LncRNA KCNQ1OT1 accelerates fracture healing via modulating miR-701-3p/FGFR3 axis

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Abstract
Emerging evidence highlights the role of the long noncoding RNA (lncRNA) KCNQ1OT1 in fracture healing. Osteoblast proliferation, migration, and survival are pivotal during this process. In this study, we aimed to improve our understanding of the regulatory role of lncRNA KCNQ1OT1 during osteoblast proliferation, migration, and survival. We searched the gene expression omnibus databases and LncBase Experimental V.2 to identify key microRNAs (miRNAs) targets of KCNQ1OT1. MiR-701-3p was selected as a differentially expressed miRNA and RNA immunoprecipitation assays were performed to verify its interaction with KCNQ1OT1. Fibroblast growth factor receptor 3 (FGFR3) was also identified as a target of miR-701-3p. We further identified KCNQ1OT1 as a competing endogenous RNA of miR-701-3p that could influence osteoblast proliferation, migration, and apoptosis in vitro and in vivo. Taken together, our results indicate that the KCNQ1OT1/miR-701-3p/FGFR3 axis is an important regulator of osteoblast proliferation, migration, and apoptosis, and provide a new therapeutic avenue for fracture healing.

Keywords
apoptosis, FGFR3, lncRNA, migration, miRNA, osteoblast proliferation

Abbreviations: BMD, bone mineral density; BSA, bovine serum albumin; BV, bone volume; ceRNA, competing endogenous RNA; FGFR3, fibroblast growth factor receptor 3; FISH, fluorescence in situ hybridization; GEO, gene expression omnibus; H&E, hematoxylin & eosin; lncRNA, long noncoding RNA; miRNAs, microRNAs; PCNA, proliferating cell nuclear antigen; PTGER4, prostaglandin E receptor 4; qRT-PCR, quantitative real-time PCR; RIP, radioimmunoprecipitation; SD, standard deviation; SDC4, syndecan 4; TV, trabecula volume.

Lang Chen, Yuan Xiong and Chenchen Yan are contributed equally to this work.
1 | INTRODUCTION

Fracture healing, which is a natural phenomenon that occurs in response to traumatic bone injury, comprises anabolic and prolonged catabolic phases.1,2 Fracture repair is a complex process due to the multi-stage integration of genetic, cellular, and physiological factors.3,4 The control of osteoblast proliferation and apoptosis are key to bone remodeling and fracture healing.5 MicroRNAs (miRNAs) are evolutionally conserved, single-stranded RNAs approximately 23 nucleotides (nts) in length that regulate posttranscriptional gene expression.6,7 Each miRNA targets an array of transcripts directly or indirectly, producing a complex network of gene expression regulation. Suppressive miRNAs have been shown to exert regulatory functions during osteoblast proliferation and differentiation through their ability to target osteogenic genes in vitro.8

Long noncoding RNAs (lncRNAs) are a class of transcribed RNAs (≥200 nts) that regulate posttranscriptional gene expression, nuclear organization, epigenetic mechanisms, intercellular signaling, and other biological processes.9-12 Accumulating evidence suggests that lncRNAs act as competing endogenous RNA (ceRNA) to sponge miRNAs.13 For example, lncRNA FOXD3-AS1 was recently shown to function as a ceRNA that sponges miR-135a-5p to promote colon adenocarcinoma.14 KCNQ1OT1 is a well-studied lncRNA that has profound effects on tumorigenesis, colon cancer development, non-small-cell lung carcinoma, cerebral ischemic stroke, and the regulation of osteogenic differentiation.15-18 Recently, the upregulation of lncRNA KCNQ1OT1 via the Wnt/β-catenin signaling pathway was shown to accelerate osteoblast differentiation.19 KCNQ1OT1 silencing inhibits osteogenic differentiation and downregulates BMP2.20 However, the relationship between KCNQ1OT1 and the proliferation, migration, and survival of osteoblastic cells remains poorly defined.

In this study, we initially investigated a direct role of KCNQ1OT1 in the modulation of MC3T3-E1 cell proliferation, migration, and apoptosis in vitro and in vivo. We further explored the underlying relationship between KCNQ1OT1 and miR-701-3p by RNA radioimmunoprecipitation (RIP) and pull-down assays, the results of which indicated that KCNQ1OT1 exert its regulatory effects by acting as a sponge of miR-701-3p.21 Through luciferase reporter assays, Fibroblast growth factor receptor 3 (FGFR3) was verified as a target gene of miR-701-3p, the downregulation of which enhanced FGFR3 expression. FGFR3 overexpression significantly enhanced osteoblast proliferation and migration, and inhibited apoptosis.

In summary, we report that KCNQ1OT1 promotes MC3T3-E1 cell proliferation and migration and prevents apoptosis through its regulation of the miR-701-3p/FGFR3 axis. This provides novel therapeutic avenues for fracture healing.

2 | MATERIALS AND METHODS

2.1 | Animal fracture model

Male C57BL/6J mice (n = 60; aged 6 weeks) were purchased from the Center of Experimental Animal, Tongji Medical College, Huazhong University of Science and Technology (China). Chloral hydrate (450 mg/kg body weight, 6%) was used for anesthesia. All animal studies were performed according to the guidelines of the Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology. After disinfection with 0.5% of povidone iodine solution, and a posterolateral incision and blunt separation were performed to establish the mouse right femoral fracture model on a sterile bench. A diamond disk was used to cut the femur in the mid-diaphysis region and the fracture was stabilized with 0.6 mm of intramedullary needles.21 Half of the mice were sacrificed on postfracture day 14 to obtain bone calciuses for further analysis. The remaining mice were sacrificed for subsequent trials.

2.2 | Treatment of fracture model mice

In control mice, PBS (100 µL) was administered at the fracture site, while other mice received 100 µL 200 μM of small siRNA-Negative Control (siRNA-NC), silncRNA KCNQ1OT1, agomiR-701-3p or antagonir-701-3p on postoperative day 0, day 4, and day 7.

2.3 | Imaging of small animals in vivo

On postfracture days 7, 14, and 21, anesthetized mice were placed in the lateral position (right side down) and were scanned with a 30 s exposure time on an in vivo FX PRO imaging system (BRUKER, Karlsruhe, Germany).
2.5 | **Micro-CT analysis**

On postoperative days 7, 14, and 21, five of the mice in each group were sacrificed and to obtain fractured femurs without soft tissue. The fracture site was scanned using the BRUKER SkyScan 1176 scanner mCT system with the following settings: 2,400 views, five frames/view, 37 kV, and 121 mA. Reconstructed (3D) images were generated using CT-Vox 2.1 version (BRUKER Minimal Intensity Projection Software, Karlsruhe, Germany). Parameters including bone volume (BV), trabecula volume (TV), BV/TV, and bone mineral density (BMD) were measured and analyzed with CTAN 1.12 version (BRUKER Minimal Intensity Projection Software, Karlsruhe, Germany). Bone calluses were also collected for subsequent PCR and western blot analyses.

2.6 | **Histologic and immunohistochemistry analyses**

After micro-CT analysis, preserved calluses were fixed in 4% of paraformaldehyde overnight. Sections (5 μm thick) of paraffin-embedded callus tissues before hematoxylin & eosin (H&E), Alcian blue, and proliferating cell nuclear antigen (PCNA) staining was performed. For immunohistochemistry, callus sections were sequentially deparaffinized, rehydrated, treated with Antigen Retrieval Solution (pH 6.0 EDTA citrate buffer; Servicebio, G1202, China), and blocked with bovine serum albumin (BSA) (Servicebio, G5001, China). Sections were then incubated with the primary detection antibody (anti-PCNA, ab29, Abcam, USA) overnight at 4°C. After 50 minutes incubation with secondary goat anti-mouse immunoglobulin, sections were mounted. Sections were then imaged with an Olympus BX51 microscope and a DP73 CCD Olympus Imaging System (Olympus Corporation, Tokyo).

2.7 | **Cell culture and transfection**

The mouse osteoblastic cell line, MC3T3-E1, was donated from the Orthopedic Laboratory, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (China), and cultured in α-MEM (Hyclone, Utah, USA, #SH30265.01B) with 10% of fetal bovine serum (Gibco, Grand Island, USA, #10099141) plus 1% of penicillin and streptomycin (Hyclone, Utah, USA, #SV30010) at 37°C under 5% of CO₂.

AgomiR-701-3p, agomiR-Negative Control (agomiR-NC), antagomiR-701-3p, antagomiR-Negative Control (antagomiR-NC), siRNA-Negative Control (si-NC), and siRNA KCNQ1OT1 (KCNQ1OT1 WT, and NC) were synthesized at 200 μM by GenePharma (Shanghai, China), and transfected into MC3T3 cells using Lipofectamine 3000 (ThermoFisher Scientific, MA, USA, #L3000001). All procedures were performed according to the manufacturer's recommendations. Transfected cells were harvested after incubation for a further 48 hours for RNA and protein analyses.

2.8 | **Quantitative real-time PCR (qRT-PCR)**

Total RNA was isolated from cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Purified RNA was reverse-transcribed into cDNA using qPCR-RT Master Mix (Toyobo, Osaka, Japan). GAPDH and U6 genes were used as internal controls. Relative miRNA expression levels were normalized to internal controls and calculated according to the $2^{-\Delta\Delta CT}$ method.

2.9 | **Western blot analysis**

Cells were washed in PBS and lysed in radio immunoprecipitation assay buffer (Beyotime) to extract total proteins. Cell lysates were subjected to 10% of SDS-PAGE, and proteins were transferred onto PVDF membranes (Millipore, Schwalbach, Germany). Membranes were probed with the following primary detection antibodies: anti-Cyclin D1 (1:1,000, Abcam, MA, USA, # ab40754), anti-Cyclin D3 (1:1,000, Abcam, MA, USA, #2936), anti-FGFR3 (1:1,000, Abcam, MA, USA, #ab133644.), and anti-GAPDH (1:10,000, Abcam, MA, USA, #ab37168). After incubation with the appropriate secondary antibody and protein bands were visualized via chemiluminescence (Canon, Tokyo, Japan, #LiDE110).

2.10 | **Luciferase reporter assay**

MC3T3-E1 cells were co-transfected with dual-luciferase reporter constructs (FGFR3 WT, FGFR3 Mut, lncRNA KCNQ1OT1 WT, and lncRNA KCNQ1OT1 Mut) and miR-701-3p mimics or negative controls (NC). Luciferase activity was measured 24 hours post-transfection using the dual-luciferase reporter assay system (Promega, Wisconsin, USA) according to the manufacturer's instructions.

2.11 | **CCK-8 assay**

Cells in the logarithmic growth phase were seeded into 96-well plates (1 × 10³ cells/well) and 10 μL of CCK-8 solution was added to each well. Plates were incubated at 37°C for 4 hours before absorbance at 450 nm was measured using a microplate reader (Synergy 4; BioTek, Winooski, VT, USA) to indirectly identify the number of viable cells.
FIGURE 1  KCNQ1OT1 promotes osteoblast proliferation and migration, and inhibits apoptosis. MC3T3-E1 cells were transfected with lipofectamine 3000 only, siRNA-NC or siRNA KCNQ1OT1, respectively, for 48 hours. A, qRT-PCR analysis. Level of lncRNA KCNQ1OT1 decreased after transfected with siRNA KCNQ1OT1 (mean ± SD, ***$P$ < .001). B, An Edu assay. The proliferation rate of MC3T3-E1 cell was decreased by siRNA KCNQ1OT1 (scale bar = 100 μm). C, CCK-8 assay. KCNQ1OT1 knockdown significantly suppressed cell proliferation. D, Cell cycle analysis. Compared to control groups, fewer cells progressed to the S phase in siRNA KCNQ1OT1 groups (mean ± SD, ***$P$ < .001). E, qRT-PCR analysis of cyclin D1 and cyclin D3. F, Apoptosis analyses. After transfection, MC3T3-E1 cells were harvested and stained with annexin V-FITC and PI, the quantitative outcome revealed higher rates of apoptosis in following KCNQ1OT1 silencing (mean ± SD, ****$P$ < .0001). G, Western Blotting of Bcl-2 and Bax. H, Transwell assay. The influence on cell migration ability of siRNA KCNQ1OT1 was assessed by transwell migration (scale bar = 100 μm)
2.12 | EdU assay

Cell proliferation was analyzed using Cell-Light EdU Apollo 567 in vitro kits (RiboBio Corporate, Guangzhou, China). Transfected cells were cultured in 96-well plates (1 × 10⁴/well). After 48 hours, 50 μM of EdU labeling media was added and cells were incubated for 2 hours. Cells were then fixed in 4% of paraformaldehyde (PFA), permeabilized in 0.5% of TritonX-100/PBS, and stained with Apollo staining solution and Hoechst33342. Images of cells were obtained under an inverted fluorescence microscope IX73-AIZFL/PH (OLYMPUS Corporation, Japan).
2.13 | Flow cytometry

Cells were seeded in 6-well plates (1 × 10^6 cells/well) and trans plated for 48 hours. The cell cycle status was analyzed using cell cycle detection kits (KeyGEN BioTECH, Jiangsu, China). Cells were detached and fixed in 70% of ice-cold ethanol overnight at 4°C. Cells were washed with PBS and labeled with 500 μL of reaction solution (RNAse: propidium iodide = 1:9) for 30 minutes. Subsequently, cells were analyzed by flow cytometry (Accuri C6, BD Biosciences) using ModFit LT software.

2.14 | RNA immunoprecipitation (RIP) assay

RIP assays were performed using RIP RNA-binding protein immunoprecipitation kits (Millipore, Billerica, MA, USA, #17-701) according to the manufacturer's instructions. Briefly, MC3T3-E1 cells were collected and lysed in RIP lysis buffer (Solarbio, Beijing, China, #N8031). Antibodies to AGO2 and IgG were purchased from Abcam (MA, USA, #ab5072). The quality of the purified RNA was assessed by qRT-PCR analysis.

2.15 | RNA pull-down assays

Purified RNAs were labeled and transcribed with Biotin RNA Labeling Mix (Roche, Basel, Switzerland, #11685597910) and T7 RNA polymerase (Ambion Life, TX, USA). The resulting RNAs were further purified using an RNeasy Plus Mini Kit (Qiagen, Munich, Germany, #74134) and DNase I (Qiagen, Munich, Germany, #19101). RNAs were mixed and incubated with AGS lysates. Magnetic beads were added to each binding reaction at room temperature. RNA complexes bound to these beads were eluted and extracted for qRT-PCR analysis.

2.16 | Fluorescence in situ hybridization (FISH)

Cy3-labeled-KCNQ1OT1 (GenePharma, Shanghai, China) and DAPI-labeled U6 probes (RiboBio, Guangzhou, China) were designed and synthesized. RNA-FISH assays were performed using FISH kits (ThermoFisher Scientific, MA, USA, #F32954) according to the manufacturer's protocol.

2.17 | Transwell migration assay

Cells (1.5 × 10^4 cells/well, three replicates per group) were resuspended in low serum (5% of FBS) medium and seeded into the upper chamber of 24-well Transwell plates (Corning, Corning, NY, USA) containing 8 μm of pore filters. The lower chambers were filled with complete medium (containing 10% of FBS) supplemented with test compounds. After 12 hours, cells on the upper surface of the filter membranes were washed and cells that had migrated to the lower surface were stained with 0.5% of crystal violet. Migration was observed under an optical microscope (Leica DMI6000B, Germany).

2.18 | Statistical analysis

Data are expressed as the mean ± standard deviation (SD), and analyzed with GraphPad Prism 7.0 (GraphPad Software, Inc, La Jolla, CA), unless otherwise stated. Statistical significance of differences between groups was assessed with an ANOVA or Student’s t test. *P < .05 was considered to indicate statistical
3 RESULTS

3.1 KCNQ1OT1 promotes osteoblast proliferation, and migration and inhibits apoptosis

To investigate the effects of KCNQ1OT1, MC3T3-E1 cells were transfected with siRNA-NC or siilncRNA KCNQ1OT1 for 48 hours and silencing was confirmed by qRT-PCR analysis (Figure 1A). EdU assay and CCK-8 assays demonstrated that KCNQ1OT1 knockdown significantly suppressed cell proliferation compared to NC cells (Figure 1B-C). Moreover, cell cycle analysis suggested that IncRNA KCNQ1OT1 silencing induced cell cycle arrest at the G1 phase in MC3T3-E1 cells (Figure 1D). The levels of cyclin D1 and cyclin D3 were lower in the silncRNA-KCNQ1OT1 groups than those in control cells (Figure 1E). In addition, staining with annexin V-FITC and PI revealed higher rates of apoptosis in the silncRNA-KCNQ1OT1 groups compared to those in the control cells (Figure 1F). The pro-apoptotic significance. Schematic diagram were prepared using Pathway Builder Tool 2.0 and Adobe Photoshop CC 2017.
signaling protein Bax and the antiapoptotic signaling protein Bcl-2 are downstream of P53 and play a significant role in regulating cell apoptosis. Bcl-2 levels were lower in silcRNA- KCNQ1OT1 groups compared to those in the controls, while Bax expression was increased (Figure 1G). Transwell migration assays indicated that KCNQ1OT1 knockdown significantly attenuated the MC3T3-E1 cell motility (Figure 1H). These results revealed that KCNQ1OT1
promotes MC3T3-E1 cell proliferation and migration, and inhibits apoptosis.

3.2 | KCNQ1OT1 in vivo assessments

To directly investigate the effects of KCNQ1OT1 on in vivo fracture healing, we constructed a mouse femur fracture model. KCNQ1OT1 levels during fracture healing were assessed by qRT-PCR. The relative levels of KCNQ1OT1 peaked on postfracture day 7 and gradually decreased thereafter (Figure 2A). Mice received PBS (control), siRNA-NC or silncRNA-KCNQ1OT1 at the fracture sites on days 0, 4, and 7. The effects of fracture healing progression were assessed visually by small animal imaging and micro-CT. Fracture healing was evidently slower in silncRNA-KCNQ1OT1 groups compared to that in the control and siRNA-NC groups (Figure 2B). The fracture line and osteotomy gap remained visible in the silncRNA-lncRNA KCNQ1OT1 groups on postfracture day 21. BV, TV, BV/TV, and BMD were calculated via 3D visualization and analysis software. A smaller callus volume and larger fracture gap were observed in the silncRNA-KCNQ1OT1 group compared to those in the control and siRNA-NC groups (Figure 2C). The BV, TV, and BV/TV of the callus and the relative BMD were similar among these three groups on postfracture day 7; however, the values of these parameters were lower in the silncRNA-KCNQ1OT1 groups on postfracture day 21 than those in the control groups (Figure 2D). Bone and callus samples were collected for the histologic and immunohistochemistry analyses. Quantification of the PCNA staining results showed that there were fewer PCNA-positive nuclei in the silncRNA KCNQ1OT1-treated group than in the control or siRNA-NC groups on postfracture day 7; however, the values of these parameters were lower in the silncRNA-KCNQ1OT1 groups on postfracture day 21 than those in the control groups (Figure 2E). H&E and Alcian blue staining showed that the groups were similar in terms of cartilage matrix content and bone area on day 7. However, although gradual formation of new bone formation was observed in the mice in the control and siRNA-NC groups on day 14 and day 21, cartilaginous callus was still clearly present in the mice in the silncRNA KCNQ1OT1 on day 21; these findings were confirmed by quantification of the results (Figure 2F). Taken together, these results indicated that KCNQ1OT1 promotes fracture healing in vivo.

3.3 | KCNQ1OT1 acts as a sponge for miR-701-3p

To explore the downstream mechanisms of KCNQ1OT1 action, the Gene expression omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) was used to screen potential miRNA targets. Microarray data (GSE76197) were obtained from the GEO database. GSE76197 is a free microarray data that evaluate the miRNAs expression profiles of mice femoral fracture callus on day 0, day 3, day 5, day 7, day 10, and day 14. The down-regulated, differentially expressed miRNAs were identified by \( P \leq 0.01 \) and \( \log_{2}FC \leq -2 \). Compared to intact control cells, miR-701-3p was selected as the most differentially expressed miRNA and its association with lncRNA KCNQ1OT1 was verified using LncBase Experimental V.2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Find_experimental) (Figure 3A). The subcellular localization of lncRNAs is closely related to function and activity. RNA-FISH assays showed that both KCNQ1OT1 and miR-701-3p were predominantly cytoplasmic on MC3T3-E1 cells (Figure 3B). FISH assays were also used to assess the localization of KCNQ1OT1 and miR-701-3p in the bone or calluses of animal models on day 0, 7, and 14 postfracture. Both KCNQ1OT1 and miR-701-3p were localized in the cytoplasm of the bone tissues and calluses (Figure 3C). Luciferase reporter assays with either wild-type (WT) KCNQ1OT1 or 3′-UTR mutant (Mut) KCNQ1OT1 were constructed to verify the role of miR-701-3p. As shown in Figure 3D, luciferase activity in the agomiR-NC + Luc-UTR-WT group was significantly higher than that in the agomiR-701-3p + Luc-UTR-WT group (\( P < 0.001 \)). To further explore the ability of lncRNA KCNQ1OT1 to sponge miR-701-3p, miR-701-3p levels were assessed after transfection with lncRNA KCNQ1OT1, siRNA-NC, or silncRNA-lncRNA KCNQ1OT1 (Figure 3E). Furthermore, the interaction between KCNQ1OT1 and miR-701-3p was assessed by transwell migration (scale bar = 100 μm).
demonstrated via RNA RIP assays, which revealed that KCNQ1OT1 increased Ago2-containing miRNA ribonucleoprotein complex enrichment compared to control IgG (Figure 3F). In accordance with these findings, pull-down assays showed that KCNQ1OT1 bound to biomiR-701-3p-WT (Figure 3G), but not to the mutant version. Taken together, these data demonstrated that KCNQ1OT1 acts as a sponge of miR-701-3p.
To investigate how miR-701-3p affects fracture healing, we assessed the relative levels of miR-701-3p using fracture gene chips (Figure 5A). Analysis of calluses from the fracture sites harvested from the murine fracture model revealed that miR-701-3p levels decreased during the early fracture healing stages (days 1-14) (Figure 5B). In addition, we administered PBS (control), Cy5-labeled agomiR-701-3p, and antagoniR-701-3p at the right femoral fracture site on days 0, 4, and 7 post-injury. Small animal imaging showed that Cy5-labeled agomiR-701-3p was absorbed at the fracture site (BRUKER in Vivo FX PRO) (Figure 5C). Mice were sacrificed and bone callus were collected to evaluate agomiR-701-3p absorption. qRT-PCR analysis confirmed that the relative miRNA-701-3p levels were significantly higher on days 7 ($P < .001$) and 14 ($P < .01$) after local administration of agomiR-701-3p (Figure 5D). X-ray imaging showed that fracture healing was evidently slower in the agomiR-701-3p groups compared to that in the controls (Figure 5E), with a clearly visible fracture line remaining on day 21 in the agomiR-701-3p group. Micro-CT scans were performed on postfracture days 7, 14 and 21 and BV, TV, BV/TV, and BMD were calculated via 3D visualization and analysis software. A smaller callus volume and larger fracture gap were observed in the agomiR-701-3p group (Figure 5F). Compared to the control groups, the BV/TV of calluses and BMD were lower in agomiR-701-3p groups on postfracture days 14 and 21 (Figure 5G). PCNA-positive nuclei were more abundant in the tissue sections of the antagoniR-701-3p groups (Figure 5I). n = 5 mice, per group (scale bar = 100 μm/500 μm; mean ± SD, ns for not significant, *$P < .05$, **$P < .01$, ***$P < .001$).}

### 3.5 AgomiR701-3p inhibits fracture healing

To investigate how miR-701-3p affects fracture healing, we assessed the relative levels of miR-701-3p using fracture gene chips (Figure 5A). Analysis of calluses from the fracture sites harvested from the murine fracture model revealed that miR-701-3p levels decreased during the early fracture healing stages (days 1-14) (Figure 5B). In addition, we administered PBS (control), Cy5-labeled agomiR-701-3p, and antagoniR-701-3p at the right femoral fracture site on days 0, 4, and 7 post-injury. Small animal imaging showed that Cy5-labeled agomiR-701-3p was absorbed at the fracture site (BRUKER in Vivo FX PRO) (Figure 5C). Mice were sacrificed and bone callus were collected to evaluate agomiR-701-3p absorption. qRT-PCR analysis confirmed that the relative miRNA-701-3p levels were significantly higher on days 7 ($P < .001$) and 14 ($P < .01$) after local administration of agomiR-701-3p (Figure 5D). X-ray imaging showed that fracture healing was evidently slower in the agomiR-701-3p groups compared to that in the controls (Figure 5E), with a clearly visible fracture line remaining on day 21 in the agomiR-701-3p group. Micro-CT scans were performed on postfracture days 7, 14 and 21 and BV, TV, BV/TV, and BMD were calculated via 3D visualization and analysis software. A smaller callus volume and larger fracture gap were observed in the agomiR-701-3p group (Figure 5F). Compared to the control groups, the BV/TV of calluses and BMD were lower in agomiR-701-3p groups on postfracture days 14 and 21 (Figure 5G). PCNA-positive nuclei were more abundant in the tissue sections of the antagoniR-701-3p groups (Figure 5H). Furthermore, H&E and Alcian blue staining of the antagoniR-701-3p group showed more new bone and less cartilage matrix content on postfracture day 14 and day 21 compared with the other groups (Figure 5I). n = 5 mice, per group (scale bar = 100 μm/500 μm; mean ± SD, ns for not significant, *$P < .05$, **$P < .01$, ***$P < .001$).
MiR-701-3p regulates proliferation, migration, and apoptosis by targeting FGFR3

To identify the targets of miR-701-3p, we synthesized the relative outcome from miRDB, fracture-related genes, and TargetScan7.1. Three target genes were identified: prostaglandin E receptor 4 (PTGER4), syndecan 4 (SDC4), and FGFR3 (Figure 6A). Following a literature review, FGFR3 was selected as the target gene of miR-701-3p due to its related osteogenic characteristics. Subsequently, luciferase reporter assays with either WT FGFR3 3'-UTR or a mutant-type (Mut) FGFR3 3'-UTR were constructed to investigate the ability of miR-701-3p to target FGFR3 directly. As shown in Figure 6B, luciferase activity of both the Luc-UTR-WT and Luc-UTR-Mut decreased after transfection with agomiR-701-3p. The luciferase activity
of agomiR-701-3p + Luc-UTR-WT was also significantly lower in agomiR-NC + Luc-UTR-WT groups (P < .001). Bone calluses were then collected to analyze FGFR3 expression during fracture healing. In contrast to the relative miR-701-3p levels, FGFR3 expression peaked on post-fracture day 7 and gradually decreased on subsequent days (Figure 6C). MC3T3-E1 cells were cultivated for a further 48 hours after transfection with control, agomiR-NC, agomiR-701-3p, agomiR-NC, or agomiR-701-3p in vitro. qRT-PCR analysis revealed lower relative FGFR3 mRNA levels in the agomiR-701-3p group compared to those in the other groups (Figure 6D). To investigate the potential for reversal of these effects, MC3T3-E1 cells were transfected with control, siRNA-NC, siRNA-FGFR3, antagomiR-701-3p + siRNA-NC, or antagomiR-701-3p + siRNA-FGFR3. qRT-PCR analysis showed that transfection with antagomiR-701-3p had positive effects on FGFR3 expression in MC3T3-E1 cells transfected with siRNA-NC or siRNA-FGFR3 (Figure 6E).

The associations between FGFR3 and cell proliferation, migration, and apoptosis were then evaluated in CCK-8 assays, EdU staining, cell cycle assays, and qRT-PCR analysis. FGFR3 silencing negatively impacted cell proliferation in comparison with the effects on the other groups and this effect was rescued by transfection with antagomiR-701-3p (Figure 6F, H-J). Compared with the controls, the relative antiapoptotic signal protein Bcl-2 gene expression levels in the siRNA-FGFR3 groups decreased, while the apoptosis rates and pro-apoptotic signal protein Bax expression increased; these effects were also rescued by antagomiR-701-3p transfection (Figure 6G, K). Similarly, Transwell assays showed the migration rate of MC3T3-E1 cells transfected with siRNA-FGFR3 was slower than that in the control groups, and this effect was also rescued by antagomiR-701-3p transfection (Figure 6L). These results revealed that miR-701-3p regulates MC3T3-E1 cell proliferation, migration, and apoptosis by targeting FGFR3.

**FIGURE 7** A Diagrammatic sketch of lncRNA KCNQ1OT1/miR-701-3p/FGFR3 axis mediated osteoblast proliferation, migration, and survival. LncRNA KCNQ1OT1 acts as a ceRNA competitively inhibiting the expression of miR-701-3p, whereas elevates FGFR3 mRNA level, and thereby promotes proliferation, migration, and survival of osteoblast MC3T3-E1 cells.
the lncRNA KCNQ1OT1 accelerates osteoblast differentiation via the Wnt/β-catenin pathway. However, the effects of KCNQ1OT1 on the proliferation, migration, and apoptosis of osteoblasts have not been described. Based on the increased expression of KCNQ1OT1 during the fracture healing process, we hypothesized a role for this lncRNAs in the regulation of fracture healing. LncRNAs play significant roles in modulating mRNA stability, functioning as miRNA sponges that compete for endogenous RNAs. To further explore whether specific miRNAs are sponged by KCNQ1OT1, we identified miRNAs that were differentially expressed between intact control bone and postfracture calluses in a mouse model of fracture healing. Based on searches of the GEO database, we selected miR-701-3p. Robust evidence of the association between lncRNA KCNQ1OT1 and miR-701-3p was shown using LncBase Experimental V.2.

To further investigate the molecular mechanisms by which miR-701-3p regulates the proliferation, migration, and apoptosis of MC3T3-E1 cells, Target Scan Mouse 7.2 and other databases were utilized to predict target miR-701-3p genes. PTGER4, SDC4, and FGFR3 were identified. FGFR3 was first linked to bone development in 1994 when Shiang et al. reported a mutation in FGFR3 in patients with achondroplasia. In recent years, FGFR3 has been found to function in many biological fields. Mao et al. showed that the circular RNA hsa_circ_0068871 targets miR-181a-5p to promote bladder cancer progression by regulating FGFR3 expression and STAT3 activation. Moreover, the PRMT5/miR-99 family/FGFR3 axis was shown to have a profound association with lung cancer progression and PRMT5 was consequently suggested as a prognostic biomarker and therapeutic target.

In this study, luciferase reporter assays performed with WT FGFR3 3′-UTR or Mut FGFR3 3′ UTR fused to luciferase reporters showed that agomiR-701-3p attenuated WT FGFR3 3′ UTR reporter activity. These results provided evidence of the association between miR-701-3p and FGFR3. We found that KCNQ1OT1 promoted MC3T3-E1 cell proliferation, migration, and inhibited apoptosis. Subsequently, the effects of miR-701-3p on MC3T3-E1 cell proliferation, migration, and apoptosis were confirmed. FISH assays showed that KCNQ1OT1 and miR-701-3p were both predominantly localized in the cytoplasm. RIP and pull-down assays further demonstrated an interactive relationship between lncRNA KCNQ1OT1 and miR-701-3p. Luciferase reporter assays confirmed that miR-701-3p, FGFR3 and KCNQ1OT1 interact to regulate the proliferation, migration, and apoptosis of MC3T3-E1 cells. To verify these findings in vivo, femoral fracture models were assessed through small animal imaging in vivo and micro-CT. The in vivo data were consistent with our in vitro findings.

However, several limitations of this study should be noted. We did not investigate the concentration-dependent effect of local administration of KCNQ1OT1 and miR-701-3p and the most suitable concentrations require further assessment. Moreover, clinical trials of the effects of KCNQ1OT1 on fracture healing have not been assessed.

In summary, we have identified KCNQ1OT1 as a sponge for miR-701-3p that promotes MC3T3-E1 cell proliferation, migration, and survival through FGFR3 activation (Figure 7). This represents a novel potential therapeutic agent for fracture healing.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

G. Liu, B. Mi, and L. Chen designed the study; L. Chen, Y. Xiong, and C. Yan were responsible for the animal experiments; L. Chen, C. Yan, and J. Liu were responsible for cell cultures and relative experiments; W. Zhou, H. Xue, and Y. Hu supervised the project; L. Hu and Z. Lin collated the data and carried out data analyses; L. Chen and Y. Xiong wrote the manuscript; B. Mi, X. Leng, and Yori Endo contributed to revise the manuscript.

REFERENCES

1. Shi Z, Zhou H, Pan B, et al. Effectiveness of teriparatide on fracture healing: a systematic review and meta-analysis. PLoS ONE. 2016;11:e0168691.
2. Einhorn TA, Gerstenfeld LC. Fracture healing: mechanisms and interventions. Nat Rev Rheumatol. 2015;11:45-54.
3. Giganti MG, Tresoldi I, Masuelli L, et al. Fracture healing: from basic science to role of nutrition. Front Biosci (Landmark Ed). 2014;19:1162-1175.
4. Niyibizi C, Kim M. Novel approaches to fracture healing. Expert Opin Investig Drugs. 2000;9:1573-1580.
5. Chen G, Li P, Liu Z, et al. Enrichment of miR-126 enhances the effects of endothelial progenitor cell-derived microvesicles on modulating MC3T3-E1 cell function via Erk1/2-Bcl-2 signalling pathway. PRION. 2019;13:106-115.
6. Dong H, Lei J, Ding L, Wen Y, Ju H, Zhang X. MicroRNA: function, detection, and bioanalysis. Chem Rev. 2013;113:6207-6233.
7. Xu HJ, Liao W, Liu XZ, et al. Down-regulation of exosomal microRNA-224-3p derived from bone marrow-derived mesenchymal stem cells potentiates angiogenesis in traumatic osteonecrosis of the femoral head. FASEB J. 2019;33:8055-8068.

8. Wei J, Shi Y, Zheng L, et al. miR-34s inhibit osteoblast proliferation and differentiation in the mouse by targeting SATB2. J Cell Biol. 2012;197:509-521.

9. Lin P, Wen D, Li Q, He Y, Yang H, Chen G. Genome-wide analysis of prognostic lncRNAs, miRNAs, and mRNAs forming a competing endogenous RNA network in hepatocellular carcinoma. Cell Physiol Biochem. 2018;48:1953-1967.

10. Schmitz SU, Grote P, Herrmann BG. Mechanisms of long noncoding RNA function in development and disease. Cell Mol Life Sci. 2016;73:2491-2509.

11. Geisler S, Coller J. RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. Nat Rev Mol Cell Biol. 2013;14:699-712.

12. Liang T, Zhou B, Shi L, et al. IncRNA AK017368 promotes proliferation and suppresses differentiation of myoblasts in skeletal muscle development by attenuating the function of miR-30c. FASEB J. 2018;32:377-389.

13. Yang C, Shen S, Zheng X, et al. Long noncoding RNA HAGLR acts as a microRNA-143-5p sponge to regulate epithelial-mesenchymal transition and metastatic potential in esophageal cancer by regulating LAMP3. FASEB. 2019;33(9):10490-10504.

14. Wu Q, Shi M, Meng W, Wang Y, Hui P, Ma J. Long noncoding RNA FOXD3-AS1 promotes colon adenocarcinoma progression and functions as a competing endogenous RNA to regulate SIRT1 by sponging miR-135a-5p. J Cell Physiol. 2019.

15. Weksberg R, Nishikawa J, Caluseriu O, et al. Tumor development in the Beckwith-Wiedemann syndrome is associated with a variety of constitutional molecular 11p15 alterations including imprinting defects of KCNQ1OT1. Hum Mol Genet. 2001;10:2989-3000.

16. Xiao D, Zhao Y. LncRNA KCNQ1OT1 enhanced the methotrexate resistance of colorectal cancer cells by regulating miR-760/PPP1R1B via the cAMP signaling pathway. J Cell Mol Med. 2019;23:3808-3823.

17. Yu S, Yu M, He X, Wen L, Bu Z, Feng J. KCNQ1OT1 promotes autophagy by regulating miR-200a/FOXE3/ATG7 pathway in cerebral ischemic stroke. Aging Cell. 2019;18:e12940.

18. Dong Z, Yang P, Qu X, et al. KCNQ1OT1 facilitates progression of non-small-cell lung carcinoma via modulating miRNA-27b-3p/HSP90AA1 axis. J Cell Physiol. 2019;234:11304-11314.

19. Gao X, Ge J, Li W, Zhou W, Xu L. LncRNA KCNQ1OT1 promotes osteogenic differentiation to relieve osteolysis via Wnt/beta-catenin activation. Cell Biosci. 2018:8:19.

20. Wang CG, Liao Z, Xiao H, et al. LncRNA KCNQ1OT1 promoted BMP2 expression to regulate osteogenic differentiation by sponging miRNA-214. Exp Mol Pathol. 2019;107:77-84.

21. Xiong Y, Cao F, Hu L, et al. miRNA-26a-5p accelerates healing via downregulation of PTEN in fracture patients with traumatic brain injury. Mol Ther Nucleic Acids. 2019;17:223-234.

22. Liu XY, Zhang FR, Shang JY, et al. Renal inhibition of miR-181a ameliorates 5-fluorouracil-induced mesangial cell apoptosis and nephrotoxicity. Cell Death Dis. 2018;9:610.

23. Huang Y, Zheng Y, Jia L, Li W. Long noncoding RNA H19 promotes osteoblast differentiation via TGF-beta1/Smad3/HDAC signaling pathway by deriving miR-675. Stem Cells. 2015;33:3481-3492.

24. Wang XN, Zhang LH, Cui XD, Wang MX, Zhang GY, Yu PL. IncRNA HOXA11-AS is involved in fracture healing through regulating miR-124-3p. Eur Rev Med Pharmacol Sci. 2017;21:4771-4776.

25. Rashid F, Shah A, Shan G. Long non-coding RNAs in the cytoplasm. Genomics Proteomics Bioinf. 2016;14:73-80.

26. Shao J, Pan X, Yin X, et al. KCNQ1OT1 affects the progression of diabetic retinopathy by regulating miR-1470 and epidermal growth factor receptor. J CELL PHYSIOL. 2019;234:17269-17279.

27. Foldynova-Trantirkova S, Wilcox WR, Krejci P. Sixteen years and counting: the current understanding of fibroblast growth factor receptor 3 (FGFR3) signaling in skeletal dysplasias. Hum Mutat. 2012;33:29-41.

28. Wang X, Qi H, Wang Q, et al. FGFR3/fibroblast growth factor receptor 3 inhibitors inhibits autophagy through decreasing the ATG12–ATG5 conjugate, leading to the delay of cartilage development in achondroplasia. Autophagy. 2015;11:1998-2013.

29. Huch K, Kleffner S, Stove J, Puhl W, Gunther KP, Brenner RE. PTHR1, PTHR, and FGFR3 are involved in the process of endochondral ossification in human osteophytes. Histochem Cell Biol. 2003;119:281-287.

30. Mao W, Huang X, Wang L, et al. Circular RNA hsa_circ_0068871 regulates FGFR3 expression and activates STAT3 by targeting miR-181a-5p to promote bladder cancer progression. J Exp Clin Cancer Res. 2019;38:169.

31. Jing P, Zhao N, Ye M, et al. Protein arginine methyltransferase 5 promotes lung cancer metastasis via the epigenetic regulation of miR-99 family/FGFR3 signaling. Autophagy. 2018;14:73-80.

32. Osawa Y, Matsushita M, Hasegawa S, et al. Activated FGFR3 promotes colon adenocarcinoma progression through regulating LAMP3. FASEB J. 2018;34:5208–5222. https://doi.org/10.1096/fj.201901864RR