


ORIGINAL ARTICLE

Targeting ROR α in macrophages to boost diabetic bone regeneration

Yufeng Shen^{1,2,3,4} | Qingming Tang^{1,2,3} | Jiajia Wang^{1,2,3} | Zheng Zhou⁴ |
Ying Yin^{1,2,3} | Yifan Zhang^{1,2,3} | Wenhao Zheng^{1,2,3} | Xinyuan Wang^{1,2,3} |
Guangjin Chen^{1,2,3} | Jiwei Sun^{1,2,3} | Lili Chen^{1,2,3} 

¹Department of Stomatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

²School of Stomatology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

³Hubei Province Key Laboratory of Oral and Maxillofacial Development and Regeneration, Wuhan 430022, China

⁴Department of Stomatology, The First Affiliated Hospital, School of Medicine, Shihezi University, Shihezi 832000, China

Correspondence

Lili Chen, Department of Stomatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China.

Email: chenlili1030@hust.edu.cn

Funding information

Hubei Provincial Natural Science Fund for Creative Research Groups, Grant/Award Number: 2020CFA014; National Natural Science Foundation of China for Key Program Projects, Grant/Award Number: 82030070; the National Natural Science Foundation of China for General Program, Grant/Award Number: 82270950; the Youth Clinical Research Fund of Chinese Stomatological Association, Grant/Award Number: CSA-Q2020-10; Young Talents Project by the Health Commission of Hubei Province, Grant/Award Number: WJ2021Q059

Abstract

Diabetes mellitus (DM) has become a serious threat to human health. Bone regeneration deficiency and nonunion caused by DM is perceived as a worldwide epidemic, with a very high socioeconomic impact on public health. Here, we find that targeted activation of retinoic acid-related orphan receptor α (ROR α) by SR1078 in the early stage of bone defect repair can significantly promote in situ bone regeneration of DM rats. Bone regeneration relies on the activation of macrophage ROR α in the early bone repair, but ROR α of DM rats fails to upregulation as hyperglycemic inflammatory microenvironment induced IGF1-AMPK signalling deficiency. Mechanistic investigations suggest that ROR α is vital for macrophage-induced migration and proliferation of bone mesenchymal stem cells (BMSCs) via a CCL3/IL-6 depending manner. In summary, our study identifies ROR α expressed in macrophages during the early stage of bone defect repair is crucial for in situ bone regeneration, and offers a novel strategy for bone regeneration therapy and fracture repair in DM patients.

1 | INTRODUCTION

Diabetes mellitus (DM) is one of the most common chronic metabolic diseases and the global prevalence of DM in 2019 is estimated to be 9.3% (463 million people), rising to 10.2% (578 million) by 2030 worldwide.¹ The disruption of skeletal system is documented as the one of most common complications of DM.^{2,3} DM patients often suffer from an explosive increase of bone fracture, and excessive hyperglycemia delays bone healing and high risk of bone graft implantation failure.^{4–6}

Underlying mechanisms involved in diabetic bone regeneration deficiency are gradually recognized, for example dysfunction of immune cells (e.g., macrophages) and impaired osteoblast function, etc.⁶ Accordingly, some therapeutic intervention approaches to facilitate bone healing under diabetic conditions have been proposed, including local application of anti-inflammatory cytokines, hyperbaric oxygen therapy or implantation of isogenic adult stem cells (ASCs).^{7–9} Unfortunately, bone regeneration of DM remains a clinical challenge, with defect of stem cells in a high-glucose microenvironment being the primary obstacle.¹⁰ Hence, it is imperative to develop an effective strategy to recruit autologous stem cells to improve osteogenesis in DM patients.

Yufeng Shen, Qingming Tang and Jiajia Wang contributed equally to this work.

This is an open access article under the terms of the [Creative Commons Attribution](https://creativecommons.org/licenses/by/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2023 The Authors. *Cell Proliferation* published by Beijing Institute for Stem Cell and Regenerative Medicine and John Wiley & Sons Ltd.

Persistent non-resolving inflammation, characterized by explosive increase of leukocytes (e.g., macrophages) and proinflammatory cytokines, is the main underlying mechanism to defect of stem cells and impaired healing of DM.^{11,12} Paradoxically, trauma microenvironment of DM is completely different during the acute phase of healing in that it suffers from inadequate macrophages and insufficient inflammatory response.^{13–16} What causes macrophages hypofunction and stem cell deficiency in diabetic bone defect during the acute phase of healing remains unknown, prompting us to revisit this issue? Retinoic acid-related orphan receptor α (ROR α) is a multi-faceted nuclear receptor in tissue regeneration beyond an ability to regulate immune signalling.¹⁷ A significant body of work has focused on the roles of the RORs, and elegant genetic studies have established that ROR α expression is closely related to DM and is indispensable to orchestrate immune microenvironment and osteogenesis.^{18–21} Therefore, we speculated that ROR α may be a vital factor in regulating inflammatory microenvironment in the early stage of bone defect and inflammatory imbalance in DM, providing a novel target for treating diabetic bone regeneration deficiency.

In this study, we found that ROR α expressed in macrophages is essential for in situ bone regeneration. Targeted activation of ROR α by SR1078 in the early stage of bone defect boosts bone regeneration of DM rats. Macrophage ROR α fails to upregulate as hyperglycemic inflammatory microenvironment induced insulin-like growth factor 1 (IGF1) scarcity and 5'-AMP-activated protein kinase (AMPK) signalling inactivation in the early stage of bone defect repair from DM rats, which causes regeneration deficiency severely. ROR α is vital for macrophage-induced migration and proliferation of BMSCs via a C-C motif chemokine 3 (CCL3)/interleukin-6 (IL-6) depending manner. Overall, our study thus provides newly fundamental insights into the osteogenesis under DM conditions and offers a novel strategy for bone regeneration therapy in diabetic patients.

2 | RESULTS

2.1 | Activation of ROR α by SR1078 boosts in situ bone regeneration of DM rats

To test whether activation of ROR α could promote DM bone regeneration, we established a calvarial defect model in type 2 DM rats and SR1078, a selective agonist of ROR α , was administered to activate ROR α driven transcription (Figure 1A). *Bmal1* and *Clock* are the main target genes of ROR α , and qRT-PCR assays firstly indicated that the mRNA transcript of *Bmal1* and *Clock* in the calvarial bone was increased 2-h after SR1078 injection, and the increase was more significant after 8-h, suggesting that SR1078 was existing in the calvarial defect (Figure 1B). Micro-CT analysis showed limited bone healing in the DM rats, with less than 30% new bone in the defect area after 28 days (Figure 1C,D). Significantly, the amount of new bone in the defect area at day 14 in the SR1078 group was comparable to that in the vehicle group at day 28, indicating an accelerated osseous regeneration by SR1078, which was evidenced by bone volume per

tissue volume (BV/TV) and trabecular thickness (Tb.Th) measurement (Figure 1C,D). Masson staining showed that the newly formed bone marked by red was much more in the SR1078 group (Figure 1E). To further assess the osteogenesis at molecular biology, we conducted alkaline phosphatase (ALP) and type I collagen (COL1A1) IHC staining, which were the markers of early and late osteogenesis, respectively. The staining data showed remarkably higher osteogenesis activities in the SR1078 group during the whole healing period (Figure 1F–H). QRT-PCR data showed that the mRNA levels of osteogenesis indicators *Osx*, *Alp*, Bone morphogenetic protein 2 (*Bmp2*), Runt-related transcription factor 2 (*Runx2*) and Osteocalcin (*Ocn*) were obviously up-regulated in the SR1078 group compared with the Vehicle group (Figure 1I). Taken together, these results suggested that functional activation of ROR α by SR1078 can significantly promote in situ bone regeneration of DM rats.

2.2 | ROR α expressed in macrophages of DM rats is deficient in early bone repair

To reveal the underlying pro-regenerative effect of SR1078, we detected the expression change of ROR α in the cranial defect tissue of normal rats and DM rats at 3, 7, 14 and 28 days post-operatively. IHC staining showed low expression of ROR α in the normal control rats and positive expression of ROR α could be seen as early as 3 days after calvarial defect (Figure 2A,B). Marked increase of ROR α continued to day 7 and decreased afterwards (Figure 2A,B). As ROR α -positive cell morphology was biased towards macrophages, we surmised that ROR α in calvarial tissue is mainly derived from macrophages. To test this, IF double staining for CD68, a pan macrophage marker, and ROR α was carried out. We found that the overlap rate of the two fluorescence is high and CD68-positive cells showed absolutely high ROR α level in contrast to the stroma cells (Figure 2C,D). ROR α staining intensity peaked at day 7-post modelling in the normal group consistent with the IHC results and the percent of double positive cells within ROR α -positive cells showed the same tendency (Figure 2C,D). In DM rats, the proportion of CD68-positive cells in the bone defect area was not significantly decreased compared to normal mice. However, ROR α expressed in CD68-positive cells in the DM group was lower than that in the normal group at all time points and lacked an early tendency to increase (Figure 2E,F), suggesting that ROR α in macrophages is inhibited by DM microenvironment. Together, we speculated that ROR α fails to increase physiologically in the early stage of bone defect repair from DM rats, which may be a vital cause of diabetic regeneration deficiency.

2.3 | Inhibition of ROR α by SR3335 impedes physiological in situ bone regeneration

To further test the function of ROR α in the physiological bone regeneration process, SR3335, an inverse agonist of ROR α , was performed to suppress the constitutive transactivation activity of ROR α during

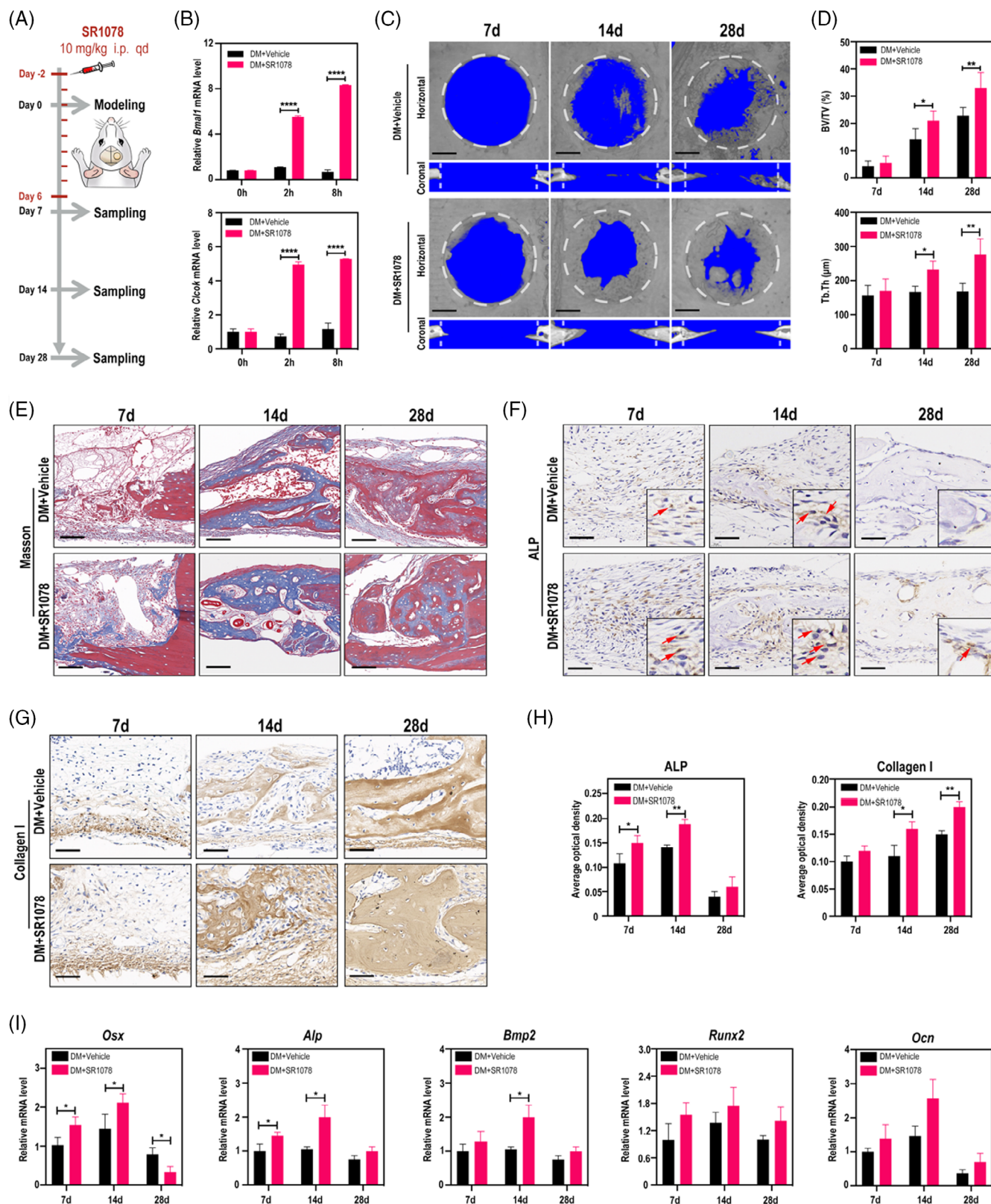


FIGURE 1 Activation of ROR α by SR1078 Boosts in situ bone regeneration of DM rats. (A) Schematic diagram of the experiment. (B) qRT-PCR analysis of *Bmal1* and *Clock* mRNA in calvarial bone tissues of DM rats at 0, 2 and 8 h post SR1078 injection. (C) Micro-CT scanning of calvarial defects on days 7, 14 and 28 post surgery. The 4 mm-diameter defect area (white dashed lines) was selected as the region of interest (ROI). Scale bar = 1 mm. (D) BV/TV and Tb.Th analysis of the selected ROI. (E) Masson staining of calvarial defects on days 7, 14 and 28 post surgery. Scale bar = 100 μ m. (F-H) IHC staining of ALP (F) and Collagen I (G) in calvarial defects and corresponsive quantity analysis (H). Scale bar = 50 μ m. (I) qRT-PCR analysis of *Osx*, *Alp*, *Bmp2*, *Runx2* and *Ocn* in calvarial bone tissues on days 7, 14 and 28 post surgery. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

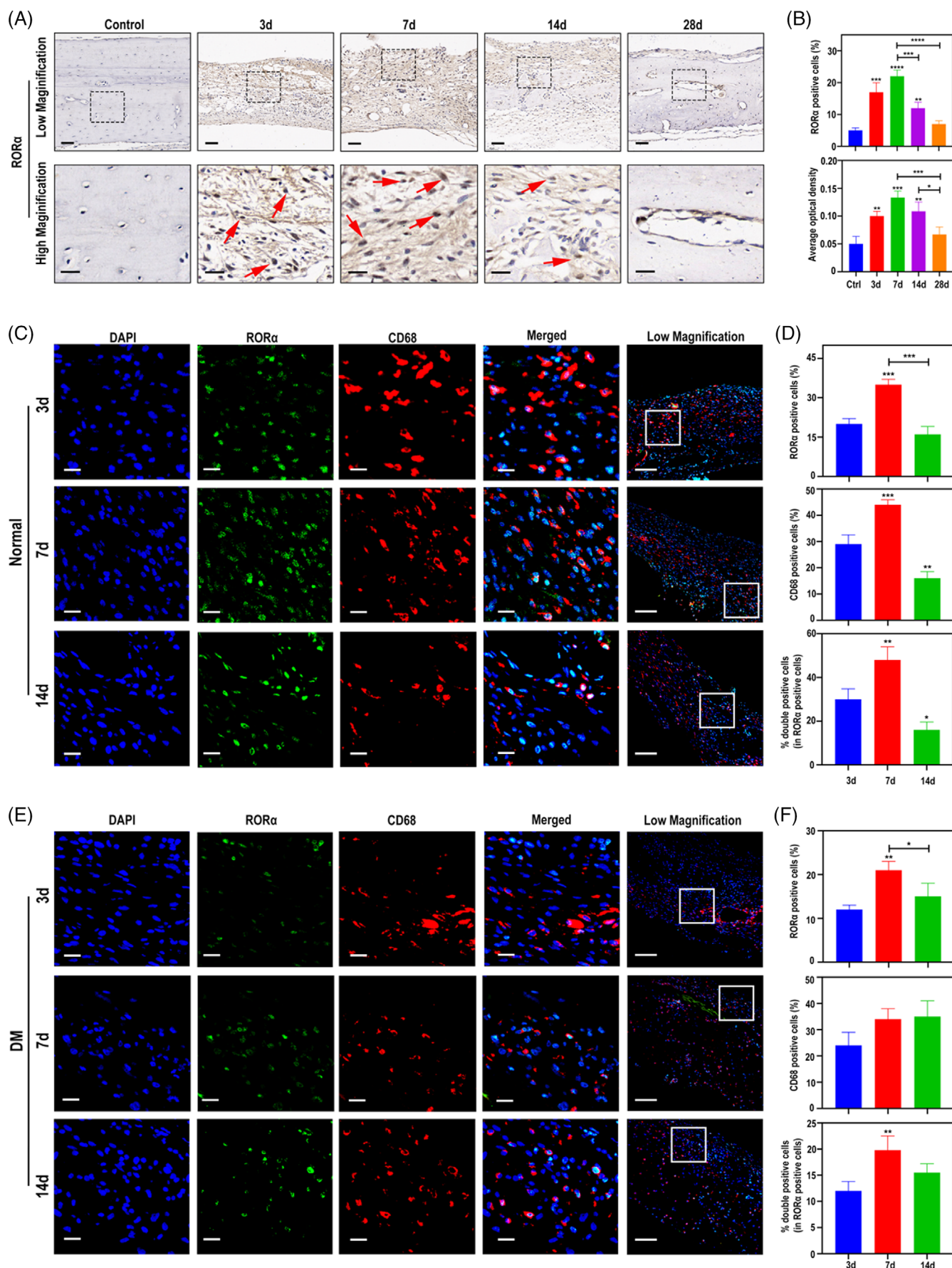


FIGURE 2 Legend on next page.

the early stage of bone healing in the normal rats (Figure 3A). qRT-PCR results of *Bmal1* and *Clock* in the calvarial bone confirmed the efficacy of SR3335 (Figure 3B). Micro-CT analysis showed that the amount of new bone in the defect area of the rats in the vehicle group increased significantly while no notable rise was observed in the SR3335 group from day 14 to 28, suggesting an impeded bone

repairing process (Figure 3C,D). Masson staining indicated that the newly formed bone marked by red in the SR3335 group was less than that in the vehicle group at day 28 (Figure 3E). We speculated that the difference in the different groups may be due to the impact on osteogenesis of bone defects after intervention of ROR α . To confirm this hypothesis, IHC staining of RUNX2 was carried out and the

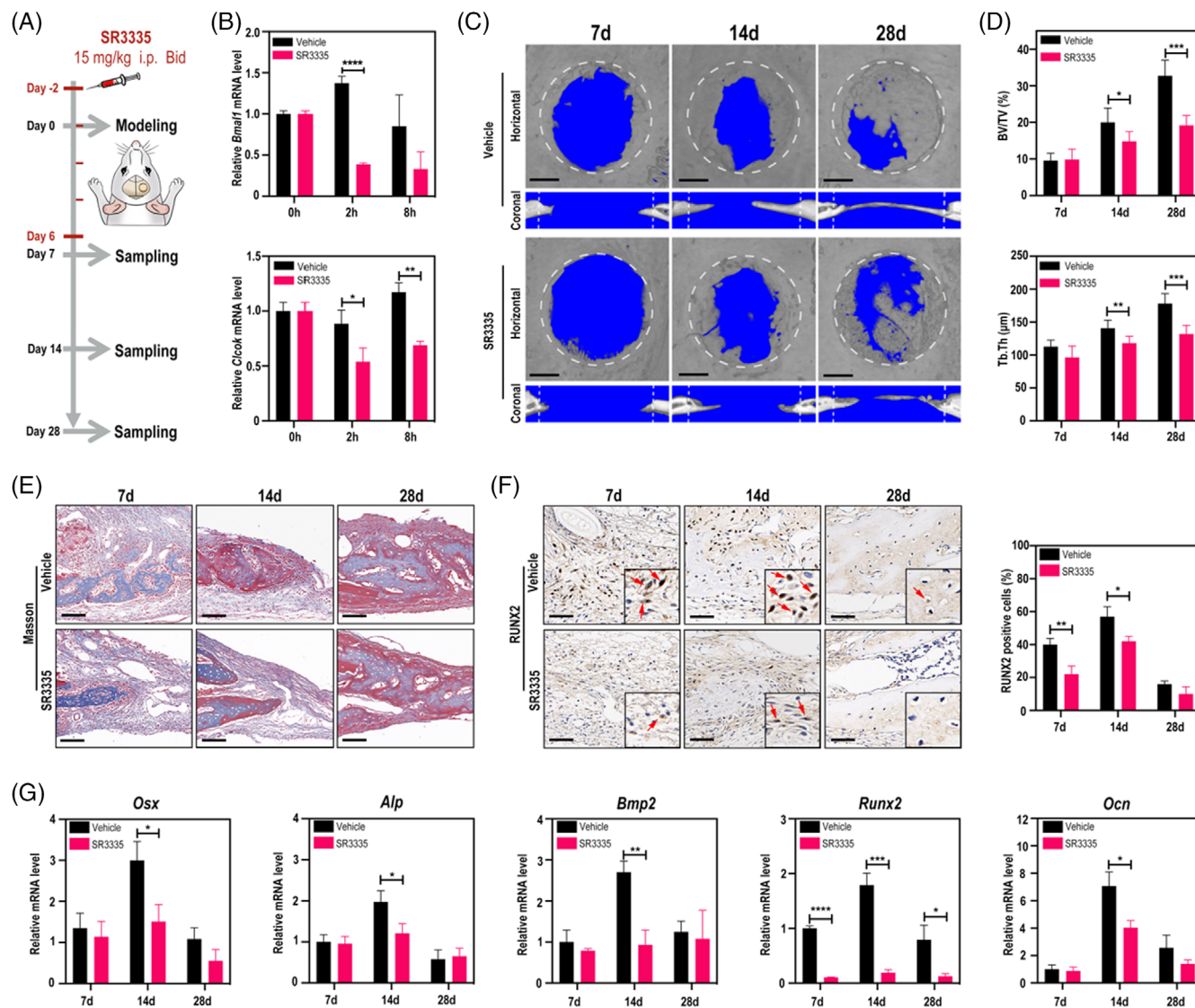


FIGURE 3 Inhibition of ROR α by SR3335 impedes physiological in situ bone regeneration. (A) Schematic diagram of the experiment. (B) qRT-PCR analysis of *Bmal1* and *Clock* mRNA in calvarial bone tissues of normal rats at 0, 2 and 8 h post SR3335 injection. (C) Micro-CT scanning of calvarial defects on days 7, 14 and 28 post surgery. The 4 mm-diameter defect area (white dashed lines) was selected as the region of interest (ROI). Scale bar = 1 mm. (D) BV/TV and Tb.Th analysis of the selected ROI. (E) Masson staining of calvarial defects on days 7, 14 and 28 post surgery. Scale bar = 100 μ m. (F) IHC staining and analysis of RUNX2 in calvarial defects. Scale bar = 50 μ m. (G) qRT-PCR analysis of *Osx*, *Alp*, *Bmp2*, *Runx2* and *Ocn* in calvarial bone tissues on days 7, 14 and 28 post surgery. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

FIGURE 2 ROR α expressed in macrophages of DM rats is deficient in early bone repair. (A) IHC staining of ROR α in calvarial defects of normal rats on days 3, 7, 14 and 28 post surgery. Rats that received sham operation were taken as the control group. Red arrows show positive cells with intranuclear ROR α staining. Low magnification scale bar = 50 μ m and high magnification scale bar = 20 μ m. (B) Quantification of ROR α expression from the IHC images. (C, E) IF staining of ROR α (green) and CD68 (red) in calvarial defects of normal rats (C) and DM rats (E) on days 3, 7, 14 post surgery. Scale bar = 100 μ m. (D, F) Quantitative analysis of ROR α ⁺, CD68⁺ and ROR α ⁺CD68⁺ cells in the normal rats (D) and DM rats (F). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

results showed that RUNX2 expression in the SR3335 group was lower than that in the vehicle group (Figure 3F). qRT-PCR assays showed that osteogenesis markers *Osx*, *Alp*, *Bmp2*, *Runx2* and *Ocn*

were remarkably down-regulated after SR3335 administration (Figure 3G), suggesting attenuated osteoblast function after pharmacological inhibition of ROR α . To be summarized, these results

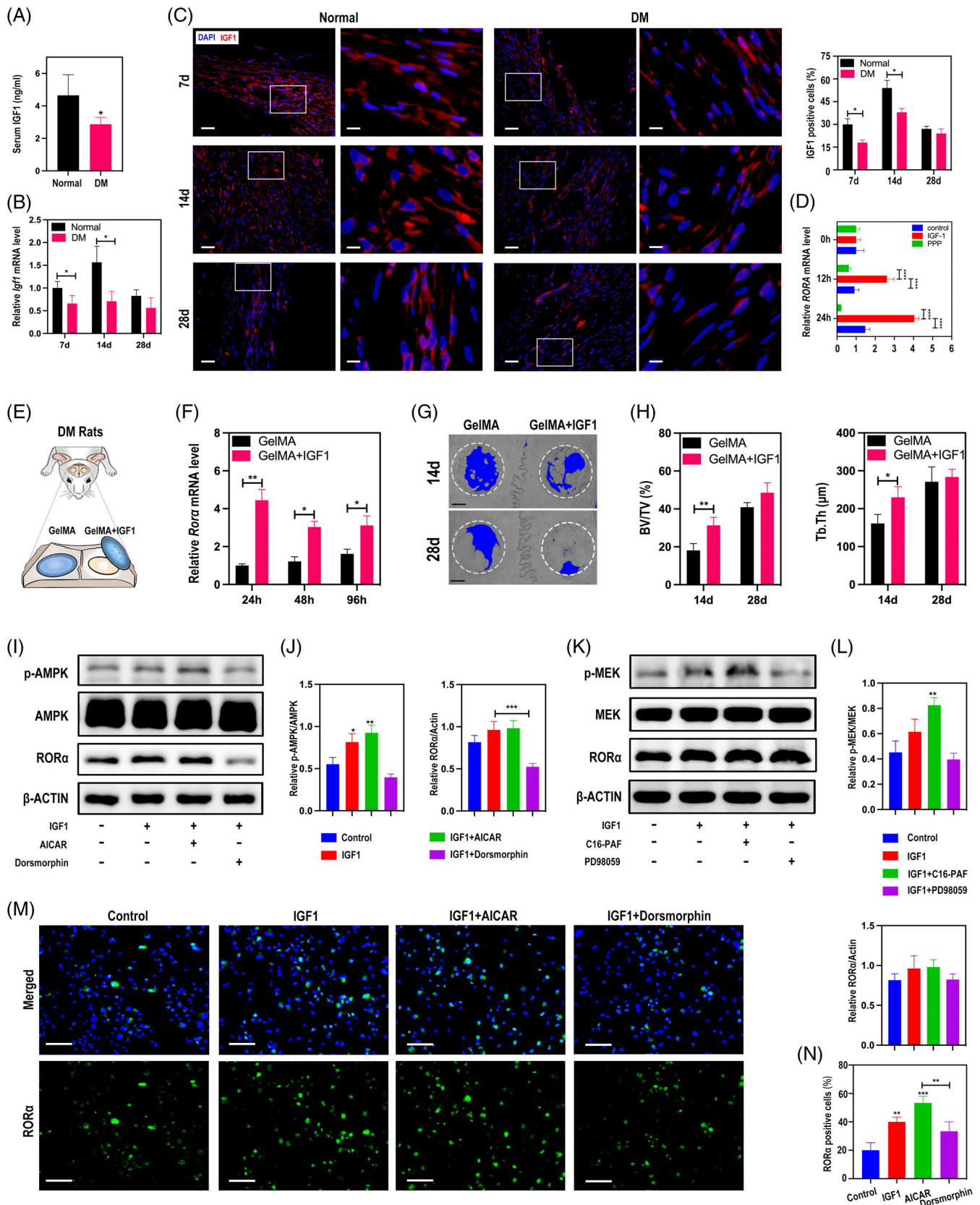


FIGURE 4 Legend on next page.

indicated that ROR α is an essential player for physiological in situ bone regeneration.

2.4 | Insufficient IGF1-AMPK signalling of DM rats blocks upregulation of ROR α

Deficiency of insulin-like growth factor 1 (IGF1) is one hallmark of the diabetic microenvironment, and its expression is sharply upregulated in early bone repair of normal individuals.^{22,23} Hereby, we supposed that inhibition of ROR α in DM may be due to IGF1 abnormality. We first detected the level of IGF1 in the serum of normal and DM rats by ELISA, and the results showed that IGF1 was significantly reduced in the serum of DM rats (Figure 4A). Further, we investigated the expression of IGF1 in calvarial defect region in rats. qRT-PCR analysis illustrated that IGF1 expression was significantly lower throughout the whole bone healing process in the DM group compared with the normal group (Figure 4B). The most significant difference was observed at day 14, with a nearly 50% decrease (Figure 4B). IF staining results showed the change more visually (Figure 4C). We next explored whether IGF1 could regulate ROR α in macrophages. THP-1, a human monocyte-derived cell line, were treated with IGF1 or IGF1 combined with IGF1R inhibitor PPP for 12, 24 h and mRNA level of ROR α was detected. qRT-PCR data indicated that IGF1 remarkably upregulated ROR α transcription, which could be eliminated by PPP administration, suggesting a positive regulation role of IGF1 on ROR α (Figure 4D). Moreover, we explored the regulation of IGF1 on ROR α in vivo. Diabetic rats received calvarial surgery and IGF1 loaded in methylpropenyl acylated gelatin (GelMA) was applied topically (Figure 4E). We could clearly see that ROR α expression increased significantly by IGF1 in newly formed tissue (Figure 4F). Consistently, Micro-CT analysis showed that more newly formed bone can be seen in the GelMA+IGF1 group at day 14 and day 28, compared with the GelMA group (Figure 4G,H). These findings suggested that IGF1 is the vital activator of ROR α in early bone repair. It is well known that adenosine monophosphate-activated protein kinase (AMPK) and mitogen-activated protein kinase (MAPK) pathways are the classical downstream intracellular signal pathways of

IGF1,^{24,25} so we tested whether IGF1 regulated the expression of ROR α by these two pathways. AMPK activator AICAR promoted phosphorylation of AMPK and ROR α expression in THP-1 cells (Figure 4I,J). Administration of AMPK inhibitor Dorsomorphin after IGF1 restrained the upward trend of AMPK phosphorylation and markedly inhibited ROR α expression (Figure 4I,J). Similarly, MAPK activator C16-PAF and inhibitor PD98059 were applied to examine the effect of MAPK on ROR α . However, no significant difference was observed in the expression of ROR α either by activation or inhibition of MAPK signalling (Figure 4K,L), suggesting that the regulation of IGF1 on ROR α was independent of MAPK pathway. Moreover, IF staining reconfirmed the IGF1-AMPK-ROR α axis (Figure 4M,N). These results indicated that IGF1 may regulate the expression of ROR α through AMPK rather than MAPK.

2.5 | ROR α actuates macrophages-induced migration and proliferation of BMSCs

After the appearance of bone defect, macrophages can rapidly recruit BMSCs through secreting chemokines, and BMSCs undergo osteogenic differentiation and exert bone regeneration effects.⁶ Therefore, we tested whether ROR α is involved in the regulation of macrophages on BMSCs. Primary bone marrow derived macrophages (BMDMs) were isolated from SD rats and identified by flow cytometry of CD68 (Figure 5A,B). We overexpressed or knocked down ROR α in BMDMs, respectively, and the efficiencies were verified by QRT-PCR (Figure 5C). Cellular supernatant of ROR α -overexpressing or ROR α -knockdown BMDMs was used as conditioned medium to incubate BMSCs (Figure 5D). Using a transwell co-culture model (Figure 5E), we found that BMDMs-conditioned medium promoted vertical migration of BMSCs (Figure 5F,G). This migration-promoting effect was dramatically enhanced by ROR α overexpression and abolished by ROR α knockdown (Figure 5F, G). Scratch assay was also performed in specially designed 6-well plates (Figure 5H). Similar with the results of transwell test, images and quantitative analysis of scratch assay showed that overexpression of ROR α strengthened BMDMs-mediated BMSCs horizontal migration whereas knockdown of ROR α inhibited this process

FIGURE 4 Insufficient IGF1-AMPK signalling of DM rats blocks upregulation of ROR α . (A) IGF1 content in the serum of normal and DM rats was detected by ELISA. (B) qRT-PCR analysis of *Igf1* mRNA levels in calvarial tissues on days 7, 4 and 28 post surgery. (C) IF staining and quantitative analysis of IGF1 in calvarial defects from normal and DM rats on days 7, 14, and 28 post surgery. Low magnification scale bar = 100 μ m and high magnification scale bar = 25 μ m. (D) THP-1 derived macrophages cultured in 25 mM glucose containing medium were treated with IGF1 (100 ng/mL) or IGF1R inhibitor PPP (5 μ M) for 12, 24 h and ROR α mRNA levels were examined by qRT-PCR. (E) Schematic illustration of topical administration of IGF1 in calvarial defects of DM rats. (F) qRT-PCR analysis of *Ror α* mRNA in calvarial bone tissues at 24, 48, 96 h post IGF1 administration. (G) Micro-CT scanning of calvarial defects on days 14, 28 post IGF1 administration. The 4 mm-diameter defect area (white dashed lines) was selected as the region of interest (ROI). Scale bar = 1 mm. (H) BV/TV and Tb.Th analysis of the selected ROI. (I) THP-1 derived macrophages cultured in 25 mM glucose containing medium were pretreated with IGF1 (100 ng/mL) for 1 h followed by administration of AMPK activator (AICAR, 0.5 mM) or AMPK inhibitor (Dorsomorphin, 2.0 μ M) for 24 h. The relative protein levels of p-AMPK α 1, AMPK α 1 and ROR α were detected by Western Blot. (J) Quantitative analysis of AMPK α 1 phosphorylation and ROR α levels. (K) THP-1 derived macrophages cultured in 25 mM glucose containing medium were pretreated with IGF1 (100 ng/mL) for 1 h followed by administration of MAPK activator (C16-PAF, 1.0 μ M) or MAPK inhibitor (PD98059, 10.0 μ M) for 24 h. The relative protein levels of p-MEK, MEK and ROR α were detected by Western Blot. (L) Quantitative analysis of MEK phosphorylation and ROR α levels. (M) IF staining of ROR α in THP-1 derived macrophages with different treatments and quantitative analysis (N). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

(Figure 5I,J). We also investigated the effect of *RORα* in BMDMs on BMSCs proliferation. CCK8 test demonstrated that after 48-h or 72-h incubation, the proliferation capacity of BMSCs treated with *Rorα*-over-expressing conditioned medium was remarkably upregulated, while *Rorα*-knockdown conditioned medium impaired BMSCs proliferation (Figure 5K). This result was further intuitively confirmed by EDU assays (Figure 5L,M). In summary, these results showed that *RORα* is vital for BMDMs to induce migration and proliferation of BMSCs.

2.6 | CCL3/IL-6 secreted by BMDMs transfer the *RORα* signalling to BMSCs

To investigate the mechanism underlying the *RORα*-induced BMSCs recruitment, we searched and obtained gene expression data for wild type (WT) and *Rorα*-deficient mice fed with a high fat diet (GSE23736). After identifying differentially expressed genes (DEGs), we performed gene ontology (GO) and Kyoto Encyclopedia of Genes and Genome

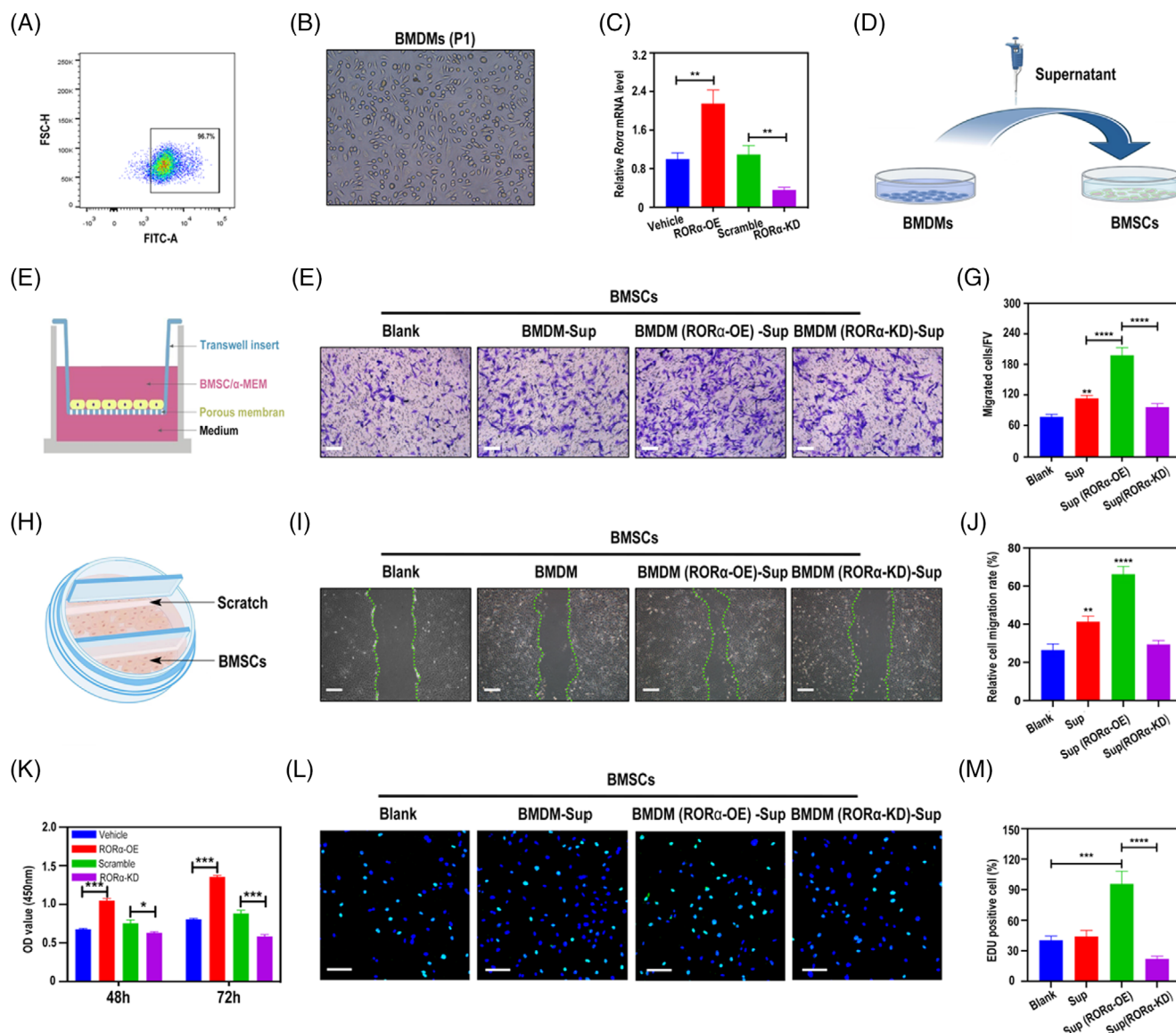


FIGURE 5 *RORα* actuates macrophages-induced migration and proliferation of BMSCs. (A) Flow cytometry was used to identify the primary cultured rat BMDMs with anti-CD68. (B) Representative images of rat BMDMs in P1 generation under light microscopy. (C) *Rorα* in BMDMs was over-expressed via lentivirus or knocked down via CRIPER/Cas 9 system and the efficiencies were examined by qRT-PCR. (D) Operation diagram of the co-culture system. *Rorα*-overexpressed or knockdown BMDMs were culture for 48 h and the supernatant was saved as conditioned medium to culture BMSCs. (E) Schematic diagram of the transwell system. (F) BMSCs were incubated in conditioned medium from *Rorα*-overexpressed or knockdown BMDMs and the vertical migrated BMSCs were stained with crystal violet. Scale bar = 200 μm. (G) Quantitative analysis of transwell assay. (H) Schematic diagram of the scratch assay. (I) Horizontal migration of BMSCs in different conditioned media was determined by scratch assay. Scratch borders were indicated by green dashed lines. Scale bar = 500 μm. (J) Quantitative analysis of scratch assay. (K) BMSCs were cultured in different conditioned media for 48, 72 h and the rates of cell growth were examined by CCK8 assay. (L) EDU staining of BMSCs cultured in different conditioned media and quantitative analysis (M). Scale bar = 100 μm. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

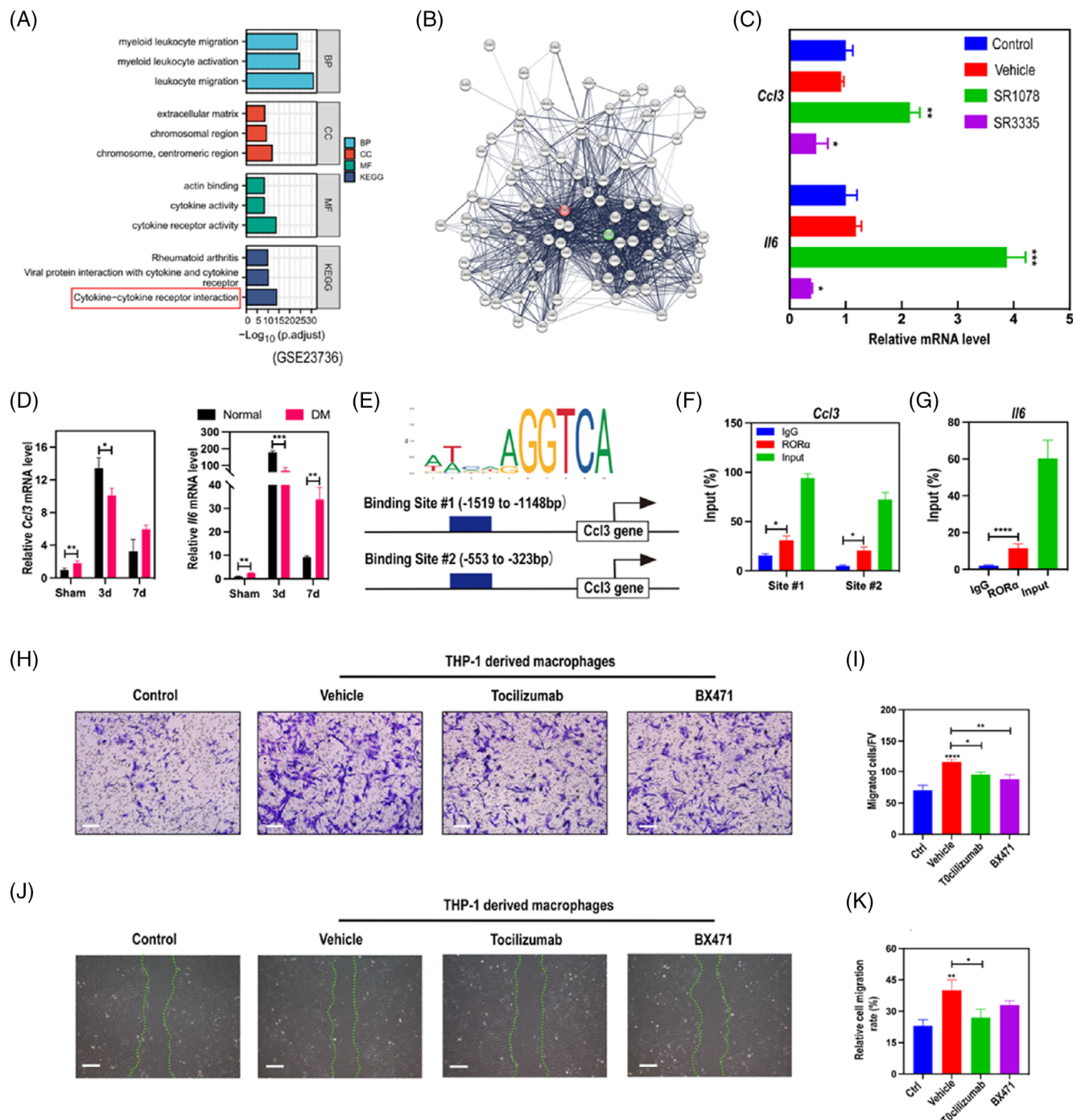


FIGURE 6 CCL3/IL-6 secreted by macrophages transfer the RORα signalling to BMSCs. (A) GO and KEGG pathway enrichment analysis of the gene expression profiles from GSE23736 dataset. (B) PPI network of the DEGs in 'Cytokine-cytokine receptor interaction' pathway. (C) RORA in THP-1 derived macrophages was activated by SR1078 (5 μ M) or inhibited by SR3335 (2 μ M) and *Ccl3*, *Il6* mRNA level were examined by qRT-PCR. (D) qRT-PCR analysis of *Ccl3* and *Il6* mRNA level in calvarial defects of normal and DM rats on days 3, 7 post surgery. (E) Schematic diagram of the potential binding site for RORα in the promoter region of CCL3 using JASPAR database. (F, G) ChIP-qPCR assay of CCL3 (F) and IL-6 (G). (H) Conditioned medium was collected from THP-1 derived macrophages treated with SR1078. Transwell assay was used to evaluate invasion ability of BMSCs incubated in conditioned medium supplemented with CCL3-neutralizing antibody (BX471, 1 μ M) or IL6-neutralizing antibody (Tocilizumab, 1 μ M) for 24 h. Scale bar = 200 μ m. (I) Quantitative analysis of the transwell assay. (J) Scratch assay of BMSCs cultured in conditioned medium with or without neutralizing antibodies of CCL3/IL-6 and quantitative analysis (K). Scale bar = 500 μ m. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

(KEGG) pathway enrichment to confirm the function of DEGs. The KEGG pathway 'Cytokine-cytokine receptor interaction' was significantly down-regulated in ROR α -deficient mice (Figure 6A). We then constructed protein-protein interaction (PPI) network to display the DEGs of "Cytokine-cytokine receptor interaction" pathway and found *Ccl3* and *Il-6* were among the most highly connected genes (Figure 6B). Based on this result, we speculated that *Ccl3* and *Il-6* may be underlying target genes that are responsible for the biological function of ROR α in BMDMs. qRT-PCR analysis indicated that SR1078 remarkably increased *Ccl3* and *Il-6* mRNA transcription in THP-1 cells while SR3335 downregulated transcription of these two genes (Figure 6C), suggesting positive transcription regulation of ROR α on *Ccl3* and *Il-6*. In the DM calvarial defect model, *Ccl3* and *Il-6* mRNA levels were lower than those in normal individuals in early bone repair, which was in line with ROR α expression (Figure 6D). Then, we performed JASPAR analysis, identified RORE sites of ROR α (Figure 6E) and predicted possible ROR α binding sites in the promoter region of *Ccl3* (Figure 6F). Further CHIP-qPCR assays confirmed ROR α -binding sites on *Ccl3* (Figure 6F). The transcriptional regulation of *Il-6* by ROR α was explored in a previous report²⁶ and we verified the binding by CHIP-qPCR analysis (Figure 6G). These results suggested that ROR α may alter the transcriptional activity of *Ccl3* and *Il-6* by direct binding. Next, we tested whether CCL3 and IL-6 are essential for ROR α -mediated recruitment of BMSCs. Conditioned medium collected from SR1078 treated macrophages was used in transwell assay of BMSCs and neutralizing antibodies against CCL3 and IL-6, BX471 and Tocilizumab, respectively, were also administrated in the transwell system. Crystal violet staining and quantity analysis illustrated that BX471 and Tocilizumab decreased the vertical migration of BMSCs induced by macrophages (Figure 6H,I). Additionally, the results from scratch assay were consistent with the transwell assay (Figure 6J,K). Together, these results demonstrate that ROR α promotes migration of BMSCs in a CCL3/IL-6 dependent manner.

3 | DISCUSSION

In this study, we outlined the role of ROR α in in situ bone healing. Under physiological conditions, significant upregulation of ROR α in macrophages was observed in the early stage of bone repair after defects. Macrophage ROR α promoted BMSCs recruitment through transcriptional activation of chemokines CCL3 and IL-6. In diabetes mellitus, ROR α was not upregulated after bone defect due to deficient upstream IGF1-AMPK signalling, resulting in impaired bone regeneration. Based on these results, we explored the potential of treating diabetic bone regeneration by targeting ROR α and found that the small molecule drug SR1078 can promote diabetic bone regeneration.

Numerous studies have proved the significant role of ROR α in regulating physiological activities of tissues and organs. As a constitutive transcription factor, ROR α is widely expressed in various tissues such as liver, kidney, skin, and adipose. In addition to its widely known involvement in the functioning of the circadian rhythm system, ROR α also plays integral regulatory roles in multiple physiological processes, such as lymphoid tissue development, lipid and glucose metabolism,

bone metabolism, and inflammation/immune response.^{27–30} Staggerer mice, which is a mutant strain with lacked functional ROR α , usually die 3–4 weeks post birth due to impeded generation of Purkinje cells,³¹ reflecting the indispensability of ROR α in maintenance of homeostasis. In adipose tissue, ROR α rhythmically inhibits the thermogenic program of white adipose tissue (WAT).³² Lau et al. reported that ROR α was a key factor in fat accumulation, staggerer mice had reduced level of serum triglycerides and exhibited resistance to diet-induced obesity.³³ Clinical studies also showed that ROR α modulated adipose tissue inflammation in obese patients.³⁴ In the content of the liver, ROR α is an essential regulator in bile acid and cholesterol homeostasis and mediates reprogramming of glucose metabolism in glutamine-deficient hepatoma cells.^{35,36} In accordance with individual performance, researchers observed abnormal thymus and spleen sizes and impaired cellularity of lymphoid tissue in staggerer mice,³⁷ so it is reasonable to assume that ROR α is critical in lymphocyte development. Widely expressed in myeloid and lymphoid cells, ROR α promotes T and B cell development by providing appropriate microenvironment and controls immune response by regulating cytokines.¹⁹ The exclusive balance of Th17/Treg cell generation is pivotal for immune homeostasis, ROR α was reported to act as an elaborate molecular switch in this teeterboard.³⁸ Another study illustrated that ROR α regulates the migration and activation of neutrophil, contributing to the host defense against microbial infection.²⁰ With the progressive exploration of the biological effects of ROR α , its role in bone metabolism is gradually revealed. Meyer et al. demonstrated that ROR α is strongly upregulated during the differentiation of BMSCs into osteoblasts. The staggerer mice of deletion within ROR α were osteopenic with thin long bones and remarkably decreased total mineral content.³⁹ Several in vitro studies have shown that ROR α regulated the metabolism of human and mouse osteoblasts and promotes osteogenic differentiation through upregulation of osteogenic mediators such as ALP, OCN, and RUNX2.^{40,41} In the current study, ROR α was inhibited in the calvarial tissue of diabetic rats after bone defects (Figure 2). Restoration of ROR α function by SR1078 promoted expression of *Col1a1*, *Alp*, *Bmp2*, *Runx2* and *Ocn*, leading to increased bone formation rate (Figure 1). This study illustrates that manipulating ROR α to promote bone repair is a viable therapeutic strategy.

Several studies suggested roles of ROR α in mesenchymal generation and differentiation. ROR α , but not ROR β was expressed in mesenchymal stem cells derived from bone marrow and ROR α acts in bone biology by direct modulation of bone matrix component.³⁹ Similarly, in human mesenchymal stem cells, ROR α was reported to act as a regulatory molecule essential for osteogenic differentiation, genetic intervention of ROR α down-regulated expression of bone sialoprotein and dentin matrix protein 1 and led to failed bone matrix formation and mineralization.⁴² Cho et al. studied ROR α in cardiac function and found that ROR α was vital in mesenchymal stem cells-mediated tissue repair.⁴³ ROR α is increased by IL-1 β and binds to angiopoietin-like 4, blunting the conversion of macrophages to the proinflammatory phenotype, ultimately facilitating regeneration under pathological conditions. Interestingly, in our study we found that ROR α expressed in macrophages promotes recruitment of BMSCs (Figure 5). Taken

together, these findings suggest that ROR α may be a key node in the crosstalk among different cells and directly or indirectly modulate the tissue regeneration microenvironment.

The molecular mechanism by which ROR α exerts its biological effects has been explored in various models. ROR α is able to response to extracellular sources of stimulus or endogenous signal mediators and conducts modulation on immune function, mainly through the most characterized pathways including NF- κ B, AMPK and IL-6/STAT3.^{44–46} The regulation role of ROR α on LPS response has been intensively studied. Staggerer mice showed elevated levels of IL-1 β , IL-6 and MIP-2 in alveolar lavage fluid and were more sensitive to LPS induced lethality.⁴⁷ In another LPS-induced septic shock model, mice exhibited reduced susceptibility in the absence of ROR α ,⁴⁸ which was due to passivated macrophages. Treatment with selective ROR α inhibitor also reduced the severity of LPS-induced endotoxemia. These seemingly contradictory results demonstrate the indispensability of ROR α in sensing inflammatory stimuli and regulating immune cell function. Specialized pro-resolving mediators (SPMs) are essential for inflammation resolution, host defense, and tissue regeneration.⁴⁹ ROR α was reported to recognize maresin-1, a classical SPM, activates monocyte phagocytosis and forms a positive feedback loop to promote maresin-1 expression thereby consolidating its anti-inflammatory effect.⁵⁰ These investigations indicate that ROR α can not only sense inflammatory stimuli in the early stage and activate immune response, but also promote resolution of inflammation in the late stage. Melatonin is widely distributed in the organism and has multiple effects such as rhythm regulation and anti-oxidative stress which are mediated mainly by interacting with specific receptors. Although it is still controversial whether it binds directly to melatonin, ROR α is a recognized melatonin receptor and induces the biological function of melatonin.⁵¹ Choi et al. revealed the link between cholesterol metabolism and osteoarthritis by ROR α . ROR α in chondrocytes responded to locally elevated cholesterol by upregulating matrix degradation factors MMPs and downregulating anabolic factor SOX9, promoting bone abnormalities.⁵² In the current study, we demonstrated that ROR α in macrophages receives upstream IGF1-AMPK signalling (Figure 4) and transfers the signal to BMSCs by manipulating CCL3/IL-6 secretion (Figures 5 and 6), ultimately promoting bone regeneration after defect. Under DM conditions, insufficient IGF1-AMPK signalling impairs the function of ROR α . Corroboratively, a recent research found that high glucose deactivates AMPK signalling by production of ROS⁵³ and this is consistent with our findings. Our study, along with those existing investigations, suggests that ROR α is a key signalling switch that senses microenvironmental cues and drives downstream pathways to modulate cell behaviours.

ROR α is a deeply shared molecule in a number of interlinked diseases, thus exploration of therapeutic strategies targeting ROR α has significant potential for clinical use. Nowadays, small molecule drugs are the mainstream direction of drug development. Among the new drugs approved by FDA in 2021, small molecules account for more than half of the drugs. ROR α is extremely sensitive to small molecule drugs and has potential as a drug target for the

treatment of different diseases. In this study, a selective agonist of ROR α , SR1078, was systematically administrated to diabetic rats and we did not observe unexpected abnormality in animals, indicating predictable biosafety of the drug. By examining the transcription level of well-recognized downstream genes of ROR α in calvarial tissue, we verified the efficiency of SR1078 (Figure 1). Modulation on ROR α -targeted genes sustained even 8 hr after a single injection, suggesting consistent long-term effect of SR1078. Finally, through molecular biology, histology and morphology test, we confirmed that SR1078 promotes diabetic bone repair. Overall, we made a preliminary attempt to boost bone regeneration by targeting ROR α , further studies of pharmacodynamics and pharmacokinetics are needed to develop a refined application strategy and broaden the scope of clinical applications.

4 | MATERIALS AND METHODS

Materials and methods are provided in supplementary materials.

AUTHOR CONTRIBUTIONS

Yufeng Shen, Qingming Tang and Jiajia Wang designed the experiments, analysed the data and wrote the manuscript. Zheng Zhou, Ying Yin and Yifan Zhang made suggestions to the writing of the manuscript and revisions to figures. Wenhao Zheng, Xinyuan Wang, Guangjin Chen and Jiwei Sun participated in conceptualization and methodology. Lili Chen and Qingming Tang supervised the work and critically revised the manuscript. Lili Chen acquired the fundings. All authors contributed to the article and approved the submitted version.

FUNDING INFORMATION

This research was supported by the National Natural Science Foundation of China for Key Program Projects (82030070) and General Program (82270950), Hubei Provincial Natural Science Fund for Creative Research Groups (2020CFA014), Young Talents Project by the Health Commission of Hubei Province (WJ2021Q059) and the Youth Clinical Research Fund of Chinese Stomatological Association (CSA-O2020-10).

CONFLICT OF INTEREST STATEMENT

The authors claim no competing interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

ORCID

Lili Chen  <https://orcid.org/0000-0002-9965-8990>

REFERENCES

1. Saeedi P, Petersohn I, Salpea P, et al. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045:

- results from the international diabetes federation diabetes atlas, 9 (th) edition. *Diabetes Res Clin Pract.* 2019;157:107843.
2. Janghorbani M, Feskanich D, Willett WC, Hu F. Prospective study of diabetes and risk of hip fracture: the Nurses' health study. *Diabetes Care.* 2006;29(7):1573-1578.
 3. Colleluori G, Aguirre L, Dorin R, et al. Hypogonadal men with type 2 diabetes mellitus have smaller bone size and lower bone turnover. *Bone.* 2017;99:14-19.
 4. Merlotti D, Gennari L, Dotta F, Lauro D, Nuti R. Mechanisms of impaired bone strength in type 1 and 2 diabetes. *Nutr Metab Cardiovasc Dis.* 2010;20(9):683-690.
 5. Yi M, Yin Y, Sun J, Wang Z, Tang Q, Yang C. Hormone and implant osseointegration: elaboration of the relationship among function, preclinical, and clinical practice. *Front Mol Biosci.* 2022;9:965753.
 6. Shen Y, Zhang Y, Zhou Z, et al. Dysfunction of macrophages leads to diabetic bone regeneration deficiency. *Front Immunol.* 2022;13:990457.
 7. Wallner C, Abraham S, Wagner JM, et al. Local application of isogenic adipose-derived stem cells restores bone healing capacity in a type 2 diabetes model. *Stem Cells Transl Med.* 2016;5(6):836-844.
 8. Hu Z, Ma C, Rong X, Zou S, Liu X. Immunomodulatory ECM-like microspheres for accelerated bone regeneration in diabetes mellitus. *ACS Appl Mater Interfaces.* 2018;10(3):2377-2390.
 9. Dias PC, Limirio PHJO, Linhares CRB, et al. Hyperbaric oxygen therapy effects on bone regeneration in type 1 diabetes mellitus in rats. *Connect Tissue Res.* 2018;59(6):574-580.
 10. Li Y et al. A sustained-release PDGF-BB nanocomposite hydrogel for DM-associated bone regeneration. *J Mater Chem B.* 2023;11(5):974-984.
 11. Bjarnsholt T, Kirketerp-Møller K, Jensen PØ, et al. Why chronic wounds will not heal: a novel hypothesis. *Wound Repair Regen.* 2008;16(1):2-10.
 12. Blakytyn R, Jude E. The molecular biology of chronic wounds and delayed healing in diabetes. *Diabet Med.* 2006;23(6):594-608.
 13. Ishida Y, Kuninaka Y, Nosaka M, et al. CCL2-mediated reversal of impaired skin wound healing in diabetic mice by normalization of neo-vascularization and collagen accumulation. *J Invest Dermatol.* 2019;139(12):2517-2527.e5.
 14. Wood S, Jayaraman V, Huelsmann EJ, et al. Pro-inflammatory chemokine CCL2 (MCP-1) promotes healing in diabetic wounds by restoring the macrophage response. *PLoS One.* 2014;9(3):e91574.
 15. Ferraro F, Lymperi S, Méndez-Ferrer S, et al. Diabetes impairs hematopoietic stem cell mobilization by altering niche function. *Sci Transl Med.* 2011;3(104):104ra101.
 16. Roy R, Zayas J, Mohamed MF, et al. IL-10 dysregulation underlies chemokine insufficiency, delayed macrophage response, and impaired healing in diabetic wounds. *J Invest Dermatol.* 2022;142(3 Pt A):692-704.e14.
 17. Hams E, Roberts J, Bermingham R, et al. Role for retinoic acid-related orphan receptor alpha (ROR α) expressing macrophages in diet-induced obesity. *Front Immunol.* 2020;11:1966.
 18. Benderdour M, Fahmi H, Beaudet F, Fernandes JC, Shi Q. Nuclear receptor retinoid-related orphan receptor α 1 modulates the metabolic activity of human osteoblasts. *J Cell Biochem.* 2011;112(8):2160-2169.
 19. Dzhagalov I, Giguère V, He YW. Lymphocyte development and function in the absence of retinoic acid-related orphan receptor alpha. *J Immunol.* 2004;173(5):2952-2959.
 20. Hsu AY, Wang T, Syahirah R, et al. Rora regulates neutrophil migration and activation in zebrafish. *Front Immunol.* 2022;13:756034.
 21. Ortega R, Hueso L, Benito E, et al. The nuclear retinoid-related orphan receptor ROR α controls adipose tissue inflammation in patients with morbid obesity and diabetes. *Int J Obes.* 2021;45(7):1369-1381.
 22. Zeng Q, Zou D, Zeng Q, Chen X, Wei Y, Guo R. Association between insulin-like growth Factor-1 rs35767 polymorphism and type 2 diabetes mellitus susceptibility: a meta-analysis. *Front Genet.* 2021;12:774489.
 23. Park Y, Lin S, Bai Y, et al. Dual delivery of BMP2 and IGF1 through injectable hydrogel promotes cranial bone defect healing. *Tissue Eng Part A.* 2022;28(17-18):760-769.
 24. Zhang XZ, Sun Y, Zhang MH, Jin Z. Insulin-like growth factor-1 inhibits apoptosis of rat gastric smooth muscle cells under high glucose condition via adenosine monophosphate-activated protein kinase (AMPK) pathway. *Folia Histochem Cytobiol.* 2022;60(1):74-88.
 25. Liu Z, Meng D, Wang J, et al. GASP1 enhances malignant phenotypes of breast cancer cells and decreases their response to paclitaxel by forming a vicious cycle with IGF1/IGF1R signaling pathway. *Cell Death Dis.* 2022;13(8):751.
 26. Journiac N, Jolly S, Jarvis C, et al. The nuclear receptor ROR(alpha) exerts a bi-directional regulation of IL-6 in resting and reactive astrocytes. *Proc Natl Acad Sci U S A.* 2009;106(50):21365-21370.
 27. Hamilton BA, Frankel WN, Kerrebrock AW, et al. Disruption of the nuclear hormone receptor RORalpha in staggerer mice. *Nature.* 1996;379(6567):736-739.
 28. Han YH, Kim HJ, Kim EJ, et al. RORalpha decreases oxidative stress through the induction of SOD2 and GPx1 expression and thereby protects against nonalcoholic steatohepatitis in mice. *Antioxid Redox Signal.* 2014;21(15):2083-2094.
 29. Jetten AM, Joo JH. Retinoid-related orphan receptors (RORs): roles in cellular differentiation and development. *Adv Dev Biol.* 2006;16:313-355.
 30. Gold DA, Gent PM, Hamilton BA. ROR alpha in genetic control of cerebellum development: 50 staggering years. *Brain Res.* 2007;1140:19-25.
 31. Steinmayr M, André E, Conquet F, et al. Staggerer phenotype in retinoid-related orphan receptor alpha-deficient mice. *Proc Natl Acad Sci U S A.* 1998;95(7):3960-3965.
 32. Monnier C, Auclair M, le Cam G, Garcia MP, Antoine B. The nuclear retinoid-related orphan receptor RORalpha controls circadian thermogenic programming in white fat depots. *Physiol Rep.* 2018;6(8):e13678.
 33. Lau P, Fitzsimmons RL, Raichur S, Wang SCM, Lechtken A, Muscat GEO. The orphan nuclear receptor, RORalpha, regulates gene expression that controls lipid metabolism: staggerer (SG/SG) mice are resistant to diet-induced obesity. *J Biol Chem.* 2008;283(26):18411-18421.
 34. Ortega R, Hueso L, Benito E, et al. The nuclear retinoid-related orphan receptor RORalpha controls adipose tissue inflammation in patients with morbid obesity and diabetes. *Int J Obes (Lond).* 2021;45(7):1369-1381.
 35. Byun JK, Choi YK, Kang YN, et al. Retinoic acid-related orphan receptor alpha reprograms glucose metabolism in glutamine-deficient hepatoma cells. *Hepatology.* 2015;61(3):953-964.
 36. Pathak P, Li T, Chiang JY. Retinoic acid-related orphan receptor alpha regulates diurnal rhythm and fasting induction of sterol 12alpha-hydroxylase in bile acid synthesis. *J Biol Chem.* 2013;288(52):37154-37165.
 37. Trenkner E, Hoffmann MK. Defective development of the thymus and immunological abnormalities in the neurological mouse mutation "staggerer". *J Neurosci.* 1986;6(6):1733-1737.
 38. Lardone PJ, Guerrero JM, Fernández-Santos JM, Rubio A, Martín-Lacave I, Carrillo-Vico A. Melatonin synthesized by T lymphocytes as a ligand of the retinoic acid-related orphan receptor. *J Pineal Res.* 2011;51(4):454-462.

39. Meyer T, Kneissel M, Mariani J, Fournier B. In vitro and in vivo evidence for orphan nuclear receptor RORalpha function in bone metabolism. *Proc Natl Acad Sci U S A*. 2000;97(16):9197-9202.
40. Benderdour M, Fahmi H, Beaudet F, Fernandes JC, Shi Q. Nuclear receptor retinoid-related orphan receptor alpha1 modulates the metabolic activity of human osteoblasts. *J Cell Biochem*. 2011;112(8):2160-2169.
41. Min HY, Son HE, Jang WG. Estradiol-induced RORalpha expression positively regulates osteoblast differentiation. *Steroids*. 2019;149:108412.
42. Miyamoto S, Cooper L, Watanabe K, et al. Role of retinoic acid-related orphan receptor-alpha in differentiation of human mesenchymal stem cells along with osteoblastic lineage. *Pathobiology*. 2010;77(1):28-37.
43. Cho DI et al. Antiinflammatory activity of ANGPTL4 facilitates macrophage polarization to induce cardiac repair. *JCI. Insight*. 2019;4(16):e125437.
44. Han S, Li Z, Han F, et al. ROR alpha protects against LPS-induced inflammation by down-regulating SIRT1/NF-kappa B pathway. *Arch Biochem Biophys*. 2019;668:1-8.
45. Ding S, Lin N, Sheng X, et al. Melatonin stabilizes rupture-prone vulnerable plaques via regulating macrophage polarization in a nuclear circadian receptor RORalpha-dependent manner. *J Pineal Res*. 2019;67(2):e12581.
46. Kim JY, Han YH, Nam MW, Kim HJ, Lee MO. RORalpha suppresses interleukin-6-mediated hepatic acute phase response. *Sci Rep*. 2019;9(1):11798.
47. Stapleton CM, Jaradat M, Dixon D, et al. Enhanced susceptibility of staggerer (RORalphasg) mice to lipopolysaccharide-induced lung inflammation. *Am J Physiol Lung Cell Mol Physiol*. 2005;289(1):L144-L152.
48. Hams E, Roberts J, Bermingham R, Fallon PG. Functions for retinoic acid-related orphan receptor alpha (RORalpha) in the activation of macrophages during lipopolysaccharide-induced septic shock. *Front Immunol*. 2021;12:647329.
49. Basil MC, Levy BD. Specialized pro-resolving mediators: endogenous regulators of infection and inflammation. *Nat Rev Immunol*. 2016;16(1):51-67.
50. Im DS. Maresin-1 resolution with RORalpha and LGR6. *Prog Lipid Res*. 2020;78:101034.
51. Slominski RM, Reiter RJ, Schlabritz-Loutsevitch N, Ostrom RS, Slominski AT. Melatonin membrane receptors in peripheral tissues: distribution and functions. *Mol Cell Endocrinol*. 2012;351(2):152-166.
52. Choi WS, Lee G, Song WH, et al. The CH25H-CYP7B1-RORalpha axis of cholesterol metabolism regulates osteoarthritis. *Nature*. 2019;566(7743):254-258.
53. Jiang P, Ren L, Zhi L, et al. Negative regulation of AMPK signaling by high glucose via E3 ubiquitin ligase MG53. *Mol Cell*. 2021;81(3):629-637.e5.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Shen Y, Tang Q, Wang J, et al. Targeting ROR α in macrophages to boost diabetic bone regeneration. *Cell Prolif*. 2023;56(10):e13474. doi:[10.1111/cpr.13474](https://doi.org/10.1111/cpr.13474)