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Research article

AdipoRon accelerates bone repair of calvarial defect in diet-induced obesity mice

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ABSTRACT

Objectives: To investigate the role of AdipoRon in bone wound healing of calvaria critical-sized defects (CSD) in diet-induced obesity (DIO) mice.
Materials and methods: After establishing the calvaria CSD in normal-chow (NC), DIO and Adiponectin knockout (APNKO) mice, AdipoRon or vehicle was orally gavaged for 3 weeks. The bone defects were analyzed by micro-CT and H&E staining. The expression of osteogenesis-related factor in the defect area, and the chemotactic gradient of SDF-1 between bone marrow and bone defect area were further analyzed.
Results: AdipoRon downregulated body weight and alleviated fasting blood glucose level of DIO mice after treatment with AdipoRon in 14 and 21 days. Newly formed bone was significantly increased in the defect area of DIO and APNKO mice after treatment with AdipoRon compared with vehicle treatment. No significant difference was shown in NC mice. Furthermore, compared with NC mice, a significant decrease of BV/TV%, Tb.N value and formed bone percentage were shown in DIO and APNKO mice. The treatment with AdipoRon could reverse of decreased value and increase the newly formed bone in those mice. AdipoRon promoted col-1α expression in wound sites in DIO and APNKO mice. AdipoRon nearly quadrupled the chemotactic gradient of SDF-1 by decreasing SDF-1 expression in bone marrow and increasing it in the bone defect area in APNKO and DIO treated mice.
Conclusion: AdipoRon alleviates the obesity status in DIO mice with calvarial defect and increase new bone formation in calvarial defects in DIO and APNKO mice by modulating chemotactic gradient of SDF-1.

1. Introduction

Diabetic bone disease is a common complication of diabetes, involving cartilage, cancellous bone, cortical bone, and bone joints

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[1], that could delay bone healing in fractures [2]. Twice as prevalent in diabetics as non-diabetics, periodontitis is the sixth most common complication of diabetes [3,4]. A large number of clinical epidemiological surveys have found that the bone mineral density (BMD) of patients with type 2 diabetes is slightly higher than that of normal people, but the type I collagen (col-1α) is reduced [1], and bone strength index is decreased while the risk of fracture is significantly higher than that of normal subjects [5]. Unlike endochondral osteogenesis on long bone, the alvealar bone healing suffered from periodontitis is categorized to intramembranous osteogenesis. Our previous studies found that alveolar bone repair in diabetic mice with periodontitis was prolonged compared with that in normal mice [6] , and titanium implantation in the femurs of diabetic mice showed less bone-implant osseointegration campared with normal mice [7]. Due to the tiny size and inconvenience usage of micro-CT on the irregular edges of the alveolar bone, the classic intramembranous osteogenesis model, calvaria defect, could be a supplementary and representative study on the alverlar bone regeneration.

In response to bone damage, the body would produce mesenchymal stem cell mobilization factors, such as stromal cell-derived factor-1 (SDF-1) [8,9]. SDF-1 is mainly secreted by bone marrow mesenchymal stem/stromal cells (BM-MSCs), osteoblasts and endothelial cells [10]. When the cellular surface receptor binded to SDF-1, it mobilized cells to migrate from the bone marrow cavity with low SDF-1 concentration to the blood circulation with high SDF-1 concentration [11] , then cells were recruited to the bone defect with higher SDF-1 concentrations to repair bone tissue [9]. Type 2 diabetes-related bone disease is associated with disordered osteogenesis and ineffective mobilization of mesenchymal stem cells from the bone marrow cavity to the site of bone defect. This inability to efficiently mobilize stem cells is known as diabetic stem-cell “mobilopathy” [11,12]. Our previous study found that the expression of SDF-1 in the bone marrow cavity of diabetic mice was significantly higher than that of normal wide-type mice, which led to a decrease in the chemotaxis of stem cells and impair bone function and regeneration [13]. From above, diabetes could undermine bone formation through inhibiting osteoblast function, the migration and chemotaxis of stem cells, resulting in delayed bone healing.

Adiponectin (APN) is an adipokine secreted by adipocytes that can sensitize insulin and improve insulin resistance. It has many biological effects such as lowering blood glucose and inhibiting inflammatory process [14]. However, since the monomeric APN has no obvious physiological effect, the unstable synthetic APN multimer structure and relative higher physiological concentrations of APN in serum [15] make APN-based clinical therapy a great challenge, including potential immune adverse reactions, continuous high-dose intravenous injection, difficulties in industrial production of complex protein structures, etc. that all limit the practical clinical application of APN.

Okada-Iwabu and other colleagues reported an oral effective synthetic small molecule, AdipoRon (APR) [16], could specifically bind to and activate APN receptors on the cell surface, showing anti-diabetic effects similar to APN, including reducing insulin resistance and blood glucose intolerance [16]. In addition to its anti-diabetic effects, scientists have found that APR can alleviate some of the diabetic complications, such as cardiovascular disease [17,18] and kidney disease [19,20]. Our previous study found that APR could inhibit osteoclast differentiation and promote osteoblast differentiation *in vitro*, moreover, APR could alleviate alveolar bone resorption and reduce the release of inflammatory factors in gum in diabetic mice with experimental periodontitis [6]. Recently, it was found that APR could promote the implant-bone osseointegration of femoral titanium implants in diabetic mice through reducing the ratio of RANKL/OPG [7]. In 2021, Liu et al. found that APR could accelerate bone repair of femoral defects by promoting the migration of bone marrow mesenchymal stem cells and adipocyte-derived stem cells in wild-type young mice [21]. As we know, intramembranous osteogenesis is different from endochondral ossification. Whether APR could facilitate intramembranous osteogenesis on diabetic mice is still need further research. In this study, we established calvarial CSD model on normal-chow (NC), diet-induced Obesity (DIO) and adiponectin knockout (APN KO) mice with oral gavage of APR or vehicle. Micro-CT and histological assay were used to analyze newly formed bone. Furthermore, osteogenesis related factor was evaluated using realtime-qPCR, and chemotactic gradient of SDF-1 expression from bone marrow cavity to calvarial defect were investigated.

2. Materials and methods

2.1. Animal model

Wide type (C57BL/6J, Jax #000664, male), DIO (C57BL/6J background, Jax #380050, male) and APN KO (Jax#008195, male) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, http://www.jax.org/). After acclimatization, DIO mice were fed a high-fat diet (containing 60% kcal from fat, ResearchDiet, D12492) from 6 weeks of age onwards. Wide type (Normal-chow group, NC) and APN KO mice were fed a normal chow diet (containing 10% kcal from fat, ResearchDiet , D12450J). Calvarial defect was prepared according to our previous study [13]. Briefly, 18 weeks age of mice were anesthetized with ketamine/xylazine. Then, they were fixed on the operating table with their backs facing upwards. After skin preparation and disinfection, the calvarial skin was cut to reach periosteum. A critical bone defect with a diameter of 2 mm was created on the left and right sides of the midcranial suture with a dental ball drill. Sterile silk scaffolds [22] (disk-shaped, pore size 500–600 μm, 2 mm in diameter and 1 mm thick) were placed in all defects. The wound was sutured with 5-0 silk suture and mice were resuscitated on a thermostatic pad. The ethical approval of this study was provided by the Tufts IACUC. The care and use of laboratory animals and Animal Research was performed in accordance with NIH guidelines at Tufts University (Boston, MA, USA).

Two days rehabilitated after the establishment of the calvarial defect, the mice were orally gavaged with APR or vehicle at 50 mg/kg body weight daily for 3 weeks. The mice were divided into 6 groups : DIO + APR , DIO + Vehicle , NC + APR , NC + Vehicle , APN KO + APR , APN KO + Vehicle.

2.2. Body weight and fasting blood glucose determination

Body wight of mice were scaled and recorded at 1, 4, 7, 10, 14, 21 days during oral gavage. Before blood glucose test, DIO mice were kept fasting overnight (12–14 h). Blood samples were collected from tail vein. Fasting blood glucose levels were determined by blood glucose monitoring system (FreeStyle Libre, Abbott Laboratories, Abbott Park, IL, USA).

2.3. Micro-CT analysis

After 3 weeks' gavage administration, the mice were sacrificed. One side of calvarial bone including defect area was collected and immediately immersed in 4% paraformaldehyde, and then replaced with 70% ethanol at 4 °C overnight. The calvarial samples were scanned by the Bruker Skyscan micro-CT system (Bruker, Kontich, Belgium). Ctan software was used to analyze the bone volume/total volume (BV/TV), trabecular number (Tb. N) and Trabecular thickness (Tb.Th).

2.4. H&E staining

The samples fixed with 4% paraformaldehyde were washed with water and then replaced with 19% EDTA for decalcification for 3 weeks. After rinsing the samples with running water, they were dehydrated step by step, soaked in xylene, liquid paraffin, embedded and sliced. Then, they were stained with hematoxylin and eosin (H&E), photographed with a microscope, and analyzed with imageJ software.

2.5. Tissue collecting and realtime quantitative PCR

Collected the other side of calvarial bone with defect, and trimmed into a circular shape with a diameter of 2.5 mm, placed in liquid nitrogen, and stored at −80 °C. At the same time ，quickly separated the bilateral femur and tibia, peeled off the muscles, periosteum and other soft tissues as much as possible, cut off the metaphysis at both ends of the bone with scissors, rinsed the bone marrow with PBS, collected and centrifuged at 3000 RPM for 5 min, discard the supernatant, and immediately placed the bone marrow in liquid nitrogen, and stored at −80 °C.

Total RNA was isolated from hard and soft tissues using TriZol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions [6]. Reverse transcription and the realtime-qPCR analyses were performed as described earlier [6]. Primers used for the PCR amplification are listed in Table 1.

2.6. Cell culture and in vitro assays for BM-MSCs migration

6-week-old C57BL/6 mice were used for the isolation of BM-MSCs, which were maintained in DMEM with 10% FBS, 1% penicillin-streptomycin as we described previously [13]. The third passage of BM-MSCs was used for following experiment. Wound-healing assay and transwell were used for investigating the cell migration ability of BM-MSCs. For wound-healing assay, 200 μL pipette tip was used to make a scratch when the cells reached 100% confluence. After 36 h' low-serum treatment with or without AdipoRon, particular points of the scratch were photographed. For transwell assay, cells were performed as previously described [13], which were plated on the top chamber overnight, then the medium of top chamber was changed to serum-free medium and the medium of bottom was changed to 10% fetal bovine serum medium with or without AdipoRon. Following incubation for 36 h, the upper layer cells were removed by sterile cotton, then the filters were removed and fixed with 4% formaldehyde and stained with 0.5% crystal violet, three different fields were photographed, counted and measured by imageJ software.

2.7. Statistical analysis

Data are presented as mean ± SD. Statistical significance between the two groups was evaluated using the student's t-test, and one- or two-way ANOVA was performed followed by Tukey's multiple comparisons test to evaluate the statistical significance among three or more groups. GraphPad Prism 7.0 software was used for all calculations. Values of $P < 0.05$ were considered statistically different.

Table 1
List of the primers used in realtime-qPCR.

Primer	Forward 5'-3'	Reverse 5'-3'
SDF-1	CTGCATCAGTGACGGTAAACC	CAGCCGTGCAACAATCTGAA
Col-1α	TGACTGGAAGAGCGGAGAGT	GTTCGGGCTGATGTACCAG
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

3. Result

3.1. AdipoRon reduced body weight and fasting blood glucose level in DIO mice

To simulate diabetic bone defects, calvarial bone defects were created. APR-treated DIO mice showed significantly decreased body weight at 14 and 21 days compared with vehicle-treated DIO mice (Fig. 1A). No significant difference was observed between APR- and vehicle-treated group in NC and APN KO mice, respectively. Meanwhile, APR significantly reduced fasting blood glucose in DIO mice compared with the Vehicle group at 14 days and 21 days, respectively (Fig. 1B).

3.2. AdipoRon promoted bone repair in calvarial defects in DIO mice

Micro-CT three-dimensional reconstruction was performed on the calvarial samples. As shown in Fig. 2A, APR treatment had no obvious effect on calvarial bone repair in NC mice. Bone repair of calvarial defects in DIO mice was slower than that in NC mice, while APR treatment could accelerate bone repair in calvarial defects in DIO mice. Meanwhile, bone repair of calvarial defects in APN KO mice was less than that in NC mice, APR treatment could promote bone repair in APN KO mice. However, APR-treatment had no significant effect on BV/TV% and Tb.N in NC mice (Fig. 2B, E). BV/TV% and Tb.N of defects in DIO and APN KO mice respectively showed significant declined compared with NC mice, while 3 week APR-treatment could increase the BV/TV% and Tb.N in DIO (Fig. 2C, F)and APN KO (Fig. 2D, G)mice, respectively. No significant difference was shown in Tb.Th among NC, DIO and APN KO mice (data not shown).

In addition, H&E staining was used to observe the histomorphology of the calvarial defect. As shown in Fig. 3, after three weeks' treatment, the newly formed bone of calvarial defect in APR-treated NC mice showed no significant difference compared with the vehicle-treated group (Fig. 3A and B). However, after three weeks, new bone formation in the calvarial defect of DIO mice was significantly reduced compared with NC mice, whereas APR-treatment could significantly enhance new bone formation in DIO mice (Fig. 3A, C). Similarly, new bone formation in the calvarial defect of APN KO mice was significantly declined compared with NC mice, and APR-treatment could strengthen new bone formation in APN KO mice (Fig. 3A, D).

3.3. AdipoRon promoted the expression of collagen in the calvarial defect of DIO mice

As the expression of col-1α in bone tissue was lower in patients with type 2 diabetes compared with normal subjects we also performed realtime-qPCR detection on col-1α expression in calvarial bone samples. As shown in Fig. 4, after APR treatment, the mRNA expression level of col-1α in the calvarial defect samples of DIO (Fig. 4A) and APN KO (Fig. 4B) mice was significantly increased compared with the vehicle-treated groups.

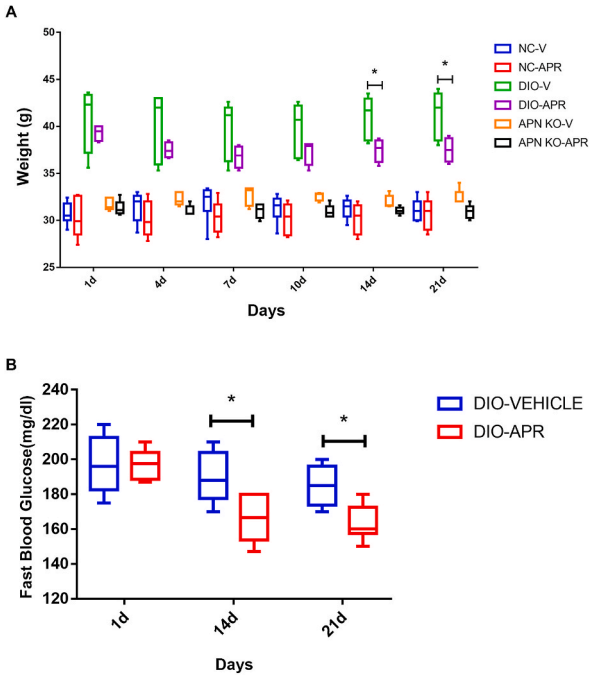

Fig. 1. AdipoRon alleviated body weight and fasting blood glucose in DIO mice. (A) Body weight of Vehicle and AdipoRon (APR)-treated NC (Normal-chow), DIO (Diet induced obesity) and APN KO (adiponectin knockout) mice after the establishment of calvarial defect. (B) Fasting blood glucose in DIO mice after the establishment of calvarial defect treated with vehicle and APR, $n \geq 5$. * , $p < 0.05$; ** , $p < 0.01$; *** , $p < 0.001$.

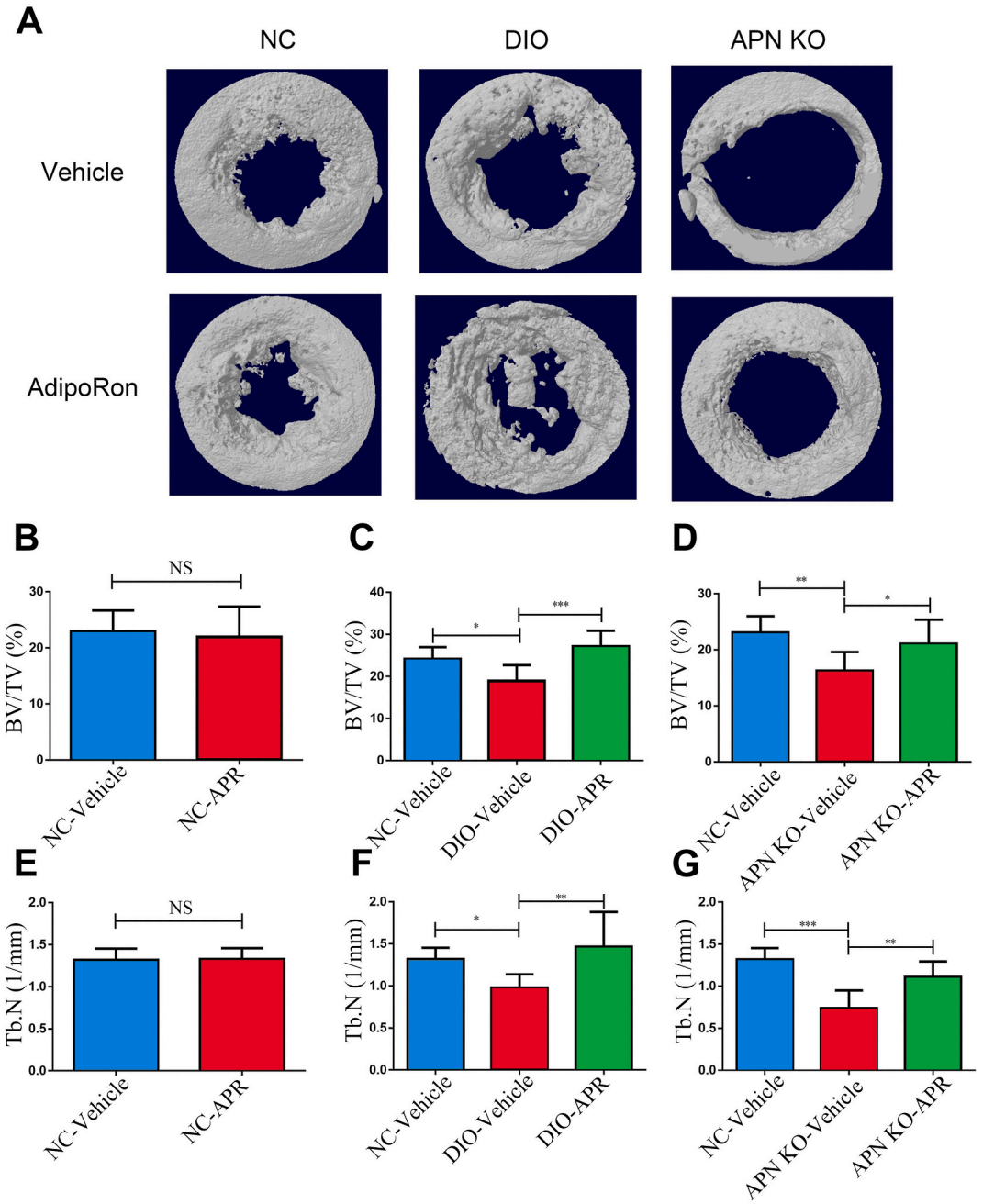

Fig. 2. AdipoRon promoted bone calcification of calvarial defect in DIO mice. (A) Representative images of microCT 3D reconstruction of calvarial defects in NC, DIO, and APN KO mice, with a Region of Interesting (ROI) of 2.5 mm in diameter. (B) (C) (D) Bone volume fraction (BV/TV%) in the calvarial defect of NC, DIO and APN KO mice, respectively. (D) (E) (F) The number of trabecular bone (Tb.N) in the calvarial defect of NC, DIO and APN KO mice, respectively. $n \geq 5$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.4. AdipoRon enhanced the chemotactic gradient of SDF-1 from the bone defect area to the bone marrow area

In DIO mice, ARP treatment significantly decreased SDF-1 in bone marrow compared with vehicle group, however, it significantly increased SDF-1 in calvarial defect area compared with control group (Fig. 5A). In the control group, the SDF-1 expression level in the calvarial defect area was 2.7 times higher than that in the bone marrow area; while, in the APR-treated group, the SDF-1 expression level in the calvarial defect area was 10.6 times higher than that in the bone marrow area. That is, APR treatment made the chemotactic gradient of SDF-1 from the calvarial defect region to the bone marrow region of DIO mice be 3.9 times as much as that of the vehicle group.

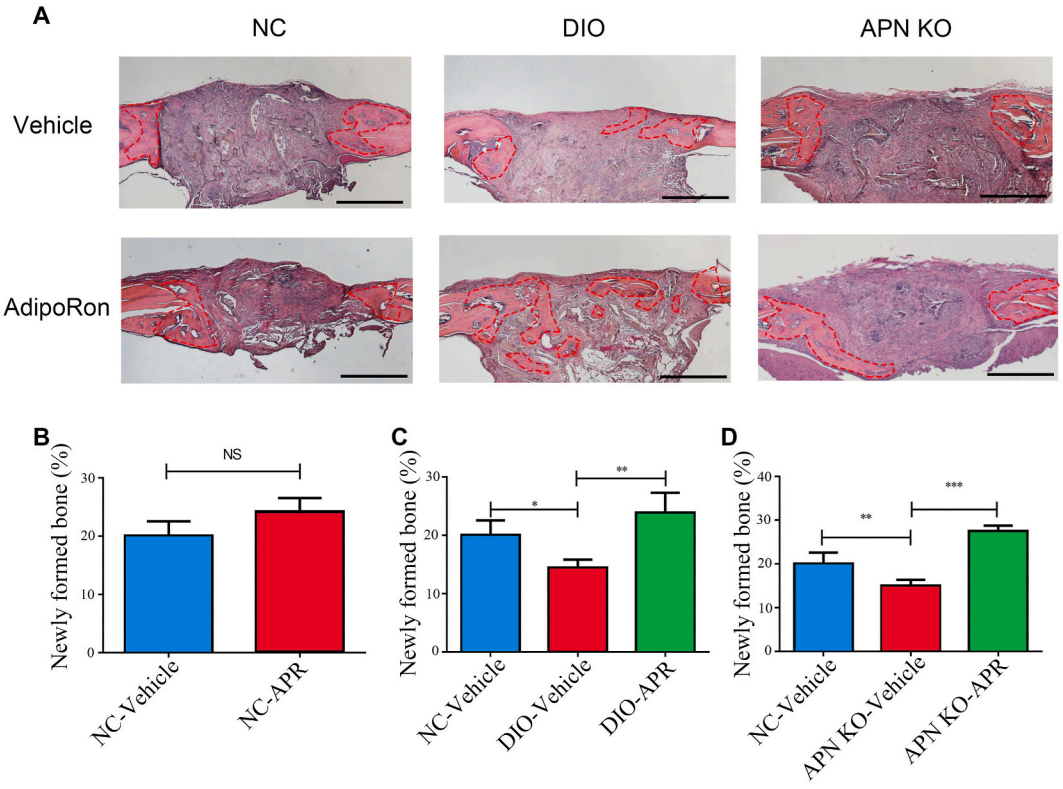

Fig. 3. AdipoRon strengthened newly formed bone of calvarial defect in DIO mice. (A) Typical H&E staining of bone tissue 3 weeks after establishment of calvarial defect in NC, DIO, and APN KO mice, where the area of new bone formation is outlined in red. (B) (C) (D) Percentage of newly formed bone area compared to the total defect area in the calvarial defect of NC, DIO and APN KO mice, respectively, $n \geq 5$, scale bar = 500 μm, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

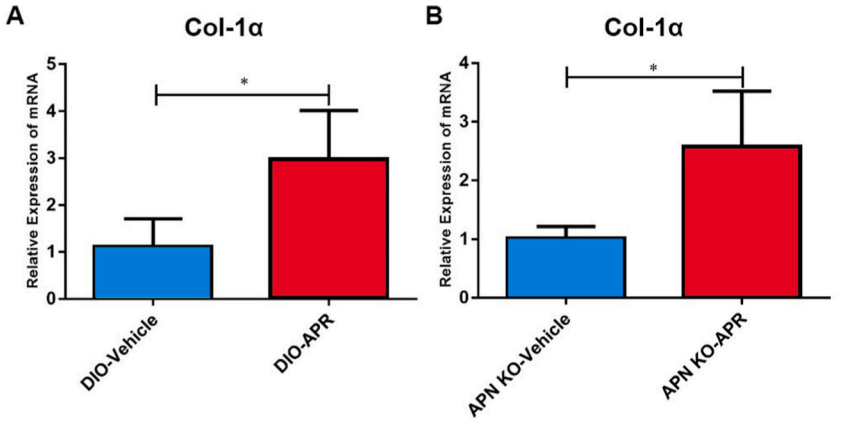

Fig. 4. AdipoRon increased col-1α expression in calcarial defect in DIO mice. The differences in gene expression of osteogenesis-related factor, as col-1α, in calvarial defect of DIO mice (A) and APN KO mice (B) were detected by realtime-qPCR. $n \geq 5$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Similarly, ARP treatment significantly decreased SDF-1 in bone marrow, but increased SDF-1 in calvarial defect site compared with vehicle group in APN KO mice (Fig. 5B). In the vehicle-treated group, the expression level of SDF-1 in the calvarial defect area was 2.5 times higher than that in the bone marrow area. In the APR-treated group, the SDF-1 expression level in the calvarial defect area was 9.3 times higher than that in the bone marrow area. That is, APR treatment made the chemotactic gradient of SDF-1 from the calvarial defect region to the bone marrow region of DIO mice be 3.7 times as much as that of the vehicle group.

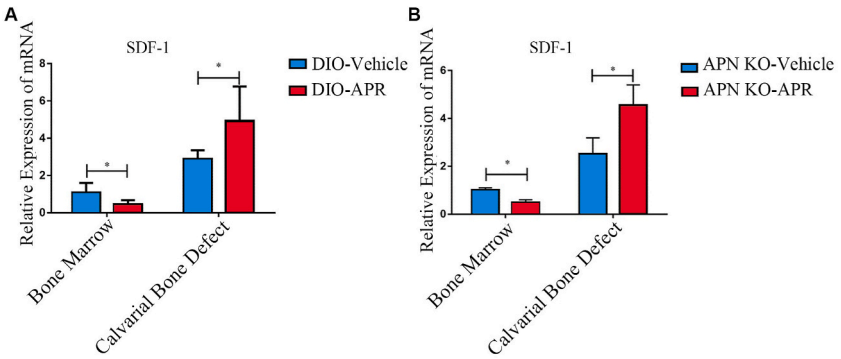

Fig. 5. AdipoRon expanded chemotactic gradient of SDF-1 from defect bone to bone marrow in DIO mice. The differences in gene expression of SDF-1 in calvarial defect area and bone marrow area of DIO mice (A) and APN KO mice (B) were detected by realtime-qPCR, with the expression of SDF-1 in the bone marrow samples of the vehicle group as 1. $n \geq 5$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.5. AdipoRon promoted migration of BM-MSCs

Wound healing test and transwell assay suggested that 36 h treatment of APR could significantly increase the migration of BM-MSCs (Fig. 6A–D). The CXCR4 expression of BM-MSCs was inhibited by APR treatment in in vitro experiment (Fig. 6E).

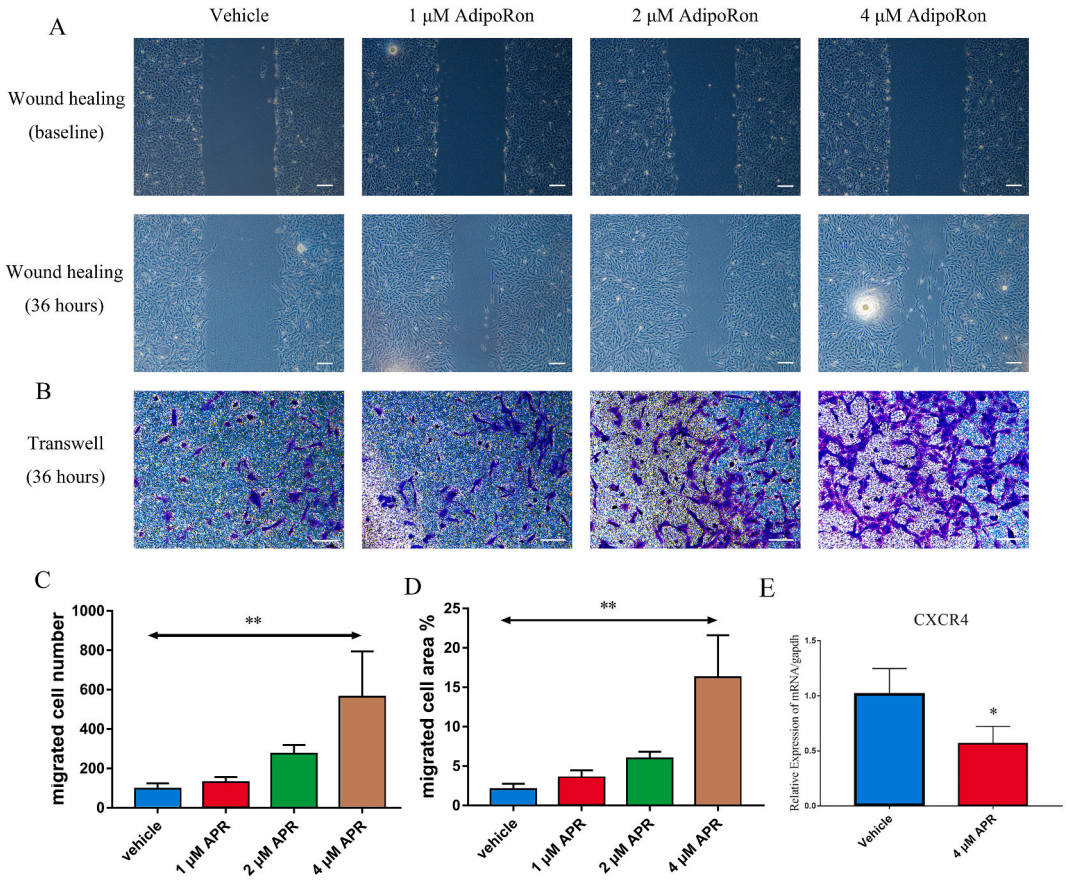

Fig. 6. AdipoRon promoted migration of BM-MSCs *in vitro*. (A) Representative images of wound healing test for BM-MSCs in 36 h treatment of APR under low-serum condition, scale bar = 100 μm. (B) Transwell assay for BM-MSCs after 36 h treatment of APR under low-serum condition, scale bar = 100 μm, and related quantitative analysis with 3 random fields of migrated cell counting (C) and migrated cell area percentage (D). Expression of CXCR4 detected by RT-PCR (E). Data shown as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

4. Discussion

Abnormal bone metabolism in patients with type 2 diabetes includes disorders of osteoblast generation and inability to effectively mobilize mesenchymal stem cells from the bone marrow cavity to the site of injury, a so-called "mobilopathy" [11,12]. Reduced circulating level of APN in obesity and type 2 diabetes [23,24] is associated with stem cell "mobilopathy" [25]. Moreover, osseous microarchitectural abnormalities and prolonged post-fracture healing time in patients with type 2 diabetes [1,26] may be related to the reduction of stem cells in the circulation leading to impaired bone regeneration [27]. Thus, elevating APN level or stimulating its corresponding receptors *in vivo* to treat disorders of osteogenesis and diabetes-related stem cell "mobilopathy" are worthy of further study.

The present study found that APR, an APN receptor agonist, could reduce fasting blood glucose and body weight in DIO mice in 14 and 21 days, which was partly consistent with previous study, that APR could reduce blood glucose tolerance, and insulin sensitivity in diabetic db/db mice [16]. Our results also showed that APR could enhance intramembranous osteogenesis in a calvarial defect model, in agreement with the previous study using a long bone fracture model in which cell proliferation at the bone fracture, mechanical properties of bone, and new bone formation in diabetic rats with optimal blood glucose control by treating with insulin are strengthened than those in diabetic rats with poor blood glucose control, indicating that ideal blood glucose control promotes bone fracture healing in diabetic rats [28].

Clinical investigations have also found that in patients with type 2 diabetes, the col-1α of bone tissue is greatly reduced [5,29–31], which is significantly lower than that of healthy people, and combined with the increase in bone mineral density, indicating increased bone fragility. The present study found that the application of APR could promote the formation of col-1α in the bone defect area, which may alleviate the increased bone fragility to a certain extent, and help reduce the risk of bone fracture in patients with type 2 diabetes.

APN could promote the osteogenesis in pre-osteoblast and BM-MSCs [32–34], and implantation of APN-overexpressing BM-MSCs as seed cells could also promote bone repair in calvarial defects [35]. Our previous study has found that APR could activate APN receptors and promote osteoblast mineralization *in vitro* [6]. In order to exclude the confounding effects of endogenous APN on APR application, we introduced adiponectin knockout mice in establishing the calvarial defect model. Previous study showed that the circulating concentration of APN in DIO mice was lower than that in NC mice [36], while almost no APN was detected in APN KO mice. After oral administration of APR, the APN receptors are fully activated, which is equivalent to supplementing the lack of APN in circulation. We found that in APN KO mice, similar to DIO mice, the process of bone repair in calvarial defects was slower than that in NC mice, and APR treatment could improve this delayed bone repair in calvarial defects, which is coordinate with our previous study that systemic APN infusion could accelerate calvarial defect healing [13].

SDF-1 is one of stem cell mobilization factors. When it binds to the cellular surface receptor CXCR4 on BM-MSCs, it would chemotactically facilitate cells migrating from an environment with a low concentration of SDF-1 to a site with a high concentration of SDF-1 [13,37–40]. In this way, BM-MSCs are mobilized to migrate from the bone marrow cavity to the blood circulation [11], and then recruited to the area of bone injury to promote bone repair [8,9]. Stem-cell mobilopathy in diabetic patients impairs and prolongs the healing of bone defects. If there is a way to improve the mobilization of stem cells, it will be an excellent approach to alleviate bone disease in diabetic patients. In this study, we found that APR could increase the migration of BM-MSCs *in vitro*, and in DIO and APN KO mice, APR treatment enhanced the chemotactic gradient of SDF-1 from the bone defect area to the bone marrow area, which was nearly four times as much as that of the vehicle group. The results suggest that APR application modulates the chemotactic gradient of SDF-1 in DIO mice, which promotes the migration of BM-MSCs and the recruitment of stem cells to the bone defect area, and empowers bone repair and bone regeneration.

This study had a limitation that, we did not use the genetic mice with specially labeled BM-MSCs to directly confirm AdipoRon effect on facilitating MSCs migration through CXCR4/SDF-1 axis.

This study found that APR could promote intramembranous osteogenesis in calvarial defects in DIO mice through modulating the chemotactic gradient of SDF-1 leading to the inhibition of diabetes stem-cell mobilopathy, which implying a promising therapeutic treatment approach to target diabetes and alverlar bone healing. This is one of the important mechanisms underlying using APR to improve the repair of bone defects in diabetes, providing a new viewpoint for the prevention and treatment strategies for diabetic bone diseases.

Author contribution statement

Xingwen Wu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Danting Zhu: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Le Shi: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Qisheng Tu, Youcheng Yu, Jake Chen: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no competing interests.

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