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BMAL1 deficiency promotes skeletal mandibular hypoplasia via OPG downregulation

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Abstract

Objectives: Skeletal mandibular hypoplasia (SMH), a common type of developmental deformities, results in impaired aesthetics of facial profile, occlusal dysfunction and poor life quality. In this study, BMAL1 deficiency leads to SMH formation, and we aim to investigate the mechanism by which BMAL1 deficiency induces SMH.

Materials and methods: Circadian rhythm-disordered mouse models were constructed by placing animals in a jet lag schedule of 6-h light advance every 7 days for 4 or 8 weeks. The OPG expression was evaluated by histomorphometry, immunohistochemistry and western blot analysis. The mechanism by which BMAL1 affects OPG expression was investigated by chromatin immunoprecipitation and luciferase reporter assays. The phenotypes caused by BMAL1 knockout can be rescued by exogenous supplementation with OPG.

Results: We demonstrate that the expressions of BMAL1 and OPG decreased in SMH patients. Circadian rhythm-disordered mice and *Bmal1*$^{-/-}$ mice exhibited decreased expression of OPG, reduced bone mass and bone size of mandibles. Our results revealed that BMAL1 bound directly to the *Opg* promoter and upregulated its expression, thus inhibiting osteoclast differentiation. BMAL1 deficiency increased osteoclast differentiation by downregulating OPG expression. In vitro, the enhancement effect of osteoclast differentiation caused by BMAL1 knockdown was significantly reversed by exogenous supplementation with OPG. Importantly, bone loss caused by BMAL1 knockout can be partially reversed by injecting OPG Intraperitoneally.

Conclusions: These results indicate that the circadian clock plays a critical role in the growth and development of mandible by regulating OPG expression, and present a potential therapeutic strategy to prevent SMH.

1 | INTRODUCTION

Skeletal mandibular hypoplasia (SMH), a common type of craniofacial deformities, can cause serious complications by causing airway obstructions, gastric disturbance, immune deficiencies and delayed developmental growth.[1-3] The development of facial morphology is influenced by both congenital factors and epigenetic factors.[4] The clarification of SMH etiology is the key to effective prevention and treatment. Previous studies have provided some insights into the significance of congenital factors,[5] but the effects of epigenetic factors on mandibular development have not yet been elucidated.

The circadian rhythm allows life to adapt to periodic changes in the environment and participates in many physiological and behavioural processes, such as cell cycle regulation, energy

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metabolism, the immune response[6] and bone remodeling.[7] The circadian clock is involved in the regulation of bone development and bone homeostasis. Molecules associated with osteoblast differentiation are under circadian control, and genes involved in mineral deposition occur in a circadian pattern.[7-10] Brain and muscle ARNT-like1 (BMAL1) is the core and irreplaceable component of the circadian molecular oscillator, playing critical roles in the regulation of bone resorption[11] and bone formation.[12] William et al found that femoral bone mass was significantly reduced in BMAL1-knockout mice and that osteogenic differentiation was decreased in bone marrow stromal cells (BMSCs).[12] BMAL1 also affected nucleus pulposus cells and regulated the bone mass of intervertebral discs, which is closely linked with disc height.[13] However, whether BMAL1 disturbance could lead to mandibular deformity remains unknown.

Bone development and bone homeostasis are tightly regulated by hormones either systemically or by factors locally secreted by either osteoblasts or osteoclasts, such as leptin, bone morphogenetic proteins (BMPs), osteoprotegerin (OPG) and RANK ligand (RANKL). Systemic leptin causes a shift in mesenchymal stem cell (MSC) lineage and results in decreased osteogenesis.[14] BMPs promote osteogenesis as growth factors.[15] OPG is a member of the tumour necrosis factor receptor superfamily that inhibits the formation and maturation of osteoclasts by preventing RANKL interaction with its receptor RANK, which plays a critical role in osteoclastogenesis.[16] OPG-deficient mice develop early-onset osteoporosis.[17,18] In contrast, OPG overexpression suppresses bone resorption and increases bone mass in transgenic mice.[19] Additionally, OPG-deficient male mice can serve as a model for studying severe alveolar bone loss.[18] We previously deployed a protein chip analysis and found that OPG expression in the mandible was decreased significantly in *Bmal1*$^{-/-}$ mice compared with wild-type mice.[20]

In the current study, we found that BMAL1 and OPG expressions decrease concomitantly in clinical cases with SMH. To explore the correlations among circadian dysrhythmia, OPG down-regulation and SMH, circadian rhythm-disordered mouse models were builded by a jet lag schedule. Reduced bone mass and bone size are observed in circadian rhythm-disordered mice, OPG expression decreasing concomitantly. Reduced bone mass and OPG expression of mandibles are also observed in *Bmal1*$^{-/-}$ mice. We further revealed that BMAL1 can bind to the promoter of *Opg* and consequently modulates osteoclast differentiation. Importantly, bone loss caused by BMAL1 knockout can be partially reversed by injecting OPG intraperitoneally. Taken together, our findings provide new insights into the pathogenesis of SMH and present a potential therapeutic strategy.

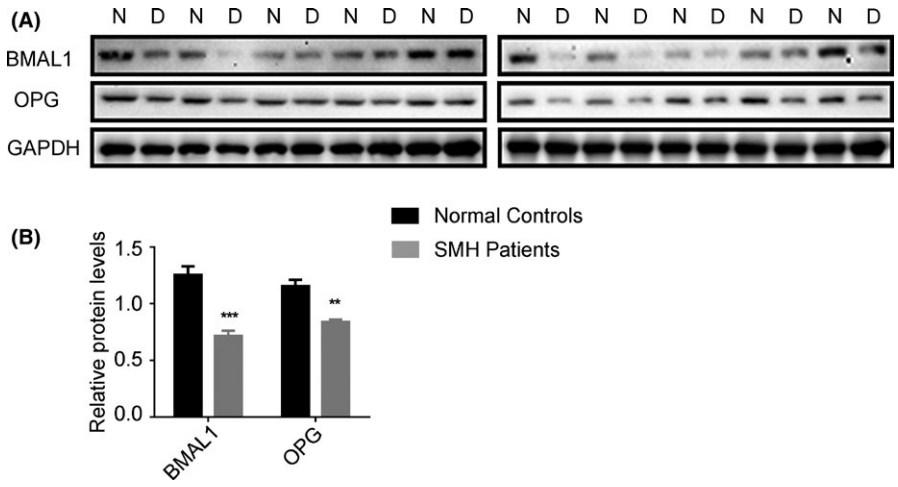


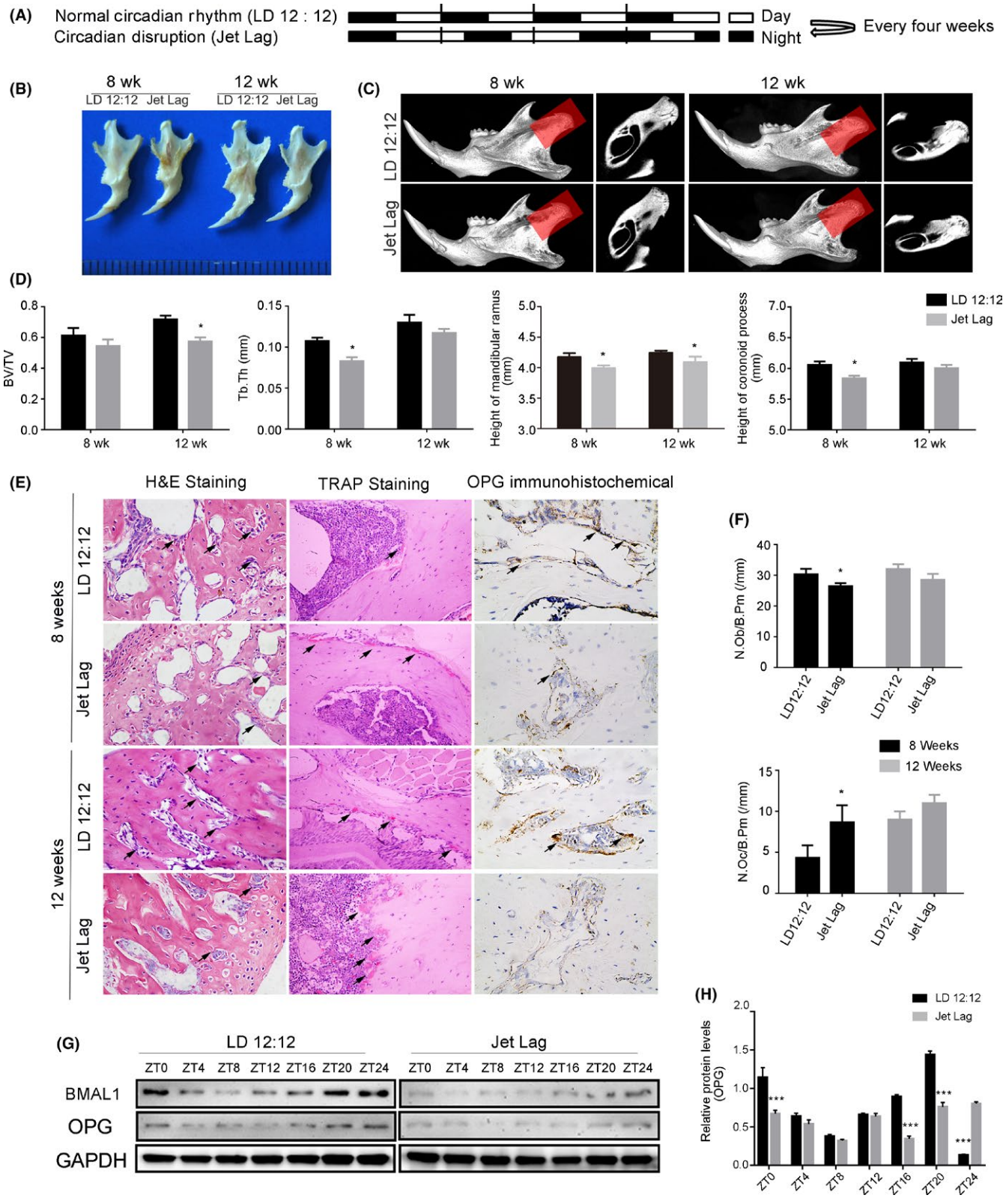
FIGURE 1 Circadian dysrhythmia may be involved in the pathogenesis of skeletal mandibular hypoplasia. (A-B) Western blot analysis of BMAL1 and OPG expression in mandibles from skeletal mandibular hypoplasia patients and normal peers. Data represent the mean ± SD (n = 10 individuals per group). $^{**}P < .01$ and $^{***}P < .001$ (compared with control), from Student's *t* tests

FIGURE 2 Circadian dysrhythmia contributes to skeletal mandibular hypoplasia and OPG expression decreased correspondingly. (A) A schematic depiction of C57 mice subjected to light/dark 12:12-h (upper panel) and 6-h light advanced every week for 4 or 8 wk (lower panel). (B) Photograph of mandibles dissected from 8- and 12-wk-old jet lag C57BL/6J or control mice. (C) Representative images of micro-CT reconstructing the mandibles of 8- and 12-week-old mice in the normal or disrupted status. Shadow areas show cross-sections of the condylar regions. (D) Micro-CT reconstruction analysis of mandibles harvested from 8- to 12-wk-old mice in the normal or disrupted status (BV/TV, Tb.Th, height of the mandibular ramus, and height of the coronoid process) (n = 5 per group). (E) Representative images of H&E and TRAP staining and OPG immunohistochemistry from normal or jet lag mouse mandibles. The arrows indicate osteoblasts in the H&E staining images, and arrows indicate osteoclasts in the TRAP staining images. (magnification, 400x). (F) Osteoblast number (N.Ob/B.Pm), osteoclast number (N.Oc/B.Pm) were measured (n = 5 per group). (G) Western blot analysis of BMAL1 and OPG expression in mandibles from 12-wk-old jet lag C57BL/6J or control mice. N = 3 independent experiments. (H) Relative protein level of OPG in mandibles from 12-wk-old jet lag C57BL/6J or control mice. N = 3 independent experiments. BV/TV, bone volume/total volume; Tb.Th, trabecular thickness; N.Ob/B.Pm, number of osteoblasts/bone perimeter; N.Oc/B.Pm, number of osteoclasts/bone perimeter. Data represent the mean ± SD. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. Two-tailed Student's *t* test was used

2 | MATERIALS AND METHODS

2.1 | Patient tissue specimens

All the twenty human mandible tissue specimens were collected from the patients at Union Hospital of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Ten specimens were obtained from patients with skeletal mandibular hypoplasia and the other ten specimens were obtained from controlled sex-matched normal peers. All specimens were critically obtained in the period between 9:00 AM and 11:00 AM. All



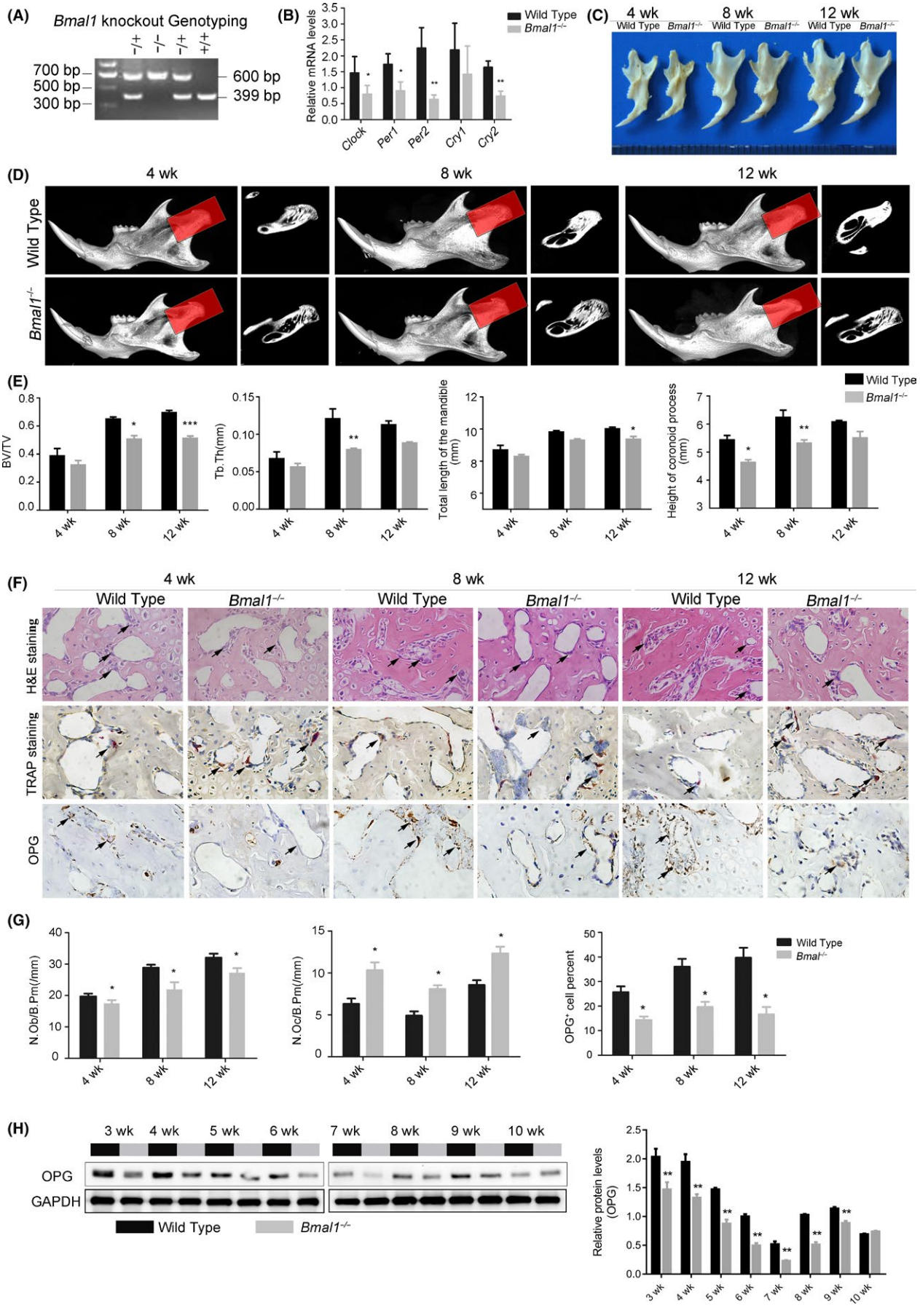

(A)
Bmal1 knockout Genotyping
-/+
-/-
-/+
+/+
700 bp
500 bp
300 bp
600 bp
399 bp
(B)
Wild Type
Bmal1-/-
Relative mRNA levels
Clock
Per1
Per2
Cry1
Cry2
(C)
4 wk
8 wk
12 wk
Wild Type
Bmal1-/-
(D)
4 wk
8 wk
12 wk
Wild Type
Bmal1-/-
(E)
Wild Type
Bmal1-/-
BV/TV
Tb.Th(mm)
Total length of the mandible (mm)
Height of coronoid process (mm)
4 wk
8 wk
12 wk
(F)
4 wk
8 wk
12 wk
Wild Type
Bmal1-/-
H&E staining
TRAP staining
OPG
(G)
N.Ob/B.Pm(/mm)
N.Oc/B.Pm(/mm)
OPG+ cell percent
Wild Type
Bmal-/-
4 wk
8 wk
12 wk
(H)
3 wk
4 wk
5 wk
6 wk
7 wk
8 wk
9 wk
10 wk
OPG
GAPDH
Wild Type
Bmal1-/-
Relative protein levels (OPG)

FIGURE 3 Skeletal mandibular hypoplasia in *Bmal1*$^{-/-}$ mice with decreasing OPG expression. (A) Genotype determined by PCR amplification in *Bmal1*$^{-/-}$, *Bmal1*$^{+/-}$ and *Bmal1*$^{+/+}$ mice (n = 5 per genotype). (B) mRNA levels of circadian clock genes *Clock*, *Per1*, *Per2*, *Cry1*, and *Cry2* in wild-type and *Bmal1*$^{-/-}$ mice, as quantified by qRT-PCR. (C) Photograph of mandibles dissected from 4-, 8-, and 12-week-old *Bmal1*$^{-/-}$ or wild-type mice. (D) Representative images of micro-CT reconstructing the mandibles of 4-, 8-, and 12-week-old *Bmal1*$^{-/-}$ or wild-type mice. Shadow areas show cross-sections of the condylar region. (E) Micro-CT reconstruction analysis of mandibles harvested from 4-, 8-, and 12-wk-old *Bmal1*$^{-/-}$ or wild-type mice (BV/TV, Tb.Th, total length of the mandible, and height of the coronoid process) (n = 5 per group). (F) Representative H&E and TRAP staining and OPG immunohistochemistry images from wild-type or *Bmal1*$^{-/-}$mice mandibles. The arrows indicate osteoblasts in the H&E staining images, and arrows indicate osteoclasts in the TRAP staining images. (magnification, 400x). (G) Osteoblast number (N.Ob/B.Pm), osteoclast number (N.Oc/B.Pm), and OPG expression level were measured (n = 5 per group). (H) Western blot analysis of OPG expression in the mandibles of 3-, 4-, 5-, 6-, 7-, 8-, 9-, and 10-week-old *Bmal1*$^{-/-}$ and wild-type mice. Data represent the mean ± SD, **P* < .05, ***P* < .01, ****P* < .001, from Student's *t* test

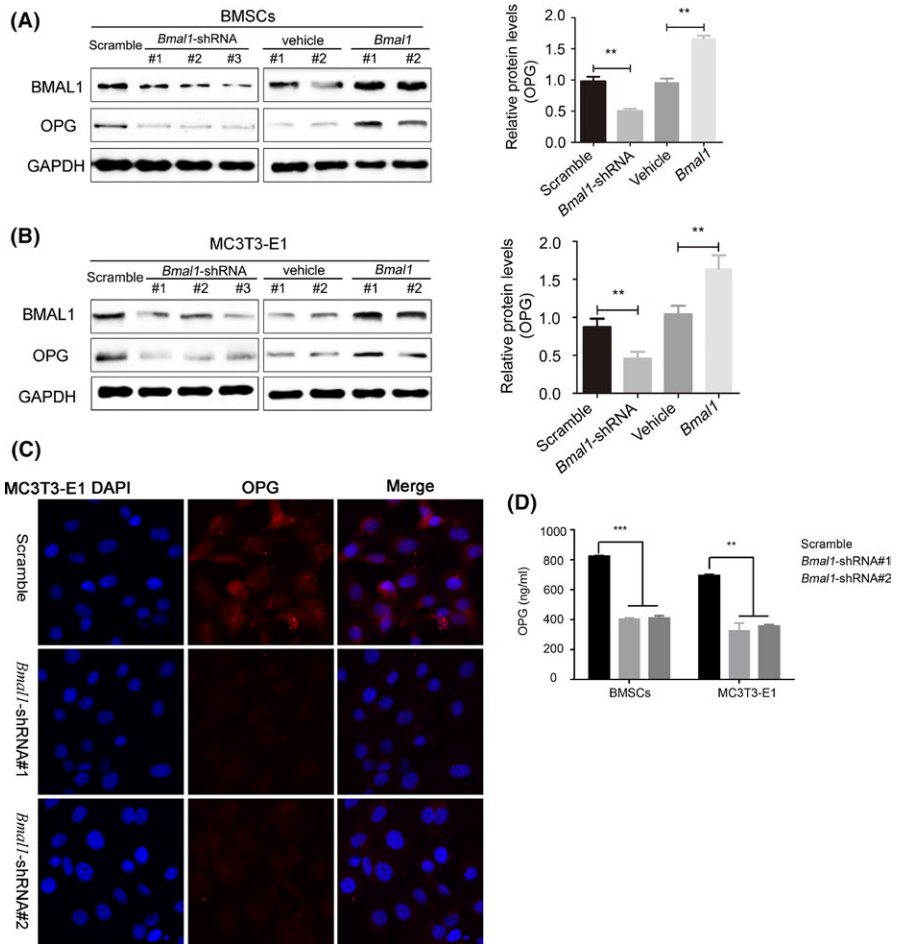


FIGURE 4 BMAL1 knockout leads to decreased OPG expression. (A-B) Western blot analysis of BMAL1 and OPG expression in BMAL1-overexpressing or BMAL1-knockdown mBMSCs and MC3T3-E1 cells. (C) Confocal microscope images of OPG expression in BMAL1-knockdown or scrambled shRNA-transfected MC3T3-E1 cells. (magnification, 600x). (D) Enzyme-linked immunosorbent assay shows the OPG concentration in the supernatant of BMAL1-knockdown mBMSCs and MC3T3-E1 cells. N = 3 independent experiments, data represent the mean ± SD, ***P* < .01, ****P* < .001. Two-tailed Student's *t* test was used

subjects' age was ranged from 10 to 12 years old and their skeletal maturation had not reached at the growth and development peak according to the quantitative cervical vertebral maturation (QCVM) measurement method. Inclusion criteria of the experimental group are listed in Table S1. Any possible impact factors, including whether treated with orthodontics or suffered from maxillofacial trauma and temporomandibular joint disorder, were excluded.

2.2 | Animals

C57BL/6J mice were purchased from the Beijing HFK Bioscience Co., Ltd. (Beijing, China). Mice were placed under strict 12-h light/12-h dark cycles, with lights on at 8 AM and off at 8 PM. For induction of the jet lag group, mice were placed under alternating light-cycle conditions with 6-h light advanced every week for 4 or 8 weeks. Five mice per group were sacrificed to collect the mandibles at the indicated time point (ZT0, ZT4,

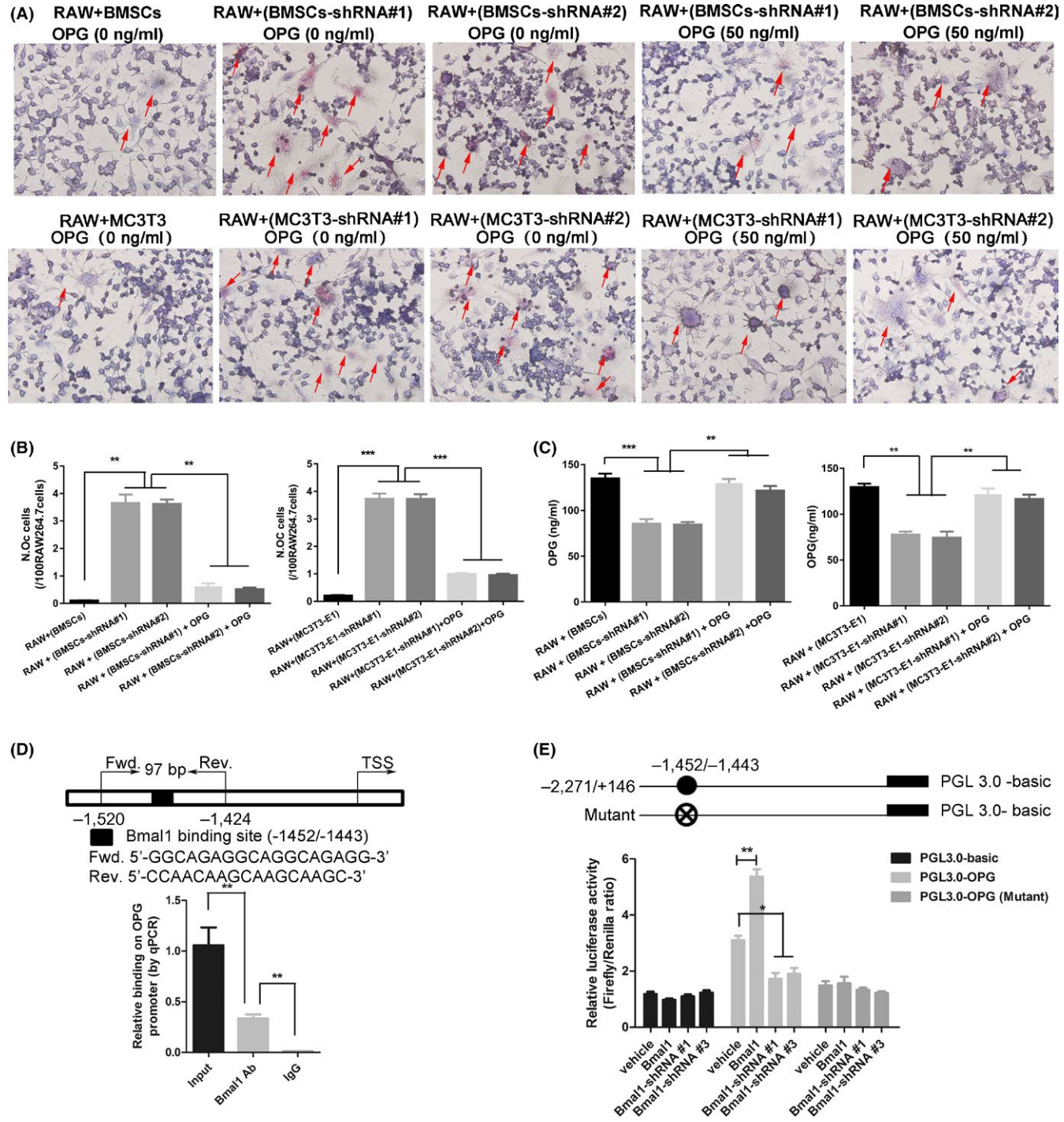


FIGURE 5 BMAL1 upregulates OPG expression by directly binding to the *Opg* promoter. (A-B) Representative images of TRAP staining of RAW 264.7 cells co-cultured with BMAL1-knockdown mBMSCs or MC3T3-E1 cells in the absence or presence of recombinant OPG. (magnification, 400x). (C) OPG concentration in the supernatants of co-cultured RAW 264.7 cells quantified by enzyme-linked immunosorbent assay. (D) The transcription factor BMAL1 bound to the *Opg* promoter in MC3T3-E1 cells. Chromatin immunoprecipitation assays were performed using anti-IgG as a negative control. (E) Luciferase reporter assays were performed to measure the activities of the wild-type or mutated BMAL1-binding site at the *Opg* promoter in BMAL1-overexpressing or BMAL1-knockdown MC3T3-E1 cells. N = 3 independent experiments, data represent the mean ± SD, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. Analysis of variance with Tukey's post-hoc test was used

ZT8, ZT12, ZT16, ZT20 and ZT24, 8 AM was set as the zeitgeber time 0: ZT0). Homozygous *Bmal1*-deficient (*Bmal1*$^{-/-}$) mice were produced by breeding heterozygous BMAL1-deficiency mating pairs (*Bmal1*$^{+/-}$) and *Bmal1* deficiency was confirmed by PCR as described by Bunger et al.[21]

2.3 | Study approval

Human mandible specimens were acquired from patients at Department of Stomatology, Union Hospital of Tongji Medical College,

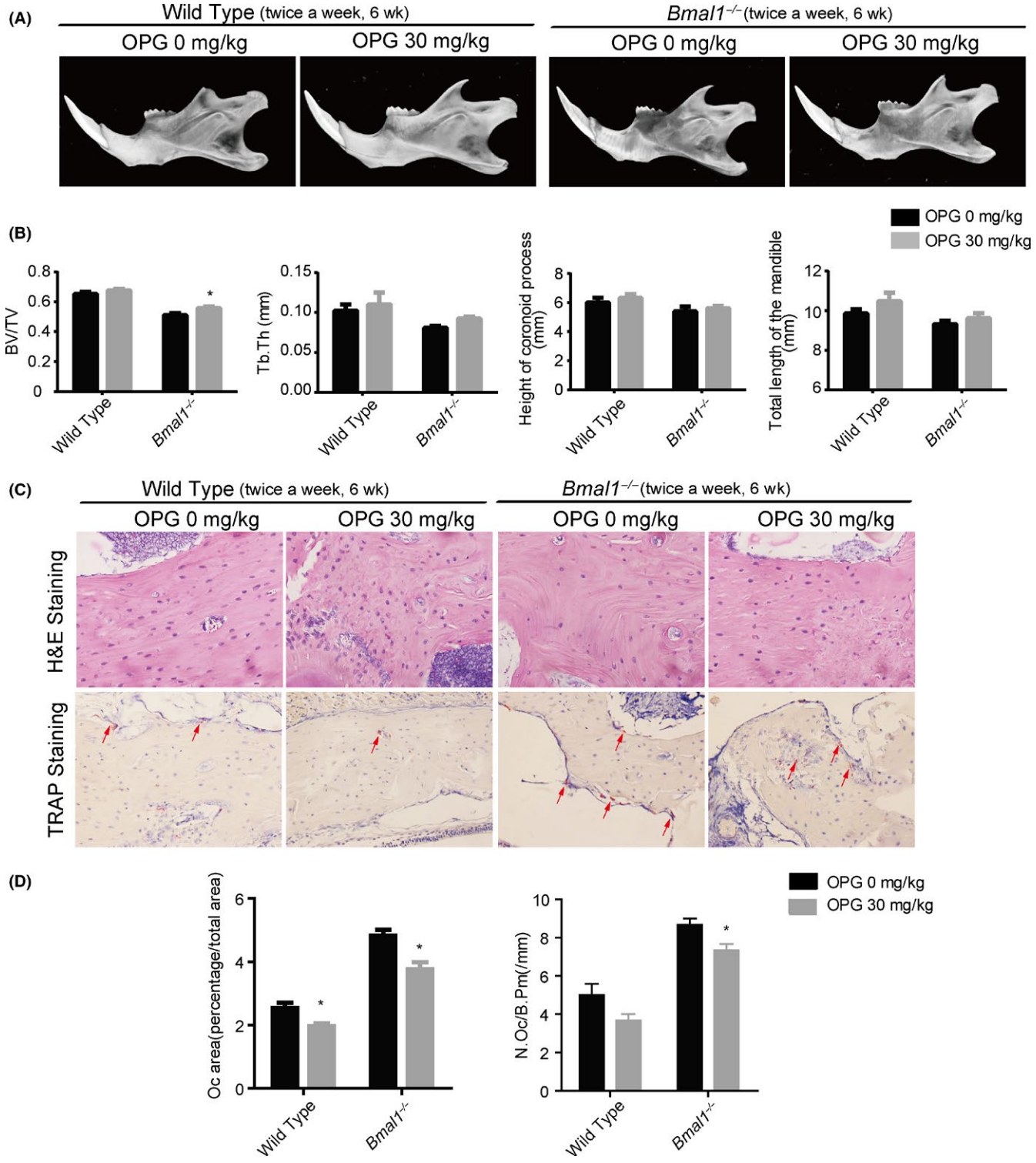


FIGURE 6 Supplemental OPG mitigates skeletal mandibular hypoplasia caused by BMAL1 knockout. (A) Representative images of micro-CT reconstructing the mandibles of wild type and *Bmal1*$^{-/-}$ mice treated with or without intraperitoneal injections of OPG (30 mg/kg, twice a week) for 6 weeks. (B) Micro-CT reconstruction analysis of mandibles of wild type and *Bmal1*$^{-/-}$ mice treated with or without intraperitoneal injections of OPG (30 mg/kg, twice a week) for 6 weeks(BV/TV, Tb.Th, total length of the mandible, and height of the coronoid process) (n = 5 per group). (C) Representative images of H&E staining (magnification, 400x) and TRAP staining (magnification, 320x) from wild-type or *Bmal1*$^{-/-}$ mice mandibles treated with or without intraperitoneal injections of OPG (30 mg/kg, twice a week) for 6 wk. (D) Oc area (percentage/total area) and osteoclast number (N.Oc/B.Pm) were measured (n = 5 per group). Oc area (percentage/total area) = area of osteoclast/total area, data represent the mean ± SD. *$P < .05$, from Student's *t* test

Huazhong University of Science and Technology, which was approved by the Institutional Research Ethics Committee of Tongji Medical College (Wuhan, China). This study was conducted in accordance with the Declaration of Helsinki. Get written informed consent before data collection. All procedures involved with animals were committed to abide by the ethical guidelines and get approved of the Institutional Animal Care and Use Committee of Tongji Medical College.

2.4 | Other methods

For other Material and methods, please see Data S1.

2.5 | Statistical analysis

All statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA). The two-tailed, unpaired Student's *t* test was used to analyse differences between groups. *P* values were considered significant at <.05.

3 | RESULTS

3.1 | Circadian dysrhythmia may be involved in the pathogenesis of skeletal mandibular hypoplasia

The circadian clock plays a key role in the regulation of bone development and bone homeostasis.[12] To determine the correlation between circadian clock and mandible development, we measured the expression levels of clock genes in human mandibular tissues and found that, in general, the protein levels of BMAL1, CLOCK, PER1, PER2 and CRY2 were changed obviously in SMH patients.[20] The core circadian molecular oscillator BMAL1 is essential for circadian pace making, which was lower in the mandibular tissues of SMH patients than in normal controls. OPG is a member of the tumour necrosis factor receptor superfamily that inhibits the formation and maturation of osteoclasts. We also found that OPG protein expression was concomitantly decreased in SMH patients (Figure 1A,B). These data suggest that the expression disruption of BMAL1 and OPG may be involved in SMH formation.

3.2 | Circadian dysrhythmia contributes to skeletal mandibular hypoplasia and OPG expression decreased correspondingly

Our data showed that the expression levels of clock genes were changed significantly in the mandibles of SMH patients, indicating that circadian dysrhythmia could be involved in the pathogenesis of SMH. To determine the role of circadian dysrhythmia in SMH formation, we constructed a jet lag mouse model in which mice were under shifting light-dark cycles of 6-h light advance every week for 4 or 8 weeks (Figure 2A). We examined the expressions of clock genes in mandibular specimens from jet lag group at several time points, and we confirmed that the circadian rhythm of jet lag mice was indeed disrupted (Figure S1). The mandibles of jet lag mice were significantly smaller than those of the normal group visually (Figure 2B). The heights of the mandibular ramus and the coronoid process were also reduced. Micro-computed tomography (CT) and three-dimensional (3D) reconstruction revealed that the bone volume/total volume (BV/TV) and trabecular thickness (Tb.Th) in the condylar regions of the jet lag mice were much lower compared with the control group (Figure 2C,D). Serial sections of mandible tissues were stained with haematoxylin and eosin (H&E). Staining revealed that the number of osteoblasts was decreased in the jet lag group. Tartrate-resistant acid phosphatase (TRAP) staining was performed to quantify osteoclast-like cells, which revealed that the osteoclasts were distributed more abundantly in jet lag group than control group (Figure 2E,F). Our findings showed that OPG protein expression was concomitantly reduced in the mandibles of SMH patients, suggesting that OPG may play a role in regulating mandible development. The expression of OPG was further assessed by immunohistochemistry and western blot, which revealed that OPG expression was significantly decreased in the mandibles of jet lag group compared to the control group (Figure 2E, G, H), implying a critical role for OPG in the growth and development of the mandible by responding to circadian clock outputs.

3.3 | Skeletal mandibular hypoplasia in *Bmal1*$^{-/-}$ mice with decreasing OPG expression

Since *Bmal1* is the core and irreplaceable component of the circadian molecular oscillator, we constructed BMAL1-knockout mice to assess the effects of the circadian rhythm on mandible development. The BMAL1 knockout genotype was identified by polymerase chain reaction (PCR) (Figure 3A). The mRNA levels of *Clock*, *Per1*, *Per2*, *Cry1* and *Cry2* were significantly decreased in the mandibles of *Bmal1*$^{-/-}$ mice, confirming the circadian rhythm disorder (Figure 3B).

Overall, we observed that the mandibles of *Bmal1*$^{-/-}$ mice were significantly smaller than those of the wild-type mice (Figure 3C). The height of the coronoid process and the total length of mandible were consistently decreased in *Bmal1*$^{-/-}$ mice (Figure 3E). Micro-CT and 3D reconstruction revealed that the BV/TV, Tb.Th and bone mineral density (BMD) were decreased in the condylar regions of *Bmal1*$^{-/-}$ mice compared with the age-matched wild-type mice (Figure 3D,E; Figure S2). H&E staining revealed that the number of osteoblasts was decreased in mandible sections of *Bmal1*$^{-/-}$ mice compared with wild-type mice. TRAP staining revealed an increased number of TRAP^{+} cells in *Bmal1*$^{-/-}$ mice comparatively. OPG immunohistochemistry revealed that the OPG protein level was decreased in the mandibles of *Bmal1*$^{-/-}$ mice relative to wild-type mice (Figure 3F,G). Next, we examined OPG expression in the mandibles of 3-, 4-, 5-, 6-, 7-, 8-, 9-, and 10-week-old wild-type and *Bmal1*$^{-/-}$ mice. The decreased OPG protein level was consistent in the mandibular tissues of *Bmal1*$^{-/-}$ mice throughout the entire growth period (Figure 3H).

3.4 | BMAL1 knockout leads to decreased OPG expression

To determine whether *Bmal1* regulates OPG expression, we knocked down BMAL1 in murine BMSCs (mBMSCs) and MC3T3-E1

cells, and found that OPG proteins were highly downregulated. In BMAL1-overexpressing mBMSCs and MC3T3-E1 cells, OPG proteins were clearly upregulated (Figure 4A,B). Immunofluorescence revealed that OPG expression was decreased in BMAL1-knockdown MC3T3-E1 cells (Figure 4C). Similarly, the levels of secreted OPG were reduced in the supernatant of BMAL1-knockdown mBMSCs and MC3T3-E1 cells (Figure 4D).

3.5 | OPG is involved in the pathway cascade of BMAL1 regulation on osteoclast differentiation

To investigate the effects of reduced OPG expression on osteoclast differentiation, we co-cultured RAW 264.7 cells with BMAL1-knockdown mBMSCs (or BMAL1-knockdown MC3T3-E1 cells), and found that osteoclast differentiation was enhanced compared to cells co-cultured with control mBMSCs (or control MC3T3-E1 cells) (Figure 5A,B). Additionally, the levels of OPG in the supernatant were lower in RAW 264.7 cells co-cultured with BMAL1-knockdown mBMSCs (or BMAL1-knockdown MC3T3-E1 cells) (Figure 5C). Next, we determined that the enhancement effect of osteoclast differentiation caused by BMAL1 knockdown was significantly reversed by exogenous supplementation with OPG (Figure 5A,B).

3.6 | BMAL1 upregulates OPG expression by directly binding to the *Opg* promoter

As a widely acknowledged transcription factor, BMAL1 exerts its function by activating the transcription of its downstream target genes.[22] To explore how BMAL1 regulates OPG expression, we predicted the BMAL1 binding site in the promoter of *Opg*, and chromatin immunoprecipitation (ChIP) was performed in MC3T3-E1 cells. The ChIP assay revealed that BMAL1 selectively bound to the promoter of *Opg* at putative E-box (CACGTG) site (Figure 5D). We then used luciferase assays to test whether BMAL1 could alter *Opg* transcriptional activity. The luciferase assay revealed that BMAL1 directly activated the *Opg* promoter, whereas the activation effect was abolished when the BMAL1-binding site was mutated or when BMAL1 was knocked down (Figure 5E).

3.7 | Supplemental OPG mitigates skeletal mandibular hypoplasia caused by BMAL1 knockout

Based on these observations, we further explored the prevention potential of OPG for SMH. 4-week-old male mice were given intraperitoneal injections of OPG (30 mg/kg, twice a week, 6 weeks). Micro-CT analyses of the mandibles showed that treatment with the OPG significantly increased BV/TV, Tb.Th and BMD (Figure 6A,B; Figure S3). In addition, the osteoclast number (N.Oc/B.Pm) was significantly suppressed by OPG administration (Figure 6C,D). Taken together, our results suggest that OPG may be a potential prevention approach for SMH caused by Bmal1 knockout.

4 | DISCUSSION

Skeletal mandibular hypoplasia (SMH) is a common type of developmental deformities, results in impaired aesthetics of facial profile, occlusal dysfunction and poor life quality. In addition to congenital factors such as genetic mutations and maternal pregnant abnormalities,[23] epigenetic factors such as chronic malnutrition[24,25] and vitamin D deficiency[26,27] can affect the normal and coordinated development of bone, causing jaw deformity. Currently, the human circadian rhythm has been greatly affected due to active or passive staying up at night, night light pollution, night shifts, etc.[28] Circadian dysfunction is closely associated with bone destruction. Indeed, previous studies have described the roles of circadian clock in developing femurs.[12,29] BMAL1 deficiency results in a low bone mass phenotype of femurs. An earlier closure and reduced thickness of growth plates in BMAL1-deficient mice compared with wild-type mice suggest alterations in endochondral bone formation.[12] Furthermore, the current study demonstrated that the circadian rhythm can affect the growth and development of the mandible. Circadian dysrhythmia significantly inhibits bone growth of the mandible. Based on mandibular morphological measurements, micro-CT analysis and histomorphometric examination, we found that circadian dysrhythmia can promote bone resorption, suggesting that circadian dysrhythmia is an important acquired factor that may cause jaw deformity. The mandibles of *Bmal1*$^{-/-}$ mice were significantly smaller than those of the wild-type mice, further confirming the effect of the circadian rhythm on mandible development.

In bone tissue, OPG competitively binds to RANKL, thereby blocking RANKL binding to RANK on osteoclast precursor cells, the only known receptor for RANKL, thus inhibiting osteoclast differentiation and maturation.[16] OPG-overexpressing mice exhibit osteopetrosis via decreased osteoclast differentiation, while OPG-knockout mice are osteoporotic with decreased total bone density and a high incidence of bone deformities and fractures.[17] In our study, BMAL1 and OPG expression was lower in SMH patients than in normal controls, and mandibular OPG expression was significantly decreased in jet lag mice. Furthermore, concomitant decreased OPG expression was present in the mandibular tissues of *Bmal1*$^{-/-}$ mice throughout the entire growth period, and osteoclast differentiation was significantly promoted in the mandibles of *Bmal1*$^{-/-}$ mice. These findings suggest that OPG is a key factor in BMAL1 deficiency-mediated SMH.

BMAL1 is the core component of the circadian molecular oscillator because it is essential for circadian behavior,[21] and the absence of BMAL1 will result in expression disorders of other clock genes in the circadian clock system. BMAL1 is a basic helix-loop-helix PAS domain transcription factor that exerts its function by binding to the E-box elements of CACGTG-type (or CACGTT-type-like) in the promoters of its downstream target genes.[30,31] For example, BMAL1 is a direct regulator of insulin-mediated osteoblast differentiation by increasing the promoter activity of *Bmp2* in MC3T3-E1 cells.[32] OPG is secreted by a variety of MSC-derived cells, such as osteoblasts and BMSCs,[33] which are regulated by various cytokines and

hormones.[34] Several potential mechanisms have been identified: PGE1 stimulates OPG synthesis via the p38 MAP kinase and SAPK/JNK signalling pathways in osteoblast-like MC3T3-E1 cells[35]; HIF-1α directly interacts with the *Opg*-binding site to stimulate transcription[36]; and glucocorticoids lower *Opg* mRNA levels by binding to the *Opg* promoter,[37] as well as indirectly by modulating Wnt/β-catenin signalling.[38] In this study, we searched potential E-box enhancer elements in *Opg*. Next, ChIP-qPCR confirmed the binding of BMAL1 to this E-box region (-1520/-1424, 97 bp) of *Opg*. Functional luciferase assays validated the BMAL1-mediated activation of this E-box sequence in cultured cells. To our knowledge, this is the first study to report that BMAL1 directly upregulates *Opg* transcription to inhibit osteoclast differentiation, highlighting the importance of the circadian rhythm in the bone-volume balance. Previous studies have reported that a change in sleep patterns can increase the risk of hip fractures in southern European men,[39] and that the risk of hip and wrist fractures is increased in postmenopausal women who experience at least three work shift changes per month.[40] Our results support that bone metabolism is directly connected to changes in the circadian rhythm. Thus, circadian rhythm dysfunction is a direct environmental factor that can cause SMH.

Due to its complex aetiology, most current studies mainly focus on the therapeutic approaches of orthodontic treatment or orthognathic surgery for SMH. However, few studies have examined the prevention of SMH. OPG is reportedly a potent inhibitor of bone resorption in human subjects. Supplemental OPG prevented the development of osteoporosis.[19] Treatment with OPG-Fc reduced the formation of evident osteolytic lesions radiographically in tumour-bearing animals.[41] In our in vivo experiment, exposure to recombinant OPG led to increased bone density in normal mice, due to a decrease in osteoclast numbers. Intraperitoneal injection of OPG can partially rescue bone loss in *Bmal1*$^{-/-}$ mice. Our results suggest that supplemental OPG may mitigate jaw deformity formation caused by circadian rhythm dysfunction to a certain extent.

Collectively, our data demonstrate that the circadian rhythm is closely associated with mandible development, and that circadian dysfunction can induce SMH formation by decreasing OPG expression. BMAL1 directly interacts with the *Opg*-binding site to stimulate *Opg* transcription and suppresses osteoclast differentiation. The results of our study will enhance our understanding of the aetiology of SMH, thereby providing us with a better understanding of the molecular targets for prevention and therapeutic approaches. OPG may serve as an important target molecule for the prevention of SMH caused by circadian dysrhythmia.

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AUTHOR CONTRIBUTIONS

L. L. Chen, contributed to conception, design, study supervision, data analysis, and interpretation, drafted and critically revised the manuscript; X. Zhou, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; R. Yu, Y. L. Long, contributed to data acquisition and analysis, critically revised the manuscript; J. J. Zhao, S. L. Yu, contributed to data acquisition, critically revised the manuscript; Q. M. Tang, contributed to data analysis, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of work.

CONFLICT OF INTEREST

The authors have no competing interests.

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SUPPORTING INFORMATION

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

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