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# MicroRNAs-141 and 200a regulate the SVCT2 transporter in bone marrow stromal cells

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## Abstract

Vitamin C is a micro-nutrient which plays an important role in bone marrow stromal cell (BMSCs) differentiation to osteogenesis. This vitamin is transported into the BMSCs through the sodium dependent vitamin C transporter 2 (SVCT2). We previously reported that knockdown of the SVCT2 transporter decreases osteogenic differentiation. However, our understanding of the post-transcriptional regulatory mechanism of the SVCT2 transporter remains poor. MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate the messenger RNAs of protein-coding genes. In this study, we aimed to investigate the impact of miR-141 and miR-200a on SVCT2 expression. We found that mouse BMSCs expressed miR-141 and miR-200a and repressed SVCT2 expression at the functional level by targeting the 3'-untranslated region of mRNA. We also found that miR-141 and miR-200a decreased osteogenic differentiation. Furthermore, miRNA inhibitors increased SVCT2 and osteogenic gene expression in BMSCs. Taken together, these results indicate that both miRNAs are novel regulators of the SVCT2 transporter and play an important role in the osteogenic differentiation of BMSCs.

### Keywords

Vitamin C transporter; SVCT2; miRNA-141; miRNA-200a; BMSCs; Osteogenic differentiation

## Introduction

Bone marrow stromal cells (BMSCs) are progenitor cells that differentiate into osteoblasts, osteocytes, adipocytes, and chondrocytes (Prockop, 1997; Pittenger et al., 1999). Differentiation of BMSCs is an important aspect of musculoskeletal development and

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maintenance during a lifetime. Lineage progression of BMSCs is controlled by various factors including DNA methylation, microRNA, transcription, growth factors and nutrition. Vitamin C is an essential nutrient that is required for normal bone marrow stromal cell differentiation, collagen synthesis, and bone formation (Bellows et al., 1986, Lee et al 1992, Choi et al 2008, Maehata et al 2007, Urban et al 2012, Wei et al 2012). It is readily available in the diet and its deficiency is generally rare in developed countries (Richardson et al 2002), except in certain groups such as the elderly, alcoholics and smokers (Alcantara-Martos et al 2007, Oeffinger et al 1998, Schectman et al 1989, Schleicher et al 2009, We et al 2001, Lykkesfeldt et al 1989). Vitamin C is highly water-soluble; it cannot simply diffuse across the hydrophobic lipid bilayer of the plasma membrane to gain access into cells; specific transport systems exist in the plasma membrane to mediate the entry process. We recently reported that vitamin C is transported into BMSCs through a sodium-dependent Vitamin C Transporter 2 (SVCT2) (Fulzele et al 2013). We also reported that this transporter isoform is expressed in bone and intervertebral disc cells (Fulzele et al 2013, Chothe et al 2013). Importantly, we demonstrated that knockdown of the SVCT2 transporter inhibits differentiation of BMSCs into the osteogenic lineage (Fulzele et al 2013).

Given the importance of the SVCT2 transporter in osteogenic differentiation, understanding the molecular mechanisms underlying the regulation of its expression is necessary. Previous studies have shown the regulation of SVCT2 expression at a transcriptional level in various cell types (Savini et al 2007, Portugal et al 2012, Qiao et al 2011, 2012). So far, regulation of SVCT2 expression at a post-transcriptional level has not yet been explored. Post-transcriptional regulation of gene expression can be controlled by various mechanisms such as RNA interference (Smialowska et al 2014, Cernilogar et al 2013), ribozymes (Klauser et al 2014) and microRNAs (miRs) (Zeng et al 2003).

In the last decade, miRNAs have emerged as important regulators of gene expression. MicroRNAs are small noncoding RNAs that down-regulate expression of their target genes by sequence-specific binding to the 3’-untranslated regions (3’-UTRs) of target mRNAs through inducing mRNA degradation or inhibiting translation (Zeng et al 2003). Although the role of most miRNAs remains elusive, several studies indicate that miRNAs act as key regulators in cell differentiation (Chen et al 2014, Kane et al 2014,), cell proliferation (Selcuklu et al 2012, Li et al 2013), and bone formation (Hwang et al 2014, Xie et al 2014). In the present study, we investigated the post-transcriptional regulation of the SVCT2 transporter in mouse BMSCs.

## Materials and Methods

### Isolation of mouse Bone Marrow Stromal Cells (BMSCs)

the mouse BMSCs were isolated according to our published method (Fulzele et al 2013). Briefly, mouse BMSCs were isolated from the long bones of 6 month-old C57BL/6 mice (n=6). The mice were euthanized and the femurs and humeri removed. The marrow was flushed with phosphate-buffered saline (PBS) and the cellular material harvested. The cellular material was centrifuged, the supernatant discarded and the pellet washed with PBS. The cells were plated in 100-$cm^2$ culture plates with DMEM, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/mL penicillin/streptomycin, and 2 mML-

glutamine. After 24 h, the supernatant was removed and the adherent stromal cells trypsinized for negative selection. A negative selection process was used to deplete hematopoietic cell lineages (T- and B-lymphocytic, myeloid and erythroid cells) using a commercially available kit (BD biosciences), thus retaining the progenitor (stem) cell population. The positive fractions were collected using the following parameters: negative for CD3e (CD3 ε chain), CD11b (integrin αM chain), CD45R/B220, Ly-6G and Ly-6C (Gr-1), and TER-119/Erythroid Cells (Ly-76). Positive selections were then performed using the anti-Stem cell antigen-1 (Sca-1) column magnetic bead sorting kit (Miltenyi Biotec, San Diego, CA).

### Quantitative real-time PCR for RNA and miRNA

For RNA analysis, RNA was isolated and real time PCR was performed as per our published method (Fulzele et al 2013, 2014). Briefly, RNA was isolated using the Trizol method following manufacturer's instructions. The RNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using iScript reagents from Bio-Rad on a programmable thermal cycler (PCR-Sprint, Thermo Electron, Milford, MA). 50 ng of cDNA was amplified in each real-time PCR using a Bio-Rad iCycler, ABgene reagents (Fisher scientific) using appropriate primers (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control for normalization.

For miR-141 and miR-200a quantitation, miRNA was isolated from cells using the miRNA easy Isolation kit and reverse-transcribed into cDNA using miScript reagents (Qiagen, Valencia, California). Fifty pg of cDNA were amplified in each qRT-PCR using SYBR Green I and miR-141 or miR-200a specific primers (Qiagen, Valencia, California). The average of RNU6 (RNA, U6 small nuclear 2) and SNORD (small nucleolar RNA, C/D box ) was used as normalization reference genes for miRs. For initial identification of miRNA in BMSCs, the amplified products were run on polyacrylamide electrophoresis gels. Samples were run on a denaturing polyacrylamide gel (12% polyacrylamide, 8 M urea) in 1× TAE buffer; electrophoresis was performed at 250 V for 40 min, followed by staining with ethidium bromide. The gels were scanned and photographed.

### MiRNA mimic and inhibitor (antagomir) transfection Assay

negative control (NC), miRNA mimics of miR-141, 200a and miRNA inhibitor’s (miR-141 and 200a) were purchased from QIAGEN. Lipofectamine 2000 was utilized for transfecting BMSCs according to the manufacturer’s instructions. Transfected cells were used for mRNA analysis and vitamin C uptake assay.

### Vitamin C uptake assay

Functional uptake studies on BMSCs were performed as described previously (Fulzele et al., 2013). Briefly, BMSCs were seeded in 24-well plates at an initial density of $1 \times 10^4$, cells/well. Uptake of [14C] ascorbic acid was measured after 24hrs of miRNA treatment. The medium was removed by aspiration and the cells washed with uptake buffer once. Uptake was initiated by adding 0.25 mL of uptake buffer containing [14C] ascorbic acid (20 nM). Uptake measurements were made with a 15 min incubation representing initial uptake rates. Uptake was terminated by aspiration of the uptake buffer from the cells. The cell monolayers

were quickly washed twice with ice-cold uptake buffer without the radiolabeled substrate. Cells were then lysed in 0.5 mL of 1% SDS/0.2 N NaOH and the radioactivity associated with the cells was quantified. Samples were analyzed using a liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA, USA, model LS-6500). The rate of uptake was normalized to the protein content of each well.

### Vector construction and luciferase reporter assay

For luciferase reporter analyses, the 3′UTR of the mouse SVCT2 gene amplified by PCR from mouse cDNA was cloned into the pEZX-MT05 (GeneCopoeia). BMSCs were seeded into 24-well plates ($5\times10^4$cells/well) and co-transfected with various concentrations of miR-141 and 200a mimic or negative control miRNA and 1 μg of pEZX-MT05 containing or not containing the 3′UTR of the mouse SVCT2 gene. After 24 h, culture media was refreshed and after another 48 h, activities of Gaussia Luciferase (GLuc) and Secreted Alkaline Phosphatase (SEAP) were determined with a luminometer. The relative reporter activity was obtained by normalizing the GLuc activity against SEAP activity.

### Osteogenic differentiation and miRNA treatment

For osteogenic differentiation, BMSCs were trypsinized, harvested, washed and plated in 24-well plates at a density of 5000 cells/$cm^2$. The cells were allowed to adhere and grow to ~90% confluence before being changed to an osteogenic medium (OM). The OM contained DMEM supplemented with 5% FBS, 50 U/mL penicillin/streptomycin, 10 nM dexamethasone, 0.25 mM L-ascorbic acid and 10 mM β-glycerophosphate. The mRNA and miRNA were analyzed at different time intervals. For miRNA treatment experiments, the cells were transfected (only once) with 50uM of miR-141 and 200a mimic on day 0 and osteogenic medium was supplemented the next day (day 1) and changed every 2 days. The mimic was transfected into cells by Lipofectamine 2000. The cells were used for mRNA analysis after 4 day and mineral deposition by alizarin red assay after 21 days of treatments.

### Alizarin red assay

Alizarin red assays were performed according to our published method (Fulzele et al 2013). In brief, BMSCs (5000 cells/$cm^2$) were plated in 24-well plates with osteogenic media as described above. Alizarin red staining was performed after 21 days. The medium was removed, and the cells washed twice with PBS. The cells were fixed with 70% ethanol for 30 min at 4 °C. The fixed cells were stained with Alizarin red solution (40mM; 300 μl/well) for 10 min and then washed with PBS until the supernatant was clear. The staining extent was recorded by photography and the retained dye was extracted with 0.25 ml of 10% (wt./vol.) cetylpyridinium chloride solution for 10 min. The solution was diluted at a ratio of 1:10 and read at 570 nm with a spectrophotometer.

## Results

### MiRNAs that target the SVCT2 transporter 3’UTR

To explore the posttranscriptional regulation mechanism of SVCT2, we performed bioinformatics analyses using TargetScan software to predict putative miRNAs that bind to the SVCT2 3′ UTR. We conducted a database search for conserved miRs that would target

both the human and mouse SVCT2 3'UTR. There were multiple miRs that targeted the SVCT2–3'UTR of both species. We selected miR-141 and 200a for further study. These miRs were selected based on their previously known role in stem cell biology and differentiation (Qiu et al 2014, Cao et al 2013, Itoh et al 2009, Chen et al 2013). MiR-141 and 200a are conserved between human and mouse; alignment of the human and mouse SVCT2–3'UTRs at the 836–842 position is shown in (Fig.1).

### Expression of miR-141 and 200a in BMSCs

Our published study shows that the SVCT2 transporter is functionally expressed in mouse BMSCs (Fulzele et al 2013). Our bioinformatics analyses predict that miR-141 and 200a target the SVCT2–3'UTR. The expressions of miRNAs are tissue specific (Liang et al 2007, Sood et al 2006) and regulate gene expression depending on their low or high abundance in a tissue type. To identify the expression of these miRs in BMSCs, we performed PCR. Here, we show that miR-141 and 200a are expressed in primary mouse BMSCs. To evaluate the specificity of the amplified real time products, melting curve analyses were performed. The melting curves for all the amplified products contained single peaks, indicating specific PCR amplification (Fig.2a). More than one peak in a melting curve usually represents mixed PCR products containing non-specific amplification. The presence of PCR product was further checked by polyacrylamide gel electrophoresis (Fig.2b).

### SVCT2 is a target of miR-141 and 200a

After bioinformatics analysis, initial experiments were performed to determine whether miR-141 and 200a target the SVCT2 transporter at a functional level in mouse primary BMSCs. The BMSCs were transfected with the miR-141 or 200a mimic and vitamin C uptake was performed after 24 and 48hrs hrs. We found that there was a significant ($p \leq 0.001$) reduction of vitamin C uptake in 24 and 48hr post transfected cells (Fig.3). To determine whether miR-141 and 200a directly regulates SVCT2, we used a SVCT2 3'UTR reporter construct consisting of firefly luciferase reporter followed by the 3'UTR of SVCT2 (Fig.4a). MiR-141 or 200a mimics were co-transfected with the SVCT2–3'UTR reporter into BMSCs to measure the effect of their presence on luciferase reporter activity. We found that the miR-141 and 200a mimics were able to repress luciferase activity in a dose dependent manner ($p \leq 0.0001$) relative to the control miRNA (Fig.4b). The negative control miR did not have a target site in the SVCT2–3'UTR and did not have an effect on luciferase activity (Fig.4b). Thus, SVCT2 is a direct regulatory target of miR-141 and 200a through the specific interaction between the miRs and the SVCT2–3'UTR.

### MiR-141 and 200a suppresses osteogenic differentiation

Our published study demonstrated that knockdown of SVCT2 inhibits in-vitro osteogenesis in BMSCs (Fulzele et al 2013). We hypothesized that miR-141 and 200a target the SVCT2 transporter and inhibit osteogenesis. To investigate the effects of miR-141 and 200a on osteogenic differentiation, we transfected negative control, miR-141 and 200a mimic into BMSCs and performed osteogenic differentiation studies. Real time PCR analysis on osteogenic genes showed significant down regulation of ALP, BMP2, collagen type 1, OPN, and OSF2 were significantly down-regulated (Fig.5c). Our results showed a significant ($p \leq 0.01$) decrease in mineralization nodule formation in response to miR-141 and 200a (Fig.5a).

The alizarin red quantification also showed similar results (Fig.5b). These data suggest that miR-141 and 200a participate as a suppressor in the osteogenic differentiation of BMSCs.

### MicroRNAs (miR-141 and 200a) inhibitors regulates bone markers in non- differentiating BMSCs

To determine whether miR-141 and 200a are involved in gene regulation of non-differentiating BMSCs, the cells were treated with miR inhibitors (miR-141 and 200a) for 36 hrs and bone markers genes (ALP, BMP-2, collagen type 1 and SVCT2) were analyzed. Quantitative real time PCR showed that miR inhibitors of miR-141 and 200a significantly up-regulated the expression of ALP, BMP-2, collagen type 1 and SVCT2 genes (Fig.6).

### Statistics

The experiments were performed in triplicates and results were shown as mean ± standard deviation. GraphPad Prism 5 (La Jolla, CA) was utilized to perform ANOVA with Bonferroni pair-wise comparison or unpaired t-tests as appropriate. A P value of $<0.05$ was considered significant.

## Discussion

Bone formation is a complex process involving differentiation of BMSCs to osteoblasts and osteocytes. A number of studies have demonstrated that vitamin C is an important micronutrient that is required for normal BMSC differentiation and bone formation (Bellows et al., 1986, Lee et al 1992, Urban et al 2012, Choi et al 2008, Spindler et al 1989). We have previously reported that vitamin C is transported into BMSCs by a sodium-dependent vitamin C transporter (SVCT), specifically the SVCT2 isoform (Fulzele et al 2013). Furthermore, we report that SVCT2 plays an important role in BMSC migration and in BMSC differentiation along the osteogenic pathway (Fulzele et al 2013, 2014). Consequently, understanding the regulation of the SVCT2 transporter becomes important in BMSC tissue engineering, specifically in directing differentiation toward osteogenesis.

A growing body of evidence has demonstrated that miRNAs play important roles in BMSC differentiation (Chen et al 2014, Kane et al 2014, Dong et al 2012) and pathogenesis, by negatively regulating gene expression (Liang et al 2013, Ceribelli et al 2011, Kapinas et al 2011). In the present study, we carried out bioinformatics analysis to predict the miRNAs that would bind to the SVCT2 3′ UTR. Among the candidates, miR-141 and 200a were selected for further study. MiR-141 and -200a are a part of the 200 miRNA family, which is composed of five distinct miRNAs (miR-200a, miR-200b, miR-429, miR-141 and miR-200c). These miRs are classified into two subfamilies according to the sequence homology in their seed region (Park et al 2008, Davalos et al 2012, Vrba et al 2010). The miRs of the 200 family are located within two clusters on different chromosomes. The miR-200a, miR-200b, and miR-429 are located on chromosome 4 in the mouse genome and chromosome 1 in humans, whereas miR-141 and miR-200c are on mouse chromosome 6 and human chromosome 12 (Davalos et al 2012, Vrba et al 2010, Korpal et al 2008). Initial experiments verified the effect of miR-141 and -200a on vitamin C uptake; i.e., transfection of miR-141 and -200a significantly reduced vitamin C uptake in BMSCs (Fig 3). Moreover,

SVCT2 was identified as a target gene for these miRNAs using 3'SVCT2 luciferases reporter assay (Fig 4). Ours is the first report to demonstrated post-transcriptional regulation of SVCT2 transporter in BMSCs. Previous studies report that expression of the SVCT2 transporter gene is tightly controlled by epigenetic (Qiao et al 2011) and transcription factors (Qiao et al 2011, 2012). For example, SVCT2 transcription is controlled by AP-1 (Savini et al 2007), NF-kappaB (Savini et al 2007, Portugal et al 2012) and zinc-finger transcription factors (Qiao et al 2011) in various cell types. From our studies, it is clear that both miR-141 and -200a target the SVCT2 3' UTR and regulate its expression at a functional level.

BMSCs are pluripotent mesenchymal precursor cells that have the potential to differentiate into osteoblasts, chondroblasts, myoblasts, or adipocytes (Prockop, 1997; Pittenger et al., 1999). We previously reported that knock-down of the SVCT2 transporter inhibits BMSCs differentiation (Fulzele et al 2013). Consequently, we speculate that these miRNA may have anti-osteogenic properties. In this study, we used osteogenic media to induce the osteogenic differentiation of BMSCs; successful induction was demonstrated by extensive formation of mineralized nodules and dramatic increases in osteoblast markers. To investigate the role of miR-141 and -200a in osteogenic differentiation, miR-141 and miR-200a mimics were transfected into BMSCs and osteogenic studies were performed. Our studies showed significant suppression of osteogenic genes and mineral deposition, suggesting inhibition of osteogenic differentiation of BMSCs. MicroRNA inhibitors (of miR-141 and miR-200a) showed increase in osteogenic gene expression in non-differentiating cells. Taken together, these results demonstrate that miR-141 and -200a are involved in SVCT2-dependent osteogenesis.

Studies involving miRs are very complex, because miRs target more than one gene, and one gene is targeted by a number of miRs. We speculate that the inhibitory effect of miR-141 and miR-200a on osteogenic differentiation of BMSCs may not only due to inhibition of the SVCT2 gene but also to targeting other osteogenic genes. It has been previously reported that, miR-141 and -200a target Dlx5 and inhibit bone formation (Itoh et al 2009). It is not new that one miR target multiple genes. Zhang and his co-workers reported that miR-20a promotes osteogenic differentiation of human mesenchymal stem cells by directly targeting more than one gene (PPARγ, Bambi and Crim1 genes) (Zhang et al 2011). MiRs are the important features of gene regulation because it "fine-tune" by directly interacting with multiple genes and signaling pathways. MiR-141 and -200a themselves are shown to influence a wide range of functions from cell proliferation, differentiation, self-renewal, epithelial-to-mesenchymal transition, and cancer biology (Liu et al 2013, Qiu et al 2014, Cao et al 2013, Itoh et al 2009, Chen et al 2013, Gregory et al 2008, Lu et al 2014, Park et al 2008, Davalos et al 2012, Vrba et al 2010). Liu and his co-worker demonstrated that the 200 family directly suppresses the differentiation of mouse embryonic stem cells into endoderm and mesoderm by targeting growth factor receptor-bound protein 2 (Grb2) (Liu et al 2013).

In conclusion, our study is the first report indicating that miR-141 and -200a are regulators of SVCT2 at the posttranscriptional level and play an important role as a suppressor in the osteogenic differentiation of BMSCs. Our findings provide novel insights into the roles of SVCT2-dependent osteogenic differentiation.

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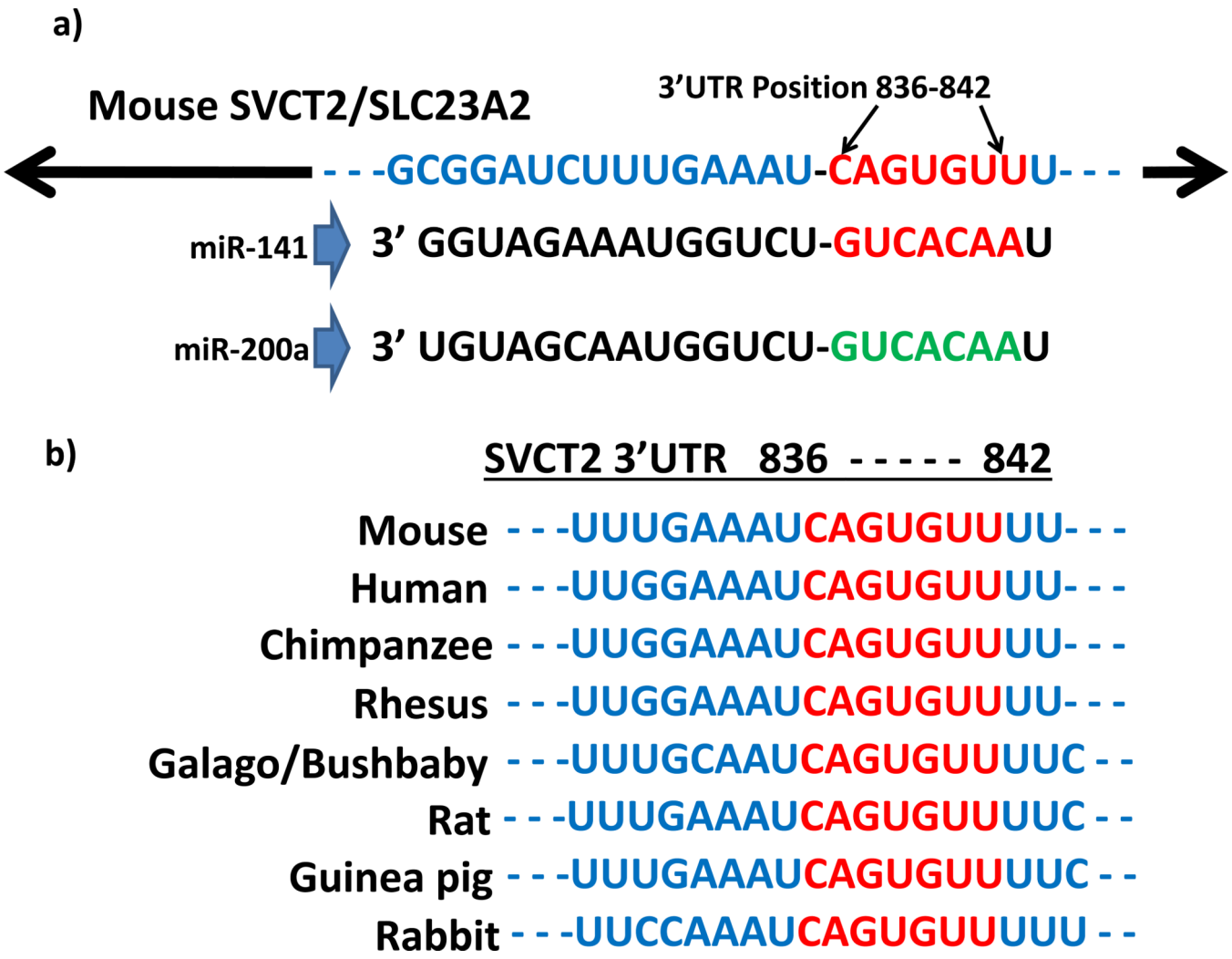


**Figure 1.**

Putative miR-141 and miR-200a binding sites within the mouse SVCT2. a) Schematic representation of the 3′ UTR of mouse SVCT2 mRNA with the putative complementary sequences to miR-141/miR-200a and b) Interspecies conservation of putative miR-141/200a binding sites within the SVCT2.

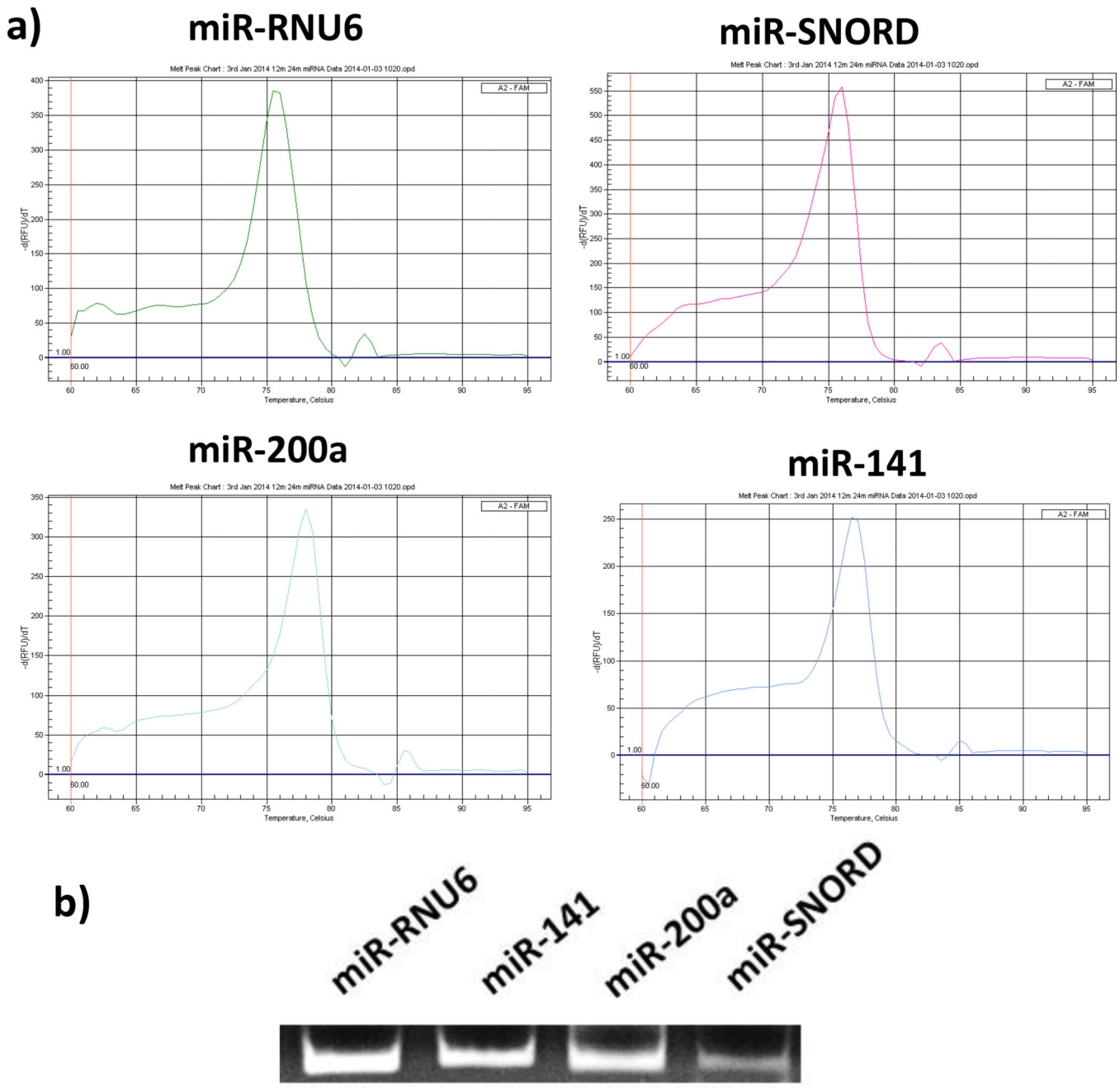


**Figure 2.**

Specific amplification of miR-141 and miR-200a in mouse bone marrow stromal cells. (a) Melting curve analysis of miR-141, 200a, SNORD and RNU6 demonstrates a single peak. (b) Polyacrylamide gel electrophoresis of the miR PCR products.

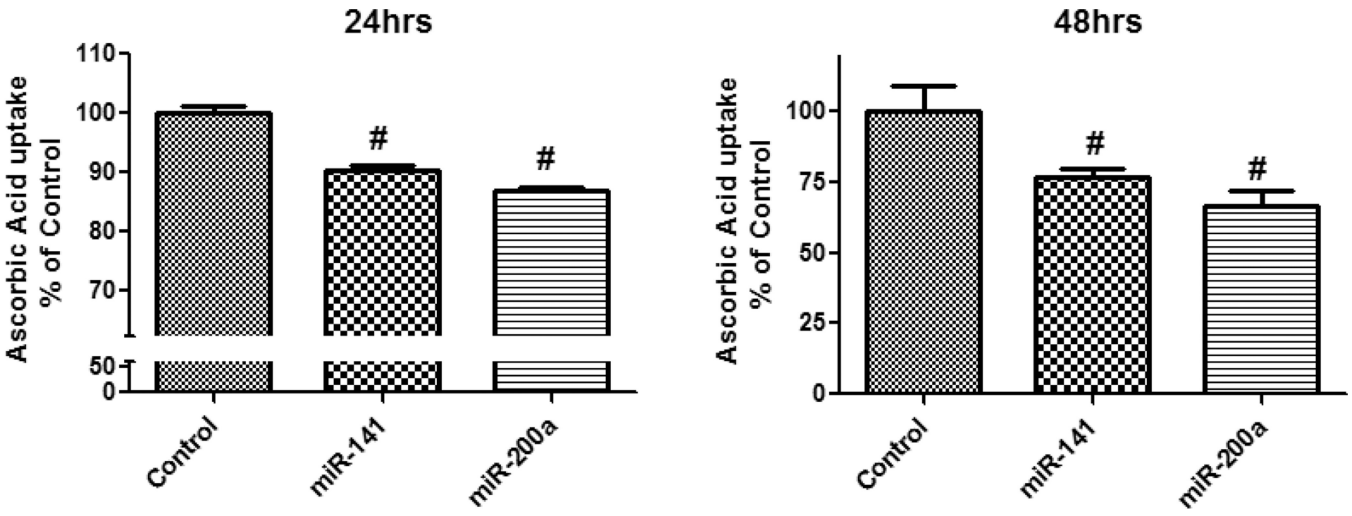


**Figure 3.**

MiR-141 and miR-200a reduce vitamin C uptake in BMSCs: Mouse BMSCs were transfected with miRNA mimic (miR-141 and miR-200a) and control miR for 24hrs and 48hrs. These cells were then used for vitamin C uptake measurements. Uptake of [14C]-ascorbic acid (20 nM) was measured for 15 min in the presence of NaCl. Data (means ± SD, n=6) are expressed relative to the control. Data were analyzed by ANOVA followed by Bonferroni post hoc test (# $p < 0.01$).

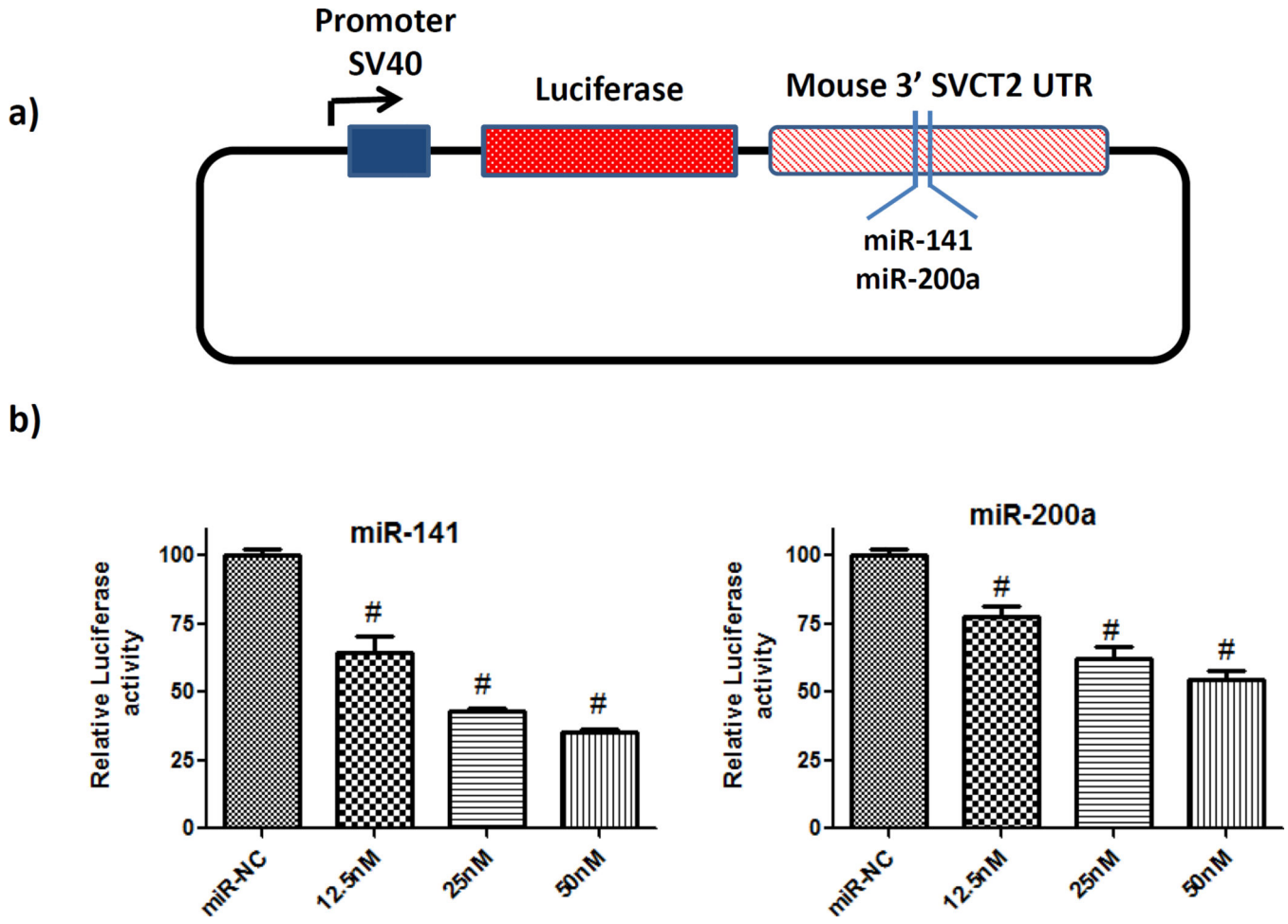


**Figure 4.**

MiR-141 and miR-200a target the 3'UTR of SVCT2 mRNA: Mouse BMSCs were co-transfected with miRNA mimic (negative control miR, miR-141 and miR-200a) and plasmids encoding a luciferase SVCT2 3′UTR for 48hrs. Luciferase activity was determined 48 hours after transfection and normalized to β-galactosidase activity. The control mimic was independently set to 100%. Data are presented as mean ± SEM (n=6). Data were analyzed by ANOVA followed by Bonferroni post hoc test (# $p < 0.01$).

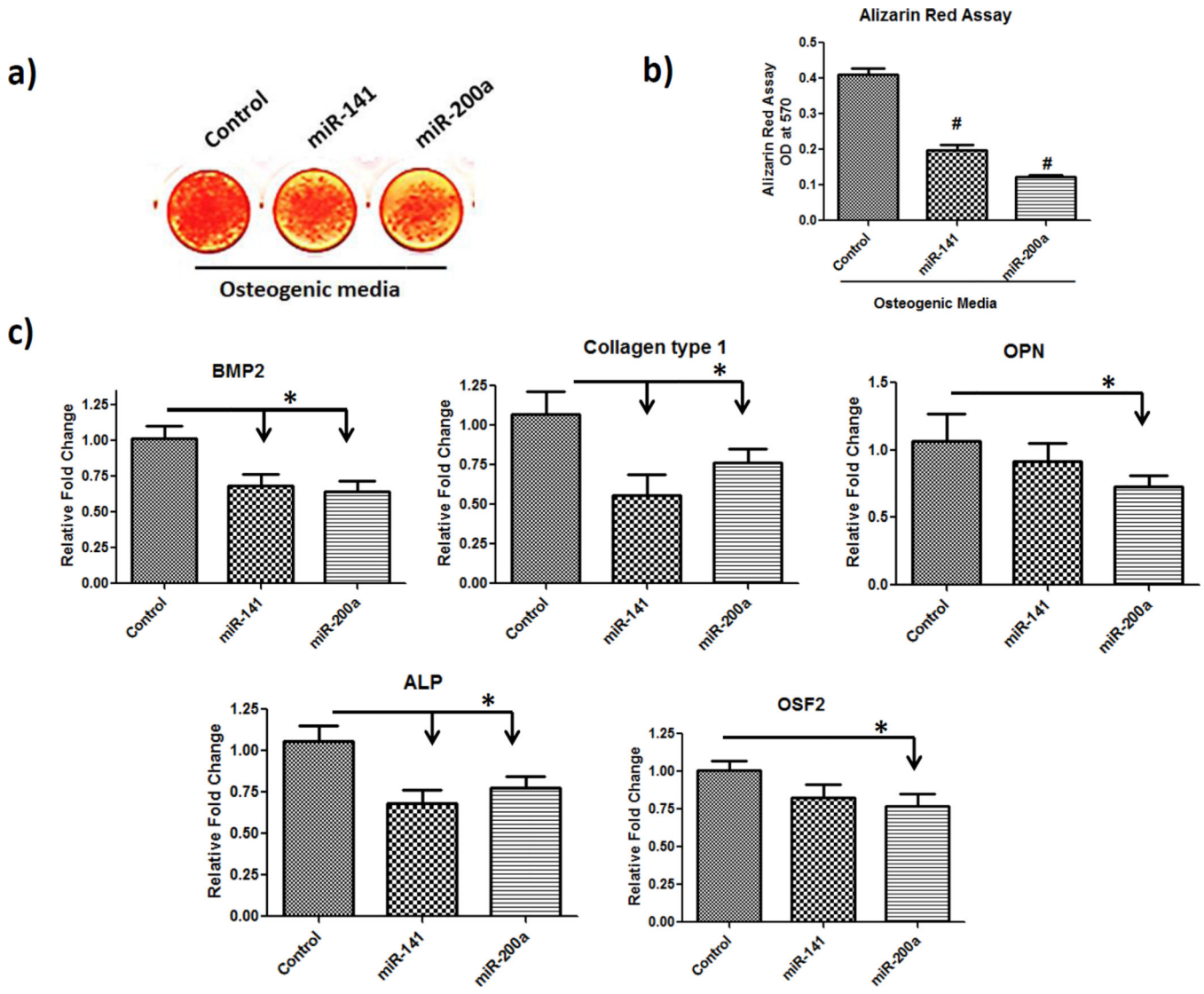


**Figure 5.**

MiR-141 and -200a regulates in-vitro osteogenesis: (**a**) MiR-141 and -200a decrease in-vitro mineral nodules formation during osteogenesis: Mouse BMSCs transfected with miR-141, miR-200a and negative control miR (control) mimics and cultured in presence of osteogenic medium for 21 days followed by Alizarin red S staining for mineralized nodules formation. (**b**) Quantitative analysis of the extent of mineralization in alizarin red S assay using elution of dye by 10% (wt./vol.) cetylpyridinium chloride (means ± SD, n=5). Data (means ± SD, n=5) * $P$<0.05, ** p< 0.01. (**c**) Mouse BMSCs transfected with miR-141, miR-200a and negative control miR (control) mimics and cultured in presence of osteogenic medium for 4 days followed by real time PCR on. (*a*) BMP-2, (*b*) Collagen type 1, (*c*) ALP, (*d*) OPN, and (*e*) OSF2. Data (means ± SD, n=5,) are represented as the fold change in expression compared to control (* $P$<0.05, ** p< 0.01).

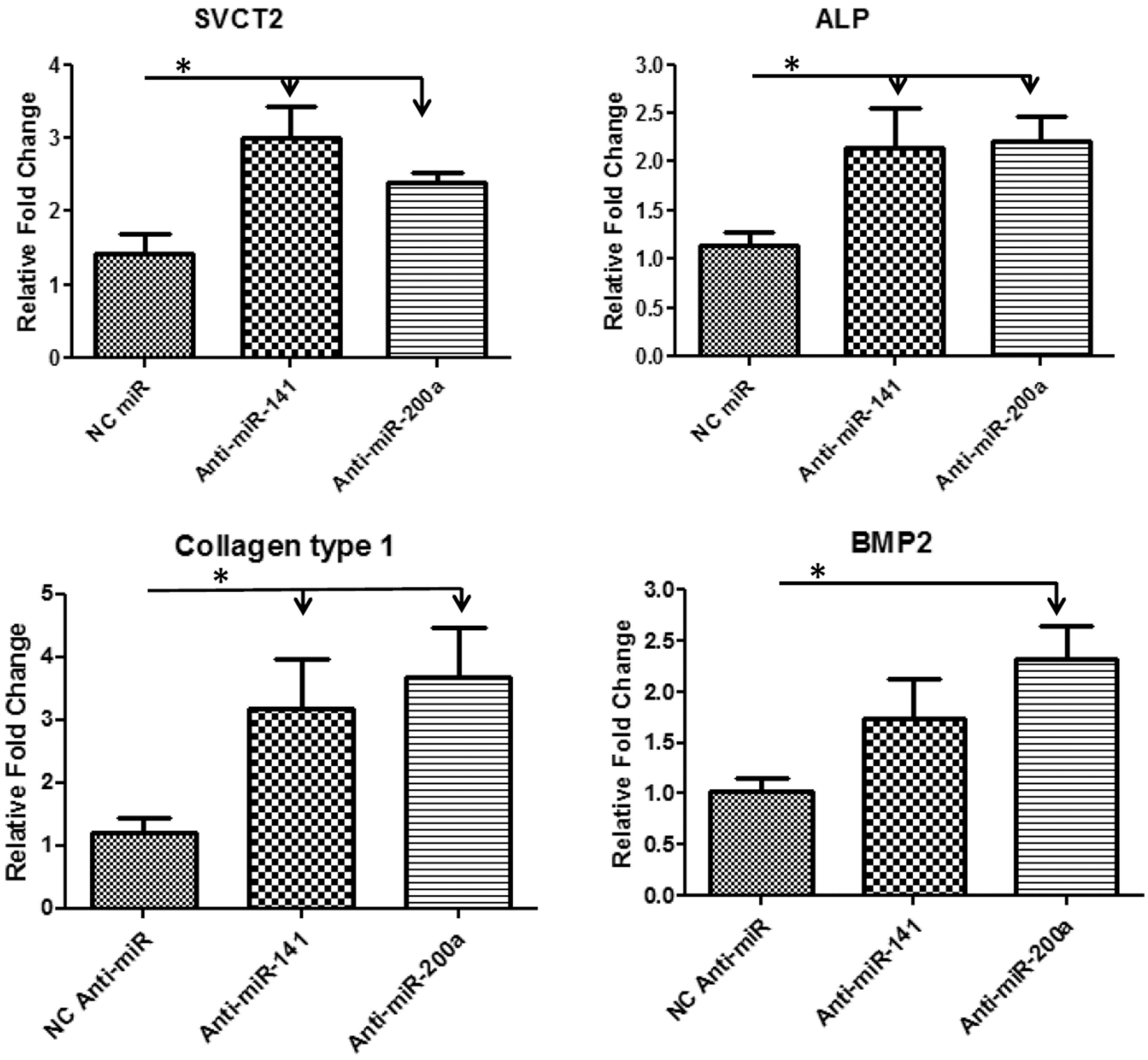


**Figure 6.**

MicroRNA inhibitor of miR-141 and -200a regulates bone marker genes in BMSCs: Mouse BMSCs transfected with miRNA inhibitors (miR-141, miR-200a) and negative control miR (control) for 36hrs followed by quantitative real time PCR on (a) SVCT2, (b) BMP-2, (c) Collagen type 1, and (d) ALP. Data (means ± SD, n=5) are represented as the fold change in expression compared to control (*$P$<0.05).

**Table 1**

Nucleotide sequences of mouse primers used for RT-PCR

| Gene | Primer | Product size in base pair | Annealing temperature (°C) | Reference/Accession Number |
|---|---|---|---|---|
| GAPDH | CAT GGC CTC CAA GGA GTA AGA<br>GAG GGA GAT GCT CAG TGT TGG | 105 | 60 | M32599 |
| SVCT2 | TAA TCC TGG CTA TCC TCG TG<br>CAT CTG TGC GTG CAT AGT AGC | 105 | 60 | Kuo et al 2004 |
| Collagen type I | GCC CAT TAG CCG GTA TGT TAT TA<br>TCC CTG GTA CCT ATG GAG ACT GT | 112 | 60 | U50767.1 |
| BMP-2 | TGT TTG GCC TGA AGC AGA GA<br>TGA GTG CCT GCG GTA CAG AT | 83 | 60 | NM_007553.2 |
| ALP | AGA GTA CGC TCC CGC CAC T'<br>CCT TAC CTG CAG GCA CTC GT | 84 | 60 | X53659 |
| OPN | AGG GCA GCC ATG AGT CAA GT<br>ATC CGA CTG ATC GGC ACT CT' | 98 | 60 | AF515708 |
| OSF2 | CTG CTT CAG GGA GAC ACA CC<br>AAC GGC CTT CTC TTG ATC GT | 85 | 60 | D13664 |