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Original Article

Chk2 deletion rescues Bmi1 deficiency-induced mandibular osteoporosis by blocking DNA damage response pathway

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Abstract: Objectives: Bmi1 deficiency has been proved to be able to cause mandibular osteoporosis through suppressing oxidative stress. However, the role of DNA damage response pathway in this pathogenesis had not been well understood. In this study, we investigate whether mandibular osteoporosis induced by Bmi1 deficiency could be rescued by blocked DNA damage response pathway. Methods: The protein expression levels of antioxidant enzymes and DNA damage and damage response pathway molecules in mandibular tissue were examined using Western blots. Double knockout mice that lacked both Bmi1 and Chk2 were generated and their mandibular phenotypes were compared at 6 weeks old to wild-type, Chk2$^{-/-}$, and Bmi1$^{-/-}$ mice using radiograph, micro-CT, histopathology, cellular and molecular techniques. Results: Bmi1 deficiency induces oxidative stress and DNA damage and activates DNA damage response pathways in mouse mandibles. Chk2 deletion rescued mandibular osteoporosis through promoting formation of osteoblastic bone as well as decreasing osteoclastic bone resorption. Mechanistically, Chk2 deletion suppressed oxidative stress, DNA damage, as well as cell senescence. In addition, it boosted proliferation of bone marrow mesenchymal stem cells (BM-MSCs) that derived from mandible through blocking the DNA damage response pathway. Conclusion: Abolish the expression of Chk2 could rescue Bmi1 deficiency-related mandibular osteoporosis through promoting BM-MSC proliferation and osteoblastic bone formation, reducing osteoclastic bone resorption, decreasing oxidative stress, inhibiting damage of DNA and associated response pathways, suppressing cell senescence as well as senescence-associated secretory phenotype (SASP). These findings offer a theoretical basis for using Chk2 or p53 inhibitors to prevent and treat age-related mandibular osteoporosis.

Keywords: Bmi1, mandibular osteoporosis, Chk2, DNA damage, DNA damage response pathway

Introduction

It has been well established that as an extensive skeletal disease that often involve multiple sites, osteoporosis is characterized by reduced bone mass, bone structure destruction, as well as an elevated fracture risk [1]. Osteoporosis can affect bones throughout the body, including the maxilla and mandible, causing tooth loss, gum disease, and an increased risk of infections and abscesses [2].

Bmi1 is a protein that belongs to the polycomb-repressor-complex-1 and regulates gene silencing through maintaining the normal structure of chromatin. Bmi1 also participates in stem cell self-renewal [3]. Even though many previous studies have conducted examine Bmi1 functions, including regulation of response upon DNA damage, cellular energetics, and its roles in various pathogenesis, much remains to be explored [4]. A study using a Bmi1 knockout mouse model showed that these mice exhibit a premature aging phenotype and reduced self-renewal of neural, hematopoietic, and mesenchymal stem cells [5-8]. Recent research has found that overexpression of Bmi1 in mesenchymal stem cells can inactivate p16-p19 signal and decrease oxidative stress which results

in an anti-aging and anti-osteoporosis effects [9, 10]. Bmi1 deletion, on the other hand, has been linked to mandibular osteoporosis [11, 12], but supplementing antioxidants, deleting p16, or using MSC-based therapy has been shown to improve this condition [11-15]. However, it is unknown whether Bmi1 deficiency induced mandibular osteoporosis by activating DNA damage response pathway.

It has been known that many exogenous induces, such as UV radiation as well as radiation from ionize, and endogenous induces like reactive-oxygen-species (ROS) produced during normal respiration can induce DNA damage [16]. Many pathways are involved in response of DNA damage such as the ATM-Chk2-p53 axis as well as the ATR-Chk1-CDK axis [17]. Collectively, these signal pathways can induce arrestment of cell cycle and cell senescence and/or cell apoptosis when receiving signals related to the damage [18]. Chk2 is a key mediator of DNA damage signaling in response to double-strand DNA breaks induced by ionizing radiation. Chk2 is constitutively expressed throughout the cell cycle. In cells without DNA damage, Chk2 exists as an inactive monomer. However, in response to double-strand DNA breaks, the ATM (phosphatidylinositol 3-kinase family checkpoint protein kinase ataxia telangiectasia mutated) phosphorylates Chk2, triggers dimerization and autophosphorylation, and then dissociates into active monomer [19]. Active Chk2 phosphorylates proteins that function in DNA repair, cell cycle regulation, and apoptosis. Thus, target proteins phosphorylated by Chk2 affect genome maintenance, cell cycle progression, and cell viability [20]. Studies have shown the phenotypes of Bmi1 knockout mice can be at least partially rescued by Chk2 knockout which also increases the survival time [21]. A recent study found that deletion of Chk2 in parathyroid hormone-related peptide 1-84 knockin mice enhances endochondral bone formation and osteogenesis [22], but it is unclear whether this will also rescue the premature osteoporosis phenotypes in Bmi1 deficient mice.

Current study was designed to decipher whether Bmi1 deficiency induces oxidative stress and DNA damage as well as its role in response pathway activation upon DNA damage in mouse mandibles. We will compare the mandibular phenotypes of 6 weeks old wild-type, Bmi1-deficient, Chk2-deficient, and Chk2-Bmi1 double knockout mice using radiographs, micro-CT, histology, and histochemical staining. Additionally, we will examine the changes of indicators that associated with oxidative stress, DNA damage, cell proliferation as well as senescence in vivo and ex vivo with mandibular tissue from these mice to better understand whether Chk2 deletion can rescue the mandibular osteoporosis caused by Bmi1 deficiency.

Materials and methods

Animal model

All mice were housed at the certified experimental animal facility of Nanjing Medical University. The protocol of this study was endorsed by the ethics committee of the university. The $Chk2^{+/-}$ C57BL/6J mice [23] we used in this study was a generous gift offered by Professor Noboru Motoyama from the Longevity Sciences National Institute of Japan. The $Bmi1^{-/-}$ mice applied in the study were generated in previous studies which described the process in detail [24]. Both models were fertile and were crossed with each other to generate the $Chk2^{-/-}Bmi1^{-/-}$ mice.

Imaging analysis

The mandibles were carefully removed and separated from all soft tissues, as described in [25]. The samples were then subjected to radiography and micro-CT analysis.

Histology examination

The tissue was collected and processed as previously introduced [26, 27]. Paraffin embedded block was cut in sections and were stained for Hematoxylin and Eosin (H&E) staining and histology which aimed to determine total collagen or tartrate-resistant acid phosphatase (TRAP) expression, as introduced in previous studies [26, 27].

Immunohistochemical staining

Sections were collected from the paraffin-embedded sample and were stained for following proteins: Osterix, SOD1, SOD2, γ-H2AX, ATM, p53, p16, and TNFα. The staining of the section followed the protocol introduced in a

Table 1. Primers used for quantitative real-time PCR

Name	S/AS	sequence
P21	S	CCTGGTGATGTCCGACCTG
	AS	CCATGAGCGCATCGCAATC
P16	S	CGCAGGTTCTTGGTCACTGT
	AS	TGTTCACGAAAGCCAGAGCG
IL-1α	S	CGAAGACTACAGTTCTGCCATT
	AS	GACGTTTCAGAGGTTCTCAGAG
IL-1β	S	GCAACTGTTCCTGAACTCAACT
	AS	ATCTTTTGGGGTCCGTCAACT
IL-6	S	TGTATGAACAACGATGATGCACTT
	AS	ACTCTGGCTTTGTCTTTCTTGTTATCT
NF-κB-p65	S	AGGCTTCTGGGCCTTATGTG
	AS	TGCTTCTCTCGCCAGGAATAC
GAPDH	S	CTTGCCAGACACAGATGATCG
	AS	GGGGACAGAAGTTGAGTTTC

previous study [28]. Briefly, the sections were first dewaxed and rehydrated before blocking with hydrogen peroxide (6%). The slide was then rinsed with PBS staining with following primary antibodies overnight at 4°C: SOD1/2 (from Abcam, MA), γ-H2AX (from Cell Signaling Technology, Danvers, MA), ATM (from Abcam, MA), p53 (from Cell Signaling Technology, Danvers, MA), p16 (from Abcam, MA), and TNFα (from Santa Cluz). After staining with the primary antibody, the slides were then rinsed with PBS before incubating with secondary antibodies (biotinylated goat anti-rabbit IgG and goat anti-mouse IgG from Sigma). Next, slides were rinsed before incubating 30 minutes with Vectastain Elite ABC reagent (from Vector Laboratories). Finally, the labeling process was completed by adding 3,3-diaminobenzidine (2.5 mg/ml) and counterstaining with Mayer's Hematoxylin.

Western blot analysis

Total protein was collected from the mandibles of the animal. To examine the expression levels of targeted protein, equal amount (20 μg) of total protein lysates were loaded and separated on SDS-PAGE gel before transferring to polyvinylidene fluoride (PVDF) membranes. Next, blocking buffer that containing 5% BSA was used to block the membrane for one hour at room temperature. The membrane was rinsed and stained by following primary antibodies: SOD1/2 (from Abcam, MA), γ-H2AX (from Cell Signaling Technology, Danvers, MA), ATM (from Abcam, MA), Chk2/p-Chk2 (from Abcam, MA), p53 (from Cell Signaling Technology, Danvers, MA), p21 (from Santa Cruz Biotechnology), p16 (from Abcam, MA) as well as p19 (from Abcam, MA). The beta-actin (from Bioworld Technology, St. Louis Park, MN) was applied as an internal control. After staining, the member was rinsed and stained with secondary antibodies before sending for chemiluminescent examination as previously described [22]. All experiments were triplicated to avoid potential error.

Real-time PCR analysis

Gene expressions were analyzed by extracting total RNA from freshly isolated tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) following producer's recommendation. The PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan) was used to generate the cDNA which was used for real-time PCR [25]. The primers used in this study were summarized in **Table 1**.

Cell culture

Bone marrow mesenchymal stem cells (BM-MSCs) were obtained from the mandibles of the mice as described previously [29]. In brief, the mandibles were collected and trimmed to remove surrounding soft tissues and teeth. The tissue was first digested with type I collagenase (Sigma-Aldrich, St. Louis, MO) at 3 mg/mL and dispase-II (Sigma-Aldrich, St. Louis, MO) for 60 minutes at 37°. The lysate was then passed through 70-mm cell mesh to obtain suspension with single cells before seeding. The cells were passed every 7 days and the third-passage of BM-MSCs was used for immunofluorescence staining for γ-H2AX, ATM, or for the ethynyl deoxyuridine (EdU) incorporation assay or beta-galactosidase (SA-β-gal) as described previously [25].

Statistical analysis

All experiments were triplicated with at least 5 samples in each group. The data was expressed as mean ± SEM. The ANOVA and Bonferroni tests were used to compare the differences as appropriate. *P* value less than 0.05 was considered as statistically significantly.

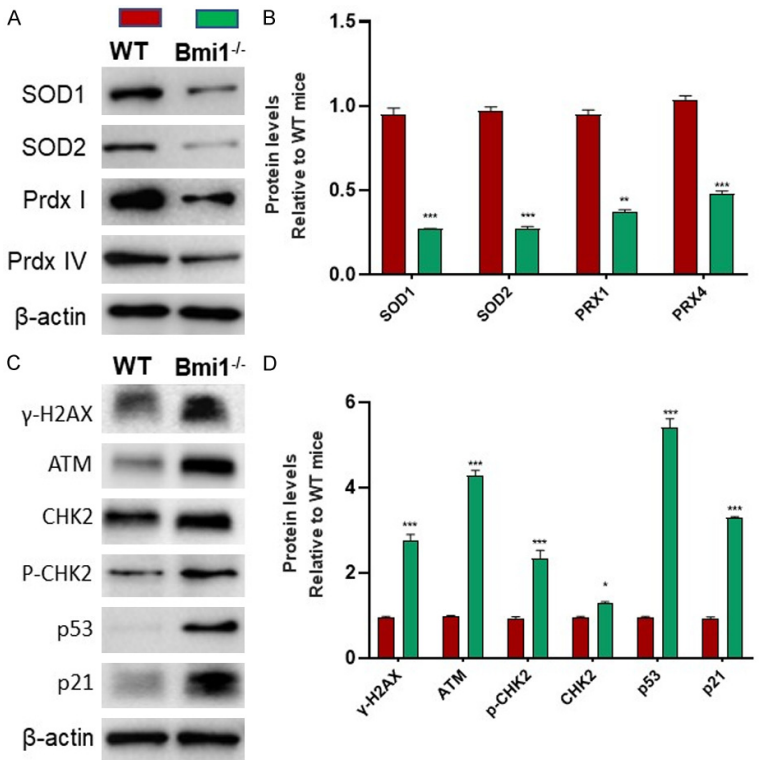

Figure 1. Bmi1 deficiency induces oxidative stress and DNA damage and activates DNA damage response pathways in mouse mandibles. A. Western blots of the protein expression levels of antioxidant enzymes in mandibular extracts from 6-week-old WT and *Bmi1*$^{-/-}$ mice. B. Protein expression levels relative to β-actin, assessed by densitometric analysis and expressed as relative levels to WT mice. C. Western blots of the protein expression levels of DNA damage and damage response pathway molecules in mandibular extracts from 6-week-old WT and *Bmi1*$^{-/-}$ mice. D. Protein expression levels relative to β-actin, assessed by densitometric analysis and expressed as relative levels to WT mice. Values are mean ± S.E.M. of 3 determinations per group. **: P<0.01, ***: P<0.001 compared with WT mice.

Results

Bmi1 deficiency induced oxidative stress as well as DNA damage and activated the associated pathways in mouse mandibles

To examine the role of Bmi1 in regulating oxidative stress and DNA damage, as well the activation of associated pathways in mouse mandibles, we analyzed the expressions of related protein. Results showed that protein expressions of SOD1/2 and Prdx I/IV were significantly downregulated, while levels of γ-H2AX, ATM, Chk2, p-Chk2, p53, and p21, were significantly upregulated in Bmi1$^{-/-}$ mice compared to controls (**Figure 1A-D**). This result demonstrated Bmi1 deficiency could trigger oxidative stress as well as DNA damage and activate related pathways in mouse mandibles.

Chk2 deletion rescued Bmi1 deficiency-induced mandibular osteoporosis by boosting the bone formation while decreasing bone resorption

To investigate the ability of Chk2 deletion to rescue Bmi1-induced mandibular osteoporosis, double knockout mice (Chk2$^{-/-}$Bmi1$^{-/-}$) were generated and their mandibular phenotypes were compared to wild-type, Chk2$^{-/-}$, and Bmi1$^{-/-}$ mice of 6 weeks old using radiographs, micro-CT scans, histochemical staining, and immunostaining. Results showed that alveolar bone mineral density, total collagen areas, as well as the ratio of osterix-positive cells were increased in Chk2$^{-/-}$ group and significantly decreased in Bmi1$^{-/-}$ group compared to wild-type controls. On the other hand, these parameters significantly elevated in Chk2$^{-/-}$Bmi1$^{-/-}$ mice comparing to the Bmi1$^{-/-}$ mice. Additionally, the surface of osteoclast was reduced in Chk2$^{-/-}$ mice and increased in Bmi1$^{-/-}$ mice compared to wild-type controls, but significantly reduced in the double knockout mice compared to the Bmi1$^{-/-}$ mice (**Figure 2A-I**). These findings demonstrate that Chk2 deletion rescues Bmi1-induced mandibular osteoporosis by boosting the formation of osteoblastic and reducing osteoclastic bone resorption.

Chk2 deletion rescues oxidative stress as well as DNA damage in mandibles of Bmi1$^{-/-}$ mice

To investigate if the rescue of Bmi1 deficiency-induced mandibular osteoporosis by Chk2 deletion is linked to the reduced oxidative stress as well as DNA damage in mandibles, the alterations of expressions of SOD1/2 and γ-H2AX were analyzed using immunohistochemical staining and Western blot analysis in mandibular tissue from the four genotypes of mice. The results indicated that the SOD1/2

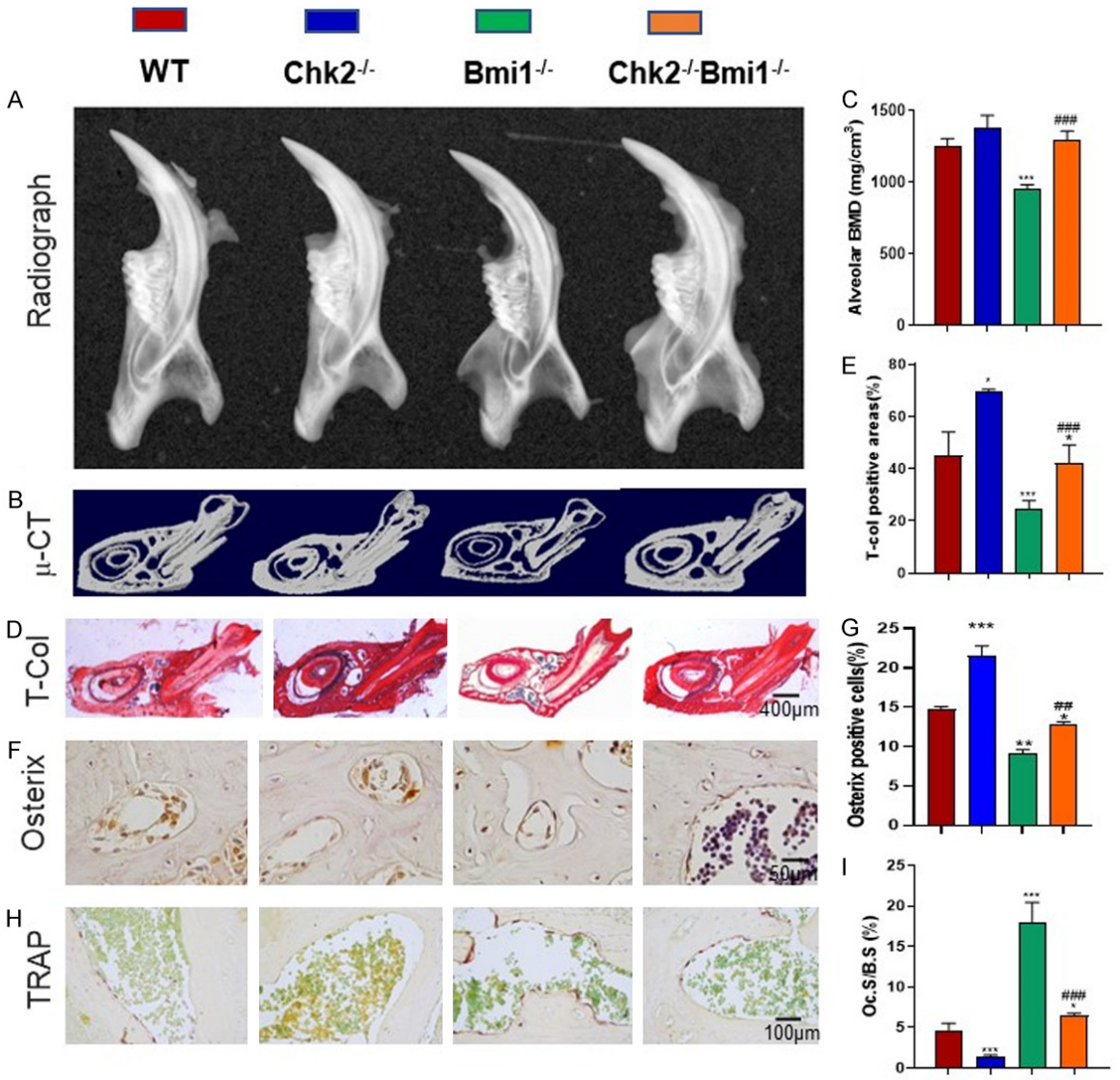

Figure 2. Chk2 deletion rescues mandibular osteoporosis induced by Bmi1 deficiency by stimulating osteoblastic bone formation and inhibiting osteoclastic bone resorption. (A) Representative radiographs of mandibles from 6-week-old WT, Chk2$^{-/-}$, Bmi1$^{-/-}$ and Chk2$^{-/-}$Bmi1$^{-/-}$ mice. (B) Representative images of micro-CT-scanned sections through the first molar. (C) Alveolar BMD. (D) Representative micrographs of decalcified paraffin-embedded sections through the first molars and the incisors from above 4 genotype mice were stained histochemically for total collagen (T-Col) and (E) T-Col positive area relative to tissue area. (F) The sections were stained immunohistochemically for osterix and (G) the percentage of osterix positive cells. (H) The sections were stained histochemically for tartrate-resistant acid phosphatase (TRAP) and (I) Osteoclastic surface relative to bone surface (Oc.S/B.S, %). Values are mean ± S.E.M. of 6 determinations per group. *: P<0.05, **: P<0.01, ***: P<0.001 compared with WT mice; ###: P<0.001 compared with Bmi1$^{-/-}$ mice.

positive cells as well as the protein expressions significantly increased in Chk2$^{-/-}$ mice and significantly decreased in Bmi1$^{-/-}$ mice compared to controls, but were significantly higher among Chk2$^{-/-}$Bmi1$^{-/-}$ mice compared to the Bmi1$^{-/-}$ mice (**Figure 3A-D**, **3G** and **3H**). On the other hand, the ratio of γ-H2AX expressing cells and protein expressions were significantly lower among Chk2$^{-/-}$ mice and significantly higher in Bmi1$^{-/-}$ mice comparing with the wide type controls, but significantly lower among Chk2$^{-/-}$Bmi1$^{-/-}$ mice comparing with the Bmi1$^{-/-}$ mice (**Figure 3E-H**). These findings suggest that Chk2 deletion can rescue oxidative stress and DNA damage in mandibles of Bmi1$^{-/-}$ mice.

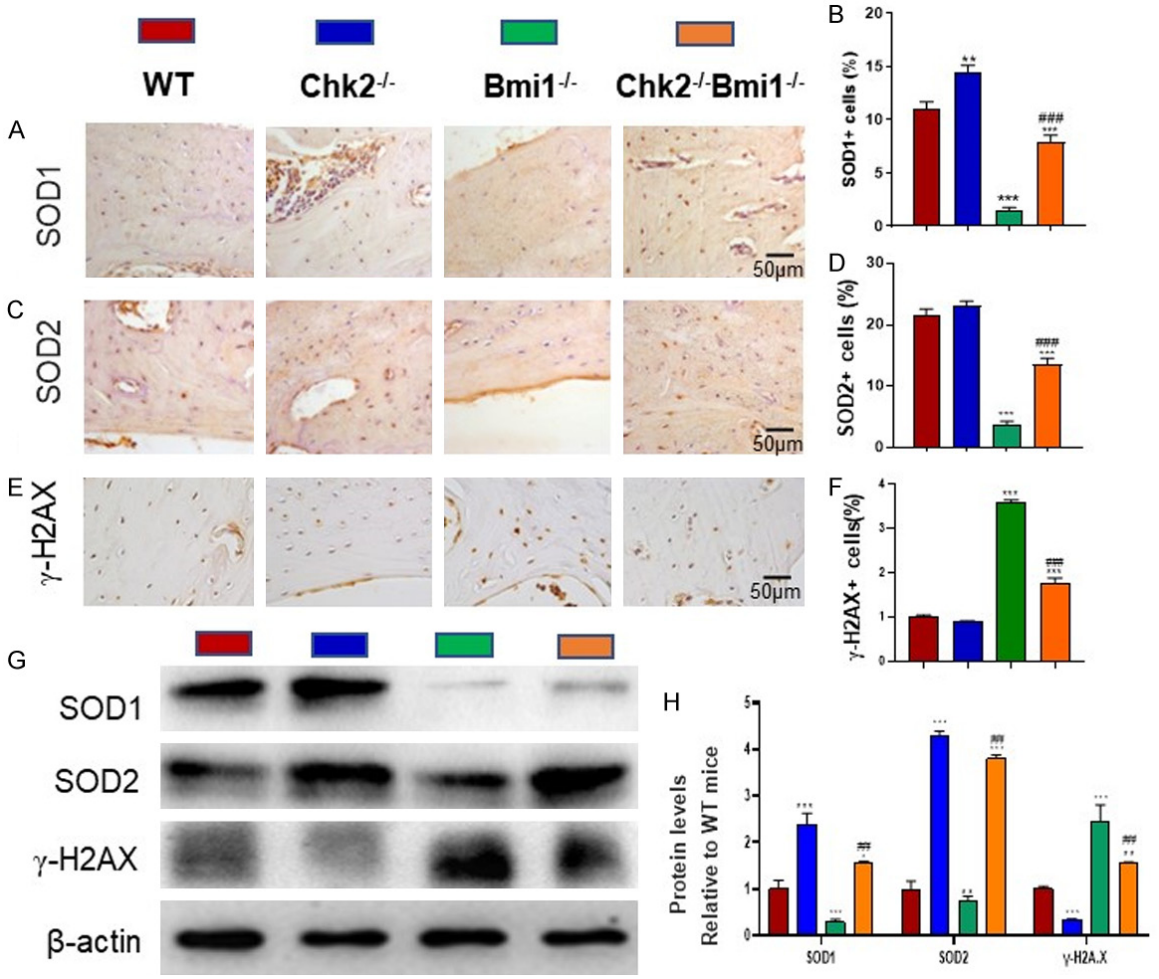

Figure 3. Chk2 deletion rescues oxidative stress and DNA damage in mandibles induced by Bmi1 deficiency. Representative micrographs of decalcified paraffin-embedded sections through the first molars and the incisors from 6-week-old from above 4 genotype mice were stained immunohistochemically for (A) SOD1, (C) SOD2 and (E) γ-H2AX. The percentages of (B) SOD1 positive cells, (D) SOD2 positive cells and (F) γ-H2AX positive cells. (G) Western blots of mandibular extracts were carried out for expression of SOD1, SOD2 and γ-H2AX. (H) Protein levels relative to β-actin were assessed by densitometric analysis and expressed as relative levels to WT mice. Values are mean ± S.E.M. of 6 determinations per group. **: P<0.01, ***: P<0.001 compared with WT mice; ###: P<0.001 compared with $Bmi1^{-/-}$ mice.

Chk2 deletion inactivates DNA damage response pathway in mandibles of Bmi1 deficient mice

To determine if the rescue of Bmi1 deficiency related phenotypes by knocking out Chk2 was related to pathway inactivation, expressions of ATM, p53, as well as p21 were measured using immunohistochemical staining and Western blot analysis in mandibular tissue from the four genotypes of mice. Our data demonstrated that the percentage of ATM and p53 positive cells, as well as ATM, p53, and p21 expressions were decreased in $Chk2^{-/-}$ mice and increased in $Bmi1^{-/-}$ mice comparing with controls, but significantly lower in $Chk2^{-/-}Bmi1^{-/-}$ mice comparing with $Bmi1^{-/-}$ mice (**Figure 4A-F**). Above discoveries demonstrated that deleting Chk2 blocked the DNA damage response pathway in mandibles of Bmi1 deficient mice.

Chk2 deletion rescues cell senescence and SASP in mandibles induced by Bmi1 deficiency

To determine the association between Chk2 deletion and the rescue of Bmi1 deficiency related phenotypes, we next studied its impact on cell senescence and SASP using immuno-

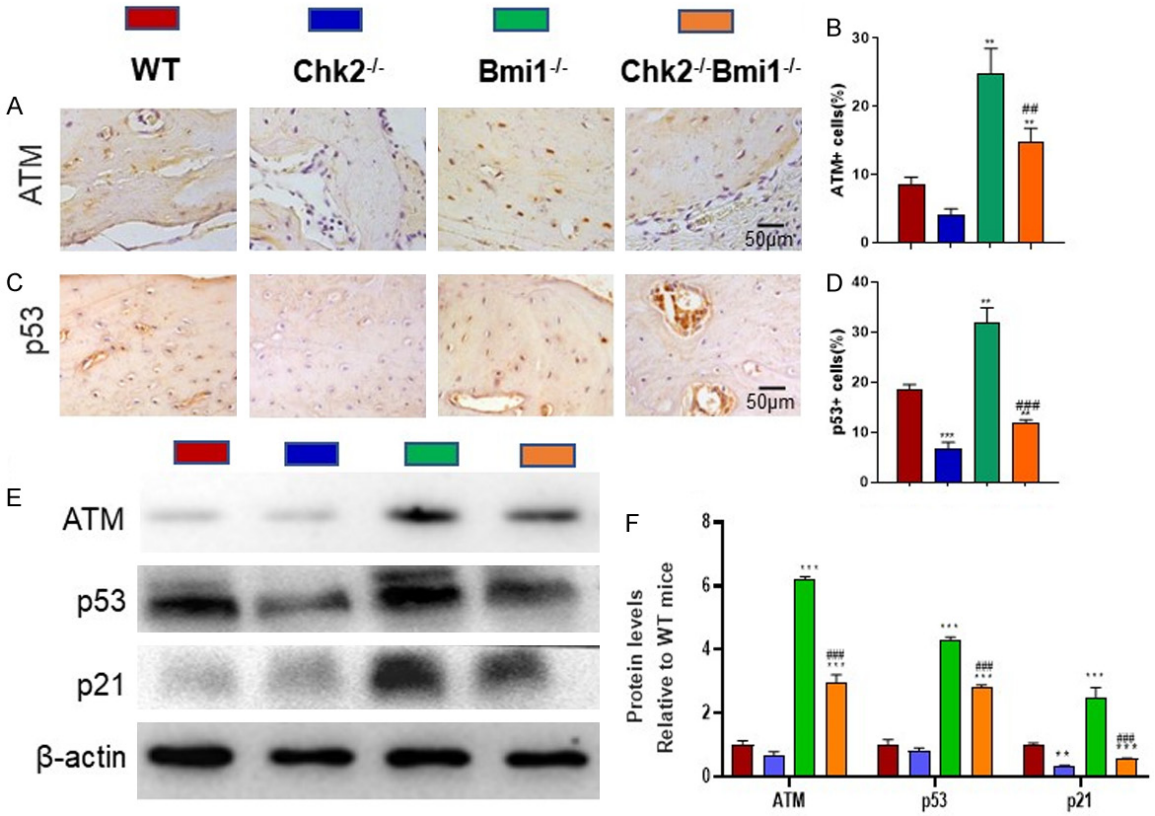

Figure 4. Chk2 deletion inactivates DNA damage response pathway in mandibles of Bmi1 deficient mice. Representative micrographs of decalcified paraffin-embedded sections through the first molars and the incisors from 6-week-old from above 4 genotype mice were stained immunohistochemically for (A) ATM and (C) p53. The percentages of (B) ATM positive cells and (D) p53 positive cells. (E) Western blots of mandibular extracts were carried out for expression of ATM, p53 and p21. (F) Protein levels relative to β-actin were assessed by densitometric analysis and expressed as relative levels to WT mice. Values are mean ± S.E.M. of 6 determinations per group. **: P<0.01, ***: P<0.001 compared with WT mice; ##: P<0.01; ###: P<0.001 compared with $Bmi1^{-/-}$ mice.

histochemistry, western-blot, as well as real-time PCR on mandibular tissue from four different genotype mice. Results showed that the ratio of cells expressing p16 as well as TNFα, levels of p16 and p19 protein, and expressions of p16, p19, IL-1α, IL-1β, IL-6, and NFκB-65 mRNA all significantly increased in $Bmi1^{-/-}$ mice comparing with controls, but significantly decreased in $Chk2^{-/-}Bmi1^{-/-}$ mice comparing with $Bmi1^{-/-}$ mice (**Figure 5A-G**). These results indicated that Chk2 deletion rescues Bmi1 deficiency related osteoporosis through reducing p16/p19-mediated cell senescence and SASP.

Chk2 deletion stimulates proliferation and inhibits cell senescence of mandible derived BM-MSCs by blocking DNA damage response pathway in Bmi1 deficient mice

To determine the impact of Chk2 deletion on stimulating proliferation and inhibiting cell senescence in mandible derived BM-MSCs from Bmi1 deficient mice, we evaluated alterations in DNA damage, the DNA damage response pathway molecule, proliferation, and senescence using immunofluorescence staining for γ-H2AX and Atm, EdU assay, as well as cytochemical SA-β-gal staining on mandible-derived BM-MSCs from the above-mentioned four genotype mice. Results showed a dramatic increase of $γ\text{-H2AX}^+$, Atm^+ and $SA\text{-β-gal}^+$ cells in $Bmi1^{-/-}$ mice comparing with wild-type controls while significantly decreased in $Chk2^{-/-}Bmi1^{-/-}$ mice comparing to $Bmi1^{-/-}$ mice (**Figure 6A-D**, **6G** and **6H**). Conversely, the percentages of EdU^+ cells significantly reduced in $Bmi1^{-/-}$ mice comparing to controls, but significantly increased in $Chk2^{-/-}Bmi1^{-/-}$ mice comparing to $Bmi1^{-/-}$ mice (**Figure 6E** and **6F**). These results demonstrate that Chk2 deletion stimulates proliferation and inhibits cell senescence in

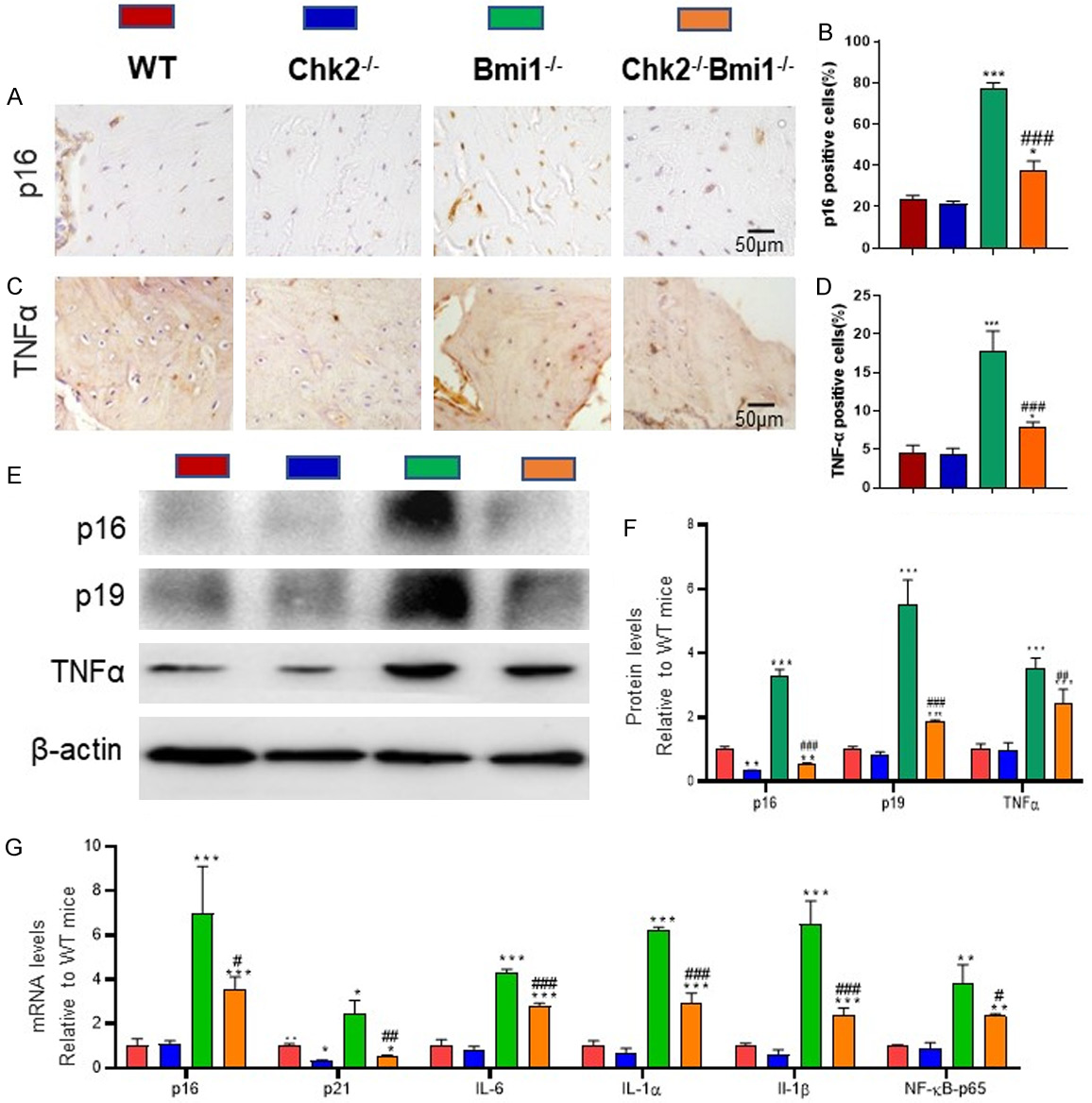

Figure 5. Chk2 deletion rescues cell senescence and SASP in mandibles induced by Bmi1 deficiency. Representative micrographs of decalcified paraffin-embedded sections through the first molars and the incisors from 6-week-old from above 4 genotype mice were stained immunohistochemically for (A) p16 and (C) TNFα. The percentages of (B) p16 positive cells and (D) TNFα positive cells. (E) Western blots of mandibular extracts were carried out for expression of p16, p19 and TNFα. (F) Protein levels relative to β-actin were assessed by densitometric analysis and expressed as relative levels to WT mice. (G) RT-PCR of tissue extracts of mandibles for expression of *p16* and *p19*, *IL-1α*, *IL-1β*, *IL-6*, *NFκB-65*. Messenger RNA expression assessed by real-time RT-PCR is calculated as a ratio relative to Gapdh, and expressed relative to WT mice. Values are mean ± S.E.M. of 6 determinations per group. *: P<0.05; **: P<0.01; ***: P<0.001 compared with WT mice; #: P<0.05; ##: P<0.01; ###: P<0.001 compared with Bmi1$^{-/-}$ mice.

Bmi1 deficient mice mandible-derived BMMSCs by blocking the pathways that related to DNA damage.

Discussion

It has been well established Bmi1 deficiency contributes to mandibular osteoporosis through increasing oxidative stress, as evidenced by the improvement seen in osteoporosis with antioxidant supplementation [11, 12]. However, the impact of Bmi1 deficiency on responding pathways that related to DNA Damage in mandibular osteoporosis was not previously known. This study confirmed that Bmi1 deficiency not only reduces antioxidant expression and induc-

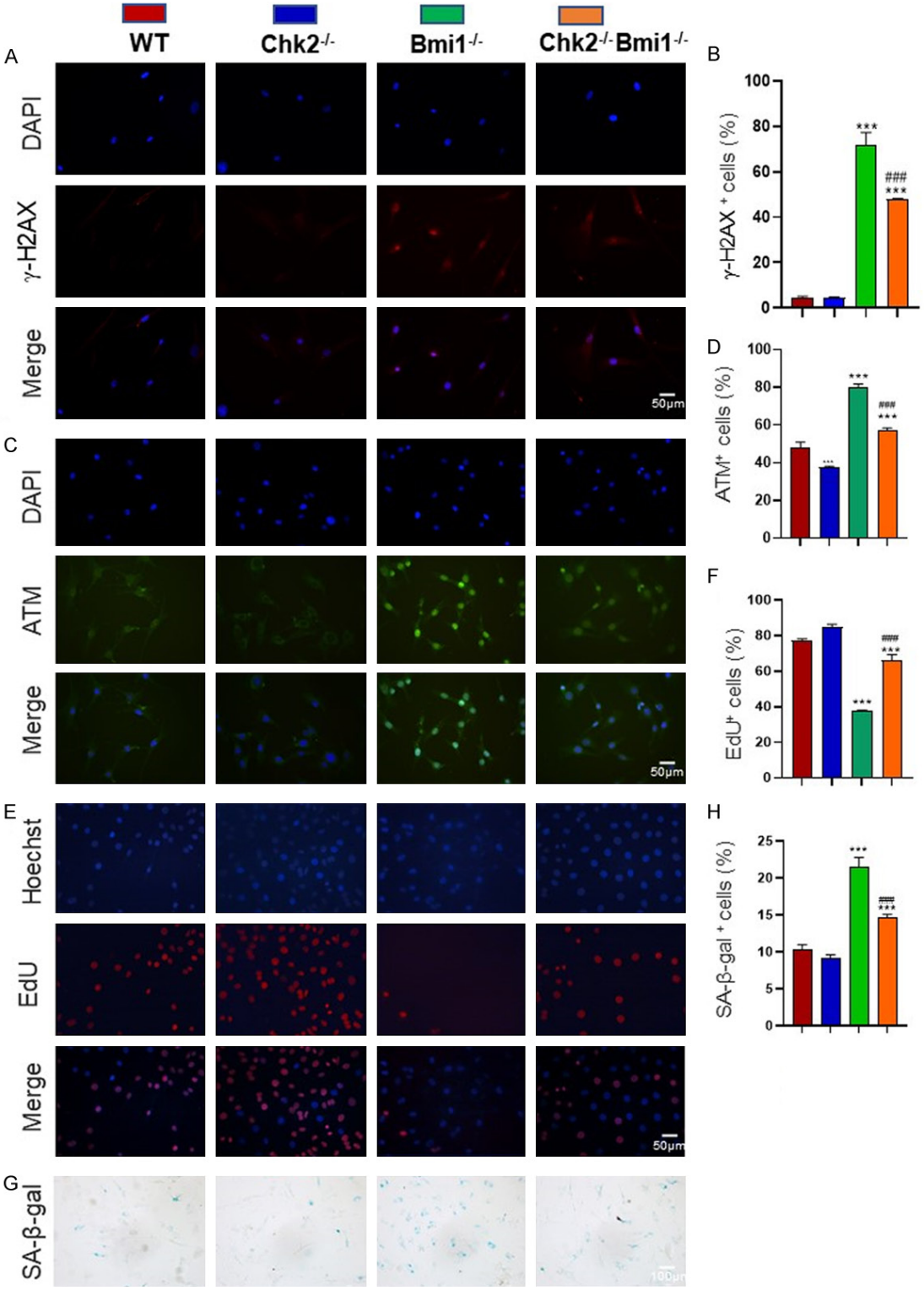

Figure 6. Chk2 deletion stimulates proliferation and inhibits cell senescence of mandible derived BM-MSCs by blocking DNA damage response pathway in Bmi1 deficient mice. Mandible-derived BM-MSCs from above 4 genotype mice were cultured, (A) Representative micrographs of immunofluorescence staining for DAPI (blue), γ-H2AX (red) and merge. (B) The percentages of γ-H2AX$^+$ cells relative to total cells. (C) Representative micrographs of immunofluorescence staining for DAPI (blue), ATM (green) and merge. (D) The percentages of ATM$^+$ cells relative to total cells. (E) EdU incorporation assay were performed, representative micrographs of immunofluorescence staining for Hoechst (Blue), EdU (Red) and merge. (F) The percentages of EdU positive cells relative to total cells. (G) Representative images of cells stained cytochemically for SA-β-gal. (H) The percentages of SA-β-gal positive cells relative to total cells. Values are mean ± S.E.M. of 3 determinations per group. ***: P<0.001 compared with WT mice; ###: P<0.001 compared with Bmi1$^{-/-}$ mice.

es oxidative stress, but also causes DNA damage as well as the activation of related signal pathways, as evidenced by the upregulation of γ-H2AX, ATM, Chk2, p-Chk2, p53, and p21 in mandibular tissue.

Premature aging has been previously observed in Bmi1 deficient mice, along with reduced capability of self-renewal in neural and hematopoietic stem cells as well as reduced MSC self-renewal and osteoblast differentiation capacity [5-7]. On the other hand, Bmi1 overexpression in MSCs reversed the delay of skeletal growth and osteoporosis, as well as ameliorated premature aging in Bmi1 deficient mice [9]. Our data indicates Bmi1 has an important role in protecting against mandibular osteoporosis by inactivating signal pathways that associated with DNA damage. Given the beneficial impact of Chk2 deletion on multi-organ phenotypes and increased survival time in mice with Bmi1 deficiency [21], as well as its ability to improve the bone formation of osteoblasts as well as growth [22], we next examined if Chk2 deletion could rescue Bmi1 ablation-induced osteoporosis by blocking the activation of signal pathways that related to DNA damage. Our current results demonstrated that Chk2 deficiency indeed rescued mandibular osteoporosis in Bmi1 deficient mice through increasing the bone formation and suppressing the bone resorption.

Bmi1 deficiency can result in premature aging, female infertility, and osteoporosis in long bones and mandibles by inducing oxidative stress and mitochondrial dysfunction [21, 30, 31]. Supplementation with antioxidants such as N-acetylcysteine (NAC) or pyrroloquinoline quinone (PQQ) can partially or largely rescue these effects of Bmi1 deficiency [11-13, 21, 30, 31]. In this study, we found that deletion of the Chk2 gene largely rescued the mandibular osteoporosis in Bmi1$^{-/-}$ mice through reducing oxidative stress as well as DNA damage.

p53 mainly maintains expressing of genes that related to anti-oxidant under physical conditions [32]. However, under severe and sustained stress, it promotes the expressions of genes that related to pro-oxidant that can cause cell death [33]. Our study found that Bmi1 deficiency caused a dramatic increase in p53 expression levels in the mandible, but these levels returned to normal in Chk2 knockout mice, suggesting that Chk2 deletion may protect against oxidative stress that occurs in Bmi1 deficient mandibles by downregulating p53. However, the specific mechanism of p53's role in regulating oxidative stress in the mandible remains to be investigated.

Accumulating gene mutations as well as DNA damage over time contributes significantly to cell aging. It has been known that DNA is constantly exposed to external and internal threats, leading to genetic damage, including exogenous agents such as biological particles, physical destruction, as well as chemistry products, and internal threats like abnormal DNA replicates, ROS, as well as responses that related to hydrolytic activities [16]. Evidence that DNA damage causes skeletal defects comes from the observation that mutations in genes that regulate DNA repair or the DNA damage response result in impaired bone development and disrupted bone homeostasis [34]. It has been known that, as an important component of the repair pathways, the ATM-Chk2-p53 axis is activated upon DNA damage as well as cell cycle errors. Chk2 and p53protein are phosphorylated by ATM when double-stranded DNA breaks (DSBs) occurs and this process can help initiating the repair activations [35]. Bmi1 has been shown to be able to participate in double-stranded DNA break repairment through the Bmi1/Rin1b E3 ubiquitin ligase [36]. Studies have also shown that ATM positively regulates osteoblast differentiation and bone formation, while p53 negatively regulates osteoblast proliferation and differentiation, and participates in bone formation [37]. Our data showed that ATM and p53 positive cells, expressions of ATM, p53, as well as p21 were reduced among Chk2$^{-/-}$ mice while increased among Bmi1 deficient mice. However, in Chk2 and Bmi1 double knockout mice, these levels were significantly decreased compared to Bmi1 single knockout mice. This study suggests that deletion of Chk2 rescues Bmi1 deficiency-induced mandibular osteoporosis by blocking pathways that related to DNA damage. The selective inhibition of Chk2 using Chk2 inhibitors may protect normal cells from the toxic effects that associated with polymerase inhibitors [38]. In addition, applicating p53 inhibitors has been proposed to protect normal tissues from chemo- and radiotherapy and to treat other stress-mediated p53-related pathologies [39]. Therefore, our findings provide a theoreti-

cal basis for the potential use of Chk2 or p53 inhibitors in preventing and treating senile osteoporosis.

It has shown that Bmi1 can regulate stem cell self-renewal. This is achieved by decreasing oxidative stress as well as DNA damage and suppressing the activation of pathways such as p16/Rb and p19/p53/p21 [21, 40, 41]. Bmi1 also helps maintain adult stem cells in some tissues by repressing genes that trigger cellular senescence and cell death. Compared to the replicating capacity of normal mouse embryonic fibroblasts (MEFs), which often can pass on to the 7 generations, Bmi1 deficient MEFs exhibit a premature ageing feature that can only pass up to the third generation. This unique characteristic has been linked to elevated p16Ink4a expression. Reexpression of Bmi1 in Bmi1-deficient MEFs can prevent premature senescence [42]. Bmi1 deficiency causes renal tubulointerstitial injury, female infertility, and osteoporosis through increased oxidative stress and activation of pathways that related to p16/Rb and p19/p53/p21 axis [11-13, 31, 43]. Deleting p16 has been shown to alleviate renal tubulointerstitial injury and mandibular osteoporosis [15, 43]. Meanwhile, Bmi1 overexpression in MSCs has anti-aging and anti-osteoporosis effects through reducing oxidative stress and deactivating the p16/p19 signal pathway [9]. We found that Bmi1 deficiency led to dramatic elevations of p16 and p19 protein expression and mRNA expression of p16 and p19, IL-1α, IL-1β, IL-6, and NFκB-65 comparing with wild-type controls. Nevertheless, these changes were reduced among Chk2 and Bmi1 double knockout mice comparing with Bmi1 deficient mice. Above observations demonstrate that deleting Chk2 can rescue Bmi1-deficiency-induced osteoporosis by inhibiting p16/p19-mediated cellular senescence and the Secretion of Pro-inflammatory Cytokines (SASP).

In conclusion, this study has confirmed that Bmi1 deficiency causes oxidative stress, DNA impairment, and activating related pathways. Additionally, it has demonstrated that Chk2 deletion can rescue mandibular osteoporosis in Bmi1-deficient mice by promoting BM-MSCs proliferation as well as bone formation and reducing bone resorption, oxidative stress, DNA damage, SASP as well as blocking the DNA Damage Response Pathway. These findings provide a theoretical basis for exploring the potential use of Chk2 or p53 inhibitors in diseases such as age-related osteoporosis.

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Disclosure of conflict of interest

None.

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