Rb1 negatively regulates bone formation and remodeling through inhibiting transcriptional regulation of YAP in Glut1 and OPG expression and glucose metabolism in male mice

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ABSTRACT

Objective: Bone is a highly dynamic organ that undergoes constant bone formation and remodeling, and glucose as a major nutrient is necessary for bone formation and remodeling. Retinoblastoma (Rb1) is a critical regulator of mesenchymal stem cells (MSCs) fate, but how Rb1 regulates bone formation and remodeling is poorly understood.

Methods: We generated MSCs- and osteoprogenitors-specific Rb1 knockout mouse models and utilized these models to explore the function and mechanism of Rb1 in regulating bone formation and remodeling in vivo and in vitro primary cell culture.

Results: Rb1 deficiency in MSCs significantly increased bone mass and impaired osteoclastogenesis. Consistently, depletion of Rb1 in osteoprogenitors significantly promoted bone formation. Mechanistically, loss of Rb1 in MSCs elevated YAP nuclear translocation and transcriptional activity of YAP/TEAD1 complex, thereby increasing the transcriptional expression of Glut1 and OPG. Moreover Prx1-Cre; Rb1f/f mice displayed hypoglycemia with increased systemic glucose tolerance instead of increased insulin level. In vitro data revealed that Rb1-mutant MSCs enhanced glucose uptake and lactate and ATP production. Increased osteogenesis caused by increased glucose metabolism and decreased osteoclastogenesis caused by increased expression of OPG eventually resulted in increased bone formation and remodeling.

Conclusions: Collectively, these findings demonstrated that Rb1 in MSCs inhibits YAP-mediated Glut1 and OPG expression to control glucose metabolism, osteogenesis and osteoclastogenesis during bone formation and remodeling, which provide new insights that controlling Rb1 signaling may be a potential strategy for osteoporosis.

Keywords Rb1; Bone formation; Bone remodeling; MSCs; Glucose metabolism

1. INTRODUCTION

Growing evidence reveals that bone formation and remodeling throughout life are maintained by glucose metabolism [1–3]. Patients with diabetes display increased bone resorption at the expense of bone formation by inhibition of osteogenesis, thereby enhancing the risk of osteoporosis and bone fractures [4,5]. Bone cells preferred the glucose as the major source of energy to fuel aerobic glycolysis and generate adenosine triphosphate (ATP) and cellular metabolites for new biomass generation, which ultimately promotes bone formation [1,3,6,7]. In addition, bone is recognized as a pivotal regulator of systemic glucose homeostasis [8,9]. The activity of glucose uptake in osteoblast lineage cells is detected to be the earliest determinant of osteogenesis and bone formation, which is also necessary for whole-body glucose homeostasis [10]. MSCs as multipotent stromal cells harbor the potentials to differentiate into several lineages such as chondrocytes, osteoblasts and adipocytes, and directly regulates bone development and homeostasis [11–14]. Previous studies have showed that glucose metabolism directs MSCs’ fate and bone formation regulated by a series of glycolytic proteins such as glucose transporter 1 (Glut1), 6-phosphofructo-2-kinase 3/4 (Pfkf3/4), hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA) [3,15]. However, how glucose metabolism is regulated in MSCs during bone formation and remodeling remains largely unknown. Rb is a critical mediator of self-renewal, cellular differentiation, and development besides tumorigenesis [16–18]. Numerous studies demonstrated that Rb1 signaling is indispensable for normal cellular metabolic balance, and dysfunction of Rb1 could result in reprogramming of specific pathways [19–21]. Recent evidence showed that Rb regulates the lineage commitment and fate choice between brown adipose tissue and bone in vivo [17]. Moreover, loss of Rb1 in osteoblasts was reported to promote osteoblast proliferation and differentiation in vitro [18]. Noteworthily, Rb1 has been established as a predictive biomarker

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for Glut1 sensitivity [21]. In triple negative breast cancer, Rb1 expression strongly correlates with the degree of sensitivity to Glut1 [21]. Interestingly, our previous studies also found double deletion of Rb1 and transformation-related protein 53 (Trp53) in cathepsin K (Ctsk) expressing cells-derived mesenchymal cells contributed to osteosarcoma formation through activation of glucose metabolism and yes-associated protein (YAP) signaling [22,23], a critical regulator of glucose metabolism through regulation of Glut1 expression [24–26]. However, whether and how Rb1 regulates glucose metabolism and bone formation and remodeling remain largely unknown.

In this study, we explored the role and molecular mechanism by which Rb1 regulates bone formation and remodeling. Our data provide the first evidence that Rb1 plays an essential role in bone formation and remodeling through inhibiting glucose metabolism via transcriptional regulation of YAP in Glut1 and osteoprotegerin (OPG) expression in MSCs.

2. METHODS

2.1. Mice

Prx1-Cre, OSX-Cre and Rb1fl mice were purchased from The Jackson Laboratory (Bar Harbor, USA). All the mice in this study were bred and maintained under specific pathogen-free conditions at the animal facility of University of Pennsylvania.

2.2. Reagents and antibodies

The fluorescent analog 2-NBDG and Alizarin Red S powder were obtained from Cambay Chemical Company (USA). Calcein was purchased from Fisher ScientificTM, 1, 25-dihydroxyvitamin D3, PGE2, trtarate-resistant acid phosphatase (TRAP) staining kit and Glut1 antibody (#SAB4502803; dilution 1:1000) were obtained from Sigma (USA). The hematoxylin & eosin (H&E) staining kit and the secondary fluorescent antibodies were from Abcam (USA). The YAP antibody (#14074; dilution 1:1000) was purchased from Cell Signaling Technology (USA). The actin antibody (sc-47778; dilution 1:1000), Lamin B1 antibody (sc-374,015; dilution 1:1000) was purchased from Cell Signaling Technology (USA). The ALP activity was determined at OD405 nm.

2.3. Cells and cell culture

The isolation of primary MSCs was performed as we previously described [3]. Briefly, all tissues around the fresh femurs and tibiae from 3-month-old Prx1-Cre; Rb1fl mice and age-matched controls were harvested with the harvest buffer (0.2% NP-40 and 2mM PMSF in 10mM Tris-Cl, pH 10.5), and then the supernatants were collected and incubated with assay buffer (100mM glycine (pH 10.5), 50mM p-nitrophenyl phosphate solution, and 1mM MgCl2). After incubation for 15 min at 37°C, the reaction was stopped by 0.1N NaOH solution. And then, the ALP activity was determined at OD405 nm as we previously described [3,34]. After incubation of osteogenic medium for 14 days, the cells as indicated were stained by Alizarin Red S staining solution as we previously reported [3,34].

2.4. Plasmids and transfection

The plasmids including flag-YAP, shYAP1, shYAP2 and 8xGTF2C-luciferase were ordered from Addgene (USA). Briefly, the MSCs (1 × 10^5 cells/well) were firstly seeded in 6-well plates with α-MEM medium.

After culture of 24 h at 37°C with 5% humidified CO2, the MSCs were transfected with indicated plasmids using FuGENE® HD transfection reagent (Promega Corporation, USA). After transfection of 48 h, the expression of target proteins was identified we described previously [29–31].

2.5. qRT-PCR, ChiP-qPCR and western blot

Briefly, the total RNA from the cells or tissues was extracted by TRIzol reagent (Invitrogen, USA), and then the cDNA was prepared using the PrimeScript™ RT Kit (Invitrogen, USA) according to the manufacturer’s instructions. The cDNA as the templates was subsequently used for performing qRT-PCR analysis with SYBR reagents. The sequences of primers are listed in Supplementary information, Table S1. The ChiP-qPCR and western blot assays were carried out as we previously reported [3,29,32,33].

2.6. Alkaline phosphatase (ALP) activity, osteogenic differentiation and Alizarin red S staining

After incubation with osteogenic medium (5mM β-glycerophosphate, 50μg/mL L-ascorbic acid and 100 nM dexamethasone) for 5 days, ALP activity was measured by BioRad microplate reader at OD405 nm. Briefly, primary MSCs from 3-month-old Prx1-Cre; Rb1fl mice and controls were harvested with the harvest buffer (0.2% NP-40 and 2mM PMSF in 10mM Tris-Cl, pH 7.4), and then the supernatants were collected and incubated with assay buffer (100mM glycine (pH 10.5), 50mM p-nitrophenyl phosphate solution, and 1mM MgCl2). After incubation for 15 min at 37°C, the reaction was stopped by 0.1N NaOH solution. And then, the ALP activity was determined at OD405 nm as we previously described [3,34]. After incubation of osteogenic medium for 14 days, the cells as indicated were stained by Alizarin Red S staining solution as we previously reported [3,34].

2.7. Whole-mount skeletal staining

Briefly, the newborn pups were euthanized and fixed by 100% ETOH for overnight at room temperature. After the fixation, the newborn pups were treated by acetone for 12 h, washed by 100% ETOH for 2 times, and then stained by alcin blue solution and alizarin red solution respectively as we previously reported [3,11,30].

2.8. Calcium labeling

Calcine (20 mg/kg) was intraperitoneally injected at Day 2 and Day 7 before harvesting Prx1-Cre; Rb1fl and control mice at 3 months of age. The Tibia and femurs were collected, fixed in 4% paraformaldehyde (PFA) for 12 h at dark, embedded by paraffin, and cut to 6-μm sections. And then, the bone formation rate per bone surface (BFR, μm3/μm2 per day) and mineral apposition rate (MAR, μm per day) were analyzed by the OsteoMeasure analysis system as we previously reported [3,11,30].

2.9. Histology

The femurs and tibiae were collected and fixed in 4% PFA solution for overnight at 4°C, decalcified in PBS with 14% ethylene-diamine-tetraacetic acid (EDTA) (pH, 7.4) for 6 weeks and then embedded in paraffin. 6-μm sections of these samples were prepared, for analyses of H&E, von Kossa, Safranin O/fast green, and TRAP staining as we previously reported [3,11,29,31,34].

2.10. Metabolite measurements

Briefly, MSCs were isolated from 3-month-old Prx1-Cre; Rb1fl mice and age-matched controls, and then the lactate production and
glucose consumption were measured in those cells using the L-lactate Assay Kit (Eton Biosciences, USA) and Glucose (HK) Assay Kit (Sigma, USA), respectively. After incubation with 100 μM 2-NBDG for 8 h, the glucose uptake was measured in MSCs as we previously reported [3]. ATP production was quantified in MSCs by the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, USA) according to the manufacturer’s instructions. Serum levels of OPG, RANKL, insulin and periodic acid-Schiff (PAS) staining were analyzed by OPG ELISA Kit (Boster Biological Technology, USA), RANKL ELISA Kit (Boster Biological Technology, USA), Insulin ELISA Kit (Crystal Chem, USA) and PAS kit (Sigma, USA) as we described previously [3,22].

2.11. Blood glucose test
After fast for overnight, 3-month-old Prx1-Cre; Rb1<sup>1f</sup> mice and age-matched controls were injected intraperitoneally with sterile glucose (2 g/kg), and then the blood glucose level was measured by the OneTouch® Ultra®/2 blood glucose meter as previously reported [3,9].

2.12. Microcomputed tomography (micro-CT)
The distal femurs from 3-month-old Prx1-Cre; Rb1<sup>1f</sup>, OSX-Cre; Rb1<sup>1f</sup>, OSX-Cre mice and age-matched controls were analyzed by a high-resolution micro-CT system at facility core of University of Pennsylvania as we described previously [3,11].

2.13. Statistical analysis
The data of this study was analyzed using Student’s t-test and reported as mean ± SEM. The statistical significance of multiple groups was determined by 2-way ANOVA. P values < 0.05 were considered significantly.

3. RESULTS

3.1. Rb1 deficiency in MSCs increased bone mass with increased osteogenesis of MSCs
Previous findings showed that Prx1-Cre recombinase mainly expresses in the limb mesenchyme and skull, and Prx1 was generally used as a genetic marker for MSCs in the skeletal studies [35–37]. To explore the
function of Rb1 in MSCs, we first generated a conditional knockout mouse model by crossing Rb1f/f mice with Prx1-Cre mice (hereafter named Prx1-Cre; Rb1f/f mice). qRT-PCR verified that Rb1 was efficiently extinguished in MSCs instead of osteoblasts from Prx1-Cre; Rb1f/f mice compared to that in controls (Supplemental Figure S1). Intriguingly, whole-mount skeletal staining data showed that the newborn Prx1-Cre; Rb1f/f mice displayed a significant increase in mineralized bone matrix of limbs and calvarium (Figure 1A,B). Moreover, von Kossa staining results further showed a remarkable increase in the bone mineralization of tibia from newborn Prx1-Cre; Rb1f/f mice compared to those in the Cre control mice (Figure 1C). To further get insight into the mechanism for the increased bone mass in Prx1-Cre; Rb1f/f mice, we isolated MSCs from Prx1-Cre; Rb1f/f and control mice to characterize MSC osteogenic potentials in vitro. The results showed a significant increase in mineralized nodule formation and ALP activity after stimulation with osteogenic medium for 14 days in Rb1 deficient cells compared to the control cells (Figure 1D,E). To further confirm the effect of Rb1 on bone formation and skeletal development, we performed qRT-PCR assay of MSCs after stimulation with osteogenic medium for 7 days and Safranin O/Fast green staining of newborn tibia. The expressions of osteogenic markers (ALP, Runx2, OSX and OCN) were dramatically increased compared to those in the control mice (Figure 1F). Additionally, our data showed that the lengths of the growth plate and proliferation zone (PZ) were pronouncedly increased in Prx1-Cre; Rb1f/f mice compared to the controls (Figure 1G–I). The quantitative result showed that the relative length of PZ zone increased by 1.79-fold in Prx1-Cre; Rb1f/f mice compared to that in the controls (Figure 1I). However, by analysis of the length of growth plate and tibia in 6-week-old Prx1-Cre; Rb1f/f mice and controls, we found there was no obvious change, indicating that the effect of Rb1 on bone development may be limited at postnatal stage (Supplemental Figure S2). Taken together, our findings demonstrated that deletion of Rb1 in MSCs promotes bone formation.
3.2. Rb1 deficiency in MSCs increased bone formation in Prx1-Cre; Rb1<sup>fl/fl</sup> mice

To further determine the function of MSCs-derived Rb1 on bone formation and remodeling, we next analyzed the changes of bone mass in the skulls and distal femurs of Prx1-Cre; Rb1<sup>fl/fl</sup> mice and age-matched controls through micro-CT. The result showed a significant increase in mineralized tissues (Figure 2A,B and Supplemental Figure S3). Bone volume per total tissue volume (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N) from the femurs of 3-month-old Prx1-Cre; Rb1<sup>fl/fl</sup> mice increased 1.81-, 1.51- and 1.2-fold, respectively; and trabecular separation (Tb.Sp) decreased 1.85-fold compared to those in the controls (Figure 2B). These results were further confirmed by H&E staining (Figure 2C). To further examine bone dynamic changes, we conducted histomorphometric analysis of the femur metaphysis and found that BFR and MAR were significantly increased in Prx1-Cre; Rb1<sup>fl/fl</sup> mice compared to those in the controls (Figure 2D–F). Overall, these data demonstrated that Rb1 negatively regulates osteogenesis and bone formation.

3.3. Rb1 deficiency in osteoprogenitor cells promotes bone formation

Numerous evidence has proved that MSCs as progenitors of osteoblast lineage cells could differentiate into osteoprogenitor cells and regulate osteogenesis and bone formation [11,27,38]. OSX is a critical transcription factor for osteogenesis from MSCs [39,40]. To further investigate whether deletion of Rb1 in osteoblast lineage cells can cause increased bone formation, we generated an osteoprogenitor-specific knockout mouse model, namely OSX-Cre; Rb1<sup>fl/fl</sup> mice, in which Cre expression is primarily restricted to osteoblast precursors. To exclude the possibility of the phenotype from OSX-Cre, we used OSX-Cre mice as the control and harvested the bone samples at age of 3 months, which has been proved to have no bone phenotype existence at this age [41]. qRT-PCR verified that Rb1 was abrogated in osteoprogenitor cells from OSX-Cre; Rb1<sup>fl/fl</sup> mice and controls, but there was no significant change in MSCs (Supplemental Figure S4). In consistent with the phenotype of Prx1-Cre; Rb1<sup>fl/fl</sup> mice, deletion of Rb1 in osteoprogenitor cells also showed a remarkably increased bone mass compared with...
controls (Figure 3A,B), as evidenced by H&E staining (Figure 3C). To further confirm the role of Rb1 on osteogenesis and bone formation, primary osteoprogenitor cells from calvarial bones of OSX-Cre; Rb1f/f mice and controls were isolated and cultured with osteogenic medium for 14 days. The results of Alizarin red staining and ALP activity assay showed that loss of Rb1 in osteoprogenitor cells markedly increased calcium mineralization and ALP activity compared to the controls (Figure 3D). Additionally, qRT-PCR analyses demonstrated a significant increase in the transcriptional levels of osteogenic markers (ALP, Runx2, OSX and OCN) in the primary calvarial cells of 3-month-old OSX-Cre; Rb1f/f mice compared to the OSX-Cre control mice (Figure 3G). To identify whether loss of Rb1 in osteoblasts affects osteoclastogenesis, we next performed TRAP staining in OSX-Cre; Rb1f/f mice and controls. Our data showed a significant decrease in osteoclastogenesis in OSX-Cre; Rb1f/f mice compared to the controls (Figure 3H), indicating that the enhanced bone mass may be partly caused by impaired osteoclastogenesis. These data further demonstrated that Rb1 is essential for osteoblast differentiation and bone formation.

3.4. Rb1 deficiency in MSCs results in increased hypoglycemia through upregulation of YAP activity and Glut1 expression

Glucose is the major energy source for bone formation and resorption, and high-glucose microenvironment can decrease the expression of osteogenic markers such as Runx2, thereby inhibiting bone formation in mice [42]. Findings from previous studies have revealed that YAP as a transcription co-activator plays an important role in regulating glucose metabolism during bone formation and remodeling [22,27,43,44]. To determine whether Rb1 negatively regulates bone formation through altering YAP signaling and glucose metabolism, we identified transcriptional activity (nuclear translocation) of YAP in Rb1-mutant MSCs. Interestingly, we found Rb1 could associate with YAP after overexpression of YAP in MSCs, directly (Figure 4A). Moreover, our data also showed that loss of Rb1 in MSCs increased the YAP nuclear translocation (Figure 4B,C), indicating that loss of Rb1 in MSCs could enhance the transcriptional activity of YAP, which were further documented by YAP staining, luciferase analysis of the transcriptional activity of YAP/TEAD complex and YAP phosphorylation in RB1-deficient MSCs.

Figure 4: Rb1 deficiency in MSCs increases YAP activity, Glut1 expression and systemic glucose tolerance. (A) Co-IP after overexpression of YAP in MSCs as indicated. (B) Western blot analysis of YAP expression in the cytoplasm and nucleus of MSCs from Prx1-Cre; Rb1f/f mice and controls as indicated. (C) Based on the western blot data of Figure 4B, the ratio of YAP protein in the nucleus to the cytoplasm fraction. (D) Representative images of YAP staining in MSCs from Prx1-Cre; Rb1f/f mice and controls as indicated. Scale bar, 25 μm. (E) Western blot analysis of YAP phosphorylation as indicated. (F) The transcriptional activity of YAP/TEAD complex. MSCs isolated from Prx1-Cre; Rb1f/f mice and controls were co-transfected with the pRL-TK plasmid (internal control) and luciferase reporter, respectively. After transfection of 48 h, the luciferase activities were measured by the Dual-Luciferase Assay Kit. (G) Glut1 expression in MSCs from Prx1-Cre; Rb1f/f mice and controls as shown. (H) The analysis of the quantification of Glut1 as shown. (I) Glut1 expression was identified after silence with two different shYAP lentivirus in Rb1-deficient MSCs. (J) Mineralized nodule formation of MSCs from Prx1-Cre; Rb1f/f mice with/without YAP lentivirus after 14 days of osteogenic induction was evaluated by alizarin red staining. The intensity of alizarin red staining was measured as shown (N = 3).

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mutant MSCs (Figure 4D–F). Moreover, we found that loss of Rb1 in MSCs significantly upregulated the expression of Glut1, which was partly aborted by silence of YAP with shYAP lentivirus (Figure 4G–I), suggesting that YAP and glucose metabolism may be involved in Rb1 regulation in bone formation, as evidenced by analysis of mineralized nodule formation in RB1-mutant MSCs with YAP lentivirus (Figure 4J).

To further assess the regulation of Rb1 in glucose metabolism, we measured the levels of triglycerides and blood glucose in 3-month-old Prx1-Cre; Rb1f/f mice and age-matched controls under random-fasted condition. Interestingly, our data showed that Prx1-Cre; Rb1f/f mice displayed a hypoglycemic phenotype accompanied with a significant decrease in serum triglycerides and blood glucose compared to the controls (Figure 4K, L). To further investigate the regulation of Rb1 on glucose metabolism, we performed glucose tolerance tests in the Prx1-Cre; Rb1f/f mice (Figure 4M), as evidenced by decreased glycogen in liver due to Rb1 deficiency in MSCs (Figure 4N). It is well known that insulin as anabolic hormone is critical for cooperating the glucose uptake and blood glucose level [3,8,9]. However, serum insulin in the Prx1-Cre; Rb1f/f mice had no significant change under random-fasted condition (Figure 4O), which was further confirmed by the insulin secretion test after glucose injection and insulin tolerance test (Figure 4P, Q), suggesting that Rb1 deficiency in MSCs promotes glucose metabolism instead of inhibition of insulin function.

3.5. Rb1 deficiency in MSCs promotes glucose metabolism by increasing YAP-dependent Glut1 expression

Glut1 is a Glut family protein and an essential regulator for Glucose metabolism during bone formation and bone homeostasis [1,3,6,10]. To further characterize the regulation of Rb1 in glucose metabolism, we identified the expressions of critical glycolysis-regulating proteins including Glut1, HK2, Pfkfb3/4, and Ldha and found that these proteins were significantly upregulated in Rb1-mutant primary MSCs compared to the controls (Figure 5A). qRT-PCR data showed that loss of Rb1 in MSCs dramatically increased the expression of glycolysis-regulating proteins (Figure 5B). Importantly, we detected a significant elevation of glucose uptake and lactate and ATP production in the Rb1-mutant MSCs compared to those in the controls (Figure 5C–E), which was further confirmed by identification of cellular uptake of a fluorescently labeled glucose analog 2-NBDG in Rb1-mutant MSCs and controls (Figure 5F). YAP cannot directly govern its target genes’ expression due to lack of DNA binding motif. It usually binds with its major partner, the TEA domain transcription factors (TEADs) to exert its function [29]. Previous findings showed that YAP/TEAD1-Glut1 axis is critical for glucose metabolism [22,25], and loss of YAP in hepatic progenitor cells

Figure 5: Rb1 deficiency in MSCs promotes glucose metabolism by increased YAP-dependent Glut1 expression.

(A) Schematic diagram of the key enzymes in the glucose metabolism. (B) qRT-PCR analysis of the expression of the key enzymes in the glucose metabolism using MSCs from 3-month-old Prx1-Cre; Rb1f/f mice and controls as indicated. (C–E) Analyses of glucose consumption, lactate production and ATP production in MSCs respectively as indicated. (F) 2-NBDG uptake in MSCs from Prx1-Cre; Rb1f/f mice and controls. (G) Schematic diagram of DNA binding motif of YAP/TEAD1 complex in the Glut1 promoter. BS, binding site; TSS, transcriptional star site. (H) ChIP analysis. Co-occupation of YAP/TEAD1 in the Glut1 promoter after loss of Rb1 in MSCs. qPCR analysis of YAP/TEAD1 binding activity in Glut1 promoter in MSCs from Prx1-Cre; Rb1f/f mice and controls. N = 3. (I) Co-occupation of YAP/TEAD1 in Glut1 promoter after silence of TEAD1. N = 3. *P < 0.05, **P < 0.01, ***P < 0.01.
impaired glucose metabolism and tolerance through inhibition of Glut1 expression [24]. By analysis of Glut1 promoter using software Vector NTI, we found a DNA binding motif of YAP/TEAD1 complex in the Glut1 promoter (Figure 5G). Next, we employed the ChIP-qPCR assay to identify the expression level of Glut1 after immunoprecipitation with YAP antibody in primary MSCs from Prx1-Cre; Rb1<sup>1<sup>ff</sup> mice and age-matched controls. As shown in Figure 5H, the transcriptional activity of the Glut1 promoter was pronouncedly increased after ablation of Rb1 in MSCs, but significantly prohibited when TEAD1 was silenced by TEAD1 siRNA (Figure 5I). Overall, these findings demonstrated that Rb1 deficiency in MSCs promotes glucose metabolism by increased YAP-dependent Glut1 expression.

3.6. Rb1 deficiency in MSCs inhibits bone resorption through YAP regulated OPG signaling

Bone remodeling is a dynamic and coupling process maintained by osteoblasts and osteoclasts [45–48]. To determine whether osteoclasts also contribute to increased bone mass in Prx1-Cre; Rb1<sup>1<sup>ff</sup> mice, we next identified osteoclast numbers and surface areas in femurs of Prx1-Cre; Rb1<sup>1<sup>ff</sup> mice and age-matched controls by TRAP staining. The result showed that the osteoclast numbers were significantly reduced in Prx1-Cre; Rb1<sup>1<sup>ff</sup> mice compared to the controls (Figure 6A–C). OPG is a competitive inhibitor for RANKL and could attenuate the interplay and connection between RANKL and receptor activator of nuclear factor κB (RANK) on the surface of osteoclasts [49–52]. The ratio of OPG/RANKL is also reported to be an important indicator of bone remodeling [51–53]. Therefore, we further identified OPG expression in MSCs and the serum level in Prx1-Cre; Rb1<sup>1<sup>ff</sup> mice and controls. qRT-PCR data demonstrated that OPG expression and serum level were markedly increased in the MSCs with Rb1 deficiency (Figure 6D, and Prx1-Cre; Rb1<sup>1<sup>ff</sup> mice respectively (Figure 6E). No pronounced change of serum RANKL was detected in the Prx1-Cre; Rb1<sup>1<sup>ff</sup> mice (Figure 6F). These findings were further confirmed by the increased ratio of serum OPG/RANKL (Figure 6G). Moreover, the co-culture data also supported the notion that loss of Rb1 in MSCs acts primarily to orchestrate bone resorption by osteoclasts in a non-cell autonomous manner (Figure 6H, I). Studies have proved that YAP signaling is critical for the bone formation and remodeling, and loss of YAP in osteoblasts could decrease OPG expression [27,43,44]. We next analyzed the OPG promoter using software Vector NTI, and found there were two DNA binding domains of YAP/TEAD1 complex in the OPG promoter (Figure 6J). By performing Chip assay in Rb1-mutant cells and controls, we found that the transcriptional activity of the OPG promoter significantly increased in Rb1 deficient MSCs compared to the control cells (Figure 6K).

Figure 6: Rb1 deficiency in MSCs inhibits bone resorption and promotes bone remodeling through YAP regulated OPG signaling. (A) Representative TRAP staining images of Prx1-Cre; Rb1<sup>1<sup>ff</sup> mice and controls at age of 3 months. Scale bar, 100 µm. (B) Osteoclast No./BS. (C) Osteoclast surface/BS. (D) qRT-PCR analysis of OPG in MSCs from Prx1-Cre; Rb1<sup>1<sup>ff</sup> mice and age-matched controls. (E–G) The serum OPG and RANKL levels of 3-month-old Prx1-Cre; Rb1<sup>1<sup>ff</sup> mice and controls and their ratio as indicated. N = 5. (H, I) Co-culture of osteoblasts and osteoclasts in the co-culture system as described in the Methods for TRAP staining (H). N = 5. Osteoclast numbers from TRAP staining in H were quantitatively analyzed (I). (J) Schematic diagram of DNA binding motifs of YAP/TEAD1 complex in the OPG promoter. BS, binding site; TSS, transcriptional start site. (K) Chip analysis. Co-occupation of YAP/TEAD1 in the OPG promoter after loss of Rb1 in MSCs. qPCR analysis of YAP/TEAD1 binding activity in OPG promoter in MSCs from Prx1-Cre; Rb1<sup>1<sup>ff</sup> mice and controls. ns: not significant, **P < 0.01, ***P < 0.01.

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Deletion of Rb1 in MSCs promotes osteogenesis and bone remodeling by activating YAP signaling and promoting Glut1 and OPG transcription expression and glucose metabolism.
MSCs as common progenitors play essential roles in bone development, regeneration, and remodeling [12,53,54]. However, the function and mechanism that coordinates the bone formation and remodeling in MSCs are largely undefined. In this study, we found Rb1 deficiency in MSCs promotes bone formation and remodeling through increased osteogenesis and decrease of osteoclastogenesis via activating YAP signaling and it-mediated upregulation of Glut1 and OPG expression, as well as enhancing glucose metabolism (Figure 7).

Recent findings presented strong evidence that Rb is a key regulator of MSCs' fate to determine the shift between osteoblasts and adipocytes [17]. As a tumor suppressor, Rb1 has important roles in tumorigenesis in virous cancer including bone cancer [16,55,56]. Interestingly, previous studies showed that deletion of Rb1 alone in osteoblasts or MSCs does not result in osteosarcoma formation, however, combined deletion of Rb1 with Trp53 in osteblast lineages causes osteosarcoma formation [17,23,56,57], suggesting that the tumorigenesis of Rb1 inactivation alone in bone may be extremely limited compared to Trp53 deficiency in MSCs. Moreover, Gabriel M. Gutierrez et al. also reported that loss of Rb1 in osteoblasts significantly promoted calvarial cell proliferation and mineral formation in vitro [18].

Enhancement of glucose metabolism promotes the bone formation and remodeling by regulation of glucose uptake and glycolysis [1,6]. Mutations that inactivate tumor suppressors or activate oncogenes could alter the glucose metabolism [58,59]. For instance, inhibition of the tumor suppressor Trp53 promoted glucose metabolism during osteoblast differentiation [60]. Our previous findings and others showing that YAP-Glut1 axis is critical for glucose metabolism and bone formation [22,25], and global knockout of YAP in zebrafish exhibited impaired glucose metabolism and tolerance through inhibition of Glut1 expression [24]. Here, we found loss of Rb1 in MSCs significantly increased the binding activity of the YAP/TEAD1 complex on Glut1 promoter, thereby promoting Glut1 expression and glucose metabolism. This is partially supported by the findings that Rb1 expression is strongly correlated with the sensitivity to Glut1 inhibition in triple negative breast cancer, and loss of Rb1 in this cancer cells can accordingly increase the mitochondrial respiration [21]. Moreover, we found a hypoglycemic phenotype accompanied with a dramatic decrease of blood glucose and triglycerides in the Prx1-Cre; Rb1f/f mice. Insulin is a pivotal hormone that could maintain the blood glucose level through regulation of uptake and metabolism in glucose [3,9]. Intriguingly, we did not detect obvious insulin production in the Prx1-Cre; Rb1f/f mice, suggesting that Rb1 deficiency in MSCs promotes bone formation and remodeling through increased glucose metabolism instead of a reduction in serum insulin. These findings were further supported by previous evidence that loss of Vhl in osteoprogenitor cells increased bone formation and inhibited bone resorption by improving global glucose metabolism [1–3,6].

Bone formation is a dynamic process which is tightly regulated and maintained by osteogenesis and osteoclastogenesis [45–47]. Interestingly, our data suggested that loss of Rb1 in MSCs could also inhibit osteoblast-dependent bone resorption through activation of OPG signaling. Mounting evidence suggested that YAP as a transcription coactivator is crucial for bone formation and remodeling [27,43,44]. More importantly, our previous study also showed that loss of Rb1 in Ctsk and Col2 positive cells resulted in a remarkable increase of YAP transcriptional activity [22,31,51]. In line with this, we found Rb1 deficiency in MSCs promoted YAP nuclear translocation and eventually enhanced the transcriptional activity of the YAP/TEAD1 complex. Furthermore, our ChIP-qPCR data showed that OPG expression could be directly regulated by the YAP/TEAD1 complex. Supporting our findings, previous study showed that Trp53 deficiency in MSCs induced a significant increase in osteogenic differentiation and bone remodeling through negative regulation of OPG signaling [53]. Additionally, our data also showed that loss of Rb1 in MSCs slightly increased the expression of YAP, which maybe further enhanced YAP/TEAD1 complex-mediated Glut1 and OPG expression. Collectively, this study provides first evidence about Rb1 regulation in bone formation and remodeling, glucose metabolism and YAP signaling. Our finding demonstrated that modification of Rb1, YAP signaling and glucose metabolism may be a potential target for treatment of bone disease such as osteopetrosis and osteoporosis.

**AUTHOR CONTRIBUTIONS**

S.Y.Y. and Y.L. conceived this study and designed experiments. Y.L. and S.T.Y. generated knock-out mice, performed the experiments and analyzed data. Y.L. wrote the manuscript. S.Y.Y. reviewed and edited the manuscript.

**DATA AVAILABILITY**

Data will be made available on request.

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**ABBREVIATIONS**

| Abbreviation | Definition |
|--------------|------------|
| 2-NBDG       | 2-([N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxyglucose | |
| ALP          | alkaline phosphatase |
| ATP          | adenosine triphosphate |
| FBS          | fetal bovine serum |
| BFR          | bone formation rate |
| Ctsk         | cathepsin K |
| Glut1        | glucose transporter 1 |
| HK2          | hexokinase 2 |
| LDHA         | lactate dehydrogenase A |
| MAR          | mineral apposition rate |
| PFKB3/4      | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3/4 |
| PPARγ        | peroxisome proliferator-activated receptor-gamma |
| PRKRB3/4     | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3/4 |
| PFA          | paraformaldehyde |
| RANKL        | receptor activator of NF-κB ligand |
Runx2  runt-related transcription factor 2
TEADs  TEA domain transcription factors
TRAP  tartrate-resistant acid phosphatase
Trp53  transformation-related protein 53
YAP  yes-associated protein

DECLARATION OF COMPETING INTEREST

The authors declare no competing interests.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2022.101630.

REFERENCES

[1] Lee, S.Y., Abel, E.D., Long, F.X., 2018. Glucose metabolism induced by Bmp signaling is essential for murine skeletal development. Nature Communications 9.
[2] Li, B.E., Lee, W.C., Song, C., Ye, L., Abel, E.D., Long, F.X., 2020. Both aerobic glycolysis and mitochondrial respiration are required for osteoblast differentiation. FASEB Journal 34(8):11058–11067.
[3] Li, Y., Yang, S., Liu, Y., Qiu, L., Wang, Y., Yang, Z., 2022. IFT20 governs mesenchymal stem cell fate through positively regulating TGF-β-Smad2/3-Glut1 signaling mediated glucose metabolism. Redox Biology 54. https://doi.org/10.1016/j.redox.2022.102373.
[4] Jiao, H., Xiao, E., Graves, D.T., 2015. Diabetes and its effect on bone and fracture healing. Current Osteoporosis Reports 13(5):327–335.
[5] Costantini, S., Conte, C., 2019. Bone health in diabetes and prediabetes. World Journal of Diabetes 10(8):421–445.
[6] Karner, C.M., Long, F., 2018. Glucose metabolism in bone. Bone 115:2
[7] Lee, W.C., Guntur, A.R., Long, F., Rosen, C.J., 2017. Energy metabolism of the osteoblast: implications for osteoporosis. Endocrine Reviews 38(3):255–266.
[8] Matsumoto, Y., La Rose, J., Lim, M., Adissu, H.A., Law, N., Mao, X., et al., 2017. Ubiquitin ligase RNF146 coordinates bone dynamics and energy metabolism. Journal of Clinical Investigation 127(7):2612–2625.
[9] Dirckx, N., Bashir, A.B., Mercken, E.M., Vangoitsenhoven, R., Moreau-Triby, C., Breugelmans, T., et al., 2018. Vhl deletion in osteoblasts boosts cellular glycolysis and improves global glucose metabolism. Journal of Clinical Investigation 128(3):1087–1105.
[10] Wei, J., Shimazu, J., Makinotsugu, M.P., Maurizi, A., Kajimura, D., Zong, H., et al., 2015. Glucose uptake and Runx2 synergize to orchestrate osteoblast differentiation and bone formation. Cell 161(7):1576–1591.
[11] Li, Y., Yang, S.T., Gun, L., Yang, S.Y., 2021. TAZ is required for chondrogenesis and skeletal development. Cell Discovery 1(1).
[12] Bruder, S.P., Fink, D.J., Caplan, A.I., 1994. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. Journal of Cellular Biochemistry 56(3):283–294.
[13] Horie, T., Fukasawa, K., Yamada, T., Mizuno, S., Lezaki, T., Tokumura, K., et al., 2022. Erk5 in bone marrow mesenchymal stem cells regulates homeostasis by preventing osteogenesis in adulthood. Stem Cells 40(4):411–422.
[14] Qin, Y., Guan, J., Zhang, C., 2014. Mesenchymal stem cells: mechanisms and role in bone regeneration. Postgraduate Medical Journal 90(1069):643–647.
[15] Lee, S.Y., Long, F., 2018. Notch signaling suppresses glucose metabolism in mesenchymal progenitors to restrict osteoblast differentiation. Journal of Clinical Investigation 128(12):5573–5586.
[16] Chinnam, M., Goodrich, D.W., 2011. RB1, development, and cancer. Current Topics in Developmental Biology 94:129–169.

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[36] ten Berge, D., Brouwer, A., Korving, J., Martin, J.F., Meijlink, F., 1998. Prx1 and Prx2 in skeletogenesis: roles in the craniofacial region, inner ear and limbs. Development 125(19):3831—3842.

[37] Wu, Z., Wu, H., Md, S., Yu, G., Habib, S.L., Li, B., et al., 2019. Tsc1 ablation in Prx1 and Osterix lineages causes renal cystogenesis in mouse. Science Reports 9(1):537.

[38] Jiang, M., Zheng, C., Shou, P., Li, N., Cao, G., Chen, Q., et al., 2016. SHP1 regulates bone mass by directing mesenchymal stem cell differentiation. Cell Reports 17(8):2161.

[39] Tang, W.J., Li, Y., Osimiri, L., Zhang, C., 2011. Osteoblast-specific transcription factor osterix (osx) is an upstream regulator of Satb2 during bone formation. Journal of Biological Chemistry 286(38):32995—33002.

[40] Sinha, K.M., Zhou, X., 2013. Genetic and molecular control of osterix in skeletal formation. Journal of Cellular Biochemistry 114(5):975—984.

[41] Wang, L., Mishina, Y., Liu, F., 2015. Osterix-cre transgene causes craniofacial bone development defect. Calcified Tissue International 96(2):129—137.

[42] Wu, X., Zhang, Y., Xing, Y., Zhao, B., Zhou, C., Wen, Y., et al., 2019. High-fat and high-glucose microenvironment decreases Runx2 and TAZ expression and inhibits bone regeneration in the mouse. Journal of Orthopaedic Surgery and Research 14(1):55.

[43] Kegelman, C.D., Mason, D.E., Dawahare, J.H., Horan, D.J., Vigil, G.D., Howard, S.S., et al., 2018. Skeletal cell YAP and TAZ combinatorially promote bone development. FASEB Journal 32(5):2706—2721.

[44] Pan, J.X., Xiong, L., Zhao, K., Zeng, P., Wang, B., Tang, F.L., et al., 2018. YAP promotes osteogenesis and suppresses adipogenic differentiation by regulating beta-catenin signaling. Bone Research 6.

[45] Hardy, E., Fernandez-Patron, C., 2020. Destroy to rebuild: the connection between bone tissue remodeling and matrix metalloproteinases. Frontiers in Physiology 11.

[46] Niedzwiedzki, T., Filipowska, J., 2015. Bone remodeling in the context of cellular and systemic regulation: the role of osteocytes and the nervous system. Journal of Molecular Endocrinology 55(2):R23—R38.

[47] Xu, Z., Greenblatt, M.B., Yan, G., Feng, H., Sun, J., Lotrinun, S., et al., 2017. SMURF2 regulates bone homeostasis by disrupting SMAD3 interaction with vitamin D receptor in osteoblasts. Nature Communications 8.

[48] Wang, L., You, X., Zhang, L., Zhang, C., Zou, W., 2022. Mechanical regulation of bone remodeling. Bone Research 10(1):16.

[49] Kohli, S.S., Kohli, V.S., 2011. Role of RANKL—RANK osteoprotegerin molecular complex in bone remodeling and its immunopathologic implications. Indian Journal of Endocrinology and Metabolism 15(3):175—181.

[50] Boyce, B.F., Xing, L., 2007. Biology of RANK, RANKL, and osteoprotegerin. Arthritis Research & Therapy 9(Suppl 1):S1.

[51] Boyce, B.F., Xing, L., 2008. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Archives of Biochemistry and Biophysics 473(2):139—146.

[52] Infante, M., Fabi, A., Cognetti, F., Gorini, S., Caprio, M., Fabbri, A., 2019. RANKL/RANK/OPG system beyond bone remodeling: involvement in breast cancer and clinical perspectives. Journal of Experimental & Clinical Cancer Research 38(1):12.

[53] Velletri, T., Huang, Y., Wang, Y., Li, Q., Hu, M.Y., Xie, N.X., et al., 2021. Loss of p53 in mesenchymal stem cells promotes alteration of bone remodeling through negative regulation of osteoprotegerin. Cell Death and Differentiation 28(1):156—169.

[54] Crane, J.L., Cao, X., 2014. Bone marrow mesenchymal stem cells and TGF-beta signaling in bone remodeling. Journal of Clinical Investigation 124(2):466—472.

[55] Dyson, N.J., 2016. RB1: a prototype tumor suppressor and an enigma. Genes & Development 30(13):1492—1502.

[56] Walkley, C.R., Qudsi, R., Sankaran, V.G., Perry, J.A., Gostissa, M., Roth, S.I., et al., 2018. Conditional mouse osteosarcoma, dependent on p53 loss and potentiated by loss of Rb, mimics the human disease. Genes & Development 22(12):1662—1676.

[57] Berman, S.D., Calo, E., Landman, A.S., Danielian, P.S., Miller, E.S., West, J.C., et al., 2008. Metastatic osteosarcoma induced by inactivation of Rb and p53 in the osteoblast lineage. Proceedings of the National Academy of Sciences of the United States of America 105(33):11851—11856.

[58] Marbanian, C., Kma, L., 2018. Dysregulation of glucose metabolism by oncogenes and tumor suppressors in cancer cells. Asian Pacific Journal of Cancer Prevention (APJCP) 19(9):2377—2390.

[59] Jang, M., Kim, S.S., Lee, J., 2013. Cancer cell metabolism: implications for therapeutic targets. Experimental and Molecular Medicine 45.

[60] Ohnishi, T., Kusuyama, J., Bandow, K., Matsuguchi, T., 2020. Glut1 expression is increased by p53 reduction to switch metabolism to glycolysis during osteoblast differentiation. Biochemical Journal 477(10):1795—1811.

[61] Han, Y., Feng, H., Sun, J., Liang, X., Wang, Z., Xing, W., et al., 2019. Lkb1 deletion in periosteal mesenchymal progenitors induces osteogenic tumors through mTORC1 activation. Journal of Clinical Investigation 129(5):1895—1909.