Role of DNA Methylation in Adipogenesis

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by

YII-SHYUAN CHEN

Under the Direction of Bingzhong Xue

ABSTRACT

The increase in the prevalence of obesity and obesity-related diseases has caused greater attention to be placed on the molecular mechanisms controlling adipogenesis. In this study, we studied the role of 5-aza-2'-deoxycytidine (5-Aza-dC), an inhibitor of DNA methylation, on adipocyte differentiation. We found that inhibiting DNA methylation by 5-Aza-dC significantly inhibited adipocyte differentiation whereas promoting osteoblastogenesis. Wnt10a was up-regulated by 5-Aza-dC treatment and it was suggested that Wnt10a might play a vital role in suppressing adipogenesis and promoting osteoblastogenesis by inhibiting DNA methylation. In 3T3-L1 cells, Wnt signaling inhibitor IWP-2 was found to reverse the inhibitory effect of 5-Aza-dC on adipocyte differentiation, whereas in mesenchymal stem cell line, ST2 cells, IWP-2 treatment reversed the effect of 5-Aza-dC on promoting osteoblastogenesis. These studies will provide a better understanding to the cause and treatment of obesity and bone-related diseases.

INDEX WORDS: Adipogenesis, DNA Methylation, Osteoblastogenesis, ST2 Cells, Wnt10a, Wnt signaling inhibitor, 3T3-L1 Cells
ROLE OF DNA METHYLATION IN ADIPOGENESIS

by

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August 2014
DEDICATION

I would like to dedicate this work to my parents, Ying-Ju Chen and Ben P. Huang. Without their unconditional love and support, I would not have been able to accomplish so much.
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This thesis paper could not have been done if it were not for the help of several individuals. First of all, it is my great pleasure to have had Dr. Bingzhong Xue as my thesis advisor. I would like to thank her for all of the efforts in mentoring me, including pushing me to higher standards of critical thinking, and for having spent time with me on numerous discussions. I would also like to thank Dr. Timothy Jon Bartness and Dr. Hang Shi for having served as my committee members and for their contributions to this project. Ultimately, I would like to thank all my colleagues, Anubama Rajan, Emily Bruggeman, Fenfen Li, Lizhi Fu, Xiaosong Yang, Xin Cui, Qiang Cao, Rui Wu, and Zha Lin who have kindly helped me throughout the detailed experiment procedures as well as the proofreading process.
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1 INTRODUCTION

The World Health Organization (WHO) officially documented obesity as a global epidemic in 1997 (WHO, 2000). Before this, obesity was uncommon (Haslam, 2007). In 2008, the WHO estimated that over 200 million men and almost 300 million women were obese out of more than 1.4 billion overweight adults (WHO, 2013). The enormous increase in the prevalence of obesity and obesity-related diseases has encouraged much research in the molecular mechanisms controlling the translation of pre-adipocytes to mature adipocytes (adipogenesis).

Obesity is the result of an energy imbalance, when energy intake is greater than energy expenditure. Consuming too much energy in a diet can cause lipid droplet accumulation in white adipocytes, leading to two possible growth mechanisms: hyperplasia and hypertrophy. Hyperplasia is adipocyte cell number increase and hypertrophy is cell size increase with more lipid accumulation. Both hypertrophy and hyperplasia contribute to the development of obesity (Johnson & Hirsch, 1972). The most important fact is that although hypertrophy returns to normal with weight loss in obese mice, hyperplasia remains (Faust, Johnson, Stern, & Hirsch, 1978).

Important regulators in the transcriptional program controlling adipogenesis include CCAAT/enhancer-binding protein (C/EBP) family members (i.e. C/EBPα, C/EBPβ, and C/EBPδ) and the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) (Siersbaek, Nielsen, & Mandrup, 2012), which have been revealed by both in vitro and in vivo studies to control adipocyte differentiation (Farmer, 2006). There are two waves that drive these regulators in this transcriptional program. The initiation of the first wave is caused by adipogenic stimuli that spur the activation of several early adipogenic factors including C/EBPβ/δ, Krüppel-like factors (KLFs), cAMP response element binding protein (CREB), and
early growth response 2 (Krox20). The second wave is induced by the first wave, and PPARγ and C/EBPα are the most important regulators leading to mature adipocytes (Farmer, 2006; Lefterova & Lazar, 2009; Rosen & MacDougald, 2006). The negative regulators of adipocyte differentiation are C/EBP homologous protein (CHOP10), retinoic acid receptor (RAR), RAR-related orphan receptor a (RORα), forkhead box O1 (FOXO1), chicken ovalbumin upstream promoter transcription factor II (Coup-TFII), Wnt/β-catenin, GATA binding protein 2/3 (GATA2/3), Krüppel-like factor2 (KLF2), and Interferon regulatory factor 4 (IRF4).

Wnt ligand occurs mostly in stromal vascular fractions and partially in pre-adipocytes. Wnt ligand mRNA expressions decrease during the development of pre-adipocytes to mature adipocytes (Cawthorn et al., 2012). Three Wnt signaling pathways are 1) the canonical Wnt pathway, 2) the noncanonical Wnt and calcium regulation pathway, and 3) the noncanonical cell polarity regulation pathway (Nusse & Varmus, 1992). The canonical Wnt pathway, also known as the Wnt/β-catenin pathway, causes the accumulation of β-catenin in the cytoplasm and it translocates into the nucleus to perform as a transcriptional co-activator of transcription factors in the TCF/LEF family. Without the Wnt ligand (i.e., Wnt10a), a repressing complex comprised of Axin, adenomatosis polyposis coli (APC), glycogen synthase kinase 3 (GSK3), and casein kinase 1α (CK1α) phosphorylates β-catenin, and the phosphorylated β-catenin is then programmed for ubiquitin-associated proteosomal degradation. When the Wnt ligand is present, this repressing complex is phosphorylated and inhibited, resulting in dephosphorylation of β-catenin. β-catenin can then dissociate from the repressing complex, translocate into the nucleus, and activate the transcriptional co-activator (Minde, Anvarian, Rudiger, & Maurice, 2011). The other two Wnt signaling noncanonical pathways do not involve β-catenin accumulation. The Wnt and calcium regulation pathway regulates calcium release by either inhibiting or activating
phosphodiesterase (PDE) (Komiya & Habas, 2008). The noncanonical cell polarity regulation controls the shape of the cell by regulating the cytoskeleton and actin modification. The activation of all three Wnt signaling pathways is led by the binding of a Wnt protein ligand to a Frizzled family receptor, which then motivates a molecular signal to the inside of the cell (Nusse & Varmus, 1992). Wnt signaling pathways are highly evolutionarily conserved and have been studied with regard to organismal development, cancer, and stem cell biology (Nusse & Varmus, 2012). Mutation of Wnt signaling and its downstream proteins may lead to bone density defects (Fahiminiya et al., 2013; Gong et al., 2001; Styrkarsdottir et al., 2013). Up-regulation of Wnt10a might play key roles in human gastric cancer and other various cancers through activation of Wnt/β-catenin signaling pathway on the TCF/LEF level (Kirikoshi, Inoue, Sekihara, & Katoh, 2001; Kirikoshi, Sekihara, & Katoh, 2001). Over the past decade, research also has established that Wnt/β-catenin signaling is a mutually exclusive regulator of whether mesenchymal stem cells (MSCs) go through adipogenesis or osteoblastogenesis. For example, PPARγ and C/EBPα activation suppress osteoblastogenesis, whereas Wnt signaling activation suppresses adipogenesis by inhibiting PPARγ and C/EBPα (Ross et al., 2000). Wnt signaling also inhibits adipogenesis and stimulates osteoblastogenesis through a Wnt/β-catenin-dependent mechanism (Cawthorn et al., 2012).

Obesity consists of an interaction between genes and environment, and one of the mechanisms by which environmental factors (i.e., diets) regulate gene expression is through epigenetic alteration. Epigenetics is the study of heritable changes in gene expression or cellular phenotypes that are not caused by changes in the DNA sequence (Bird, 2007). Examples of mechanisms that yield such changes are DNA methylation and histone modifications including methylation, acetylation, phosphorylation, ubiquitination, sumoylation and ADP-ribosylation. In
mammals, DNA methylation adds a methyl group to the 5\textsuperscript{th} position of the cytosine or adenine residue. These methylated cytosines are usually next to a guanine residue such as in a cytosine–phosphate–guanine (CpG) dinucleotide. When clusters of CpGs (i.e., CpG islands) are unmethylated, the genes can be transcribed, but when the CpG islands become methylated, the genes are turned off (Jones & Takai, 2001). This process is catalyzed by DNA methyltransferases (DMNTs). Three active methyltransferases are DNMT1, DNMT3a, and DNMT3b. DMNT3a and DNMT3b add methyl groups in a de novo way (Okano, Bell, Haber, & Li, 1999), and DNMT1 ensures that methylation patterns are accurately passed on to the next generation through mitosis and meiosis (Leonhardt, Page, Weier, & Bestor, 1992).

Epigenetic control of adipocyte differentiation has been previously reported in the literature. Recent research has demonstrated that the maintenance of the de-acetylated local chromatin accompanied by the suppression of histone H3-Lysine-4 trimethylation (H3K4me3) suppresses expression of PPAR\textgreek{y} and subsequent adipogenesis (Okamura et al., 2009). In addition, inactivation of the H3K4me3 methyltransferase myeloid/lymphoid, or mixed-lineage, leukemia 3 (MLL3) exposes a vital role for MLL3 in PPAR\textgreek{y}-stimulated adipogenesis (J. Lee et al., 2008). Furthermore, the H3K4me3 regulator PTIP (PAXIP1-associated glutamate-rich protein 1) is essential for PPAR\textgreek{y} and C/EBP\textgreek{a} expression and adipocyte differentiation (Cho et al., 2009). This evidence clearly demonstrates the importance of epigenetics in the regulation of adipogenesis although the role of DNA methylation in regulating adipocyte differentiation is not yet clear.

5-Aza-dC is a drug that was originally developed for the treatment of myelodysplastic syndromes and later was applied to the treatment of cancer (Cihak, 1974; Sorm, Piskala, Cihak, & Vesely, 1964). It is a cytidine analog and prevents DNA methylation by inhibiting DNA
methyltransferases. As epigenetic signatures affect differentiation (Kim et al., 2011), our research group has treated 3T3-L1 cells with 5-Aza-dC. In this project, we aimed to study the role of DNA methylation in the regulation of adipogenesis.

Our preliminary data shows that 5-Aza-dC down-regulated adipocyte differentiation and up-regulated Wnt10a expression. Because activating Wnt signaling is associated with reciprocal regulation of osteoblastogenesis vs. adipogenesis (Ross et al., 2000), we therefore hypothesized that inhibiting DNA methylation inhibits adipogenesis and at the same time promotes osteoblastogenesis via activation of Wnt10a-associated canonical Wnt-β-catenin signaling. These studies will give a better understanding to the cause and treatment of obesity and bone-related diseases.
1 EXPERIMENT

1.1 Cell culture and chemicals

1.1.1 Adipogenesis

To induce adipogenesis, mouse embryo 3T3-L1 fibroblasts were cultured in a DMEM high glucose medium combined with 10% NBCS and 5% Penicillin solution, and grown at 37°C in a humidified atmosphere of 5% CO₂. Two days after confluence (post-confluence), pre-adipocytes of 3T3-L1 (designated as day 0) were in a differentiation medium (DM) containing 10% fetal bovine serum (FBS), 5% Penicillin solution, 400 µM insulin, 100 mM isobutylmethylxanthine, 10 mM dexamethasone, and 1 M HCl. After 2 days, the medium was changed to post DM containing 10% FBS and 400 µM insulin for two days, and then changed to a 10% FBS medium for additional 4 days to induce differentiation. Cells were treated with either PBS or 5-Aza-dC at different time points and were harvested at Day 8. 3T3-L1 cells were later treated with either PBS or 5-Aza-dC in the presence or absence of 10 mM IWP-2 at Day 1-2 of differentiation and were harvested at Day 8.

1.1.2 Osteoblastogenesis

To induce osteoblastogenesis, 3T3-L1 or ST2 cells were grown and incubated in a DMEM low glucose medium combined with 10% FBS and 5% Penicillin solution. Two days after confluence (post-confluence), pre-adipocytes of 3T3-L1 or ST2 (designated as day 0) were in DM containing 10% FBS, 5% Penicillin solution, 10 mM β-glycerophosphate and 20 µg/mL ascorbic acid-2-phosphate. The osteoblastogenic medium was replaced every 2 days afterwards. Cells were treated with either PBS or 5-Aza-dC throughout the differentiation process and were
harvested at different points in time. ST2 cells were later treated with either PBS or 5-Aza-dC in
the presence or absence of 10 mM IWP-2 and were harvested at different points in time.

1.2 Cell staining and quantification

Accumulation of lipids in adipocytes was assessed with Oil Red O staining (Kang et al., 2007). Dye preparation: 0.5 g of Oil Red O was dissolved in 100 mL of isopropanol in a dark bottle at room temperature. Forty mL of water was added to 60 mL of Oil Red O solution and left at least 20 minutes at room temperature. The working solution was filtered and added to fixed cells. Cells were incubated at room temperature for 10-15 minutes. Oil Red O (catalog # O0625) was purchased from Sigma-Aldrich (St. Louis, MO).

The degree of mineralization in osteoblasts was determined using Alizarin Red staining and was quantified by assaying calcium content (Kang et al., 2007). Dye preparation: 2 g of Alizarin Red S was dissolved in 100 mL of distilled water in a dark bottle at room temperature. The solution was filtered and added to fixed cells. Cells were incubated at room temperature for 30 minutes. Cells were de-stained for quantification by adding 1 mL 5% perchloric acid and absorbance was read in a 96 wells plate reader at 490 nm. Alizarin Red S (catalog # A5533) was purchased from Sigma-Aldrich (St. Louis, MO).

The activity of alkaline phosphatase (Alp) in osteoblasts was determined and quantified by assaying acid phosphatase activity (Bergmeyer, 1974). Cells were lysed by using lysis buffer (1 % v/v Igepal® CA-630; 10 mM Tris-HCl; 1 mM MgCl2, pH 7.5) with overnight freezing at -80 °C. Lysates then were centrifuged at 12,000 rpm for 10 minutes, after which 50 µl of supernatant was used to measure Alp activity by using Acid Phosphatase Assay Kit according to manufacturer’s instruction. Acid Phosphatase Assay Kit (catalog # CS0740) was purchased from Sigma-Aldrich (St. Louis, MO).
1.3 Total RNA isolation and RT-PCR

Total RNA was isolated from 3T3-L1 cells and ST2 by using Tri-Reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH). The messenger RNA of specific genes were quantified by real-time RT-PCR by using TaqMan One-step RT-PCR Master Mix (Applied Biosystems) and a Stratagene Mx3000P system (Stratagene; La Jolla, CA). Levels of mRNA were normalized to Cyclophilin. The primer and probe pairs used in the measurements were purchased from Applied Biosystems (Grand Island, NY).

1.4 Cell lysates and immunoblotting

For whole-cell lysates, cells were washed with 1X DPBS, scraped with lysis buffer and homogenized. Lysates then were centrifuged at 15,000 rcf for 30 min at 4 °C and then supernatants were transferred to fresh tubes and stored at −80 °C. Protein concentration in cell lysates was measured by using the BCA protein assay. For SDS–PAGE, 20-50 µg of protein was run on 4–15% precast polyacrylamide gel. Samples then were transferred to Immun-Blot® PVDF membrane. Membranes were blocked in 5% non-fat milk for 1.5 hours and then will be immunoblotted with the indicated primary antibodies (1:200~1:1000 dilution, each in 2% non-fat milk) overnight at 4°C, and fluorescence-conjugated secondary antibodies AlexaFluor 680 (1:5000 or 1:10000 dilution in 5% non-fat milk) at room temperature for 2 hrs. The blots were developed with a Li-COR Odyssey Infrared Imager system (Li-COR Biosciences). AlexaFluor 680-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA). Mouse β-catenin antibody (catalog # sc-7199), mouse β-actin antibody (catalog # sc-1616), PPARγ antibody (catalog # sc-7196 or sc-7273) was purchased from Santa Cruz Biotechnology (Dallas, TX). Rabbit Phospho-GSK-3α/β antibody (catalog # 9331) was purchased from Cell Signaling.
(Danvers, MA). Mouse Anti-GSK, clone 4G-1E antibody (catalog # 05-412) was purchased from Millipore (Billerica, MA). Polyacrylamide gel (catalog # 5671084) and Immun-Blot® PVDF membrane (catalog # 1620177) were purchased from Bio-Rad (Hercules, CA).
2 RESULTS

2.1 5-Aza-dC down-regulated adipocyte differentiation.

3T3-L1 cells were treated with either PBS or 5-Aza-dC at Day 1-2, Day 3-5 and Day 6-8 of differentiation and were harvested at Day 8. Real time RT-PCR results showed that adipogenesis markers such as PPARγ, C/EBPα, aP2, and FAS were down-regulated especially when L1 cells were treated with 5-Aza-dC at early differentiation period of Day 1-2 and Day 3-5, but this inhibition was lost when cells were treated with 5-Aza-dC at later differentiation period of Day 6-8. Therefore, we focused on inhibition of adipocyte differentiation by 5-aza-dC at an early stage. Consistent with the inhibition of RNA expression by 5-Aza-dC, the inhibition of PPARγ protein expression also was found in 5-Aza-dC treated 3T3-L1 cells at early differentiation period (Fig. 1A, 1B). Results of Oil Red O staining revealed less lipid accumulations in 3T3-L1 cells treated with 5-Aza-dC during an early differentiation stage of Day 1-2 (Fig. 1C). This evidence shows that adipocyte differentiation in 3T3-L1 cells is inhibited by 5-Aza-dC when treated at Day 1-2 during the early differentiation process.
Fig. 1A Real time RT-PCR results on adipogenesis markers in 3T3-L1 cells treated with 5-Aza-dC during adipocyte differentiation. 1B PPARγ protein expression in 3T3-L1 cells treated with 5-Aza-dC at Day 1-2 of adipocyte differentiation 1C Oil Red O staining results in 3T3-L1 cells treated with 5-Aza-dC at Day 1-2 of adipocyte differentiation. 3T3-L1 cells were treated with either PBS or 5-Aza-dC at Day 1-2, Day 3-5 and Day 6-8 of differentiation and were harvested at Day 8. Value in each independent experiment was normalized to the control group and presented as means ± SE (n =3-6). *P < 0.05 **P < 0.01 and vs. control group.

Figure 1: 5-Aza-dC down-regulated adipocyte differentiation
2.2 Wnt10a was up-regulated in 5-Aza-dC treated 3T3-L1 cells.

It is unknown why 5-Aza-dC causes adipocyte differentiation inhibition. DNA methylation causes gene repression. 5-Aza-dC is used to reactivate gene expression in tumor cells that were previously repressed by DNA methylation (Cihak, 1974; Sorm et al., 1964). Therefore, we hypothesized that 5-Aza-dC may inhibit 3T3-L1 adipocyte differentiation by activation of certain adipocyte differentiation repressors. We used pooled samples (n =3-6) to screen the negative regulators of adipocyte differentiation. These negative regulators are C/EBP homologous protein (CHOP10), retinoic acid receptor (RAR), RAR-related orphan receptor α (RORα), forkhead box O1 (FOXO1), chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), Wnt/β-catenin, GATA binding protein 2/3 (GATA2/3), Krüppel-like factor2 (KLF2), and Interferon regulatory factor 4 (IRF4) (Siersbaek et al., 2012). Real time RT-PCR screening on these negative regulators genes of 3T3-L1 cells showed that Wnt10a was up-regulated 10-fold in 5-Aza-dC treated 3T3-L1 cells (Fig. 2A). This evidence suggests that Wnt10a may play a key role in inhibiting adipocyte differentiation by 5-Aza-dC treatment. We confirmed this result by measuring Wnt10a expression in individual samples. We found that 5-Aza-dC induced the most significant Wnt10a increase when 3T3-L1 cells were treated at Day 1-2 during adipocyte differentiation on an mRNA level (Fig. 2B).
Figure 2A: Real time RT-PCR screening on repressor gene expression of 3T3-L1 cells treated with 5-Aza-dC during adipocyte differentiation. 2B: Real time RT-PCR result on Wnt10a expression of 3T3-L1 cells treated with 5-Aza-dC during adipocyte differentiation. 3T3-L1 cells were treated with either PBS or 5-Aza-dC at Day 1-2, Day 3-5 and Day 6-8 of differentiation and were harvested at Day 8. Value in each independent experiment was normalized to the control group. Abbreviations: DDIT3, DNA-damage inducible transcript 3; CHOP10; ERN1, endoplasmic reticulum (ER) to nucleus signalling 1; FOXO1, forkhead box O1; RARA, retinoic acid receptor, alpha; RORA, RAR-related orphan receptor alpha; WNT10B, wingless related MMTV integration site 10b; WNT10A, wingless related MMTV integration site 10a; WNT5A, wingless-related MMTV integration site 5a; WNT6, wingless-related MMTV integration site 6; WNT7B, wingless-related MMTV integration site 7b; Fzd2, frizzled homolog 2; Ctnnb1, catenin (cadherin-associated protein), beta 1; Nrrf2, nuclear receptor subfamily 2, group F, member 2; GATA2, GATA binding protein 2; GATA3, GATA binding protein 3; Klf2, Kruppel-like factor 2 (lung); Klf3, Kruppel-like factor 3 (basic); Irf4, interferon regulatory factor 4; shh, sonic hedgehog; dhh, desert hedgehog; ihh, Indian hedgehog; Tulp3, tubby-like protein 3
2.3 Wnt signaling and osteoblastogenesis markers were up-regulated in 5-Aza-dC treated 3T3-L1 cells during adipocyte differentiation.

We also measured Wnt signaling by measuring phospho-GSK3 and β-catenin proteins. Here, we demonstrate that Wnt signaling proteins, phospho-GSK3β and β-catenin, were up-regulated in 5-Aza-dC treated 3T3-L1 cells at Day 1 of the differentiation period (Fig. 3A, 3B). Over the past decade, research has established that Wnt/β-catenin signaling is a mutually exclusive regulator of whether mesenchymal stem cells (MSCs) go through adipogenesis or osteoblastogenesis. For example, Wnt signaling activation suppresses adipogenesis by inhibiting PPARγ and C/EBPα (Ross et al., 2000). Wnt signaling also stimulates osteoblastogenesis through a Wnt/β-catenin-dependent mechanism (Cawthorn et al., 2012). Real time RT-PCR results showed that osteoblastogenesis markers Bone Gamma-Carboxyglutamate (Gla) Protein/Osteocalcin (BGLAP) and Distal-Less Homeobox 5 (Dlx5) were up-regulated in 5-Aza-dC treated 3T3-L1 cells during adipocyte differentiation (Fig. 3C).
Figure 3: Results of Wnt signaling and osteoblastogenesis markers in 3T3-L1 cells
2.4 Osteoblastogenesis markers were up-regulated in 5-Aza-dC treated 3T3-L1 cells during the differentiation of osteoblastogenesis.

When 3T3-L1 cells were subjected to osteoblastogenesis differentiation in the presence of 5-Aza-dC, osteoblastogenesis markers such as Alkaline Phosphatase (Alp), BGLAP, Basic Helix-Loop-Helix Transcription Factor 1 (Twist1), and Dlx5 were up-regulated (Fig. 4).
Figure 4: Real time RT-PCR results on osteoblastogenesis markers in 3T3-L1 cells treated with 5-Aza-dC during osteoblastogenesis. 3T3-L1 cells were treated with either PBS or 5-Aza-dC and were harvested at Day 5, 9, and 17 during osteoblastogenesis differentiation. Value in each independent experiment was normalized to the control group and presented as means ± SE (n = 3-6). *P < 0.05 **P < 0.01 and vs. control group.

Figure 4: Osteoblastogenesis markers were up-regulated in 5-Aza-dC treated 3T3-L1 cells
2.5 Wnt10a was up-regulated in 5-Aza-dC treated 3T3-L1 cells during osteoblastogenesis differentiation.

Moreover, Wnt signaling molecules such as Wnt10a, Wnt10b, and Wnt6 were up-regulated in 5-Aza-dC treated 3T3-L1 cells during osteoblastogenesis differentiation, especially Wnt10a (Fig. 5).
Figure 5: Result of Wnt10a in 5-Aza-dC treated 3T3-L1 cells during osteoblastogenesis
2.6 Wnt10a and osteoblastogenesis markers were up-regulated in 5-Aza-dC treated ST2 cells.

We also used ST2 cells, one of MSCs, and differentiated ST2 cells into osteoblasts to further study the role of 5-Aza-dC in bone formation. The evidence showed that inhibiting DNA methylation by 5-Aza-dC promotes osteoblastogenesis in ST2 cells. During osteoblastogenesis differentiation, Wnt10a and osteoblastogenesis markers such as Alp, BGLAP, Twist1, and Dlx5 were up-regulated in 5-Aza-dC treated ST2 cells (Fig. 6).
Fig. 6 Real time RT-PCR results on osteoblastogenesis markers and Wnt signaling in ST2 cells treated with 5-Aza-dC during osteoblastogenesis differentiation. ST2 cells were treated with either PBS or 5-Aza-dC during osteoblastogenesis differentiation and were harvested at Day 0, 7, 14, and 21. Value in each independent experiment was normalized to the control group and presented as means ± SE (n =3-6). *P < 0.05 **P < 0.01 and vs. control group.

Figure 6: Results of Wnt10a and osteoblastogenesis markers in 5-Aza-dC treated ST2 cells
2.7 Calcium content and alkaline phosphatase activity were up-regulated in 5-Aza-dC treated ST2 cells during the differentiation of osteoblastogenesis.

We measured calcium content and alkaline phosphatase activity to confirm the phenotypes of the osteoblasts. At Day 21 of osteoblastogenesis differentiation, calcium content and alkaline phosphatase activity were significantly increased by 5-Aza-dC treatment (Fig. 7).

Figure 7: Results of Calcium content and Alp in 5-Aza-dC treated ST2 cells

*Fig. 7 Calcium content and alkaline phosphatase activity in ST2 cells treated with 5-Aza-dC during osteoblastogenesis differentiation. ST2 cells were treated with either PBS or 5-Aza-dC at Day 0, Day 1-7, Day 1-14, and Day 1-21 of differentiation and were harvested at Day 0, 7, 14, and 21. Value in each independent experiment was normalized to the control group and presented as means ± SE (n = 3-6). *P < 0.05 **P < 0.01 and vs. control group.*
2.8 IWP-2 reversed the effect of 5-Aza-dC in 3T3-L1 cells during adipogenesis differentiation.

We treated 3T3-L1 cells with 5-Aza-dC in the presence or absence of Wnt signaling inhibitors IWP-2 to see if inhibiting Wnt signaling reversed the effects of 5-Aza-dC on adipogenesis. IWP-2 inhibits Wnt signaling production through Porcupine (Porcn) function. Porcn belongs to the membrane-bound O-acyltransferase (MBOAT) family, which adds a palmitoyl group to Wnt protein ligands that are needed for Wnt signaling ability and secretion (Chen et al., 2009). IWP-2 was found to completely reverse the effect of 5-Aza-dC on the inhibition of PPARγ, aP2, and FAS mRNA levels, and partially reverse the effect of 5-Aza-dC on the inhibition of the C/EBPα mRNA level (Fig. 8A). Results of Oil Red O staining revealed less lipid accumulations in 5-Aza-dC treated 3T3-L1 cells during an early differentiation period at Day 1-2, and this effect was reversed by IWP-2 (Fig. 8B). This evidence suggests that Wnt signaling plays an important role in the inhibition of adipocyte differentiation by 5-Aza-dC, which may be mainly exerted via regulating PPARγ expression.
Figure 8: IWP-2 reversed the effect of 5-Aza-dC in 3T3-L1 cells during adipogenesis.
2.9  **Wnt signaling inhibitor IWP-2 prevented the promotion of 5-Aza-dC on osteoblastogenesis in ST2 cells under the differentiation of osteoblastogenesis.**

We also treated ST2 cells with 5-Aza-dC in the presence or absence of Wnt signaling inhibitors IWP-2 to see if inhibiting Wnt signaling reversed 5-Aza-dC’s effects on osteoblastogenesis. IWP-2 was found to reverse the effect of 5-Aza-dC on transcriptional factor Wnt10a in a mRNA level and the promotion of osteoblastogenesis markers BGLAP (Fig. 9).
Figure 9: IWP-2 prevents the promotion of 5-Aza-dC in ST2 cells under osteoblastogenesis.
Adipogenesis in 3T3-L1 cells was inhibited by 5-Aza-dC when treated at Day 1 to Day 2 during the differentiation process as evidenced by reduced expression of important adipogenesis regulators PPARγ and C/EBPα, reduced PPARγ protein levels and decreased Oil Red O staining (Fig. 1). PPARγ and C/EBPα are the most important regulators leading to mature adipocytes in the adipogenesis program (Farmer, 2006; Lefterova & Lazar, 2009; Rosen & MacDougald, 2006) and are revealed by both in vitro and in vivo studies to control adipocyte differentiation (Farmer, 2006). The present study is the first evidence showing adipocyte differentiation regulated by DNA methylation through 5-Aza-dC treatment, and also indicates that there are dynamic changes in DNA methylations in the early stage of adipogenesis. To induce adipogenesis, 3T3-L1 cells are cultured into confluence. Two days after confluence (post-confluence), pre-adipocytes of 3T3-L1 are induced into differentiation. In this period, cells undergo mitotic clonal expansion with the last run of cell division, and then cells undergo growth arrest to be induced into terminal differentiation (Tang & Lane, 2012). Examples of adipogenesis regulators controlling this stage are C/EBPβ, C/EBPδ, repressors Pref-1, CHOP10, and Wnt signaling. We have measured Pref-1, CHOP10 and Wnt signaling mRNA level in 5-Aza-dC treated 3T3-L1 cells. We found no effect of 5-Aza-dC treatment on Pref-1, a mild effect on CHOP10 (shown as DDIT3), and a significant increase on Wnt10a (Fig2A).

Our data demonstrate that inhibiting DNA methylation by 5-Aza-dC suppresses 3T3-L1 adipocyte differentiation by up-regulating Wnt10a-associated signaling. Another research group has shown that mRNA expression of Wnt6, Wnt10a and Wnt10b is maintained in pre-adipocytes and stromal vascular fractions of adipose tissue, but down-regulated through adipogenesis (Cawthorn et al., 2012). In our experiment, however, only Wnt10a was significantly increased in
5-Aza-dC treated 3T3-L1 cells, but not Wnt6 and Wnt10b. Therefore, inhibition of DNA methylation by 5-Aza-dC treatment may mainly target Wnt10a during 3T3-L1 differentiation process.

We confirmed this theory by inhibiting Wnt signaling. The Wnt signal inhibitor IWP-2 completely reversed the effect of 5-Aza-dC on the inhibition of PPARγ mRNA, and partially reversed the effect of 5-Aza-dC on the inhibition of C/EBPα mRNA (Fig. 8). This evidence suggests that Wnt signaling plays an important role in the inhibition of adipocyte differentiation by 5-Aza-dC, which may be mainly exerted via regulating PPARγ expression. To further study the role of the PPARγ and its interaction with Wnt10a, genetic deletion on PPARγ should be tested. Without knockdown of PPARγ, IWP-2 treatment is expected to rescue the inhibition of adipocyte differentiation by 5-Aza-dC. With knockdown of PPARγ, IWP-2 treatment is expected to fail to rescue the inhibition of adipocyte differentiation by 5-Aza-dC.

Adipogenesis and osteoblastogenesis are mutually exclusive processes that are regulated by Wnt/β-catenin signaling (Ross et al., 2000). Our data supported that 5-Aza-dC up-regulated Wnt signaling expression, and also supported the role of Wnt signaling in osteoblast formation. To induce osteoblastogenesis, ST2 cells are cultured into post-confluence. ST2 cells then are induced into differentiation by adding β-glycerophosphate and ascorbic acid-2-phosphate for up to 21 days. In this process, cells undergo the influence of several transcription and hormonal factors, and then form the phenotype of osteoblasts (Marie, 2008). Alp, BGLAP, Dlx5, Osx, Twist1, Runx2 and Wnt signaling are important regulators leading to mature osteoblasts and matrix mineralization in the osteoblastogenesis program (Marie, 2008). In our experiments, when ST2 cells were subjected to osteoblastogenesis differentiation in the presence of 5-Aza-dC, osteoblastogenesis markers such as Alp, BGLAP, Twist1, and Dlx5 were up-regulated (Fig. 6).
Alp is an important enzyme through osteoblastogenesis and is found to be a trigger of mineralization (Robison, 1923). BGLAP is promoted by C/EBPβ and C/EBPδ during osteoblastogenesis development and promotes osteoblastogenesis (Gutierrez et al., 2002). The family of Twist genes is expressed mostly in early stages of osteoblastogenesis. A study shows that overexpression of Twist genes keeps cells in osteo-progenitor and pre-osteoblast stages, whereas antisense of Twist genes promotes cells into mature osteoblasts (M. S. Lee, Lowe, Strong, Wergedal, & Glackin, 1999). Dlx5 is a transcription factor that contains a homeobox protein (Stock et al., 1996). It is expressed during osteoblastogenesis, and one of the functions is to promote BGLAP (Holleville, Mateos, Bontoux, Bollerot, & Monsoro-Burq, 2007). Therefore, our data suggest that inhibiting DNA methylation by 5-Aza-dC promotes osteoblastogenesis by stimulating these important regulators in the process.

Because Wnt/β-catenin signaling is a mutually exclusive regulator of MSC fate to either adipogenesis or osteoblastogenesis, research should confirm that adipogenesis inhibition through inhibiting DNA methylation by 5-Aza-dC is mediated by Wnt signaling. Future studies using genetic methods to knockdown Wnt10a and β-catenin and treat with 5-Aza-dC in 3T3-L1 and ST2 cells, would be expected to reverse the effects of 5-Aza-dC on inhibiting adipogenesis and promoting osteoblastogenesis.

Studies support the hypothesis that Wnt signaling can be regulated by epigenetic mechanisms. One example shows that Wnt10a can be regulated by histone methylation through histone methyltransferase G9a (Wang et al., 2013). Histone methyltransferase G9a deletion decreases the methylation at the repressive histone marker H3K9 at Wnt10a promoter, and increasing Wnt10a expression can inhibit adipogenesis by decreasing PPARγ expression (Wang et al., 2013). Although most Wnt signaling protein such as Wnt4, Wnt5a, Wnt 5b, Wnt
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9b and Wnt11 can be methylated on a DNA level, there is still no evidence showing Wnt10a expression can be regulated by DNA methylation (Choi et al., 2012). This thesis paper is the first evidence that Wnt10a expression can be regulated by DNA methylation. To further study the role DNA methylation of the Wnt10a promoter in adipocyte differentiation, research could be aimed at quantifying the DNA methylation level on the Wnt10a promoter during 3T3-L1 cell differentiation using pyrosequencing. Additional studies could determine how DNA methylation of the Wnt10a promoter is regulated during adipocyte differentiation. Chromatin immunoprecipitation (ChIP) could be used to study whether the binding of 5-methylcytidine, DNMT1, DNMT3a or DNMT3b on the Wnt10a promoter is regulated during 3T3-L1 adipocyte differentiation. Future studies should identify which DNMTs are important in regulating adipocyte differentiation by using gain- and loss-of-function experiments.

The development of obesity is caused by hyperplasia and hypertrophy (Johnson & Hirsch, 1972). Early treatment of 5-Aza-dC at Day 1-2 inhibits DNA methylation, which then leads to the inhibition of adipocyte differentiation. Previous data suggests that the development of white adipocytes in postnatal animal models such as A/J and B6 mice follow a similar pattern of these adipogenesis regulators during adipocyte differentiation in 3T3-L1 cells (Xue et al., 2007). For example, Pref-1 is significantly decreased in postnatal 30 days, whereas important adipogenesis regulators PPARγ and C/EBPα are significantly increased starting from postnatal day 5, achieve a peak at day 20 and continuously stay elevated. C/EBPβ and C/EBPδ both achieve a peak at day 20. The present study suggested that the in vitro program is comparable to the in vivo program. To study the effect of DNA methylation inhibition of adipocyte differentiation in terms of adiposity in animal models, tissue specific knockouts could be tested.
to demonstrate the development of adiposity, body weight, and obesity. We expect to see that tissue specific knockout of certain genes cause body weight reductions.

In conclusion, inhibiting DNA methylation inhibits adipogenesis and at the same time promotes osteoblastogenesis via activation of Wnt10a-associated canonical Wnt-β-catenin signaling. In 3T3-L1 cells, Wnt signaling inhibitor IWP-2 was found to reverse the inhibitory effect of 5-Aza-dC on Adipocyte differentiation, whereas in mesenchymal stem cell line, ST2 cells, IWP-2 treatment reversed the effect of 5-Aza-dC on promoting osteoblastogenesis. Studying the role of DNA methylation in adipogenesis and osteoblastogenesis will provide a better understanding to the cause and treatment of obesity and bone-related diseases.
REFERENCES


gene expression and repress adipogenesis. [Research Support, Non-U S Gov't]. *Proc Natl Acad Sci U S A*, 106(14), 5819-5824.


