**Glucose dependent miR-451a expression contributes to parathyroid hormone mediated osteoblast differentiation**

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**Abstract**

Parathyroid hormone (PTH; amino acid 1–34, known as teriparatide) has reported promoting differentiation and glucose uptake in osteoblasts. However, how PTH regulates glucose metabolism to facilitate osteoblast differentiation is not understood. Here, we report that PTH promotes glucose dependent miR-451a expression which stimulates osteoblast differentiation. In addition to glucose uptake, PTH suppresses AMPK phosphorylation via PI3K-mTOR-akt axis thereby preventing phosphorylation and inactivation of octamer-binding transcription factor 1 (OCT-1) which has been reported to act on the promoter region of miR-451a. Modulation of AMPK activity controls miR-451a levels in differentiating osteoblasts. Moreover, pharmacological inhibition of PI3K-mTOR-akt axis suppressed miR-451a via increased AMPK activity. We report that this glucose regulated miRNA is an anabolic target and transfection of miR-451a mimic induces osteoblast differentiation and mineralization in vitro. These actions were mediated through the suppression of Odd-skipped related 1 (Osr1) and activation of Runx2 transcription. When injected in vivo, the miR-451a mimic significantly increased osteoblastogenesis, mineralization, reversed ovariectomy induced bone loss and improved bone strength. Together, these findings suggest that enhanced osteoblast differentiation associated with bone formation in case of PTH therapy is also a consequence of elevated miR-451a levels via glucose regulation. Consequently, this miRNA has the potential to be a therapeutic target for conditions of bone loss.

**Keywords:** PTH, Glucose, AMPK, Osteoblast differentiation, MicroRNA

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**1. Introduction**

Parathyroid hormone (PTH; amino acid 1–34, known as teriparatide), with the intermittent treatment regimen, is known to increase bone formation and reduce fracture incidences in osteoporotic patients [1]. Skeletal studies based on PTH therapy show its bone anabolic effects through monitoring osteoblast cells at multiple levels; these include enhancement of osteoblast activity, promotion of osteoblast differentiation, reduction of osteoblast apoptosis, and stimulation of quiescent bone-lining cells [2–5]. Research has shown that elevated serum glucose levels observed in diabetes affect bone metabolism [6]. In response, intermittent PTH treatment has improved bone mineral density and led to a reduction in serum glucose levels and [6,7]. At the cellular level, PTH and insulin-like growth factor I (IGF-1) stimulate glucose transport in osteoblastic cells and promote expression of Glut1 which is an important glucose transporter effective during skeletogenesis [8,9]. In particular, PTH shows its bone anabolic effect by stimulating aerobic glycolysis while suppressing glucose oxidation under the regulation of IGF-1–phosphatidylinositol-3-kinase (PI3K)–mammalian target of rapamycin (mTOR) signaling [10]. Importantly, PTH enhances IGF-1 levels in osteoblast cells [11] and inhibition of either IGF-1 or IGF-1 receptor hampers PTH mediated bone anabolic effect [12]. However, potential application in bone pharmacotherapy demands exploration of additional mechanisms in PTH mediated bone...
anabolism.

Lactation and weaning are the important phases of a female reproductive system coupled with bone resorption and formation respectively. Postweaning phase is the most bone anabolic phase where bone loss during lactation is completely regained [13]. Earlier reports evidenced the link between the postweaning rise in PTH levels and skeletal recovery [14,15]. Previously we evidenced the up-regulation of a limited cohort of micro-RNAs (miRNAs) through miRNA profiling during late weaning phase [16]. Of the various candidates, one miRNA of potential interest is miR-451a. miR-451a has been well studied for its role as a tumor suppressor in osteosarcoma and found to be up-regulated during fracture healing as well as postnatal skeletal recovery [17–20]. Additionally, miR-451a transfection shows glucose dependency and found to be suppressed after AMPK activation during glucose deprivation in glioblastoma cells [21–23]. Since, both PTH and miR-451a show elevated expressions during late weaning phase and helping in fracture repair, we hypothesized that there might be a possibility of the existence of a regulation between them in context to skeletal recovery.

To validate this observation, we examined miR-451a expression during therapeutic intermitted PTH regimen in ovarioctomized mice, where osteoblast activity is increased by PTH resulting in restoration of trabecular network. Our study showed that the glucose regulated miR-451a expression which is in turn modulated specifically by PTH during osteoblast differentiation. We further investigated miR-451a action on the osteoblasts both in vitro and in vivo. Odd-skipped related 1 (Osr1) was identified as a direct target of miR-451a. miR-451a mimic represses Osr1 and promotes the BMP-4 induced osteoblast differentiation and thus bone formation. Through this study, we could establish that PTH regulates glucose dependent miR-451a that in turn increases osteoblast differentiation and finally bone formation.

2. Materials and methods

2.1. Chemicals and reagents

The miRNA isolation kit (miR-Vana), miR-451a mimic, miR-451a inhibitor, miR-control, Osr1 and OCT-1 siRNA, scrambled siRNA were purchased from Ambion (Life Technologies, USA), and the BMP-4 ELISA was from Chongqing Biospe Co., Ltd. (China). Human PTH (1–34), β-Glucose, Compound C and AICAR were purchased from Calbiochem - Merck Millipore, Germany. LY294002 and AKT 1/2 inhibitor, and its antisense called anti-miR-451a; an inhibitor (Assay ID: MC10286, Applied Biosystems; sequence of miR-451a-AAACCGUUACCAUUAC UGA-GUU) which shows activity similar to endogenous mature miRNA and its antisense called anti-miR-451a; an inhibitor (Assay ID: MH10286, Applied Biosystems; sequence-AACUCAGUAAUGGUAA-CAG GUUU) were transfected by using cationic liposomes (Lipopectamine RNAiMAX) according to the manufacturer’s lipofection protocol. Transfections of the mimic and inhibitor into mouse osteoblasts were carried out at 50–60% confluence with reduced-serum and antibiotic-free OptiMEM using the transfection agent (Invitrogen). Knockdown experiments for Osr1 (Assay ID-576718), OCT-1 (Assay ID-68746) were performed using the same protocol as above. ALP activity, mineralization, and qPCR were performed as previously published previously [16].

2.2. miRNA target site prediction and transfection studies in osteoblast cells

In silico putative targets were screened for miR-451a using TargetScan and miRanda on the basis of specific base-pairing rules [16,25,26]. The mature type of miR-451a; a mimic (Assay ID: MC10286 Applied Biosystems; sequence of miR-451a-AAACCGUUACCAUUAC UGA-GUU) which shows activity similar to endogenous mature miRNA and its antisense called anti-miR-451a; an inhibitor (Assay ID: MH10286, Applied Biosystems; sequence-AACUCAGUAAUGGUAA-CAG GUUU) were transfected by using cationic liposomes (Lipopectamine RNAiMAX) according to the manufacturer’s lipofection protocol. Transfections of the mimic and inhibitor into mouse osteoblasts were carried out at 50–60% confluence with reduced-serum and antibiotic-free OptiMEM using the transfection agent (Invitrogen). Knockdown experiments for Osr1 (Assay ID-576718), OCT-1 (Assay ID-68746) were performed using the same protocol as above. ALP activity, mineralization, and qPCR were performed as previously published previously [16].

2.3. Calvarial cell culture and alkaline phosphatase assay

For experiments, 1-day-old mice calvarial osteoblasts (MCOs) were used, harvested from nine to ten calvariae at room temperature. Initially, individual calvariae were isolated from the skull surgically followed by separating the sutures and the pooled calvariae were kept for repetitive digestion (15 min/digestion) with 0.05% trypsin and 0.1% collagenase P to release cells. The first digestion supernatant was discarded. The supernatant containing cells were collected from the next four digestions in 2 ml fetal bovine serum FBS and centrifuged. Cells were cultured in α-minimum Eagle’s medium (Gibco, USA) containing 10% FBS and 1% penicillin/streptomycin (complete growth medium). Calvarial osteoblasts were allowed to reach 70–80% confluence for the experiments [16].

For the measurement of alkaline phosphatase (ALP) activity, MCOs at ~ 80% confluence were trypsinized and 2 × 104 cells per well were seeded in 96-well plates. Cells were transfected and incubated for 48 h in α-MEM supplemented with 5% charcoal treated FBS, 10 mM β-glycerophosphate, 50 μg/ml-1 ascorbic acid, and 1% penicillin or streptomycin (osteoblast differentiation medium). At the end of the incubation period, total ALP activity was measured using p-nitrophenyl phosphate (PNPP) as substrate and absorbance was read at 405 nm using SpectraMax Paradigm Multi well Elisa plate reader (Molecular Devices) [27].

2.4. Mineralization assay

MCOs were seeded on to 12-well plates (10,000 cells per well) in osteoblast differentiation medium. MCOs were transfected with or without miR-C, miR-451a, Anti-miR-451a, stScr or siOsr1, incubated for 21 days. Media were changed and transfection was performed after every 48 h. At the end of the experiment, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 15 min. The fixed cells were stained with alizarin red-S (ARS) (40 mM, pH 4.5) for 30 min followed by washing with tap water. Stained cells were first photographed under a light microscope, and alizarin stain S was then extracted by using 10% (v/v) acetic acid with shaking at room temperature for 30 min. Cells were scraped out from wells and centrifuged (2000g for 15 min), and the supernatant was collected. To the supernatant, 10% (v/v) ammonium hydroxide was added to bring the pH of the solution to 4.5 for colour formation. Absorbance of the solution was read at 405 nm [28].

At the end of the treatments, bone marrow cells (BMCs) from the femora of mice were flushed out in osteoblast medium containing α-MEM supplemented with 5% charcoal treated FBS, 10 mM β-glycerophosphate, 50 μg/ml-1 ascorbic acid and 1% penicillin or streptomycin (osteoblast differentiation medium) and 10−7 M dexamethasone (bone marrow differentiation medium). BMCs were cultured for 21 days and mineralized nodules were stained and quantified as described above [29].

2.5. Quantitative real-time PCR (qPCR)

Cells were collected in 1 ml of TRIZol reagent (Invitrogen), and total RNA was extracted according to the manufacturer’s protocol. cDNA was synthesized using Revert Aid First Strand cDNA synthesis kit (No.K1621 Fermentas, Burlington, ON, Canada) from 1 μg of total RNA. Quantitative real-time PCR amplifications were performed in the Light Cycler 480 Real-Time PCR System (Roche Diagnostics, Indianapolis, IN, USA) using Light Cycler SYBR Green (Roche Diagnostics) according to the manufacturer’s instruction. The sequences of primer sets for Osr1, p27, BMP-4, Runx2, OCT-1 and β-actin are shown in Table 1. The quantity of each sample was normalized using the CT (threshold cycle) value obtained for the β-actin mRNA amplifications [16].
Table 1

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer sequence</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>F-CTAGGCGCAGCTGGAAGAG</td>
<td>NM_007393.5</td>
</tr>
<tr>
<td>BMP-4</td>
<td>R-ACCAGGAGCTACAGGGACA</td>
<td>NM_007554.3</td>
</tr>
<tr>
<td>Runx2</td>
<td>R-GCTCTCGCAGAGAGATCA</td>
<td>NM_00114920.2</td>
</tr>
<tr>
<td>Osr1</td>
<td>R-AGAAGGGTGACACCTGTCTGC</td>
<td>NM_011859.3</td>
</tr>
<tr>
<td>p27</td>
<td>R-GTTACGGGAGAGCTGCTCA</td>
<td>NM_009875.4</td>
</tr>
<tr>
<td>OCT-1</td>
<td>R-TGGTGGCTGACTCTTCTCTC</td>
<td>NM_198934.3</td>
</tr>
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qPCR, quantitative polymerase chain reaction; F, forward; R, reverse.

2.6. Western blot analysis

MCoS were grown to 80% confluency followed by various treatments for respective time periods. Cells were washed with cold phosphate-buffered saline (vehicle), and whole-cell lysates were prepared by the addition of lysis buffer from Sigma-Aldrich containing a protease inhibitor and phosphatase inhibitor mixture from Sigma-Aldrich. Femur was crushed in liquid nitrogen and lysis buffer followed by centrifugation [16]. Protein content was quantified by BCA method. Protein (30–50 μg) was loaded per lane and separated on a 10% polyacrylamide gel, followed by transfer to a PVDF membrane (Millipore, Billerica, MA, USA) by electroblotting. Membrane was blocked with 5% non-fat dry milk and followed by incubation with a primary antibody at 4 °C overnight. OCT-1, Osr1, mTOR, p-mTOR, Runx2, AMPK (Abcam, Billerica, MA, USA), and anti-miR-451a and were grown in Lab-Tek Chamber Slides (Nunc, Denmark) for 48 h. For immunocytochemistry, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, followed by permeabilization with 0.1% Triton X-100 and incubation in primary antibody (Runx2-mouse monoclonal antibody 1:100 dilution). Sections were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling, USA) and visualized by an enhanced chemiluminescence system (GE Healthcare Life Sciences, Bangalore, India) according to the manufacturer’s instructions [16]. Densitometry was performed using ImageJ software as previously described [30].

2.7. Immunocytochemistry

MCoS were incubated in medium with or without miR-451a mimic and anti-miR-451a and were grown in Lab-Tek Chamber Slides (Nunc, Denmark) for 48 h. For immunocytochemistry, cells were fixed with 4% paraformaldehyde (PFA) followed by permeabilization with 0.1% Triton X-100 and incubation in primary antibody (Runx2-mouse monoclonal antibody 1:100 dilution) for overnight. Alexa Fluor 488 goat anti-mouse (Invitrogen, USA) was used as secondary antibody. Slides were mounted with ProLong™ Gold Antifade Mountant with DAPI (Life Technologies, USA). Fluorescence was captured using a fluorescent microscope (Eclipse 80i, Nikon, Tokyo, Japan) with the aid of appropriate filter (excitation 490 nm and emission 525 nm) [31].

2.8. [3H] 2-Deoxyglucose uptake assay

The MCoS were cultured in 24-well plates till 70–80% confluence. Cells were treated with PTH (500 ng/ml) for 48 h in differentiation medium. Washing followed by incubation was performed with serum-free medium. Cells were incubated with Insulin (10 nM) for 30 min followed by washing with HEPES saline (pH 7.4). This is followed by incubation with 250 μl of KKH buffer (20 mM HEPES, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO4, and 1.25 mM CaCl2) containing mixture of 5 μCi of 2-[1,2-3H]-deoxy-o-glucose and 1 mM of 2-Deoxy Glucose (MP Biomedicals, California, USA) and left for 5 min. The reaction was terminated by adding stop solution (200 mM of 2-DOG) followed by two times washing with ice-cold PBS. Cells were scraped in of 0.5 M NaOH (500 μl per well) and collected in scintillation vials containing 5 ml of cocktail W scintillation grade (Sisco Research Laboratories Pvt. Ltd., Mumbai, India). Radioactivity retained by the cell lysate was measured by a liquid scintillation counter (Beckman Coulter, LS 6500, and Beckman Coulter Inc. USA) [32].

2.9. In vivo studies and assessment of bone parameters

The study was carried in accordance with current legislation on animal experiments (Institutional Animal Ethical Committee at Central Drug Research Institute, Approval Reference no. - I.AEC/2017/F-235) and performed according to the regulations of the Council for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. Female 8 weeks old BALB/c mice (18 ± 5 g) were kept in a 12-h light-dark cycle with controlled temperature (22–24 °C) and humidity (50–60%) and ad libitum access to standard rodent food and water [16]. The subcutaneous dose for PTH (40 μg·kg⁻¹·day⁻¹) used in this study is shown to have an anabolic effect [33]. Micro-computed tomography (μCT) measurements were carried out on a SkyScan 1076 CT scanner (Aartselaar, Antwerp, Belgium). miR-451a mimic and miR-C were administered (7 mg·kg⁻¹ each) through tail vein injection [16,34] with an anionic liposome (Invivofectamine 3.0, Life Technologies) which allows for the fusion of the liposome–nucleic acid complex with the cell membrane and subsequent uptake by endocytosis. Various treatments were started 6 weeks after the ovariectomy and continued for 6 weeks. Precisely, injections were given on 1st, 2nd and 3rd days of 1st, 3rd and 5th weeks. After 6 weeks of treatment, all mice were sacrificed and autopsied to collect femur (measurement of the bone parameter) and organs like liver, kidney and uterus. Blood was collected to separate serum and stored at −80 °C for further experiments. Bone marrow cells (BMCs) from femurs of vehicle and treatment groups were harvested, and mineralization was studied as described previously [29]. Briefly, femora were scanned at a nominal resolution of 18 mm. One hundred projections were acquired (angular range, 180°) and image slices were reconstructed using a modified Feldkamp algorithm (according to Sky Scan Nrecon software). Parameters, including bone volume-to-tissue volume (BV/TV), trabecular number (Tb.N), and trabecular separation were calculated [16,29]. Femora were subjected to three-point bending using a bone strength tester (model TK-252C; Muromachi Kikai, Co. Ltd., Tokyo Japan) [16].

To check the integrity of the trabecular region, 6 μm undecalciﬁed sections were prepared from distal femur metaphysis and embedded in polymerized methyl methacrylate. For osteoblast and osteoclast/bone resorption examination Goldner trichrome, Toluidine blue, Von Kossa and TRAP staining were performed according to the protocol available from Bioquant [35,36]. Total trabecular bone volume was measured by using Bioquant Osteo Software (Bioquant Image Analysis, Nashville, TN, USA).

2.10. Immunohistochemistry

Immunohistochemistry was performed to examine the expression level of Osr1 and Runx2 in the trabecular region of decalcified distal femur metaphysis. Femurs were ﬁxed in 4% paraformaldehyde in 0.1 M PBS (pH7.4). Fixed bones were decalcified by immersion in decalcifying solution (10 mM Na2EDTA, 0.1 M NaCl, 4.7 mM KCl, 1.25 mM MgSO4, and 1.25 mM CaCl2) containing mixture of 5 μCi of 2-[1,2-3H]-deoxy-o-glucose and 1 mM of 2-Deoxy Glucose (2-DOG; MP Biomedicals, California, USA) and left for 5 min. The reaction was terminated by adding stop solution (200 mM of 2-DOG) followed by two times washing with ice-cold PBS. Cells were scraped in of 0.5 M NaOH (500 μl per well) and collected in scintillation vials containing 5 ml of cocktail W scintillation grade (Sisco Research Laboratories Pvt. Ltd., Mumbai, India). Radioactivity retained by the cell lysate was measured by a liquid scintillation counter (Beckman Coulter, LS 6500, and Beckman Coulter Inc. USA) [32].
(Invitrogen, USA) for 1 h at room temperature. Sections were washed with PBS and mounted with ProLong™ Gold Antifade Mountant with DAPI (Life Technologies, USA). Fluorescence was captured using a confocal microscope (Carl Zeiss LSM510 Meta) with the aid of appropriate filter [37].

2.11. Statistical analysis

For experiments requiring multiple comparisons testing, we used one-way ANOVA followed by Newman-Keuls test of significance (GraphPad Prism v.5). Student’s t-test was used for experiments with only two treatments [16].

3. Results

3.1. miR-451a drives osteoblast differentiation by regulating Osr1

The highly conserved miR-451a is identified earlier at the late weaning phase in the reproductive window [16]. It is located on human chromosome 17 in the noncoding region and forms a characteristic miRNA precursor stem-loop secondary structure (Fig. 1A). Tissue specific miRNA profiling showed miR-451a expresses significantly high in bone and liver compared with skeletal muscle or kidney (Fig. 1B). Furthermore, miR-451a expression increased significantly during osteoblast differentiation as well as in the mineralization phase (~2 fold) (Fig. 1C).

In our attempt to predict the target for miR-451a using TargetScan and miRBase, Odd-skipped related 1 (Osr1) was considered as the putative targeted gene. Osr1 showed an 8-nt seed sequence match with miR-451a in the 3’ UTR and was highly conserved among vertebrates (Fig. 1D) [38,39]. Further, we confirmed the differential expression pattern of miR-451a during proliferation, differentiation and mineralization by western blotting for its putative target Osr1. During proliferation stage, Osr1 showed maximum expression while Runx2 showed decreased expression, Osr1 expression decreased gradually as cell started differentiation and mineralization while Runx2 increased concurrently (Fig. 1E–F). Transient transfection of MCOs with the miR-451a mimic stimulated osteoblast differentiation, ALP activity and mineralization along with increased mineralized nodules. In contrast, anti-miR-451a (a specific blocker for miR-451a) significantly reduced ALP activity and mineralization nodules (Fig. 1G–I). Quantitative PCR (q-PCR) showed increase in BMP-4, p27 and Runx2 expression with the miR-451a mimic transfection in MCOs cultures, while the modest inhibitory effects were noted with the anti-miR transfection (Fig. 1J). In concordance with the in silico data, we found that the miR-451a mimic repressed Osr1 expression, whereas Osr1 expression increased with anti-miR transfection. Immunofluorescence microscopy confirmed an increase in Runx2 expression in MCOs transfected with miR-451a mimic while a decrease in Runx2 protein expression with anti-miR-451a (Fig. 1K). These changes were further corroborated at the protein level by western blotting (Fig. 1L).

To extrapolate this study in humans, human osteoblast-like cells – MG-63 were transfected with miR-451a mimic and anti-miR. Similar results were observed in MG-63 as seen in MCOs (Fig. 1L). Taken together, the results suggest that miR-451a directly promotes osteoblast differentiation and mineralization.

We next examined whether Osr1 repression was a pre-requisite for miR-451a induced Runx2 activation leading to osteoblast differentiation. siRNA mediated knockdown of Osr1 in MCOs showed increased ALP activity and mineralization compared with scrambled siRNA transfected cells (Fig. 1M–O). Furthermore, the expression of Runx2, p27 and BMP-4 protein in Osr1-depleted cells were analyzed. A markedly increased expression of Runx2, p27 and BMP-4 proteins were observed (Fig. 1P). We next analyzed the miR-451a expression after 21 days of transfection in mice calvarial cells. miR-451a mimic transfection elevated the endogenous miR-451a levels by ~3000 folds while anti-miR-451a suppressed the endogenous miR-451a levels (Fig. 1Q). These data suggest that Osr1 down-regulation is critical for miR-451a induced Runx2 expression and osteoblast differentiation.

3.2. PTH promotes miR-451a expression via glucose regulation

Given that, miR-451a is dependent on glucose in hepatocytes and cancer cells [21–23,40], we tested the levels of mature miR-451a in MCOs cultured for 48 h at three different conditions; vehicle or 1 mM glucose (low glucose condition), 4.5 mM (physiological glucose condition) and 10 mM (high glucose condition). Medium was replenished every 12 h up to 48 h. Significant increase in miR-451a levels was observed in high glucose (10 mM) condition when compared with low glucose (1 mM) condition; whereas physiological range showed an intermediate effect (Fig. 2A). Further, MCOs were cultured for 48 h in three different regimens: G0 (Æ); continuously high glucose (medium replenished every 12 h), G1 (ÆÆ); continuously low glucose, and G2; low glucose replenished at 24 h with high glucose medium (Fig. 2B). In continuous high glucose, the expression of miR-451a was high, while in low glucose it significantly dropped. miR-451a dependency on glucose was further confirmed when an increase in miR-451a levels was observed in the osteoblasts after the glucose replishment (Fig. 2C). In order to check the glucose-sensitive response of miR-451a expression on its direct target Osr1, western blotting was performed. Notably, in high glucose conditions, the expression of Osr1 remained low while in low glucose it was significantly elevated. Moreover Osr1 level decreased after the supplementation of glucose. On the contrary, Runx2 expression showed an inverse pattern compared with Osr1 (Fig. 2D).

These results suggest that the expression of miR-451 is intrinsically linked to glucose levels.

PTH therapy is known to lower blood glucose levels in osteoporotic women [6,7] may be because PTH promotes aerobic glycolysis in osteoblast cells [10]. Thus, we investigated the change in miR-451a expression pattern during PTH supplementation. In consistent with the previous report, 500 ng·ml−1 of PTH [10] on osteoblasts for 48 h, significantly enhanced glucose uptake as confirmed by [3H] 2-deoxyglucose (2-DG) uptake assay (Fig. 2E). Dose-dependent increase in miR-451a expression was observed in MCOs during PTH treatment (Fig. 2F). We next checked whether the presence of glucose has any effect on PTH mediated miR-451a expression. Glucose showed the
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A. Glucose-regulated miR-451a expression.

B. MCoB glucose regimen.

C. miR-451a expression (fold change).

D. Western blot analysis: Osr1, Runx2, β-Actin.

E. Glucose-stimulated apoptosis.

F. miR-451a expression (fold change).

G. miR-451a expression (fold change) with PTH.

H. Relative mRNA expression of Osr1.

I. Relative mRNA expression of Runx2.

J. Relative mRNA expression of BMP-4.

K. Relative mRNA expression of BMP-2.

L. Relative mRNA expression of p27.

Caption on next page.
additive effect to PTH mediated miR-451a expression, as revealed by
quantitative real-time PCR assay (Fig. 2G). In agreement with the in-
crease in miR-451a expression by PTH, Osr1 mRNA level was decreased
whereas Runx2, BMP-4, BMP-2 and p27 transcripts were increased
(Fig. 2H-L). Taken together, these data suggest that PTH promotes miR-
451a expression via glucose uptake in osteoblast in vitro.

3.3. PTH promotes miR-451a mediated osteoblast differentiation by
regulating AMPK activation

Provided that in low glucose conditions, activated AMPK phos-
phorylates and inhibits OCT-1 (a glucose regulated transcription factor)
which in turn prevents miR-451a transcription in cancer cells [23]; we
explored the involvement of PTH with this mechanism in osteoblasts.
MCOs grown in low glucose conditions (vehicle) showed increased
phosphorylation of AMPK (T172) and OCT-1 (S336) (complementary to
OCT-1 at S335 in humans [23]) whereas in high glucose decreased
phosphorylation was observed, similar to cancer cells. Ex-
pression of downstream targets for miR-451a was further investigated
to understand the association of miR-451a with the above mechanism
in osteoblasts. Protein levels of Osr1 increased whereas, BMP-4, p27
and Runx2 levels decreased in low glucose conditions indicating de-
creased miR-451a levels and osteoblast differentiation (Fig. 3A).

Furthermore, PTH supplementation in MCOs decreased the phos-
phorylation of both AMPK as well as OCT-1 and their phosphorylation
decreased further when PTH was supplemented in high glucose
conditions (Fig. 3A). Similarly, to investigate the effect of PTH mediated
AMPK inhibition on miR-451a levels, the expression pattern of down-
stream targets for miR-451a were analyzed. PTH markedly decreased
the Osr1 levels and increased BMP-4, p27 and Runx2 protein levels
indicating elevated miR-451a levels and osteoblast differentiation.
Supplementation of glucose further boosted PTH mediated effect on
these differentiation markers.

Pharmacological inhibition of AMPK activity by the small molecule
inhibitor Compound C [41] resulted in the reduction of OCT-1 (S336)
phosphorylation (Fig. 3A) with a simultaneous decrease in Osr1 levels
leading to increasing miR-451a levels. Vice versa stimulation of AMPK
by activator AICAR showed the opposite effects (Fig. 3A). In the
agreement, we observed elevated BMP-4, p27, Runx2 levels in presence
of Compound C while these proteins were suppressed in presence of
AICAR. PTH supplementation along with Compound C or AICAR
showed additive effects. These observations confirmed the involvement
of PTH in AMPK inhibition leading to increased miR-451a expression
and osteoblast differentiation. Furthermore, quantitative real-time PCR
analysis showed increased miR-451a levels in the presence of AMPK
inhibitor (compound C) while AMPK activator (AICAR) significantly
alleviated miR-451a transcript (Fig. 3B). These results suggest that
there might be possibility of existence of a mechanism for PTH by
which it promotes miR-451a mediated osteoblast differentiation via
inhibiting AMPK activation in presence of glucose. To confirm func-
tional link between AMPK phosphorylation and osteoblast differentia-
tion we treated MCOs with increasing concentration of AICAR for 48 h
in differentiating medium. AICAR dose dependently elevated p-AMPK
(T172) levels compared to untreated cells. AMPK phosphorylation was
followed by OCT-1(S336) phosphorylation. AICAR treatment resulted in
increased Osr1 levels along with decrease in Runx2, BMP-4 and p27
levels in dose dependent manner (Fig. 3C-D). These results suggest that
reduction in p-AMPK (T172) levels were essential during osteoblast
differentiation.

3.4. Glucose uptake favors PTH mediated inhibition of AMPK activation
and osteoblast differentiation

To validate involvement of glucose uptake in PTH mediated sup-
pression of AMPK phosphorylation and osteoblast differentiation, we
treated MCOs with the glucose transporter inhibitor WZB 117 (Glut-i)
which competitively inhibits GLUT 4, GLUT 1 and GLUT 3. Glucose
alone and PTH supplemented with glucose significantly decreased p-
AMPK (T172), p-OCT-1(336) and Osr1 levels and increased osteoblast
differentiation markers Runx2, BMP-4 and p27 compared with the ve-
hicle as concurrent with the previous results. Pharmacological inhibi-
tion of glucose transporters GLUT on osteoblast by addition of Glut-i
resulted into inverse effects as observed in case of glucose and PTH
supplemented with glucose conditions. Addition of Glut-i removed the
inhibitory effect as observed in case of PTH supplemented with glucose
and elevated p-AMPK, p-OCT-1 and Osr1 levels while inhibited osteo-
blast differentiation markers Runx2, BMP-4 and p27 (Fig. 4A-B). These
findings suggest that glucose uptake is necessary for PTH mediated
AMPK inactivation during osteoblast differentiation.

3.5. OCT-1 drives miR-451a expression and promotes osteoblast
differentiation

Earlier, the possibility of OCT-1 in osteoblast differentiation has
been reported but the exact mechanism was not known. To elucidate
the role of OCT-1 or POU2F1 during osteoblast differentiation we ex-
amined whether OCT-1 repression was a pre-requisite for miR-451a
induced Runx2 activation leading to osteoblast differentiation. siRNA
mediated knockdown of OCT-1 in MCOs showed a significant decrease
in miR-451a levels compared with scrambled siRNA-transfected cells
(Fig. 5A). Quantitative PCR (q-PCR) showed an increase in Osr1 level
while a decrease in BMP-4, p27 and Runx2 expression with the OCT-1
siRNA transfection in MCOs cultures (Fig. 5B). Furthermore, the ex-
pression of Osr1, Runx2, p27 and BMP-4 protein in OCT-1-depleted
cells were analyzed. A markedly decreased expression of Runx2, p27
and BMP-4 proteins were observed thus inhibiting osteoblast differ-
entiation (Fig. 5C-D). These findings suggest that OCT-1 is crucial for
miR-451a expression and promotes osteoblast differentiation.

3.6. PTH promotes glucose mediated inhibition of AMPK activation by
P38K-mTOR signaling

Previously, PTH has been reported to promote glycolysis via P38K-
mTOR signaling [10]. The observed PTH mediated inhibition of AMPK
in presence of glucose led us to examine the involvement of P38K-mTOR
signaling in miR-451a mediated osteoblast differentiation. Involvement
of P38K-mTOR signaling pathway was clearly observed in the inhibition
of AMPK phosphorylation by PTH in presence of glucose (Fig. 6A, left
panel). This was evident from significant decrease in relative phospho-
AMPK (T172) levels with a simultaneous increase in relative p-P38K, p-
mTOR and p-AKT (S473) levels.

To further confirm this, we evaluated the signaling cascade in

Fig. 2. PTH promotes miR-451a expression via glucose regulation. (A) Expression (qPCR) of miR-451a with different glucose concentrations in MCOs; (n ≥ 3). (B-D) Glucose (10 mM) regimens used in the experiment; G ▲ (▲); continuously high glucose, G ▼ (▼); continuously low glucose and GA; low glucose replenished at 24 h with high glucose medium (B), expression (qPCR) of miR-451a expression in glucose regimens used (C); (n ≥ 3) and western blotting showing the expression of target protein Osr1 and Runx2 in glucose regimens used in the experiment (D); (n ≥ 3). (E) Glucose uptake in control and PTH (500 ng·ml⁻¹) treated osteoblasts for 48 h. The experiment was performed with or without acute insulin pulsing at 10 nM; (n ≥ 3). (F-G) Expression (qPCR) of miR-451a was assessed with different PTH concentrations in MCOs (F); (n ≥ 3) and glucose supplementation (10 mM) with PTH (500 ng·ml⁻¹) (G); (n ≥ 3). (H-L) Quantitative PCR was performed for the expression of target gene Osr1 (H), Runx2 (I), BMP-4 (J), BMP-2 (K) and p27 (L) during osteoblast differentiation with or without glucose and PTH at 48 h; (n ≥ 3). Data represented as Mean ± S.E.M. from three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001, compared with control; other comparisons,
*p < 0.05, *p < 0.1, *p < 0.05, **p < 0.01, ***p < 0.001, compared with control; other comparisons,
presence of PI3K or AKT inhibitors, along with glucose and PTH supplementation. Inhibition of PI3K by LY294002 blocked PTH mediated AMPK inactivation. This was evident in the results showing no change in relative phosphorylation of AMPK and p-PI3K, p-mTOR and p-AKT (S473) levels (Fig. 6A, middle panel, B) in case of PI3K inhibition. Moreover, glucose supplementation along with PTH could not show any additive effect in presence of PI3K inhibitor. Inhibition of AKT phosphorylation by an AKT 1/2 inhibitor abolished the effects of PTH treatment and resulted in increased phosphorylation of AMPK in the presence or absence of glucose (Fig. 6A, right panel, C). Thus, PTH
Fig. 5. OCT-1 drives miR-451a expression and promotes osteoblast differentiation. (A-D) Knockdown experiments in osteoblasts with siRNA against OCT-1 or scrambled siRNA (50 nM each) for expression (qPCR) of miR-451a after transfection in MCOs. (n ≥ 3). (A): Quantitative PCR was performed for the expression of target gene Osr1, Runx2, BMP-4, and p27. (n ≥ 3). (B): western blotting (C) and densitometry (D) respectively. (n ≥ 3). Data represented as Mean ± S.E.M. from three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001 compared with respective vehicle.
appears to promote glucose uptake thereby inhibiting phosphorylation of AMPK through PI3K-mTOR-AKT signaling.

3.7. PTH promotes miR-451a expression in ovariectomized mice

Further, in vivo efficacy of PTH in regulating miR-451a levels was evaluated in ovariectomized mice. Similar to the previous study, we observed that PTH at 40 μg·kg⁻¹ recovered bone loss [33] that happened due to ovariectomy (data not shown). In agreement to the in vitro results, PTH treatment for 6 weeks stimulated miR-451a expression in long bones both in sham (~5 to 6 fold) and Ovx (~3 to 4 fold) operated groups compared to respective vehicle treated groups (Fig. 7A). Moreover, PTH treatment reduced the Osr1 protein level and increased BMP-4, p27 and Runx2 protein levels in long bones as compared with Ovx animals (Fig. 7B). Immunohistochemical analysis further confirmed that PTH treatment reduced Osr1 and increased Runx2 protein levels in decalcified sections of distal femur metaphysis region (Fig. 7C).

Furthermore, we assessed protein levels in femur bone from these sham + V, Ovx + V and Ovx + PTH treatment groups. Consistent with in vitro data, PTH treatment to Ovx animals promoted p-PI3K, p-mTOR, p-AKT (S473) levels and reduced the p-AMPK (T172) and p-OCT (S336) levels compared to the Ovx + vehicle group (Fig. 7D–E). These findings

Fig. 6. PTH promotes glucose mediated inhibition of AMPK activation by PI3K-mTOR signaling. (A; left and middle panel) Western blotting and quantification were performed to assess the expression of a phosphorylated and non-phosphorylated form of PI3K, mTOR, AKT and AMPK with or without PTH (500 ng·ml⁻¹), glucose (10 mM), LY294002 (10 μM) in MCOs at 48 h; (n ≥ 3). (C, right panel) Western blotting was performed to assess the expression of a phosphorylated and non-phosphorylated form of AKT and AMPK with or without PTH (500 ng·ml⁻¹), glucose (10 mM), AKT 1/2 inhibitor (10 μM) in MCOs at 48 h. (n ≥ 3). Data represented as Mean ± S.E.M. from three independent experiments; Mean ± S.E.M.; *p < 0.05, **p < 0.01, ***p < 0.001 compared with respective vehicle; another comparison, ^^^p < 0.001.
Fig. 7. PTH promotes miR-451a expression in ovariectomized mice. (A) Expression (qPCR) of miR-451a from femur, (B) western blotting showing the expression of target protein Osr1 and BMP-4, p27 and Runx2 at 48 h, (C) Immunofluorescence in decalcified sections for Osr1 (Dilution 1:100) and Runx2 (Dilution 1:100), magnification-10× (4× zoom) in distal femur metaphysis, (D) Western blotting and quantification were performed to assess the expression of phosphorylated and non-phosphorylated form of PI3K, mTOR, AKT, AMPK and OCT-1 in femur bone from sham, sham + PTH (40 μg·kg⁻¹), ovariectomized and Ovx + PTH (40 μg·kg⁻¹) Balb/c mice, 8-week-old females; (n = 6). Data represented as Mean ± S.E.M. from three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001 compared with respective Ovx + vehicle group; other comparison, ^p < 0.05, ^^p < 0.01, ^^^p < 0.001.
suggest that PTH promotes miR-451a expression and bone formation in vivo.

3.8. miR-451a shows anabolic action in ovariectomized mice

To address the anabolic function of miR-451a in vivo, ovariectomized and sham-operated mice were administered with the miR-451a mimic (7 mg·kg\(^{-1}\)) or scrambled miRNA (miR-C, 7 mg·kg\(^{-1}\)) or PBS (0.2 ml) for 6 weeks. Quantitative PCR assay confirmed the overexpression of miR-451a in femur bone extracts of both Sham and Ovx animals treated with miR-451a mimic. This confirmed effective in vivo tissue delivery and uptake of miR-451a (~2 fold) in both sham-operated and ovariectomized mice (Fig. 8A). We next evaluated the \(\mu\)CT parameters of trabecular bone at the distal femoral metaphysis. Fig. 8B shows representative images of the trabecular region of distal femur metaphysis. Ovariectomy expectedly reduced trabecular BMD, BV/TV, trabecular number; Tb.N, connection density; Conn Dn, trabecular separation; Tb.Sp, Trabecular Pattern factor; Tb.Pf, Structural Model Index; SMI parameters (units shown) in both sham-operated and ovariectomized mice. This significant trabecular loss was partially restored in miR-451a mimic treated animals; whereas miR-C and vehicle treated animals showed no such regain (Fig. 8C–D). Mimic treatment also prevented trabecular bone loss at both tibia and vertebra (Fig. S1). In addition, forced expression of miR-451a, increased femoral bone strength in both

![Fig. 8. miR-451a reverses ovariectomy-induced bone loss and improves strength in mice. (A) Enhanced miR-451a expression in bone following the injection of miR-451a (mimic) using Invivofectamine 3.0 Reagent into 8-week-old female Balb/c mice. This suggests adequate tissue uptake. (B) Representative \(\mu\)CT images of the distal femur metaphysis. (C) Bone mineral density; BMD, (D) structural \(\mu\)CT parameters, including bone volume-to-tissue volume; BV/TV, trabecular number; Tb.N, Connection density; Conn Dn, trabecular separation; Tb.Sp, Trabecular Pattern factor; Tb.Pf, Structural Model Index; SMI parameters (units shown) in femur metaphysis, (E) femoral bone strength shown by breaking energy (units are shown) from sham-operated (Sham) and ovariectomized (Ovx) mice that were given PBS (0.2 ml), scrambled miR (miR-C, 7 mg·kg\(^{-1}\)) or miR-451a (mimic, 7 mg·kg\(^{-1}\)) as indicated in the 'Results' section. Data represented as Mean ± S.E.M. from three independent experiments; *\(p < 0.05\), **\(p < 0.01\) and ***\(p < 0.001\) compared with PBS-treated group; other comparison, ^\(p < 0.05\), ^^\(p < 0.01\) and ^^^\(p < 0.001\) (\(n = 6\) mice per group).]
ovariectomized and sham-operated mice as significantly higher energy was required to break their bones than bones of miR-C and vehicle treated animals (Fig. 8E).

To further explore the mechanism of the increase in bone mass by overexpression of miR-451a, bone marrow stromal cells at 6 weeks were isolated. miR-451a mimic significantly increased mineralization and Cfu-ob formation compared with the control groups (Fig. 9A–B). Overall, miR-C administration did not affect these ex vivo parameters in ovarietomized and sham-operated mice as significantly higher energy was required to break their bones than bones of miR-C and vehicle treated animals (Fig. 8E).

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**Fig. 9.** miR-451a suppresses Osr1 in vivo to stimulate osteoblastogenesis. (A) Ex vivo BMCs culture showing mineralization, (B) representative wells showing alizarin-positive colony (Cfu-ob) formation in osteoblast cells cultures at day 15 in osteogenic medium, (C) qPCR and (D) western blotting analysis was performed for Osr1, BMP-4, p27 and Runx2, in femur bone, (E) serum BMP-4 levels (pg·ml$^{-1}$) and (F) immunohistochemistry for Osr1 (magnification-20×) in distal femur metaphysis from sham-operated and ovarietomized mice that were given PBS (0.2 ml), scrambled miR (miR-C, 7 mg·kg$^{-1}$) or miR-451a (mimic, 7 mg·kg$^{-1}$) as indicated in the ‘Results’ section. Data represented as Mean ± S.E.M. from three independent experiments; ($n$ = 6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with PBS treated group; other comparisons, ^$p < 0.05$, ^^$p < 0.01$ and ^^^$p < 0.001.
Similar to in vitro transfection observations (Fig. 9J–L), mRNA and protein levels of BMP-4, p27 and Runx2 significantly increased and Osr1 levels decreased in bone extracts of mimic-treated mice compared with miR-C or PBS treated animals (Fig. 9C–D). Elevated serum BMP-4 levels were observed in miR-451a treatment groups confirming its bone anabolic effect (Fig. 9E).
Immunohistochemistry results also showed that mimic-treatment suppressed the Osr1 levels both in Sham and Ovx group (Fig. 9F). Von Kossa and Toluidine blue staining revealed sparse and discontinuous trabeculae near the distal femur metaphysis were observed in Ovx mice (Fig. 10A). Mimic treatment to Ovx animals restored distorted trabecular microarchitecture. Bone histomorphometry analysis by Goldner trichome staining showed improvement in the trabecular bone volume of mimic-treated Sham and Ovx animals compared to PBS and miR-C treated groups (Fig. 10B). No changes in number of osteoblast per bone surface (N.Ob/BS) and percentage of surface covered by osteoblasts (Ob.S/BS) were observed in both Sham and Ovx operated groups (Fig. 10C-D). On the contrary ovariectomy resulted in bone resorption, as indicated by elevated the number of osteoclasts (OCs) per bone surface (N.Oc/BS) and the higher percentage of surfaces covered by OCs (Oc.S/BS) than sham operated mice. Mimic treatment to Ovx mice significantly reduced the N.Oc/BS and Oc.S/BS compared to miR-C and PBS treated Ovx mice (Fig. 10E-F). Overall, these data suggest that the in vivo anabolic action of miR-451a is mediated through Osr1 repression and Runx2 activation.

4. Discussion

This study showed an important role for PTH in regulating glucose dependent miR-451a expression during bone anabolism. We showed that PTH favors upregulation of miR-451a via increased glucose uptake leading to osteoblast differentiation. Our data demonstrated that PTH regulates glucose dependent miR-451a expression via the PI3K-mTOR-AKT-AMPK mechanism. Furthermore, we established the uncharacterized role of miR-451a during osteoblast differentiation, consistent with existing knowledge [42-44]. Maintenance of renal calcium and a moderate rise in PTH levels after weaning might be the reason behind post weaning bone recovery [14,15,45]. Considering that this mRNA is elevated during late weaning period [16], it is possible that it plays a key role in skeletal recovery following pregnancy and lactation in mother.

Previous studies have reported the contribution of several miRNAs, such as miR-26a, -133, -135, -29b, -2861, -3960 and -874-3p in the osteoblast differentiation [16,46-49]. Based on the earlier findings regarding PTH mediated glucose metabolism [8,10] and miR-451a dependency on glucose availability [22,23], we deciphered the connecting link between PTH and glucose controlled miR-451a expression during osteoblast differentiation. To elucidate the functional aspect of miR-451a related to PTH-stimulated osteoblast differentiation, we used mouse calvarial osteoblasts. In our study, we investigated the expression profile of miR-451a in various tissues. In mouse tissues, miR-451a was preferentially expressed in bone and liver and was detected at a lower level in muscle. Indeed, we found that PTH stimulates miR-451a expression in bone tissue. This finding may explain the possibility of a rise in miR-451a levels after PTH treatment in the prevention of fracture incidence, reliable with previous studies [1,20]. We found the expression of miR-451a is stage specific in osteoblast cells; miR-451a levels were high during differentiation and mineralization stages and were low during the proliferation stage. The key finding of our study is that miR-451a regulates osteoblast differentiation and function by suppressing its conserved target Osr1, which encodes a zinc-finger transcription factor that plays important roles in embryonic, kidney, heart, urogenital development and bone [50-52] and is active during osteoblast proliferative stage [52]. The functional role of miR-451a was further confirmed by overexpression and knockdown experiments. Therefore, we tested directly the modulatory effect of miR-451a expression, using specifically designed mimics and inhibitors (or Anti-miRs). Of note is that miR-451a has been characterized as a tumor-suppressive miRNA in osteosarcoma [17-19]. We found that transfection of osteoblast precursors with the miR-451a mimic stimulated cells to differentiate faster into mature, mineralizing osteoblasts and that this effect was associated with elevated BMP-4, p27 and Runx2 expression, consistent with previous studies [9,53,54]. Moreover, transfection experiments with miR-451a mimic showed a significant increase in osteoblast differentiation marker ALP and promoted bone mineralization in vitro. Furthermore, a consequence of mimic induced osteoblast differentiation and Runx2 expression were both dependent on Osr1 repression. This is not unexpected because there is prior evidence for an interaction between Osr1 and Runx2 where they experience inverse relation [55] and Osr1 is found to repress osteoblast differentiation [51,52]. Of note is our finding that BMP-4 expression is also regulated similarly, as Osr1 is known to negatively regulate BMP-4 expression levels [56]. Referring to previous knowledge, BMP-4 stimulates osteoblast differentiation and bone formation through enhancing p27 [53,54]. We found that miR-451a mimic stimulated BMP-4 expression both in vitro and in vivo. Therefore, we hypothesize that the inhibition of Osr1 by miR-451a increases Runx2 levels, potentiating BMP-4 induced osteoblast differentiation.

Osteoblasts undergo a developmental sequence of growth and differentiation characterized by a stage-specific expression of cell growth and bone-related genes. For this osteoblast requires glucose as a source of energy [57,58]. Here, we found glucose dose dependently stimulated miR-451a expression in osteoblasts, consistent with existing findings from hepatocytes and cancer cells [21,23,40]. Time and duration of exposure of glucose affected miR-451a levels. Similarly, PTH promoted glucose uptake and dose dependently upregulated miR-451a levels in osteoblasts in vitro. In vivo results confirmed the effect, where intermittent PTH treatment to Ovx Balb/c mice upregulated miR-451a levels in long bones. Assessment of miR-451a target protein Osr1 and bone related markers from PTH treated mice confirmed the stimulatory effect of PTH on miR-451a levels in bone.

Altered glucose metabolism in bone is associated with increased risk of bone disorders [59]. Previous study has shown that insulin and glucose elevate hepatic miR-451a levels and contribute to lower blood glucose levels in type 2 diabetes [40]. In addition to this, activation of AMPK levels and inhibition of osteoblast differentiation are dependent on glucose availability [9,60]. However, osteoblast differentiation requires a reduction in AMP kinase activity and glucose uptake by osteoblasts has shown to suppress AMPK activity. Notably, Activated AMPK is found to promote proteasomal degradation of Runx2 and thus hampers osteoblast differentiation [9,60]. Moreover, AMPK activation by glucose deprivation has been shown to inactivate OCT-1 via direct phosphorylation at serine 335, thus inhibiting miR-451a transcription in glioblastoma [23]. However, the same effect is unknown in bone tissue. To this effect, we report that pharmacological inhibition and activation of AMPK in osteoblasts lead to upregulation and down-regulation of miR-451a indicated by decreased and increased Osr1 levels respectively. PTH is known to stimulate aerobic glycolysis under the regulation of IGF-PI3K-mTOR-AKT mechanism [10]. Additionally, AKT is known to negatively regulate AMPK in the heart [61] and in the
later stages of osteoblast differentiation [62]. Hence, whether the same mechanism plays a role in PTH driven miR-451a expression in differentiating osteoblasts was checked. Here we found, pharmacological suppression of PI3K-mTOR-AKT signaling blunts PTH mediated miR-451a upregulation indicated by the elevated Osr1 and activated AMPK levels, thus alleviating the osteoblast differentiation function of PTH in vitro.

Extensive research has indicated that estrogen deficiency is the major cause of post-menopausal bone loss [63]. In addition, estrogens has been reported to positively regulate miR-451a in splenic lymphocytes [64,65]. Thus, we explored in vivo efficacy of miR-451a in 8-week-old ovariectomized mice which showed severe estrogen deficiency [16]. Mice were injected with mimic or scrambled miRNA intermittently over 6 weeks [16]. Mimic treatment prevented trabecular bone loss and improved bone strength both in ovariectomized mice and the sham-operated group. The ex vivo cultures of bone marrow stromal cells from mimic treated mice showed evidence of increased osteoblastogenesis, mineralization, and BMP-4 and Runx2 expression as well as reduced Osr1, essentially confirming our in vitro data. Bone histomorphometric analysis showed increased mineralized trabecular bone after the miR-451a treatment. Therefore, these findings document miR-451a as a potential anabolic target.

Overall we conclude, in the presence of glucose, inhibition of AMPK by PTH drives OCT-1 mediated miR-451a transcription leading to osteoblast differentiation via direct targeting of Osr1. The work not only clears and expands the remarkable studies about the effect of PTH on osteoblasts but more importantly provides evidence for the existence of additional regulatory mechanism behind PTH regulated osteoblast differentiation.

Conflict of interest

The authors have no conflict of interest.

Author's contributions

RT and AK designed this study. AK, PK, NA, SA, PK, AKT and VK performed experiments. RT and AK designed the experiments. All authors analyzed the data. Experiments were performed under the supervision of RT. RT and AK wrote the manuscript.

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Appendix A. Supplementary data

Trabecular assessment of µCT parameters in Vh0 lumbar vertebrae andibia of both sham and ovariectomized mice underwent various treatments and calciel labeling in femur diaphysis (Fig. S1). Serum PINP levels (Fig. S2). Confocal imaging was performed to check BCP-4 expression after transection experiment in MCOs (Fig. S3). Haematoxylin and eosin staining was performed for 5 μM transverse sections of kidney, liver and uterus for histology (Fig. S4). Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2018.09.007.

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