MicroRNA-140 promotes adipocyte lineage commitment of C3H10T1/2 pluripotent stem cell via targeting Osteopetrosis-associated transmembrane protein 1*

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Background: BMP4 treatment induces adipocyte lineage commitment of C3H10T1/2 pluripotent stem cells.

Results: Expression of miR-140 increases significantly during adipocyte lineage commitment.

Conclusion: miR-140 promotes adipocyte lineage commitment through down-regulating Ostm1.

Significance: Understanding which miRNA and how it functions in adipocyte lineage commitment.

SUMMARY
Bone morphogenetic protein 4 (BMP4) has been shown to induce C3H10T1/2 pluripotent stem cells to commit into adipocyte lineage. Besides several proteins identified, microRNAs (miRNAs) also play a critical role in the process. In the present study, we identify microRNA-140 (miR-140) as a direct downstream component of BMP4 signaling pathway during the commitment of C3H10T1/2 into the adipocyte lineage. Over-expression of miR-140 in C3H10T1/2 would promote the commitment, while knockdown the expression would lead to impairment. Further studies indicate that Osteopetrosis-associated transmembrane protein 1 (Ostm1) is a bona fide target of miR-140, which is significantly decreased during the commitment and Ostm1 is also demonstrated to function as an anti-adipogenic factor.

Obesity has become an escalating global epidemic since people have changed diet and lifestyle in 20th century; it not only
affects people’s appearance, but also poses an array of serious disorders to health, including insulin resistance, type 2 diabetes, hypertension, and atherosclerosis (1, 2). Elucidating the mechanisms underlying obesity is critical for understanding of obesity occurrence and progression. It has been shown that adipocyte development includes two progressive stages: lineage restricted preadipocytes committed from pluripotent mesenchymal stem cells (3), lipid-laden adipocytes differentiated from growth-arrested preadipocytes (4). Researchers have applied 3T3-L1, a widely used preadipocyte cell line, to clearly understand the differentiation process (5, 6). However, there is still far more to know about the commitment process (7).

C3H10T1/2, a generally accepted mesenchymal stem cell line, has been used to elucidate the mechanisms of adipocyte lineage commitment (4). C3H10T1/2 cells are multipotent stem cells that can differentiate into various lineages including: osteocyte, chondrocyte, myocyte, and adipocyte (8, 9). BMP4 treatment can efficiently induce C3H10T1/2 to commit into adipocyte lineage through two downstream signaling pathways, Smad and p38MAPK (10). The commitment of C3H10T1/2 is accompanied by dramatic changes of cell shape and several cytoskeleton-associated proteins (i.e. lysyl oxidase (LOX), translationally controlled tumor protein 1 (TPT1), and αB-crystallin) are demonstrated to be up-regulated by BMP4 during the commitment (11).

microRNAs function at the posttranscriptional level by negatively regulating mRNA stability or translation and participate in almost every physiological and pathological process(12-15). Numerous miRNAs have been shown to be involved in the terminal adipocyte differentiation (16). For example, miR-143, a well known miRNA that enhances adipogenesis, increases after induction of differentiation and targets Pleiotrophin (PTN) to promote differentiation of 3T3-L1 preadipocytes (17), while PTN plays a negative role during adipogenesis through PTN/PI3K/AKT/GSK-3β/β-catenin signaling pathway. Stable transfection of 3T3-L1 cells with miR-17/92 cluster results in accelerated differentiation by negatively regulating the tumor suppressor protein Rb2/p130, which participates in a fundamental step for mitotic clonal expansion (18). miR-375 enhances 3T3-L1 adipocyte differentiation by suppressing phosphorylation levels of extracellular signal-regulated kinases 1/2 (ERK1/2) (19). On the other hand, the miR-27 gene family, including miR-27a and miR-27b, is down-regulated during 3T3-L1 adipocyte differentiation, and over-expression of miR-27a and miR-27b inhibits adipocyte differentiation of 3T3-L1 preadipocytes (20). The miR-27 family is also shown to elevate in obese mice and contribute to LPS-mediated inflammation by targeting PPARγ (21). However, the roles of miRNAs during adipocyte lineage commitment are barely reported.

Ostm1 is a type I transmembrane protein localizing in intracellular vesicles and is highly expressed in cartilage and generally conserved in a wide range of species including zebrafish, mice and human (25, 26). Previous studies
elucidated three biological functions of Ostm1: serving as a beta-subunit of ClC-7 to support bone resorption and lysosomal function; working as an E3 ubiquitin ligase to induce proteasome dependent degradation of Goαi3 and promoting β-catenin/Lef1 interaction (22-24). The above findings suggest that Ostm1 function an important role in the bone development. As mesenchymal stem cells can differentiate into both osteocytes and adipocytes, Ostm1 might influence cell fate determination between these cells.

In the current study, we find BMP4 treatment dramatically increases miR-140 expression, which promotes the commitment of C3H10T1/2 into adipocyte lineage. Furthermore, Ostm1 is identified as a direct target of miR-140 and functions as an anti-adipogenic factor.

EXPERIMENTAL PROCEDURES

Cell culture and induction of commitment and differentiation The C3H10T1/2 mesenchymal stem cells and 3T3-L1 preadipocytes were propagated and differentiated as described (4).

Construction of Plasmids The miR-140 expression plasmid, MSCV-miR-140, was generated using standard DNA cloning techniques. The mouse miR-140 precursor, including ~670bp of genomic flanking sequence, was cloned between the Bgl II (5’end) and Xho I (3’end) restriction sites of the MSCV vector using the following primer pair: forward 5’-TCGAGTACCTTTTCAGCTACTGTTGTCAAAACCCTCCTGGCTAAAGAGCTGGGC-3’; reverse 5’-GGCCGCCCAGGTCTTTTAGCCAAAGCAGTTGGTTGTGATCACACAGTAAGAAGGTAC-3’. Each oligo contained a predicted miR-140 binding site (underlined). The sites mutated oligo were as following: forward 5’-TCGAGTACCTTTTCAGCTACTGTTGTCACATTGGTGAGCTTTTGCTGAAGAGGTAC-3’ and reverse 5’-GGCCGCCCAGGTCTTTTAGCCAAAGCTCACAAAAGGATACACACAGTATCGAAAGGTAC-3’. Both oligos were annealed and cloned into psiCHECK2 vector downstream of the Renilla luciferase reporter gene.

Ostm1 gene CDS sequence was amplified using primers as following; forward 5’-CCGCTCGAGATGGCTCGGGACGCGGAGCT-3’ and reverse 5’-GGAATTCTCAGGTGGCATTTTCTGAAT-3’, and cloned between Xho I (5’end) and EcoR I (3’end) of mscv vector (GenBank accession no. NM172416.3). MiR-140-sponge is a generous gift from Dr. XuZhiGuo (The Bio-X center of Shanghai Jiao Tong University).

Microarray and Data Analysis Total RNA was extracted using the TRIZol method (Invitrogen) according to the manufacturer’s protocol. miRNA microarray was performed using Agilent Mouse miRNA Microarrays in triplicate.

qRT-PCR Analysis Total RNA (including total microRNA) was harvested from C3H10T1/2 cells using TRIZol (invitorgen). The miRNA was reverse
transcribed using the TaqMan miRNA reverse transcription kit (Applied Biosystems) and miRNA-specific primers (Applied Biosystems). MiRNA expression levels were then analyzed using the appropriate TaqMan miRNA assay (Applied Biosystems) according to the manufacturer’s instructions. Quantitation of the ubiquitously expressed miRNA, U6, was performed as an endogenous control.

To determine the expression level of Ostm1, the mRNA was reverse transcribed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) with random primer followed by real-time PCR with SYBR Green chemistry (Applied Biosystems). Primer sequences were as following; Ostm1: forward 5'-GAGCTGACCGCCTGTATGG-3', reverse 5'-ATGGTTTCGCTGATGGTTGTC-3', GAPDH: forward 5'-GGCAAAATTCAACGGCACAGT-3', reverse 5'-CGCTCCTGGAAGATGGTGAT-3'.

Oil Red O Staining Cells were washed 3 times with PBS and fixed for 15 minutes with 3.7% formaldehyde. Oil Red O (0.5% in isopropanol) was diluted with water (3:2), filtered through 0.45um filter, and incubated with the fixed cells for 1h at room temperature. Cells were then washed with water, and stained lipid droplets in the cells were visualized by light microscopy and photography.

Luciferase Assay 293T cells were transfected with 10 ng psiCHECK2-Ostm1 3’UTR vector in 24 h after plated, psiCHECK2-Ostm1 mut 3’UTR vector and miR-140 sponge vector for 6 h in reduced serum and antibiotics-free Opti-MEM with Lipofectamine 2000. Cells were co-transfected with the miR-140 mimic or a negative control (Genepharm) at 100nM concentration. Firefly and Renilla luciferase were measured in cell lysates using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was used for normalization and as an internal control for transfection efficiency.

Transfection Assay Transfection experiments were performed with the transfection kit Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX (Invitrogen) following the manufacturer’s instruction.

RNA interference Synthetic siRNA oligonucleotides specific for Smad4 (GenBank accession no.NM008540.2), p38 MAPK (GenBank accession no.U10871.1) and Ostm1 mRNA were designed and synthesized by Invitrogen Stealth™ RNAi. The sequences were as following:

Smad4 Stealth™ RNAi: 5’-CAUACACACCUCUUUUGCCUC ACCA-3’
p38 MAPK Stealth™ RNAi: 5’-CCUUUGAAAGCAGGGACCUC UCAA-3’
Ostm1 Stealth™ RNAi: 5’-GAGCUGCUGAUGGACUUCGCC AAUA-3’

Isolation of the Stromal Vascular Fraction Epididymal and inguinal fat pads from male C57BL/6 mice, wild type and BMP4 transgenic mice in which BMP4 was specifically over-expressed in adipose tissue were excised and minced in PBS with 0.5% BSA. Collagenase (Sigma-Aldrich) was added to 1mg/ml before incubation at 37°C for 2 hours with shaking. Suspensions were centrifuged at 1500g
for 5 min to remove cellular debris and oil; precipitations were resuspended with RBC lysis buffer, and then re-centrifuged at 4000g for 10 min. Suspensions were washed by PBS for twice and centrifuged then re-suspended with cell lysis buffer. Samples then could be heating at 110°C for 15 min and ready for western blotting.

**RESULTS**

*miR-140/140* was induced during the adipocyte lineage commitment of C3H10T1/2 by BMP4 treatment*—That BMP4 could induce C3H10T1/2 cells commitment from pluripotent stem cells to the adipocyte lineage has been repeated (4) (Fig. 1 A and B) and activation of BMP4 signaling, e.g. phosphorylation of p38MAPK and Smad1/5/8, was significantly induced (Fig. 1 D). To determine whether miRNA was involved in this process, microarray analysis was performed. And the results revealed that numerous miRNAs were positively or negatively regulated by BMP4 treatment (Fig.S1). Besides many slightly changed miRNAs, miR-140 and miR-140* increased by over 2 folds in BMP4 treated C3H10T1/2 cells compared with control cells, and were selected for further studies. Since miR-140 and miR-140* originated from two strands of one single pre-miRNA, both of their expression levels were detected by TaqMan real-time PCR assay (Fig. 1 E). As illustrated in Fig. 1F, the expression of miR-140 and miR-140* began to increase gradually during commitment, peaked on day 6 after continuous BMP4 treatment, and decreased during terminal adipocyte differentiation, which implied that they might function in the adipocyte lineage commitment. In contrast, the miRNA expression level changed slightly in control C3H10T1/2 cells compared with BMP4 treatment.

*Overexpression of miR-140 promotes the adipocyte lineage commitment of C3H10T1/2—*Since miR-140/140* were specifically enriched during BMP4 induced C3H10T1/2 commitment, we hypothesized that their introduction into C3H10T1/2 might bias cells towards a preadipocyte lineage. Since miR-140 and miR-140* come from the same precursor, their over-expression could not be separated. However, because miR* is usually degraded merely as a carrier strand, and miR-140 has been reported to function during mesoderm development, we focused our research mainly in miR-140. We constructed the miRNAs over-expression plasmid, MSCV-miR-140/140*, by cloning miR-140/140* precursor into the MSCV vector, followed by infection into C3H10T1/2 cells. Real-time PCR confirmed that the over-expression was effective (Fig. 2 A). miR-140/140* over-expressed C3H10T1/2 cells could partially differentiate into mature adipocytes even without BMP4 treatment. Furthermore, treatment with BMP4 before confluence significantly boosted their adipogenic capacity as indicated by Oil Red O staining of fat droplet accumulation and Western blotting analysis of adipocyte specific markers (Fig. 2 B and C). These results combined with the miRNAs expression pattern showed in Fig. 1E indicated that miRNAs played a critical role during the adipocyte lineage commitment of C3H10T1/2. Thereafter, miR-140 sponge vector was used to decrease miR-140 expression in miRNA
over-expressed C3H10T1/2 after BMP4 treatment (27). Sponge’s effect was validated in Fig. 3E. Consistent with our assumption, the increase in adipogenesis by miRNA over-expression was significantly decreased after the introduction of miR-140 sponge (Fig. 2 E and F). This confirmed that miR-140 plays a very important role during the commitment.

Ostm1 was identified as a target protein of miR-140 during the adipocyte lineage commitment of C3H10T1/2—PicTar, TargetScan and Miranda were used to identify putative target proteins of miR-140 of which overlapped among these three algorithms (28). We found most of the genes in the list would match the dual-luciferase experiments (Fig. S2), however, only a few of them could be eventually demonstrated to be coordinately regulated during the same physiological process. Accordingly, Ostm1 was markedly down-regulated in both protein and mRNA level when miR-140 was over-expressed in C3H10T1/2 cells (Fig. 3 A and B). Furthermore, the repressive function of BMP4 or miR-140 on the expression of Ostm1 could be predominantly reversed by miR-140-sponge, which could bind to miR-140 and diminish its repression of the target protein (Fig. 3 C).

According to the sequence analysis, Ostm1 contained two 7-nt sites within its 3’-UTR which matched the seed region of miR-140. These two putative miRNA binding sites were cloned into psiCHECK-2 vector respectively and only one site bound miR-140 (Fig. 3 D). Compared with negative control miRNA mimic, co-transfection of Ostm1 3’-UTR luciferase reporter with miR-140 mimic resulted in significantly repressed luciferase activity. Correspondingly, miR-140 had no effects on mutated-Ostm1 3’-UTR reporters. Furthermore, when miR-140 sponge vector was used to neutralize the effects of miR-140 mimic, no difference of luciferase activity could be observed between control mimic and miR-140 mimic transfected cells (Fig. 3 E). All of the above indicated that Ostm1 was a direct target of miR-140.

OSTM1 was identified as an anti-adipogenic factor and its expression is decreased by BMP4 through miR-140 during adipocyte lineage commitment of C3H10T1/2—Western blotting was used to detect Ostm1 expression during the commitment stage in C3H10T1/2 cells. Compared with control C3H10T1/2 cells, Ostm1 expression was significantly decreased in BMP4 treated cells (Fig. 4 A). In previous studies, Smad and p38MAPK were downstream of BMP signaling pathway in the commitment of C3H10T1/2 stem cells to adipocyte lineage. Specific Stealth™RNAi was used to define the regulation of BMP signaling pathway on the expression of miR-140 (10). miR-140 was observed to be significantly repressed by p38 MAPK and Smad4RNAi, respectively (Fig. 4 B). Meanwhile, these two RNAi successfully rescued BMP4-induced Ostm1 repression compared with control RNAi(Fig. 4 C). And their relationship was further confirmed in aP2-BMP4 transgenic mice, in which BMP4 is over-expressed specifically in adipocytes (Fig. 4 D). Stromal vascular fraction was isolated from adipose tissue of wild type and transgenic mice. Ostm1 expression was dramatically decreased in BMP4 over-expressed mice correlative with up-regulation of
miR-140 in the SVF (Fig. 4 E and F). These results indicate that Ostm1 is decreased by BMP4 through miR-140 during adipocyte lineage commitment. Based on the above results, we assumed Ostm1 might function as an anti-adipogenic factor. As shown in Fig. 5C and 5D, adipocyte differentiation could be significantly impaired in Ostm1 over-expressed C3H10T1/2 cells as indicated by Oil Red O staining and Western blotting analysis of adipocyte specific markers. Moreover, when Ostm1 expression was knocked down by siRNA (Fig. 5 F), apparent increase in adipogenic ability was observed. Half dosage of BMP4 resulted in over 70% adipogenesis when Ostm1 was knocked down, compared with less than 10% adipogenesis in control (Fig. 5 G and H). To further confirm that targeting Ostm1 is important for the pro-adipogenic function of miR140, knockdown of Ostm1 was performed in miR-140 over-expressed C3H10T1/2 cells. In the presence of BMP4 treatment, there’s no significant difference between the control group and the Ostm1 knockdown group (Fig.S3), possibly because BMP4 treatment in miR-140 over-expressed cells would lead to nearly total adipogenesis. However, in the absence of BMP4 treatment, knockdown of Ostm1 enhances miR140’s pro-adipogenic effect (Fig.S3), indicating that Ostm1 is an important target of miR-140 during adipocyte lineage commitment. All these results demonstrate that Ostm1 functions as an anti-adipogenic factor and is an important target gene of miR-140 in BMP4-induced commitment of C3H10T1/2 stem cells.

DISCUSSION

Mesenchymal stem cells are multipotent stem cells that can differentiate into a variety of cell types, including osteocyte, chondrocyte, myocyte, and adipocyte (8, 29). Adipogenesis includes two sequential processes, lineage commitment into preadipocytes and terminal differentiation into mature adipocytes (3, 4). Comparing with the second process, commitment leaves many questions unresolved. Previously, we have shown that C3H10T1/2 could commit into adipocyte lineage under BMP4 treatment (4). In the present study, miR-140 was identified as a downstream component of BMP4 signaling pathway during C3H10T1/2 commitment, which was recognized as a chondrocyte specific miRNA before (25-27, 30-33). We consider that it might function as one of the commitment factors. Its over-expression in C3H10T1/2 validates our hypothesis. Exogenously expressed miR-140 in C3H10T1/2 significantly increases the adipocyte differentiation even without BMP4, while miR-140 knockdown significantly decreases adipocyte differentiation. This is the first time that a particular miRNA directly involved in adipocyte lineage commitment has ever been identified. Previously studies have shown that miR-140 is prevalently expressed in normal cartilage and regulates cartilage development and homeostasis. Cartilage master regulator Sox9 promotes miR-140 expression. Besides, John H. shows miR-140 regulates palatogenesis in zebrafish via suppressing PDGFRα (30). Kobayashi uses miR-140-null mice to demonstrate that miR-140 is essential for normal endochondral bone development and miR-140’s target Dnpep reduces BMP signaling to
function in the skeletal defects in the mouse model (33). These results combined with our research indicate that miR-140 is essential for mesodermal tissue development and there is a positive mutual influence between miR-140 and BMP signaling. We then identified a direct miR-140 target protein, Ostm1, which is significantly decreased after BMP4 treatment. When we over-expressed Ostm1 in C3H10T1/2, adipogenic differentiation was significantly decreased, and knockdown led to the opposite. Therefore, we consider Ostm1 functioning as an anti-adipogenic factor in our system. Previous investigation indicates Ostm1 promote β-catenin/Lef1 interaction (24). As all know, Wnt/β-catenin signaling pathway affects multiple cellular functions, and inhibition of this pathway would favor adipogenesis from mesenchymal stem cells (34, 35). Accordingly, we predict Ostm1 repression may lead to decrease of Wnt/β-catenin signaling pathway and favor adipogenic differentiation. We also know that the crosstalk between BMP and Wnt pathways have long been known and is extensively considered as an essential growth factor crosstalk intertwining throughout the whole life of animals(36). These two signals interact at multiple levels and our work poses a possibility that Ostm1 may serve as a new linker molecule between BMP and Wnt pathways. Nonetheless, a physical process must include combinatorial growth or differentiation signaling repertoires. Other undisclosed miR-140 targets may contribute to the adipocyte lineage commitment. Meanwhile, other miRNAs may also function as promotion or suppression factors in adipocyte lineage commitment. More studies are needed to better expound the whole physiological process.

In conclusion, we demonstrate that miR-140 functions as a positive regulator of adipocyte lineage commitment in response to BMP4 treatment, which in turn, decreases Ostm1 in both mRNA and protein level, and conduces to adipogenesis. This is the first time reporting that a specific miRNA participates in BMP4-induced C3H10T1/2 adipocyte lineage commitment. Therefore, these studies offer a new insight to understanding the mechanisms of lineage commitment.

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FIGURE LEGENDS

FIGURE 1. miR-140/140* were induced during adipocyte lineage commitment of C3H10T1/2 after BMP4 treatment. C3H10T1/2 stem cells plated at low density (day -6), were treated without or with BMP4 for 6 days until post confluent, and then subjected to the adipocyte differentiation protocol till day 7. (A and B) On day 7, Oil Red O staining and Western blotting were used to confirm adipogenesis. (C) Relative intensity of western blotting in Fig. 1B was performed through three independent experiments. (D) Activation of BMP4 signal was demonstrated by phosphorylation of p38MAPK and Smad1/5/8 by Western blotting on day 0. (E) miR-140 and miR-140* expression level were detected on day 0 using qRT-PCR. Each column represents the mean ± S.D. of three independent experiments. (F) miR-140 and miR-140* expression pattern during C3H10T1/2 adipogenesis were assessed with qRT-PCR. (day -6 to day 7). Each point represents the mean ± S.D. of three independent experiments.

FIGURE 2. miR-140 promotes adipocyte lineage commitment of C3H10T1/2. C3H10T1/2 stem cells were infected with MSCV or MSCV-miR-140/140*. (A) Two days after post confluence, total RNA was isolated and subjected to qRT-PCR to confirm the over-expression of miR-140 and miR-140*. Each column represents the mean ± S.D. of three independent experiments. (B and C) Upon reaching post confluence, cells were induced to differentiate with standard adipocyte differentiation protocol, and on day 7, Oil Red O staining and Western blotting were used to analyze the adipogenesis. (D) Relative intensity of western blotting in Fig. 2C was performed through three independent experiments. (E and F) MSCV-miR-140/140* infected C3H10T1/2 were transfected with empty pEGFP or pEGFP-miR-140-sponge and treated with BMP4 or not till post confluent. Cells were subjected to the adipocyte differentiation protocol till day 7. Oil Red O staining and Western blotting were used to analyze the adipogenesis. (G) Relative intensity of western blotting in Fig. 2F was performed through three independent experiments.
FIGURE 3. Ostm1 was identified as a target protein of miR-140 during the commitment of C3H10T1/2. (A and B) Western blotting and qRT-PCR were applied to detect Ostm1 expression during the commitment of C3H10T1/2 cells. Each column represents the mean ± S.D. of three independent experiments. (C) MSCV-miR-140/140* infected C3H10T1/2 were transfected with empty pEGFP or pEGFP-miR-140-sponge and treated with BMP4 or not till post confluent. Cells were then subjected to Western blotting to detect Ostm1 expression. (D) Schematic of miR-140 putative target site in Ostm1 3′UTR. (E) 293T cells were transfected with 10 ng empty psiCHECK2 or psiCHECK2-Ostm1 or psiCHECK2-Ostm1-MUT vector and 0.8 ug empty pEGFP or pEGFP-miR-140-sponge vector. Cells were co-transfected with 100nM miR-140 mimic or a negative control. Firefly and Renilla luciferases were measured in cell lysates, and values are normalized to the psiCHECK2 vector and presented as fold change. Each column represents the mean ± S.D. of three independent experiments. ** P<0.01.

FIGURE 4. BMP4 decreases Ostm1 expression through miR-140. (A) Cell lysates were collected each day during commitment and subjected to Western blotting. (B and C) C3H10T1/2stem cells were plated at 30% confluence and transfected with Smad4 or p38 MAPK Stealth™RNAi, and 24h later treated with BMP4 or not until post confluent. Expression of miR-140 and miR-140* were determined by qRT-PCR and expression of p38 MAPK, Smad4 and Ostm1 were determined by Western blotting. Each column represents the mean ± S.D. of three independent experiments. (D) Transgenic mice over-express BMP4 in adipose tissue. Western blot is applied to detect BMP4 expression level in white adipose tissue (subcutaneous) from wild type and aP2-BMP4 transgenic mice. (E and F) SVF was isolated from wild type and transgenic mice to determine miRNAs and protein levels.* P< 0.05.

FIGURE 5. Ostm1 functions as an anti-adipogenic factor during adipocyte lineage commitment of C3H10T1/2. (A and B) C3H10T1/2 cells were infected by MSCV or MSCV-Ostm1. Two days after post confluence, qRT-PCR and Western blotting were used to confirm the over-expression of Ostm1. Each column represents the mean ± S.D. of three independent experiments. (C and D) Upon reaching post confluence, cells were induced to differentiate with standard adipocyte differentiation protocol, and on day 7, Oil Red O staining and Western blotting were used to confirm the adipogenesis. (E) Relative intensity of western blotting in Fig.5D was performed through three independent experiments. (F) C3H10T1/2 were transfected with Ostm1 Stealth™RNAi at 30% confluence and after 24 h were cultured with half dosage of BMP4 (10ng/ml) until post confluent, then induced to differentiate with standard differentiation protocol. (G and H) The accumulation of cytoplasmic triglyceride was detected by Oil Red O staining on day 7, and at which point the cells were photographed. The expression of adipocyte markers (422/aP2 and PPARγ) were also detected with cell extract on day 7. (I) Relative intensity of western blotting in Fig. 5H was performed through three independent experiments.
**Fig 3**

**A**
- MSCV
- MSCV-miR-140/140
- OSTM1
- HSP90

**B**

**C**

|          | MSCV         | MSCV-miR-140/140* |
|----------|--------------|--------------------|
| BMP4     | -            | +                  |
| Vector   | +            | +                  |
| 140Sponge| -            | +                  |
| OSTM1    | -            | -                  |
| HSP90    | -            | +                  |

**D**
- mmu-miR-140: 3'-GAUGGUAAUCCCAUUWGGUGAC -5'
- OSTM1-3'UTR-WT: 5'-AQTACTGTGTGTACAAACCACTG -3'
- OSTM1-3'UTR-Mut: 5'-AGTACTGTGTGTACATTGGTGA -3'

**E**

Relative Luciferase Activity

- NC mimic
- miR-140 mimic

|          | NC mimic | miR-140 mimic |
|----------|----------|---------------|
| WT       |          |               |
| Mut      | *        |               |
| WT+140Sponge |        |               |
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