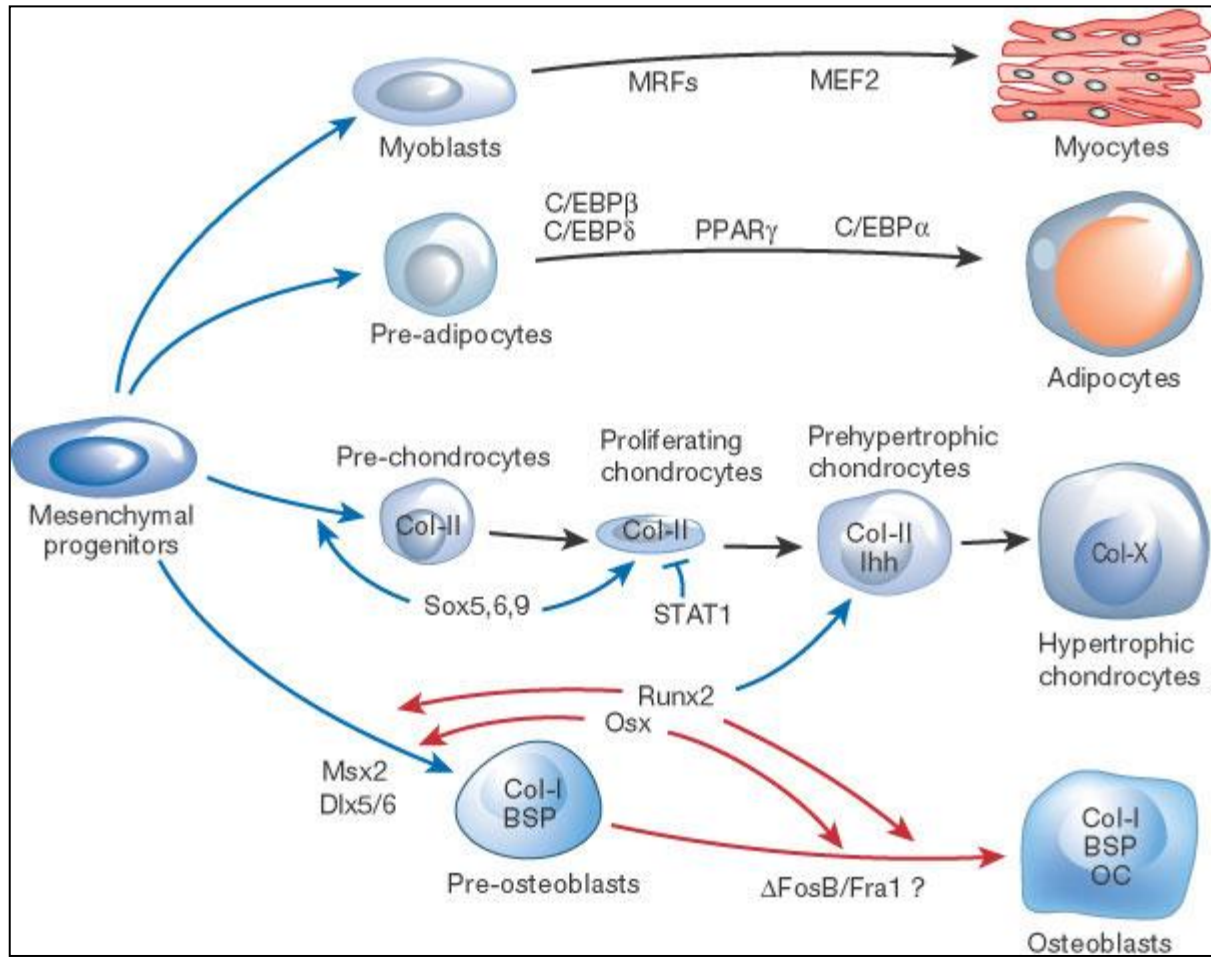
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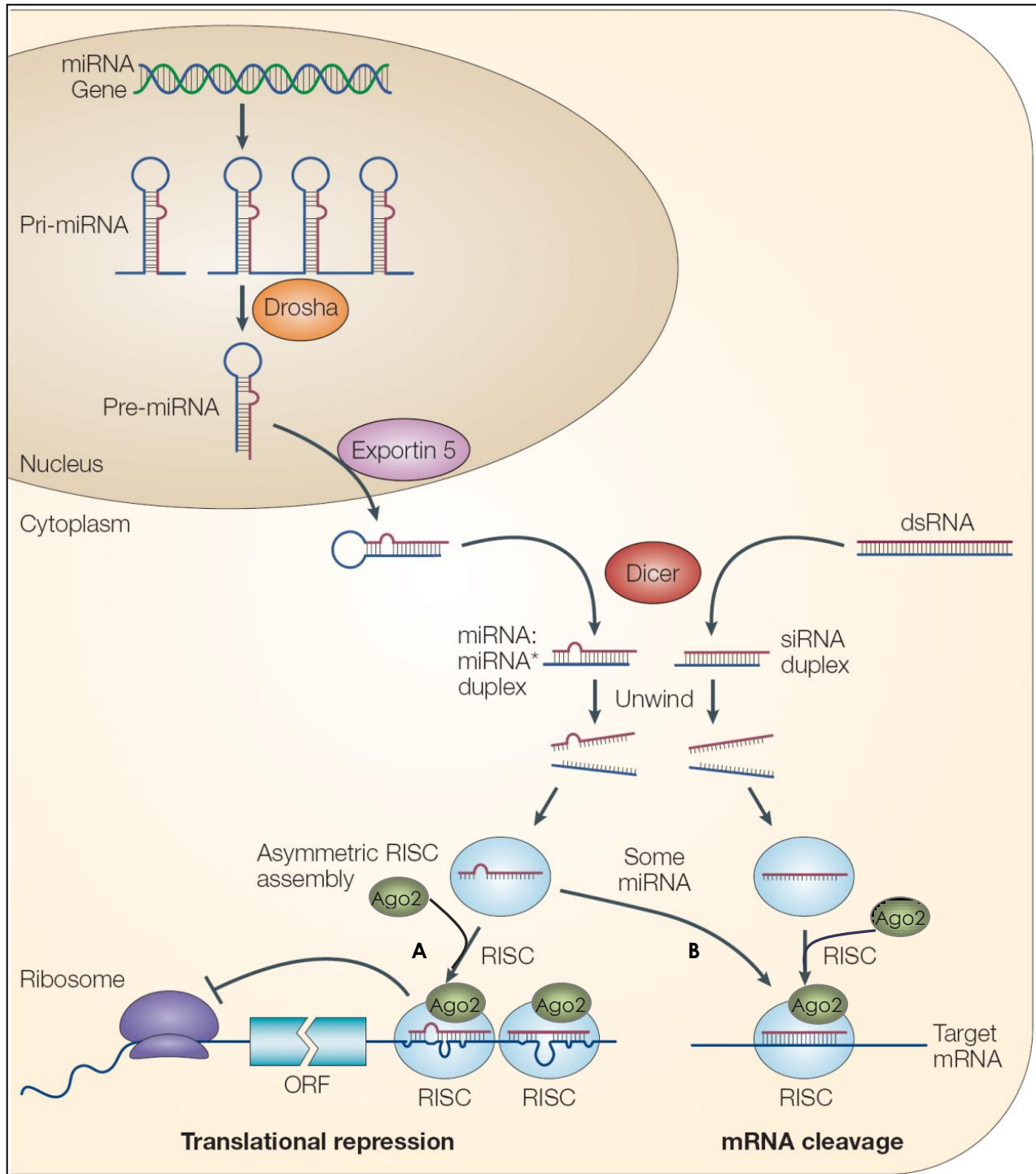
Figure 3.

A
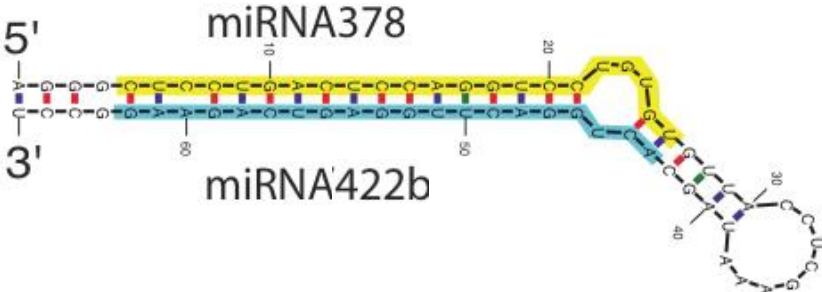
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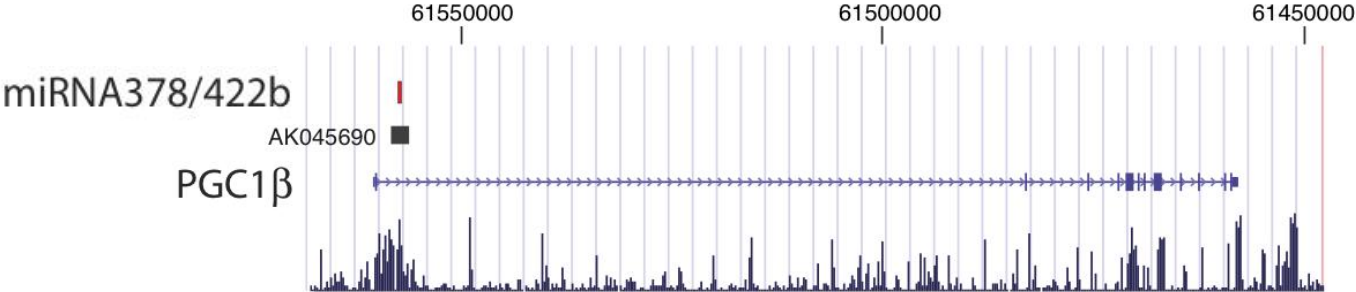
Figure 4.

A

Control

B

miR-378/422b

Figure 5.

A

B

C

D

E

pcDNA3.1/pMSCV-puro

pcDNA3.1/pMSCV-puro
+ miR-378/422b

39
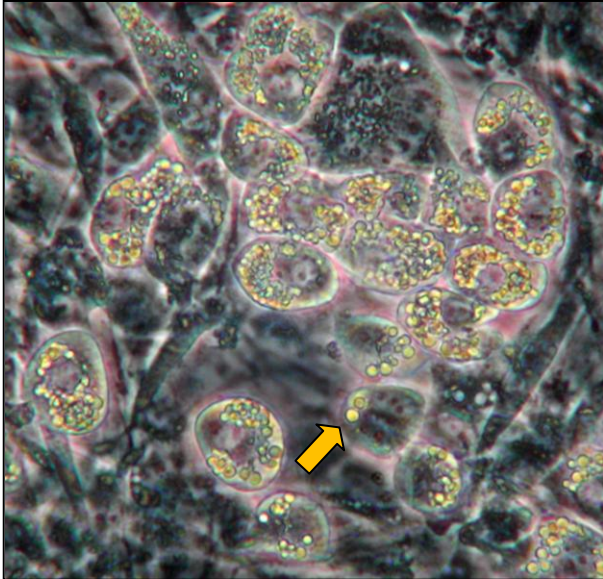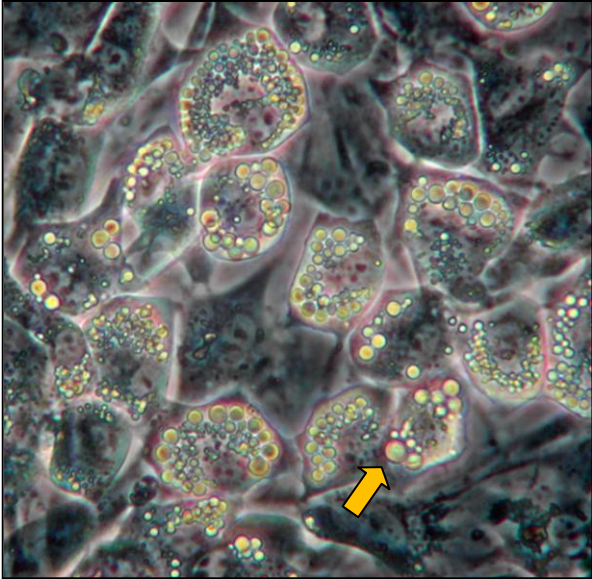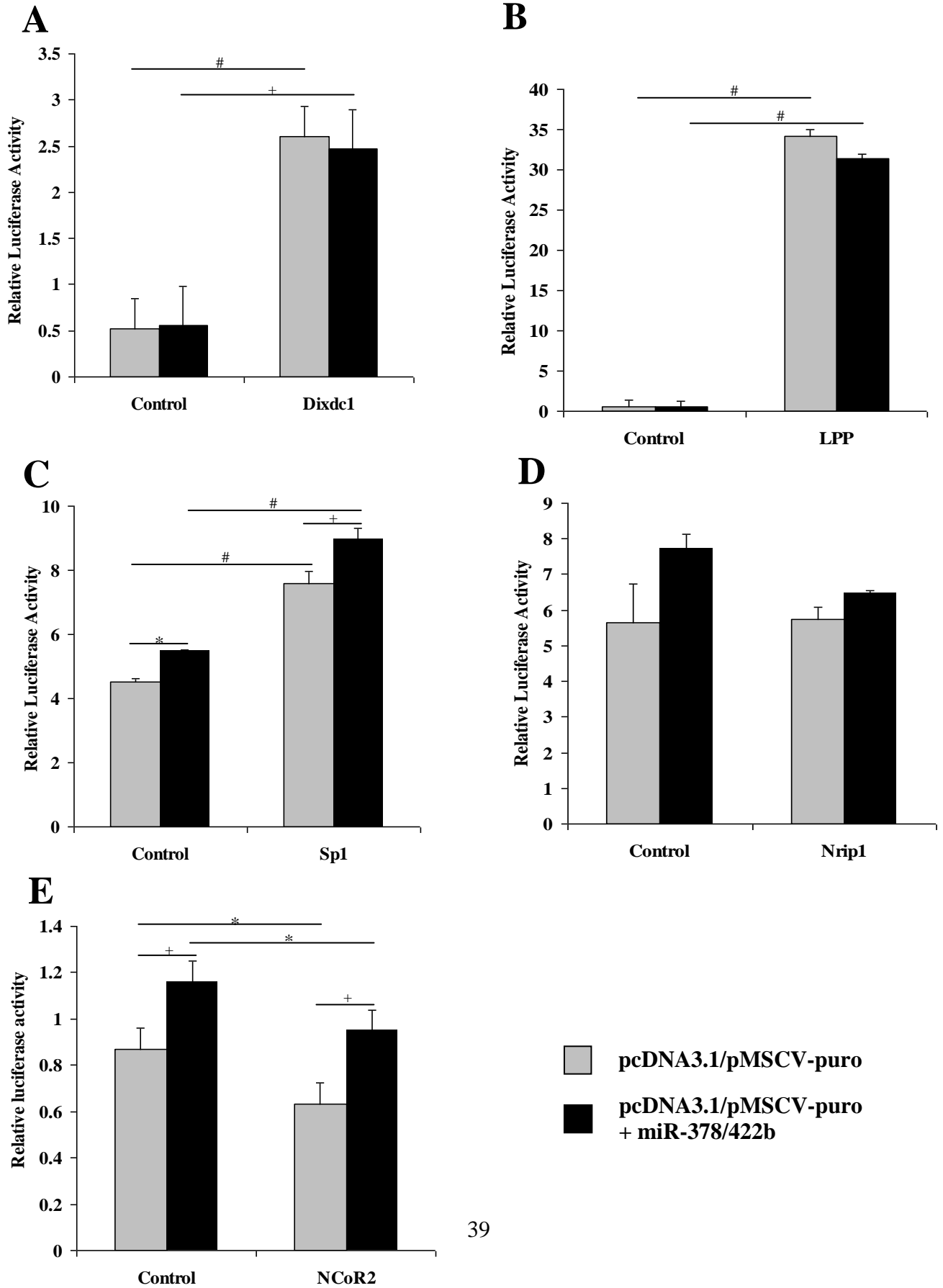
Figure 6.

NCOR2

Figure 7.

A

ST2 Cells

B

3T3-L1 Cells
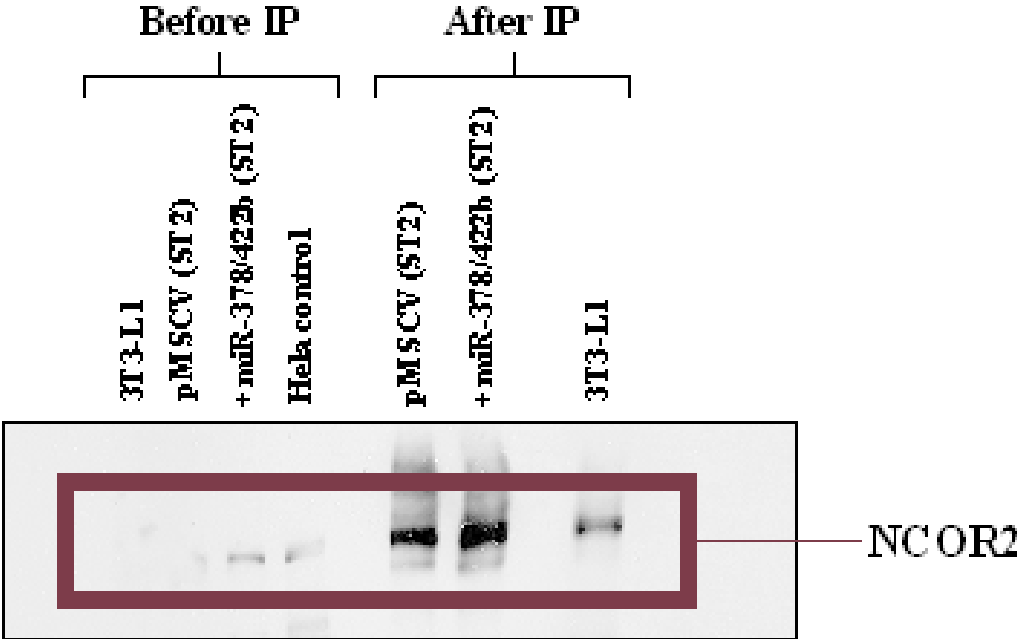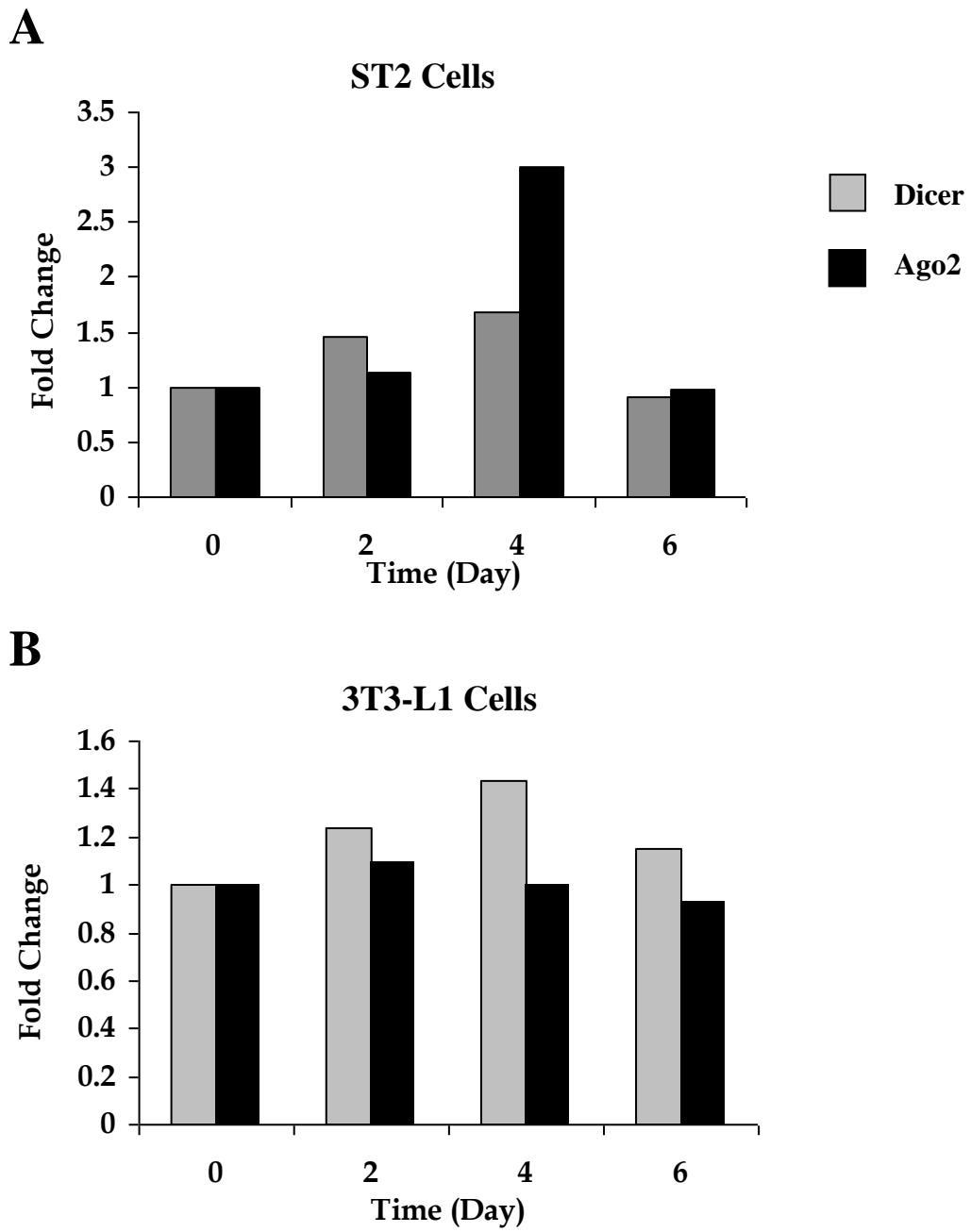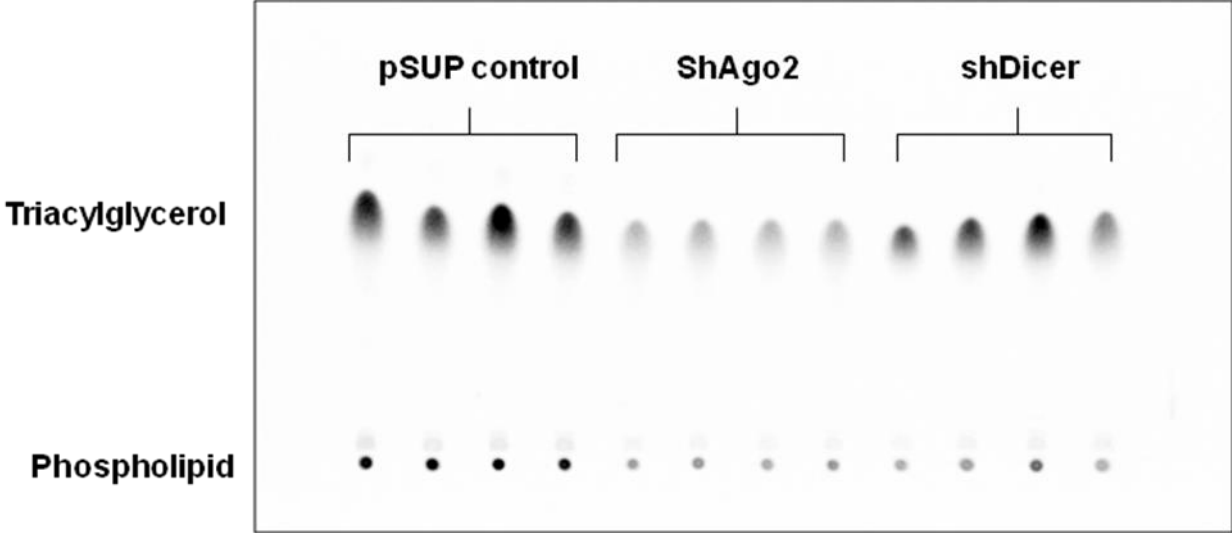
Figure 8.

Figure 9.

A

Glut4

B

FABP4

C

C/EBPα