MAFG-driven osteosarcoma cell progression is inhibited by a novel miRNA miR-4660

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Osteosarcoma (OS) is the most common primary bone malignancy in the adolescent population. MAFG (v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog G) forms a heterodimer with Nrf2 (NF-E2-related factor 2), binding to antioxidant response element (ARE), which is required for Nrf2 signaling activation. We found that MAFG mRNA and protein expression is significantly elevated in human OS tissues as well as in established and primary human OS cells. In human OS cells, MAGF silencing or knockout (KO) largely inhibited OS cell growth, proliferation, and migration, simultaneously inducing oxidative injury and apoptosis activation. Conversely, ectopic overexpression of MAFG augmented OS cell progression in vitro. MicroRNA-4660 (miR-4660) directly binds the 3’ untranslated region (UTR) of MAFG mRNA in the cytoplasm of OS cells. MAFG 3’ UTR luciferase activity and expression as well as OS cell growth were largely inhibited with forced miR-4660 overexpression but augmented with miR-4660 inhibition. In vivo, MAGF short hairpin RNA (shRNA) or forced overexpression of miR-4660 inhibited subcutaneous OS xenograft growth in severe combined immunodeficient mice. Furthermore, MAFG silencing or miR-4660 overexpression inhibited OS xenograft in situ growth in proximal tibia of the nude mice. In summary, MAFG overexpression-driven OS cell progression is inhibited by miR-4660. The miR-4660-MAFG axis could be a novel therapeutic target for human OS.

INTRODUCTION

Osteosarcoma (OS) is the most common histological form of primary bone cancer in adolescents and children. It ranks the eighth-most-common form of childhood cancer, comprising around 2.4% of all cancers in pediatric patients and around one-fifth of all primary bone malignancies.2,3 Advanced OS is characterized by high malignancy grade, dismal prognosis, early local recurrence, and distant metastasis.2,3 OS patients can develop micro-metastases in the lung with dismally low 5-year survival rates.4 The standard treatment options for OS patients include surgery combined with chemotherapy.5,6 The large cooperative group studies have implied that there has been little to no further improvement of overall survival for OS patients in the past years.6 To improve clinical outcome, there is an urgent need to explore the pathological mechanisms of OS tumorigenesis and progression.7,8 Recent molecular genetic studies of OS have changed our view on the disease.2,5 The molecularly targeted therapies are the current research focus of OS.2,5,9

MAFG (v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog G) is a small MAF protein belonging to the basic leucine zipper (bZIP) family of transcription factors.10 The MAFG bZIP structure has a basic DNA binding region and a leucine zipper structure but no canonical transcriptional activation domain.11 MAFG is broadly expressed in all human tissues but is relatively abundant in lung, lymph node, skeletal muscle, and thyroid tissues.12 MAFG knockout (KO) mice presented with a mild neuronal phenotype and mild thrombocytopenia.13 MAFG forms heterodimers with Nrf2 (NF-E2-related factor 2) protein, the latter being an essential regulator of antioxidant genes and detoxification enzymes.14,15 MAFG-Nrf2 heterodimers bind and activate the antioxidant response element (ARE) in the promoter regions of many genes involved in antioxidant defense.16 These genes including heme oxygenase 1 (HMOX-1), NAD(P)H quinone oxidoreductase 1 (NQO1), glutamate cysteine ligase catalytic subunit (GCLC), and many others.14,15 MAFG KO mouse embryonic fibroblasts

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mRNA expression is relatively low in OB-6 human osteoblastic cells versus N tissues; Figures 1F and 1G). That MAFG and HMOX1 protein upregulation was significantly detected in human OS tissues, 22–24 associated with OS tumorigenesis, progression, and therapy resistance.25,26 Here we identify a MAFG-targeting miRNA: microRNA-4660 (miR-4660). We show that miR-4660 specifically directly binds and silences MAFG to inhibit OS cell progression.

RESULTS

**MAFG overexpression in human OS**

OS comprises almost 60% of all the common histological subtypes of bone sarcoma.3 To examine MAFG expression in OS, we first consulted the TARGET Pan-Cancer (PANCAN) database to examine RNA sequencing (RNA-seq) data in children’s sarcoma tissues and normal adjacent tissues via a UCSC Xena project. As shown, in children’s sarcoma tissues (n = 1,524) MAFG mRNA expression is significantly upregulated (p < 0.05 versus normal tissues; n = 66) (Figure 1A). Furthermore, MAFG mRNA upregulation is detected in 14 cases of recurrent children’s sarcoma samples (Figure 1A). Importantly, the average survival of patients with MAFG-high sarcoma (RNA-seq ≥ 4.210) is lower than those with MAFG-low (MAFG RNA-seq < 4.210) sarcoma (p = 0.029; Figure 1B). These results show that MAFG is upregulated in children’s sarcoma tissues and is associated with poor overall survival.

To confirm the significance of the bioinformatics observations, we examined MAFG expression in human OS tissues. OS tumor tissues (T) and surrounding normal bone tissues (N), derived from a total of 12 primary OS patients, were analyzed. By quantitative real-time PCR, MAFG mRNA expression in OS tissues was 5.2-fold greater than in normal tissues (p < 0.05; Figure 1C). The mRNA expression of *heme oxygenase-1* (HMOX1), a key Nrf2 response gene,27,28 was also upregulated in OS tissues (p < 0.05 versus N tissues; Figure 1D). Western blotting studies were performed to test protein expression and showed that MAFG and HMOX1 protein expression was elevated in human OS tissues (Figure 1E). Quantification analyses integrating blotting data from 12 sets of human tissues confirmed that MAFG and HMOX1 protein upregulation was significant (p < 0.05 versus N tissues; Figures 1F and 1G).

We also examined MAFG expression in established OS cell lines (U2OS and MG63) and primary human OS cells. The primary OS cells were derived from three primary OS patients, namely pOS-1, pOS-2, and pOS-3 (see Materials and methods). As shown MAFG-HMOX1 mRNA expression is relatively low in OB-6 human osteoblastic cells and primary human osteoblasts (Figures 1H and 1I). In the established and primary human OS cells, MAFG-HMOX1 mRNA upregulation was detected (Figures 1H and 1I). Western blotting results (Figure 1J) confirmed MAFG-HMOX1 protein overexpression in established (U2OS and MG63) and primary OS cells, whereas low expression is detected in primary human osteoblasts. Together, these results confirmed MAFG overexpression in human OS.

**MAFG shRNA or KO inhibits OS cell viability, growth, proliferation, and migration**

A set of three different lentiviral short hairpin RNAs (shRNAs), targeting non-overlapping sequences of *MAFG* (by sh-MAFG-Seq3) cells and ko-MAFG cells (p < 0.05 versus shC+Cas9 cells). Furthermore, MAFG protein expression was robustly downregulated (Figure 2B). MAFG shRNA or KO led to downregulation of *HMOX1* mRNA (Figure 2C) and protein (Figure 2B) in pOS-1 cells, indicating Nrf2 signaling inhibition. Two other MAFG shRNAs, sh-MAFG-Seq1, also downregulated *HMOX1* mRNA in pOS-1 cells (Figure S1C). To further confirm Nrf2 inhibition, we showed that the relative ARE-reporter luciferase activity in pOS-1 cells was significantly downregulated by MAFG shRNA or KO (Figure S2A). Additionally, mRNA expressions of two other Nrf2-dependent genes, *NQO1* and GCLC, were decreased in MAFG-silenced/-KO pOS-1 cells (Figures S2B and S2C).

The cell growth curve results (Figure 2D) demonstrated that the growth of sh-MAFG cells and ko-MAFG cells was much slower than shC+Cas9 control cells (p < 0.05). Testing cell viability using a Cell Counting Kit-8 (CCK-8) assay, we found that MAFG silencing or KO resulted in a 60%–70% viability reduction in pOS-1 cells (Figure 2E; p < 0.05 versus shC+Cas9 cells). Nuclear 5-ethynyl-20-deoxyuridine (EdU) incorporation (% versus 4',6-diamidino-2-phenylindole [DAPI]) was also decreased in MAFG-silenced/-KO pOS-1 cells (Figure 2F; p < 0.05 versus shC+Cas9 cells), suggesting proliferation inhibition. Notably, in pOS-1 cells the two other MAFG shRNAs (sh-MAFG-Seq1/2) also led to viability (CCK-8 optical density [OD]) reduction (Figure S1D) and proliferation (EdU-positive nuclei ratio) inhibition (Figure S1E).
MAFG is upregulated in human OS tissues and cells

The TARGET Pan-Cancer (PANCAN) database shows MAFG expression (RNA-seq-TOIL RSEM) in 1,604 cases of children’s sarcoma tissues, 14 cases of recurrent sarcoma samples, and 66 cases of normal adjacent tissues (A). Kaplan-Meier survival analyses of MAFG-low (n = 160) and MAFG-high (n = 161) children’s sarcoma patients (B).

Expression of MAFG-HMOX1 mRNA and protein in OS tumor tissues (T) and the surrounding normal tissues (N) of 12 primary human OS patients was shown, with results quantified (C–G). Expression of MAFG-HMOX1 mRNA and protein in established OS cell lines (U2OS and MG63) and primary human OS cells, as well as in OB-6 human osteoblastic cells (OB-6) and primary human osteoblasts (osteoblasts) is shown, with results quantified. (H–J). Data were presented as mean ± standard deviation (SD). ***p < 0.01 versus normal tissues (A). *p < 0.05 versus N tissues/osteoblasts (C–I).
Examining cell cycle progression, using the propidium iodide-fluorescence-activated cell sorting (PI-FACS) assay, confirmed that MAFG shRNA or KO induced G1-S arrest in pOS-1 cells, as both increased the number of G1-phase cells but decreased S- and G2-phase cells (Figure 2L; p < 0.05 versus shC cells). Collectively, these results show that MAFG silencing or KO caused apoptosis activation. Significant caspase-3 activation was also detected in pOS-1 cells-bearing the MAFG-shRNA or MAGF-KO construct (Figure 3G). The two other MAFG shRNAs, sh-MAFG-Seq1/2, induced ROS production (CellROX intensity; Figure S1F), mitochondrial depolarization (JC-1 green monomer intensity increase; Figure S1G), and apoptosis activation (nuclear TUNEL ratio increase; Figure S1H) in pOS-1 cells. These results confirm that MAFG silencing/depletion induces robust oxidative injury in primary OS cells.

In OS cells, proliferation arrest and/or oxidative injury is anticipated to result in cell apoptosis. Testing the potential effect of MAFG depletion on cell apoptosis, we found that the ratio of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)-positive nuclei was significantly increased in pOS-1 cells-bearing the MAFG-shRNA or MAGF-KO construct (Figure 3E; p < 0.05 versus shC+Cas9 cells). Annexin V FACS assay results (Figure 3F) further confirmed that MAFG silencing or KO caused apoptosis activation. Significant caspase-3 activation was also detected in pOS-1 cells with MAFG silencing or KO (Figure 3G). The two other MAFG shRNAs, sh-MAFG-Seq1/2, induced ROS production (CellROX intensity increase; Figure S1F), mitochondrial depolarization (JC-1 green monomer intensity increase; Figure S1G), and apoptosis activation (nuclear TUNEL ratio increase; Figure S1H) in pOS-1 cells. These results confirm that MAFG silencing/depletion induces robust apoptosis activation in primary OS cells.

To test whether oxidative stress is the primary cause of cell apoptosis in MAFG-depleted cells, we applied several known antioxidants, including N-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC),30,31 and AGI-1067.32,33 As shown, in pOS-1 cells MAFG shRNA- and MAFG KO-induced viability (CCK-8 OD) reduction, mitochondrial depolarization, and DNA damage.
Figure 2. MAFG shRNA or KO inhibits OS cell viability, growth, proliferation, and migration

Stable primary human OS cells (pOS-1/pOS-2/pOS-3, derived from OS patients) or the established OS cell lines (U2OS and MG63), bearing MAFG shRNA (sh-MAFG), the lenti-CRISPR-Cas9-MAFG-KO construct (ko-MAFG), scramble control shRNA plus CRISPR-Cas9 empty vector (shC+Cas9), or scramble control shRNA (shC), were established and expression of listed genes tested by quantitative real-time PCR and western blotting assays (A–C and I). Cells were cultured for applied time periods, cell growth (D), viability (CCK-8 assays, E and J) and proliferation (nuclear EdU incorporation, F and K), as well as cell cycle progression (G) and cell migration (H and L) (Transwell assays) were tested by the assays mentioned in the text, with results quantified. For EdU staining assays, five random views with a total of 1,000 cell nuclei from each treatment were counted. (legend continued on next page)
proliferation inhibition (nuclear EdU ratio reduction), and cell apoptosis (increased ratio in TUNEL-positive nuclei) were largely attenuated by the ROS scavengers (Figure 3H). These results indicate MAFG silencing/KO resulted in ROS accumulation to induce OS cell apoptosis.

In other primary (pOS-2 and pOS-3) and established OS cells (U2OS and MG63), MAFG silencing by targeted lentiviral shRNA (sh-MAFG-Seq3/2) similarly induced robust ROS production (CellROX intensity increase; Figure S1I), mitochondrial depolarization (JC-1 green monomers accumulation; Figure S1J), and cell apoptosis (TUNEL-positive nuclei ratio increase; Figure S1K). These results together suggest that MAFG shRNA or KO induces oxidative injury and apoptosis in human OS cells.

**Ectopic MAFG overexpression further promotes OS cell progression in vitro**

Based on the results, we hypothesized that MAFG overexpression would promote OS cell progression in vitro. A MAFG-expressing GV369 vector was transduced into pOS-1 cells, and stable cells were established (OE-MAFG cells). Quantitative real-time PCR assay results (Figure 4A) confirmed that MAFG mRNA levels were increased over seven-fold in the OE-MAFG cells, and MAFG protein expression was significantly elevated (Figure 4B). Further, HMOX1 mRNA and protein expression was increased (Figures 4B and 4C). Overexpression of MAFG augmented ARE-reporter luciferase activity (Figure S2D), indicating Nrf2 cascade activation, which was further confirmed by increased mRNA expression of NQO1 (Figure S2E) and GCLC (Figure S2F).

Cell growth results (Figure 4D) showed that OE-MAFG cells grew significantly faster than control cells. In pOS-1 cells, ectopic overexpression of MAFG augmented cell viability (CCK-8 OD; Figure 4E) and proliferation (EdU ratio; Figure 4F). Furthermore, cell migration (Figure 4G) was enhanced in MAFG-overexpressed pOS-1 cells.

Similarly, in other primary OS cells (pOS-2/pOS-3) and established OS cell lines (U2OS and MG63), transfection of the MAFG-expressing GV369 vector resulted in upregulation of MAFG mRNA (Figure 4H). Consequently, HMOX1 mRNA levels were significantly increased (Figure 4H). Increased MAFG and HMOX1 protein levels were detected in the OS cells with MAFG-expressing vector (Figure 4I). EdU incorporation assay results (Figure 4I) demonstrated that ectopic overexpression of MAFG promoted cell proliferation in OS cells. Thus, ectopic overexpression of MAFG promotes human OS cell progression in vitro.

**miR-4660 directly binds and silences MAFG in OS cells**

To identify MAFG-targeting miRNAs that bind its 3’ UTR, we searched the microRNA database, TargetScan (V7.2). Promising MAFG-targeting miRNAs were further verified through multiple microRNA databases: mirBase (v21.0) and mirDB. Three candidate miRNAs with the context score percentage over 99% and the context++ score less than −0.5 were retrieved, including miR-4660, miR-4730, and miR-1184. Each of the three miRNA mimics (500 nM, 48 h) were individually transfected to pOS-1 cells, of which only miR-4660 mimic resulted in significant MAFG mRNA downregulation (Figure S3A). As shown, microRNA-4660 (miR-4660) putatively targets the MAFG 3’ UTR (at position 788–795; Figure 5A). The miR-4660-MAFG 3’ UTR binding context score percentage is 99%, with the context++ score at −0.53 (TargetScan V7.2;34 Figure 5A). These results indicate a high percentage of direct binding between the two.34

By employing an RNA-immunoprecipitation (RNA-IP) assay, we demonstrated that endogenous MAFG mRNA and miR-4660 were pulled down by an anti-Ago2 antibody in pOS-1 cells (Figure 5B). The non-specific anti-immunoglobulin G (IgG) antibody failed to pull down MAFG mRNA and miR-4660 (Figure 5B). RNA pull-down assay results further showed that biotinylated-miR-4660 directly associated with MAFG mRNA, but not with two other MAF family genes, MAFF mRNA and MAFK mRNA (Figure 5C). In addition, fluorescence in situ hybridization (FISH) assay results demonstrated that miR-4660 (in red) and MAFG mRNA (in green) were co-localized mainly in the cytoplasm of pOS-1 cells (Figure 5D). These results suggested a high probability for the two to bind.34

Based on the above results, we hypothesized that ectopic miR-4660 overexpression would alter MAFG expression. A lentiviral construct encoding pre-miRNA-4600 (lv-pre-miR-4660) was transduced into pOS-1 cells, and two stable cell lines, lv-pre-miR-4660-L1 and lv-pre-miR-4660-L2, were established via puromycin selection. The quantitative real-time PCR results (Figure 5E) confirmed that the expression of mature miRNA-4660 increased over 20-fold in lv-pre-miR-4660 cells (p < 0.05 versus cells with miRNA control construct/lv-miRC). A dual-luciferase reporter assay demonstrated that MAFG 3’ UTR luciferase reporter activity was significantly decreased after the forced miR-4660 overexpression in pOS-1 cells (p < 0.05 versus lv-miRC cells) (Figure 5F). Consequently, MAFG mRNA (Figure 5G) and protein (Figure 5H) expression were dramatically downregulated in miRNA-4660-overexpressed cells. Expression of MAFF mRNA and MAFK mRNA was unchanged, however (Figure 5I). In lv-pre-miR-4660-expressing pOS-1 cells, the relative

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included to calculate the average EdU/DAPI ratio (same for all figures). For Transwell/Matrigel Transwell assays, five random views were included to calculate the average number of migrated/invaded cells in each condition (same for all figures). For all the functional assays, the exact same number of viable cells of different genetic treatments were seeded initially to each well or each dish (at 0 h/day 0, same for all figures). Parental control cells (same for all figures). Expression of listed proteins was quantified and normalized to the loading control (B). Data are presented as mean ± standard deviation (SD, n = 5). *p < 0.05 versus shC+Cas9 cells/shC cells. Experiments in this figure were repeated five times, with similar results obtained.

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ARE-reporter luciferase activity (Figure S2G) as well as NQO1 (Figure S2H) and GCLC (Figure S2I) mRNA expression were largely inhibited, confirming Nrf2 cascade inhibition.

The results demonstrate that miRNA-4660 can selectively target and silence MAFG mRNA in OS cells. To further support this hypothesis, pOS-1 cells were transfected with either wild-type (WT) or the

Figure 3. MAFG shRNA or KO induces oxidative injury and apoptosis in OS cells
Stable pOS-1 cells, bearing MAFG shRNA (sh-MAFG), the lent-CRISPR-Cas9-MAFG-KO construct (ko-MAFG), and scramble control shRNA plus CRISPR-Cas9 empty vector (shC+Cas9), were cultured for applied time periods; ROS levels were tested by measuring CellROX intensity (A) and the GSH/GSSG ratio (B). Mitochondrial depolarization was tested by the fluorescence dye JC-1 (C), with single-strand DNA (ssDNA) contents measured by an ELISA kit (D). Cell apoptosis was examined and quantified by nuclear TUNEL staining (E), Annexin V FACS (F), and Caspase-3 activity (G) assays. pOS-1 cells with shC+Cas9, sh-MAFG, or ko-MAFG were treated with NAC (50 μM), PDTC (40 μM), or AGI-1067 (10 μM) and cultured for applied time periods; cell viability (CCK-8 OD), proliferation (nuclear EdU incorporation), and apoptosis (TUNEL staining) were tested, with results quantified (H). Data were presented as mean ± standard deviation (SD, n = 5). *p < 0.05 versus shC+Cas9 cells. #p < 0.05 versus PBS treatment (H).

Experiments in this figure were repeated five times with similar results obtained.
mutant (Mut) miR-4660 mimic. The mutant miR-4660 mimic (Mut-miR-4660) contains nucleotide mutations at the miR-4660 binding site to MAFG 3’ UTR (see sequence in Figure 5J). RNA-IP experiment confirmed that only WT-miR-4660 immunoprecipitated with MAFG mRNA, while the mutant failed (Figure 5K). Transfection the WT-miR-4660, but not the Mut-miR-4660, decreased MAFG 3’ UTR luciferase reporter activity (Figure 5L) as well as expression of MAFG mRNA (Figure 5M) in pOS-1 cells.

We next tested whether miR-4660 inhibition could increase MAFG expression. A lentiviral construct encoding the anti-sense of pre-miR-4660, or lv-antagomiR-4660, was transduced into pOS-1 cells, and stable cells were established. As compared to cells transduced with the lentiviral microRNA anti-sense control construct (lv-antagomiR-4660), mature miR-4660 levels decreased over 90% in lv-antagomiR-4660-expressing pOS-1 cells (Figure 5N). miR-4660 inhibition resulted in an increased MAFG 3’ UTR luciferase reporter activity (Figure 5O), as well as upregulation of MAFG mRNA (Figure 5P). Therefore, forced miR-4660 inhibition resulted in MAFG upregulation, further corroborating that miR-4660 is a MAFG-targeting miRNA in OS cells. In other primary OS cells (pOS-2/pOS-3) and established cells (U2OS and MG63), ectopic miR-4660 overexpression, using lv-pre-miR-4660 (Figure 5N), led to significant MAFG mRNA reduction (Figure 5O).

We also analyzed expression of miR-4660 in the human OS tissues described in Figure 1. The quantitative real-time PCR assay results (Figure 5P) demonstrated that miR-4660 expression in OS tumor tissues (T) was significantly lower than that in the surrounding normal tissue (N). Furthermore, low miR-4660 expression was detected in primary OS cells (pOS-1, pOS-2, and pOS-3) and established OS cell lines (U2OS and MG63) (Figure 5Q), compared to expression...
Figure 5. miR-4660 directly binds and silences MAFG in OS cells

miRNA-4660 putatively targets the 3' UTR (untranslated region) of MAFG mRNA (at position of 788–795) (A). miRNA-4660 (fluorescence-tagged) locates in the cytoplasm of pOS-1 cells (B). RNA immunoprecipitation (RNA-IP) experiment results demonstrate that the Ago2 protein immunoprecipitated with MAFG mRNA and miR-4660 in pOS-1 cells (B). RNA-pull-down assay results display that biotinylated-miR-4660 associated with MAFG mRNA (but not MAFF mRNA and MAFK mRNA) in pOS-1 cells (C). Fluorescence in situ hybridization (FISH) results show miR-4660 (in red) and MAFG mRNA (in green) cellular distribution and their co-localization in pOS-1 cells (D). The pOS-1 cells were transduced with lentiviral pre-microRNA-4660 (lv-pre-miR-4660). With selection by puromycin, two stable cell lines were established: lv-pre-miR-4660-L1/lv-pre-miR-4660-L2. Control cells were transduced with the lentiviral nonsense microRNA (lv-miRC) construct. Expression of mature miR-4660 and listed mRNAs was tested by quantitative real-time PCR (E, G, and I). The MAFG 3' UTR luciferase reporter activity was tested (F), with expression of listed proteins tested by western blotting (H). pOS-1 cells were transfected with 500 nM of nonsense microRNA control (miRC), the wild-type (WT), or the mutant miR-4660 mimic (sequences listed in J). miR-4660-MAFG mRNA binding was tested by RNA pull-down (K). The MAFG 3' UTR luciferase reporter activity (L), as well as its mRNA expression (M) were tested. The applied OS cells, including pOS-2, pOS-3, U2OS, and MG63, were infected with lv-pre-miR-4660 or lv-miRC for 72 h; expression of miR-4660 (N) and MAFG mRNA (O) is shown. Expression of miR-4660 in OS tumor tissues (T) and the surrounding normal tissues (N) of 12 primary human OS patients is shown, with results quantified (P). Expression of miR-4660 in established OS cell lines (U2OS and MG63) and primary human OS cells, as well as in OB-6 human osteoblastic cells (OB-6) and primary human osteoblasts (osteoblasts) is shown, with results quantified (Q). Data are presented as mean ± standard deviation (SD). *p < 0.05 versus IgG or bead control (B and C). *p < 0.05 versus lv-miRC/miRC/lv-anta-C cells. *p < 0.05 versus N tissues/osteoblasts (P and Q). Experiments in this figure were repeated five times with similar results obtained.
in OB-6 osteoblastic cells and primary human osteoblasts (Figure 5Q). Therefore, in human OS tissues, low miR-4660 expression is correlated with MAFG elevation (see Figure 1).

miR-4660 overexpression inhibits OS cell progression in vitro

As miR-4660 can silence MAFG expression, we tested its effect on OS cell function. EdU incorporation assay results (Figure 6A) demonstrated that forced expression of miRNA-4660, using lv-pre-miR-4660 (see Figure 5), potently inhibited pOS-1 cell proliferation (recording EdU/DAPI ratio, p < 0.05 versus lv-miRC cells). Furthermore, in vitro cell migration was largely suppressed in pOS-1 cells bearing lv-pre-miR-4660 (Figure 6B; p < 0.05 versus lv-miRC cells). ROS production was enhanced, as indicated by increased CellROX fluorescence intensity detected in miR-4660-overexpressed pOS-1 cells (Figure 6C; p < 0.05 versus lv-miRC cells). Overexpression of miRNA-4660 also induced cell apoptosis, evidenced by increased ratio of TUNEL-positive nuclei (Figure 6D; p < 0.05 versus lv-miRC cells). Conversely, miR-4660 silencing by lv-antagomiR-4660 led to increased cell proliferation (Figure S3E) and migration (Figure S3F) in pOS-1 cells.

Treatment with the antioxidants, NAC and PDTC, largely inhibited miR-4660 overexpression (lv-pre-miR-4660-L1 and lv-pre-miR-4660-L2)-induced viability (CCK-8 OD) reduction (Figure S3G), proliferation inhibition (nuclear EdU staining assay; Figure S3H), and cell apoptosis (by recording nuclear TUNEL ratio; Figure S3I). These results support the model that miR-4660 overexpression leads to MAFG downregulation, ROS production, and oxidative injury, causing significant anti-OS cell activity.

In other primary OS cells (pOS-2 and pOS-3) and established cells (U2OS and MG63), forced overexpression of miR-4660 by lv-pre-miR-4660 (see Figure 5) inhibited cell viability (CCK-8 OD; Figure 6E) and proliferation (EdU ratio; Figure 6F). Furthermore, ROS production (CellROX intensity increase; Figure 6G) and apoptosis (TUNEL ratio increase; Figure 6H) were detected in lv-pre-miR-4660-expressing OS cells. Therefore, mimicking MAFG silencing-induced actions, miR-4660 overexpression inhibited pOS-1 cell proliferation and migration but provoked oxidative injury and cell apoptosis.

Significantly, in OE-MAFG pOS-1 cells, stable transfection of lv-pre-miR-4660 induced miR-4660 overexpression (Figure S3J), causing robust MAFG mRNA depletion (Figure S3K). Importantly, miR-4660 overexpression largely inhibited high basal cell proliferation (EdU positive nuclei ratio; Figure S3L) and migration (Figure S3M) in OE-MAFG pOS-1 cells.

miR-4660-induced anti-OS cell activity is due to MAFG silencing

To further confirm that miR-4660-induced anti-OS cell activity is due to MAFG silencing, a lentiviral 3' UTR null MAFG expression vector, MAFG (UTR null), was constructed. As shown, the construct completely restored MAFG mRNA (Figure 7A) and protein (Figure 7B) expression in lv-pre-miR-4660-expressing pOS-1 cells but had no significant effect on miR-4660 expression (Figure 7C). Further, miR-4660 overexpression-induced HMOX1 downregulation (both mRNA and protein) was reversed by the MAFG (UTR null) construct (Figures 7A and 7D). These results show that MAFG (UTR null) can restore MAFG-HMOX1 expression in miR-4660-overexpressed pOS-1 cells. Functional studies demonstrated that in pOS-1 cells, lv-pre-miR-4660-induced proliferation inhibition (EdU ratio reduction; Figure 7E), ROS production (CellROX intensity increase; Figure 7F), and cell apoptosis (TUNEL staining increase; Figure 7G) were reversed by MAFG re-expression.

Based on these results, altering miR-4660 expression should be ineffective in MAFG-depleted cells. The ko-MAFG cells (see Figures 2 and 3) were transduced with lv-pre-miR-4660 or lv-antagomiR-4660, both of which significantly altered mature miR-4660 expression (Figure 7H). MAFG-KO-induced proliferation inhibition (Figure 7I), ROS production (Figure 7J), and cell apoptosis (Figure 7K) were not affected by ectopic miR-4660 overexpression or inhibition (Figures 7I–7K). These results further supported that miR-4660 silenced MAFG to inhibit OS cell progression, and MAFG KO abolished its activity.

MAFG silencing or miR-4660 overexpression inhibits growth of subcutaneous OS xenografts and in situ OS xenografts in mice

To study the potential role of the miR-4660-MAFG axis on OS cell growth in vivo, a xenograft mouse model was applied. The POS-1 cells were subcutaneously (s.c.) injected into the flanks of severe combined immunodeficient (SCID) mice. Within 3 weeks, OS xenografts were established, with each tumor close to 100 mm3. Mice were then randomly assigned into three groups (day 0), with intratumoral injection of MAFG shRNA lentivirus (MAFG shRNA; see Figure 2), pre-miR-4660 lentivirus (lv-pre-miR-4660; see Figure 5), or the control virus (miRC+shC). The tumor growth curve results confirmed that tumors bearing MAFG shRNA or lv-pre-miR-4660 grew significantly slower than the control tumors (Figure 8A). The estimated daily tumor growth, calculated by the formulation (tumor volume at day 0)/42, was significantly different between the three groups (Figure 8D), and no significant toxicity was observed.

At day 7 and day 14, one tumor of each group was isolated, with a total six tumors obtained. Western blot assay results (Figure 8E) confirmed significant MAFG-HMOX1 downregulation in tumors bearing MAFG shRNA or lv-pre-miR-4660, where MAFG mRNA depletion was detected (Figure 8F). As shown, miR-4660 overexpression was detected in tumor tissues bearing lv-pre-miR-4660 (Figure 8G). miR-4660 expression was unchanged in MAFG shRNA-expressing tumor tissues (Figure 8G). In vivo RNA-FISH experiments confirmed MAFG mRNA downregulation in pOS-1 xenografts...
Figure 6. miR-4660 overexpression inhibits OS cell progression in vitro
Stable primary human OS cells (pOS-1/pOS-2/pOS-3, derived from different OS patients) or the established OS cell lines (U2OS and MG63) were transduced with lentiviral pre-microRNA-4660 (lv-pre-miR-4660). Control cells were transfected with lentiviral nonsense microRNA (lv-miRC). With selection by puromycin, two stable cell lines were established. Cells were further cultured for applied time periods. Cell proliferation (by recording nuclear EdU incorporation, A and F) and migration (B) were tested by the assays mentioned in the text, with results quantified. Cellular ROS intensity and apoptosis were tested by CellROX staining (C and G) and TUNEL staining (D and H) assays, respectively. Cell viability was tested by CCK-8 assay (E). Data are presented as mean ± standard deviation (SD, n = 5). *p < 0.05 versus lv-miRC cells. Experiments in this figure were repeated five times with similar results obtained.
bearing MAFG shRNA or lv-pre-miR-4660 (Figure 8H). Thus, consistent with the in vitro findings, MAFG silencing or ectopic miR-4660 overexpression inhibited subcutaneous OS xenograft growth in SCID mice.

To further support a role of the miR-4660-MAFG axis in OS cell growth in vivo, genetically modified primary OS cells were injected into the proximal tibia of nude mice to establish an animal model of in situ OS. These cells included stable pOS-1 cells expressing MAFG shRNA lentivirus (MAFG shRNA), pre-miR-4660 lentivirus (lv-pre-miR-4660), or the control virus (miRC+shC). Results demonstrated that, 24 days (day 24) after the cell injection, the volumes of in situ pOS-1 xenografts bearing MAFG shRNA or lv-pre-miR-4660 were significantly lower than xenografts with control virus (Figures 8H and 8I). At day 24, the in situ pOS-1 xenografts were isolated and homogenized. As demonstrated, MAFG mRNA levels were significantly decreased in in situ pOS-1 xenograft tissues with MAFG shRNA and lv-pre-miR-4660 (Figure 8J). The mature miR-4660 levels were increased over eight-fold in lv-pre-miR-4660-expressing xenograft tissue (Figure 8K). These results demonstrate that MAFG shRNA or forced miR-4660 overexpression potently inhibits OS xenografts in situ growth in mice.

DISCUSSION
MAFG forms a heterodimer with Nrf2 to bind ARE, initiating transcription of detoxification enzymes and antioxidant genes.35–37 Recent studies suggest that MAFG could play an important role in cancer progression.38 The overexpression of MAFG in hepatocellular carcinoma
is associated with tumor progression and decreased survival. In non-small cell lung cancer, MAFG inhibition or downregulation induced ROS production, sensitizing cancer cells to cisplatin-induced apoptosis. BRAFV600E-stabilized MAFG initiated recruitment of a co-repressor complex to CpG island methylator phenotype (CIMP) gene promoters in colorectal cancer cells, which is associated with tumorigenesis and cancer growth. Silencing of MAFG potently inhibited colorectal cancer cell growth.

**Figure 8. MAFG silencing or miR-4660 overexpression inhibits growth of subcutaneous OS xenografts and in situ OS xenografts in mice**

The SCID mice bearing pOS-1 xenografts were randomly assigned into three groups, intratumorally injected with MAFG shRNA lentivirus (MAFG shRNA), pre-miR-4660 lentivirus (lv-pre-miR-4660), or the control virus (miRC+shC); tumor volumes (A) or the mice body weights (D) were recorded every 7 days. Estimated daily tumor growth was calculated (B). At the end of the experiments (day 42), tumors were isolated and individually weighed (C). At day 7 and day 14, one tumor of each group was isolated, and total six tumors were achieved; expression of listed genes in tumor tissue lysates is shown (E–G). In vivo RNA-FISH in tumor slides further confirmed MAFG mRNA silencing in MAFG shRNA- and lv-pre-miR-4660-tumors (H). At 3 × 10^6 cells per mouse, stable pOS-1 cells expressing MAFG shRNA lentivirus (MAFG shRNA), pre-miR-4660 lentivirus (lv-pre-miR-4660), or the control virus (miRC+shC) were injected into the proximal tibia of the nude mice. Twenty-four days after injection (day 24), in situ tumor volumes were recorded (I). Tumors were also isolated; expression of listed genes in tumor tissue lysates is shown (J and K). Data are presented as mean ± standard deviation (SD). *p < 0.05 versus miRC+shC tumors.
We provide evidence to show that MAFG is a novel oncogenic gene and potential therapeutic target of OS. MAFG mRNA and protein expression are significantly elevated in human OS tissues, compared to low expression in normal bone tissues and osteoblasts. In primary and established OS cells, MAFG shRNA or KO inhibited OS cell growth, proliferation, and migration, while simultaneously eliciting cell-cycle arrest and cell apoptosis. Conversely, ectopic overexpression of MAFG further promoted OS cell progression in vitro. Significantly, MAFG silencing potently inhibited growth of subcutaneous OS xenografts and in situ OS xenografts in mice.

Bai et al.41 have reported that low levels of ROS, detected in human OS tissues and cells, are important for OS cell proliferation and invasion. However, sustained ROS production and profound oxidative injury induced OS cell growth arrest and apoptosis. Wang et al.52 have shown that androgapholide induced OS cell apoptosis via ROS production and downstream JNK activation. Similarly, hyperthermia-induced OS cell apoptosis was associated with ROS production.43 Ionizing radiation (IR) was also found to induce robust ROS production to promote U2OS cell apoptosis.44 Such action by IR was attenuated by Nrf2 upregulation-induced antioxidant response.54 We found that MAFG silencing results in Nrf2-signaling inhibition, leading to significant ROS production and oxidative injury. Antioxidants, including NAD, PDTC, and AGI-1067, inhibited MAFG silencing-induced OS cell apoptosis.

miRNA binds the 3’ UTR of the complementary mRNAs, causing targeted mRNA translation inhibition and/or mRNA degradation.25,26 miRNA is often dysregulation in human OS25-28 and is associated with tumorigenesis progression.25,26 The potential function of miR-4660 is largely unknown. A recent study by Tu et al.45 has demonstrated that miR-4660 is downregulated in patients with oxalosis. It bound directly to 3’ UTR of alanine–glyoxylate aminotransferase (AGXT) to inhibit its expression.45 The results of this study demonstrate that miR-4660 is a novel MAGF-targeting miRNA in OS cells. miR-4660 is localized in the cytoplasm of OS cells, where it can bind directly to 3’ UTR of MAFG mRNA. In OS cells, ectopic overexpression of miR-4660 inhibited MAFG 3’ UTR luciferase activity and expression. Both were, however, augmented with miR-4660 inhibition. miR-4660 overexpression mimicked MAFG silencing-induced actions, inhibiting OS cell proliferation and migration and inducing ROS production and apoptosis activation. Importantly, restoring MAFG expression completely reversed miR-4660 overexpression-induced OS cell inhibition. Furthermore, exogenously altering miR-4660 expression failed to affect the function of MAFG-KO OS cells. Significantly, decreased miR-4660 expression in human OS tissues correlates with MAFG upregulation.

OS accounts for 60% of the common histological subtypes of bone sarcoma. It is the primary malignant bone tumor that commonly affects adolescents and young adults.6,46 Current chemotherapy for human OS consists of the combination of methotrexate, doxorubicin, and cisplatin (MAP).25,47 The application of molecularly targeted therapies has so far failed to significantly improve the overall survival of advanced OS patients. Hence, further exploration of the pathology of OS is essential for improving the prognosis of patients with advanced OS.2,6,47 Our results indicate that dysregulation of the miR-4660-MAFG axis is involved in the progression of OS. Targeting the miR-4660-MAFG axis could be a promising therapeutic strategy for this devastating malignancy.

MATERIALS AND METHODS

**Chemicals and reagents**

CCK-8 was obtained from Dajiindo (Kumamoto, Japan). Antioxidants including NAC, PDTC, and AGI-1067, as well as puromycin, neomycin, polybrene, and Matrigel were provided by Sigma-Aldrich Chemicals (St. Louis, MO, USA). Cell culture reagents, including fetal bovine serum (FBS), DMEM, and antibiotics, were obtained from HyClone (Logan, UT, USA). Antibodies of MAFG, HMOX1, and tubulin were provided by Cell Signalling Technology (Beverly, MA, USA).

**Cell culture**

Established human OS cell lines, U2OS and MG63, were obtained from the Cell Bank of Shanghai Institute of Biological Science (Shanghai, China). Cells were cultured using a previously described protocol.52 The primary human OS cells derived from three human OS patients, pOS-1, pOS-2, and pOS-3, were provided by Dr. Ji at Nanjing Medical University,49 and cells were cultured under the described conditions.49,50 Primary OS cells at passages 3–10 were utilized. OB-6 human osteoblastic cells and primary human osteoblasts were provided again by Dr. Ji at Nanjing Medical University, cultured as previously described.51 The protocols of the study were approved by the institutional animal care and use committee (IACUC) and ethics committee of Soochow University.

**Human OS tissues**

Human OS tissues and the matched surrounding normal bone tissues were from a set of 12 OS patients with written informed consent administrated at the Affiliated Children Hospital of Soochow University (Suzhou, China). Tissues were incubated with the tissue lysis buffer33 and stored in liquid nitrogen. The protocols of using human tissues were approved by the ethics committee of Soochow University.

**Quantitative real-time PCR**

TRIzol reagents were applied to achieve total RNA, which was quantified and reversely transcribed.54 The quantitative real-time PCR was performed by the SYBR Premix Ex TaqTM kit under the ABI-7500 PCR system (Shanghai, China).54 mRNA expression was quantified by 2−ΔΔCt protocol with glyceraldehyde-3-phosphatedehydrogenase (GAPDH) as the internal control. Mature miR-4660 expression was detected using the TaqMan microRNA assay of hsa-miR-4660 (Applied Biosystems, Shanghai, China). The TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) was used for RNA (10 ng) reverse transcription via the stem-loop primer. U6 RNA was tested as the internal control for miR-4660. mRNA primers were listed in Table 1. mRNA primers of human GCLC, NQO1, and GADPH were described previously.55 mRNA primers for MAFF and MAFK were provided by Genechem (Shanghai, China).
MAFG shRNA
OS cells were initially seeded into six-well plates at $1 \times 10^5$ cells per well. MAFG shRNA lentiviral particles (shRNA sequences are listed in Table 1; Genechem) were added to cultured OS cells. After 24 h, puromycin-containing medium was added to the OS cells, with stable cells (sh-MAFG cells) achieved within 4–5 passages. MAFG silencing was verified by quantitative real-time PCR and western blotting assays.

MAFG KO
Human OS cells were initially seeded into six-well plates at $1 \times 10^5$ cells per well. The MAFG small guide RNA (sgRNA, Target DNA sequence: TGAGAATGGCGACACCTGA, PAM Sequence: CGG) was inserted into the CRISPR-Cas9 PX458 construct.66 The construct was transfected to human OS cells by Lipofectamine 2000 (Thermo Fisher Invitrogen, Shanghai, China). The transfected cells were inoculated into 96-well plates to establish single monoclonal cells, subjected to screening of MAFG KO. The established cells were further subjected to selection by puromycin (5.0 μg/mL)-containing medium, and stable cells were established (ko-MAFG). MAFG KO was verified by quantitative real-time PCR and western blotting assays.

Forced MAFG overexpression
The full-length MAFG cDNA and the UTR null MAFG cDNA were synthesized and sequence-verified by Genechem (Shanghai, China) and separately inserted into the GV369 construct (Genechem). The construct and the lentivirus-packing plasmids (psPAX2 and pMD2.G, from Dr. Jiang57) were co-transfected to the packaging cell line (HEK293T), generating MAFG-expressing lentivirus. The viruses were then enriched, filtered, and added to OS cells (cultured in complete medium with polybrevine) for 24 h. Thereafter, puromycin (5.0 μg/mL)-containing complete medium was added to select stable cell line OE-MAFG, where MAFG overexpression was verified by quantitative real-time PCR and western blotting assays. Control cells were transduced with the empty vector.

Forced overexpression or inhibition of miR-4660
The miR-4660 precursor (pre-miR-4660) sequence (ACUCCUUCUGCAUCUCUGUGGAAAUGGAGACUUUUCGUUUCUCUGCAUCUCCCCAGGCGCUGGUGGAGU) and the corresponding anti-sense sequence were synthesized and verified by Genechem. Each was individually inserted into a GV248 lentiviral construct (Genechem, Shanghai, China). The construct and the lentivirus-packing plasmids (psPAX2 and pMD2.G) were co-transfected into HEK293T cells, establishing pre-miR-4660 expression lentivirus (LV-pre-miR-4660) or pre-miR-4660 anti-sense lentivirus (LV-antago-miR-4660). Viruses were added to cultured OS cells. To select stable cell lines, puromycin (5.0 μg/mL) was added. The mature miR-4660 (sequence: UGCGAGGUUUGAGAAAUGGAG) expression was examined by quantitative real-time PCR in stable cells. Control OS cells were transduced with lentiviral nonsense control miRNA construct (lv-miRC) or the lentiviral nonsense control miRNA inhibitor (anta-C).
Cell viability

Cell viability was assessed through a CCK-8 assay. Briefly, cells with the applied genetic modifications were seeded into the 96-well tissue culture plates (at $3 \times 10^3$ cells per well). Following incubation for 96 h, cell viability was estimated by recording the CCK-8 OD at 450 nm using a microplate reader.

EdU staining

OS cells with the applied genetic modifications were seeded into six-well plates (at $1 \times 10^5$ cells in each well) and cultured for 72 h. An EdU Apollo-567 assay kit (RiboBio, Guangzhou, China) was employed to quantitatively measure cell proliferation. The cell nuclei were stained with both EdU and DAPI, visualized under a fluorescent microscope (Leica, DM 4000, Germany). Cells in each field of view were then counted and analyzed. The nuclear EdU ratio, % versus DAPI, from at least 1,000 cells in five random views per treatment was calculated.

In vitro cell migration assay

On the upper Transwell chambers (8-μm pore, Corning, New York, NY, USA) OS cells (10,000 cells per chamber, in serum-free medium) with applied genetic modifications were plated, with the lower chambers filled with 10% FBS complete medium. After 16 h, OS cells migrating to the lower chambers were fixed, stained, and counted.

Apoptosis detection

OS cells with the applied genetic modifications were seeded into six-well plates (at $1 \times 10^5$ cells in each well) and cultured. The detailed protocols for cell apoptosis assays, including nuclear TUNEL staining, Annexin V FACS, and caspase-3 activity assay, were described in the previous studies.

ROS detection

OS cells with applied genetic treatments were seeded into the six-well plates at $1 \times 10^5$ cells per well and stained with CellROX fluorescence dye (Thermo Fisher Scientific, Shanghai, China) for 1 h in the dark. The CellROX fluorescence intensity (at 625 nm) was detected and the representative CellROX images were presented as well.

JC-1 assay

OS cells were seeded into the six-well plates and stained with JC-1 fluorescence dye for 30 min. JC-1 intensity was quantified via a fluorescence spectrophotometer (F-7000, Hitachi, Japan) at test-wavelength of 488 nm (green). The representative JC-1 images, integrating both green fluorescence (at 488 nm) and red fluorescence (at 625 nm) channels, were presented as well.

ARE reporter assay

The primary human OS cells, pOS-1, were initially seeded in six-well plates at 50%-60% confluence. Cells were then transfected with the ARE-inducible firefly luciferase vector (from Dr. Jiang at Nanjing Medical University). Following the applied genetic treatment, total cell lysates (30 μg per treatment) were subjected to ARE-reporter luciferase activity assay under a luminescence machine.

Mice xenograft assay

The CB.17 female SCID mice and the nude mice were maintained under the Animal Facility of Soochow University (Suzhou, China). For each mouse, $5 \times 10^6$ OS cells (in Matrigel-containing medium, no serum) were s.c. injected to the flanks. The subcutaneous OS xenografts were established within 3 weeks with each tumor close to 100 mm³ in volume. Tumor-bearing mice were randomly divided into three groups and were treated as described. Mice body weight and bidimensional tumor measurements were recorded every 7 days. Tumor volume was estimated using the standard formula: (length × width²)/2. For the in situ OS model, POS-1 cells ($3 \times 10^6$ cells per mouse) with applied genetic modifications were injected to the proximal tibia of the nude mice. Twenty-four days after cell injection, in situ tumors were visualized under X-ray film. Tumors were isolated and measured and tumor tissues tested by quantitative real-time PCR analyses. The animal protocols were approved by IACUC and Ethic Committee of Soochow University.

Statistical analysis

Statistical analysis was performed with SPSS version 23.0 (SPSS, Chicago, IL, USA). All quantitative data were presented as the mean ± standard deviation (SD). Differences between two groups were compared by the Student’s t test when they had a normal distribution. A one-way analysis of variance (ANOVA) followed by a Scheffé and Tukey test was used to compare data among groups when they had a normal distribution and homogeneous variances. p values less than 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.03.006.

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AUTHOR CONTRIBUTIONS

H.S., L.Z., C.Y., Q.J., X.Z., X.W., and C.C. conceived, designed, and supervised the study. H.S., L.Z., C.Y., Y.L., Z.Z., Q.J., X.Z., X.W., and C.C. collected samples, performed the experiments, and analyzed the data. Q.J., X.Z., X.W., and C.C. wrote the paper. All authors reviewed and approved the final manuscript.
DECLARATION OF INTERESTS
The authors declare no competing interests.

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