Osteoblast lineage Sod2 deficiency leads to an osteoporosis-like phenotype in mice

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Abstract
Osteoporosis is a systemic metabolic skeletal disease characterized by low bone mass and strength associated with fragility fractures. Oxidative stress, which results from elevated intracellular reactive oxygen species (ROS) and arises in the aging organism, is considered one of the critical factors contributing to osteoporosis. Mitochondrial (mt)ROS, as the superoxide anion (O$_2^-$) generated during mitochondrial respiration, are eliminated in the young organism by antioxidant defense mechanisms, including superoxide dismutase (SOD) 2, whose expression and activity are decreased in aging mesenchymal progenitor cells, accompanied by increased mtROS production. Using a mouse model of osteoblast lineage Sod2 deficiency, we observed significant bone loss in trabecular and cortical bone accompanied by decreased osteoblast activity, increased adipocyte accumulation in the bone marrow, and augmented osteoclast activity, suggestive of altered mesenchymal progenitor cell differentiation and osteoclastogenesis. Furthermore, osteoblast senescence was increased. To date, there are so far only a few studies suggesting a causal association between mtROS and cellular senescence in tissue in vivo. Targeting SOD2 to improve redox homeostasis may represent a potential therapeutic strategy for maintaining bone health during aging.

Keywords: skeletal aging; osteoporosis; mitochondrial dysfunction; reactive oxygen species; senescence

Introduction
Osteoporosis, a generalized metabolic skeletal disease, which is characterized by low bone mineral density (BMD) and structural degeneration of the bone tissue, predisposing to high fracture risk, is one of the major health problems affecting the aging society (Hendrickx et al., 2015). Epidemiological and preclinical studies
indicate that reactive oxygen species (ROS) are involved in the development of age-
related and postmenopausal osteoporosis (Almeida and O’Brien, 2013; Bonaccorsi et
al., 2018; Manolagas, 2010). The ROS level in bone increases with age as the
activity of antioxidant defense mechanisms decreases (Almeida et al., 2007; Chung
et al., 2011). ROS are mainly generated in the mitochondria during cellular
respiration and are eliminated in the young organism by antioxidant defense
systems, including the enzyme superoxide dismutase (SOD) 2 (Agidigbi and Kim,
2019; Almeida and O’Brien, 2013). Under physiological conditions, ROS are involved
in the regulation of bone remodeling where they facilitate the resorption of bone
tissue (Agidigbi and Kim, 2019; Wauquier et al., 2009). They promote bone turnover
by increasing osteoclast differentiation and activity in the healthy young organism,
whereas in the old organism, increased ROS production can induce the generation of
pro-inflammatory mediators, which both enhance osteoclastic bone degradation and
inhibit osteoblastic bone formation (Agidigbi and Kim, 2019).

Superoxide dismutases (SODs) are the first line of antioxidant defense enzymes
against ROS (Younus, 2018). They catalyze the dismutation of superoxide anion free
radical (O$_2^-$) into molecular oxygen and hydrogen peroxide; the latter is converted by
catalase to water. SODs serve as anti-inflammatory agents and SOD conjugates
have been demonstrated as potential therapeutic agents in age-related and
inflammatory diseases, including neutrophil-mediated inflammation (Fang et al.,
2009; Younus, 2018). In humans, three forms of SODs are present: the Cu- and Zn-
containing SOD1 and SOD3, which are located in the cytoplasm and the extracellular
compartment, respectively, and the Mn-containing SOD2, which is located in the
mitochondria and is necessary for eliminating superoxide radicals released mainly
from the mitochondrial complex III during cellular respiration, the main source of ROS
within a cell (Bigarella et al., 2014). During osteoblast differentiation, SOD2 is
upregulated to maintain mitochondrial function and osteoblast differentiation (Gao et al., 2018).

It has been shown that rat and human mesenchymal progenitor cell aging is associated with decreased SOD2 expression and activity and increased mitochondrial ROS (mtROS) production (Almeida and O’Brien, 2013; Chen et al., 2019; Stolzing et al., 2008; Stolzing and Scutt, 2006). Decreased SOD2 activity and mitochondrial oxidative stress have been demonstrated to be associated with senescence in the skin and brain (Flynn and Melov, 2013; Melov et al., 1999; Velarde et al., 2012). Cellular senescence is considered as a stress response normally induced by various extrinsic and intrinsic insults, including irradiation, oxidative stress and mitochondrial dysfunction, and was originally identified as a highly stable cell cycle arrest (Qadir et al., 2020). Recent studies indicated a crucial role of mitochondrial oxidative stress and functional SOD2 in implant osteointegration (Wang et al., 2020; Zhou et al, 2019). How mitochondrial oxidative damage affects bone integrity due to reduced SOD2 activity in osteoblast lineage cells is still unknown. Therefore, we established and characterized a mouse model of Sod2 deficiency in the osteoblast lineage to study the influence of ROS specifically generated in osteoblast lineage cells on bone metabolism.

The increased osteoblast lineage-specific mtROS generation resulted in an accumulation of adipocytes in the bone marrow, an altered osteoblast and osteoclast activity as well as increased osteoblast senescence and bone loss in vivo, implying SOD2 as a potential target for maintaining bone health during aging.
Results

Osteoblast lineage-specific Sod2 deficiency leads to bone loss in mice

To analyze the effect of osteoblast lineage-specific Sod2 deficiency on bone mass in mice, we deleted Sod2 by crossing Sod2<sup>fl/fl</sup> mice with Runx2<sup>Cre</sup> mice, which express Cre recombinase in osteoblast lineage cells (Rauch et al., 2010; Strassburger et al., 2005; Treiber et al., 2011). Female mice lacking Sod2 expression, hereafter referred to as Runx2<sup>CreSod2<sup>fl/fl</sup></sup>, displayed a decreased trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and an increased trabecular separation (Tb.Sp) at 12 and 52 weeks of age as determined by micro-computed tomography (µCT) in the femora, compared to their littermate control mice (Fig. 1A). Runx2<sup>CreSod2<sup>fl/fl</sup></sup> mice aged 12 weeks revealed a bone volume fraction that was not significantly different from Sod2<sup>fl/fl</sup> mice at the age of 52 weeks.

Moreover, female Runx2<sup>CreSod2<sup>fl/fl</sup></sup> mice displayed decreased tissue mineral (TMD) and cortical thickness (Ct.Th) compared to control mice (Fig. 1B, C). In vertebrae of female Runx2<sup>CreSod2<sup>fl/fl</sup></sup> mice, the trabecular bone volume fraction and Tb.N were decreased and Tb.Sp was increased (Fig. 1D,E). Male mice with osteoblast-lineage specific Sod2 deficiency also displayed a decreased BV/TV, Tb.N, Tb.Th, and an increased Tb.Sp at 12 and 52 weeks of age as determined by micro-computed tomography (µCT) in the femora, compared to their littermate control mice (Fig. S1A).

In male Runx2<sup>CreSod2<sup>fl/fl</sup></sup> mice aged 12 and 52 weeks, cortical TMD and Ct.Th were also decreased (Fig. S1B)

Because efficient recombination by Runx2<sup>Cre</sup> was also observed at sites of endochondral bone formation (Rauch et al., 2020), we analyzed the growth plate thickness and found a significantly decreased growth plate thickness in the femora of female Runx2<sup>CreSod2<sup>fl/fl</sup></sup> mice aged 12 weeks (Fig. S2), whereas male mice did not show significant growth plate thickness changes (results not shown). Because
osteoblast lineage-specific Sod2 deficiency in female mice led to a more pronounced femoral trabecular skeletal phenotype compared to male mice, we used the long bones of female mice for further analyses, unless otherwise specified.

To analyze the cellular changes underlying the skeletal phenotype of female Runx2CreSod2fl/fl mice, bone histomorphometry was performed and revealed a lower osteoblast number (Fig. 2A) and an increased osteoclast number in Runx2CreSod2fl/fl mice when aged 12 and 52 weeks (Fig. 2B). The cortical bone marrow area displayed a higher adipocyte number and adipocyte area in the femora of female Runx2CreSod2fl/fl mice aged 52 weeks (Fig. 2C,D).

**Higher ROS generation in Sod2-deficient osteoblast lineage cells impairs the proliferation and differentiation of osteoblasts and mesenchymal osteoprogenitor cells**

Dihydroethidium (DHE) staining was performed of osteoblasts in culture to analyze ROS generation due to osteoblast lineage-specific Sod2 deficiency. Osteoblasts from Runx2CreSod2fl/fl mice revealed increased ROS generation by an increased corrected total cell fluorescence (CTCF) of DHE-positive cells compared to osteoblasts from control mice (Fig. 3A). We detected significantly reduced Sod2 expression in osteoblasts isolated from the long bones of Runx2CreSod2fl/fl mice in comparison with its expression in osteoblasts from Sod2fl/fl mice (Fig. 3B). Proliferation of osteoblasts of Sod2fl/fl and Runx2CreSod2fl/fl mice was analyzed by BrdU assay. The proliferation of osteoblasts from Runx2CreSod2fl/fl mice was significantly reduced (Fig. 3C). To investigate the influence of higher ROS generation in osteoblast lineage cells on osteogenic differentiation, both, mesenchymal osteoprogenitor cells and osteoblasts from Sod2fl/fl and Runx2CreSod2fl/fl mice were cultured in differentiation medium. Mesenchymal
osteoprogenitor cells were cultured for 10 days and osteoblasts were cultured for 14 days in osteogenic differentiation medium. As expected, the expression of the osteogenic differentiation markers runt-related transcription factor (Runx)2 and alkaline phosphatase (Alpl) was increased at day 10 of differentiation in osteoprogenitor cells from Sod2\(^{fl/fl}\) mice (Fig. 3D). Runx2 and Alpl expression was decreased at day 10 in osteoprogenitor cells from Runx2CreSod2\(^{fl/fl}\) mice compared to their expression in osteoprogenitor cells from Sod2\(^{fl/fl}\) mice. At day 10, peroxisome proliferator-activated receptor gamma (Ppar\(\gamma\)) expression was upregulated in osteoprogenitor cells from Sod2\(^{fl/fl}\) mice, while the upregulation in osteoprogenitor cells from Runx2CreSod2\(^{fl/fl}\) mice tended to be higher. Moreover, adipogenesis markers CCAAT/enhancer binding protein alpha (Cebp\(\alpha\)), adipocyte P2 (aP2), perilipin1 (Plin1) were significantly upregulated in osteoprogenitor cells with Sod2 deficiency (Fig. 3E). In osteoblasts from Runx2CreSod2\(^{fl/fl}\) mice, the receptor activator of nuclear factor kappa B ligand (Rankl)/ osteoprotegerin (Opg) ratio was significantly increased compared to osteoblasts from control mice (Fig. 4A). In agreement with the decreased Runx2 and Alpl expression, osteoblast differentiation was impaired, as revealed by reduced alkaline phosphatase activity staining as well as reduced von Kossa and alizarin red staining in osteoblast cultures of Runx2CreSod2\(^{fl/fl}\) mice (Fig. 4B,C). Oil-red O staining demonstrated higher adipogenesis in mesenchymal osteoprogenitor cultures from Runx2CreSod2\(^{fl/fl}\) mice (Fig. 4B, C).
Increased senescence of osteoblasts in osteoblast lineage-specific Sod2-deficient mice

We applied the senescence-associated β-galactosidase (SA-β-Gal) activity assay to analyze the influence of osteoblast lineage-specific Sod2 deficiency on the senescence status of osteoblasts in vivo. Obvious blue staining indicating SA-β-Gal activity in osteoblasts on the surface of bone trabeculae in the femora in both, Sod2^{fl/fl} and Runx2CreSod2^{fl/fl} mice aged 52 weeks was observed, although osteoblast-specific Sod2 deficiency led to an increased osteoblastic SA-β-Gal activity staining (Fig. 5A). To verify the increased SA-β-Gal activity observed in vivo, we stained osteoblasts in cultures for SA-β-Gal activity. The number of osteoblasts with SA-β-Gal activity from Runx2CreSod^{fl/fl} mice in culture was increased compared to that of SA-β-Gal activity-positive osteoblasts from Sod2^{fl/fl} mice (Fig. 5B).

Furthermore, we analyzed senescence-associated gene marker expression. The expression of the tumor suppression markers p21 and p16^{INK4a} was significantly increased in osteoblasts from Runx2CreSod^{fl/fl} mice (Fig. 5C). Consistent with this, we detected more p16^{INK4a}-positive osteoblasts on the trabecular surfaces in the femora of Runx2CreSod^{fl/fl} mice (Fig. 5D).

We could not detect any difference regarding apoptotic osteoblasts in the bone tissue of the two mouse genotypes (Fig. 6A). Moreover, in osteoblasts we analyzed senescence-associated secretory phenotype (SASP) marker expression causing premature senescence (Qadir et al., 2020). SASP factors interleukin (IL)6 and tumor necrosis factor (TNF)α were upregulated in osteoblasts surrounding the trabecular bone in the femora of Runx2CreSod2^{fl/fl} mice in comparison with Sod2^{fl/fl} mice (Fig. 6B,C). The expression of the transcription factor FoxO1, which preserves redox balance to ensure bone redox homeostasis (Ma et al., 2020) was also upregulated in
response to ROS in osteoblasts enclosing the trabecular bone of Runx2CreSod2fl/fl mice compared to Sod2fl/fl mice (Fig. 6B,C). qRT-PCR expression analysis confirmed the upregulation of TNFα and Il6 in mesenchymal osteoprogenitor cells from Runx2CreSod2fl/fl mice (Fig. 7A). In line, by western blotting FoxO1 protein expression could be demonstrated to be upregulated in isolated osteoblasts from Runx2CreSod2fl/fl mice (Fig. 7B).

Discussion

Although it has been reported that oxidative stress in osteoblasts plays a significant role in the pathogenesis of osteoporosis (Almeida et al., 2007; Rached et al., 2010), with SOD2 being important for removal of excessive ROS (Younus et al., 2018), the exact molecular and functional consequences of mtROS accumulation in osteoblastic cells are still not completely understood. To our best knowledge, there is only one preclinical study in bone cell-specific Sod2-deficient mice (Dmp1CreSod2fl/fl), which allows investigation of oxidative stress selectively in late osteoblasts and terminally differentiated osteocytes (Kobayashi et al., 2015), but not in differentiating osteoblast precursor cells. While decreased SOD2 activity and subsequent disbalance in mtROS has previously been described in aging mesenchymal progenitor cells (Almeida and O'Brien, 2013; Chen et al., 2019; Stolzing et al., 2008; Stolzing and Scutt, 2006), the consequences on bone integrity and development of osteoporosis have not been addressed before. Using our new mouse model, which is characterized by an osteoblast lineage-specific Sod2 knockout, we here give a novel insight in consequences of Sod2 deficiency along the osteoblast lineage including progenitor cells. Overall, we demonstrated that mtROS accumulation in early osteoblast precursor cells considerably disturbed osteogenic differentiation with a switch to the adipogenic lineage, induced osteoblast senescence and promoted
osteoclast formation resulting in remarkable bone loss. These results are meaningful for a better understanding of aberrant antioxidative defense mechanisms in osteogenic cells and subsequent development of age-related osteoporosis.

Osteoblast lineage-specific Sod2 deficiency in female and male mice resulted in a low-bone-mass phenotype that was characterized by a decreased trabecular bone volume fraction, when aged 12 and 52 weeks in the femora. Interestingly, the decrease in bone mass of Runx2CreSod2fl/fl mice at the age of 12 weeks corresponded to that observed in Sod2fl/fl control mice aged 52 weeks, indicating that Sod2 deficiency in part resembles an age-related bone phenotype. As in humans, femoral trabecular bone mass decreases with age in mice, starting in early adulthood before any changes in sex steroid production occur (Jilka, 2013). We also found a decreased cortical mineral density and thickness of the femoral bone in Runx2CreSod2fl/fl mice with Sod2 deficiency in osteoblast lineage cells. These results are consistent with previous studies in mice with a global knockout of cytoplasmatic Sod1 (Nojiri et al., 2011). Sod1-deficient mice also displayed low bone mass that was accompanied by increased ROS generation in osteoblasts derived from these mice (Nojiri et al., 2011). A further study of Kobayashi et al. demonstrated that Sod2 depletion in osteocytes leads to a remarkable bone loss in an age-dependent manner (Kobayashi et al., 2015). These and our results confirm other studies demonstrating that oxidative stress is involved in age-related bone loss (Almeida, 2012; Jilka, 2013).

Clinical studies showed that elevated oxidative stress is associated with the pathogenesis of osteoporosis (Bonaccorsi et al., 2018; Deveci. et al., 2017; Domazetovic et al., 2017). In particular, one clinical study demonstrated that aged postmenopausal women exhibit a significant increase in serum lipid hydroperoxides, a ROS-induced byproduct, accompanied by a decreased BMD in vertebrae, compared to young women in the reproductive age (Cervellati et al., 2014),
suggesting that estrogen deficiency promotes oxidative stress, leading to low bone mass.

Overall, it should also be mentioned that global knockout of Sod2 results in neonatal lethality, while Sod1 deficiency leads to shortened mean lifespan and various age-related phatophysiological changes in different tissues and organs (Watanabe et al. 2014). Although both global knockout of cytoplasmic Sod1 as well as osteocyte-specific knockout of mitochondrial Sod2 seem to result in an osteoporosis-like phenotype in mice (Nojiri et al., 2011; Kobayashi et al., 2015), several studies imply differences in the expression and the role of these antioxidative enzymes in bone. While Sod1 was upregulated in bone marrow cells in response to mechanical unloading-induced ROS generation and seemed to be protective against subsequent bone loss, Sod2 was not involved in the antioxidative response (Morikawa et al, 2013). However, in contrast to mitochondrial SOD2, which plays a pivotal role in counterbalancing excessive ROS production during osteoblast differentiation and bone formation, expression of Sod1 was not found to be upregulated during this process (Gao et al, 2018). Considering the various pathological changes observed in Sod1–/– mice, which might indirectly affect bone physiology, an osteoblast-specific Sod1 knockout model might help to differentiate the respective role of SOD1 and SOD2 in bone development and metabolism.

The bone loss in Runx2CreSod2fl/fl mice may result from an impaired osteoblast and elevated osteoclast activity, because we detected a decreased osteoblast number and surface as well as an increased osteoclast number and osteoclast surface in these mice. Furthermore, adipocyte number in the bone marrow of Sod2-deficient mice was increased, suggesting altered mesenchymal progenitor cell differentiation. Consistent with the expectation that Runx2-controlled Cre expression also may occur in hypertrophic chondrocytes (Rauch et al., 2010; Elefteriou et al, 2011) we found a
significantly decreased growth plate thickness in the femora of female Runx2CreSod2\(^{fl/fl}\) mice, indicating a disturbed metabolism in the presence of increased ROS levels in these cells, too (Bai et al. 2018). Although the underlying mechanisms have not been addressed in the present study, we assume that excessive ROS accumulation associated with endochondral ossification in the growth plate and simultaneous loss of Sod2 due to Runx2 expression in hypertrophic chondrocytes, might result in increased cellular stress. This interesting finding deserves further investigation to clarify whether excessive oxidative stress results in apoptotic cell death or any other cellular alteration in the growth plate.

Supporting these in vivo findings, osteoblasts isolated from Sod2-deficient mice also exhibited an increased ROS accumulation, and mesenchymal progenitor cells isolated from these mice mice exhibited reduced Runx2 and Alpl expression with a concomitant increased Ppar\(\gamma\), Cebp\(\alpha\), aP2 and Plin1 expression, suggesting disturbed osteogenic differentiation and a switch to the adipogenic lineage. Moreover, the ratio of Rankl to Opg expression, which is important for osteoclastogenesis, was elevated in osteoblasts from Runx2CreSod2\(^{fl/fl}\) mice, which explains the higher osteoclast activity observed in these mice. These results are consistent with Kobayashi et al., who demonstrated significantly suppressed bone formation and increased bone resorption concomitant with an upregulation of the Rankl/Opg ratio in a mouse model of osteocyte-specific Sod2 deletion (Kobayashi et al., 2015) Furthermore, in our study, a lower BrdU incorporation and a decreased AP, von-Kossa and alizarin red staining of differentiated osteoblast cultures as well as a higher adipocyte number in mesenchymal progenitor cell cultures originated from Runx2CreSod2\(^{fl/fl}\) mice also indicated an impaired proliferation and osteogenic differentiation potential. Thus, osteoblast lineage-specific Sod2 deficiency resulted in
bone loss, which was closely associated with impaired osteoblast differentiation and bone marrow adipogenesis as well as with an increased osteoclastogenesis. Noteworthy, the composition of the bone marrow also changed with aging in humans, showing a high accumulation of adipocytes (Gao et al., 2014; Sebo et al., 2019). The sites of accumulated adipocytes in the bone marrow are referred to as marrow adipose tissue (MAT). MAT is an important endocrine organ, which is able to regulate the systemic metabolism. Interestingly, it was demonstrated to be inversely related to BMD in humans. Moreover, MAT is also associated with metabolic diseases, including metabolic syndrome and diabetes mellitus, and plays a crucial role in the development and progression of tumors, including bone metastasis.

Notably, we discovered more senescent osteoblasts both in osteoblast cultures and in the bone tissue of Runx2CreSod2fl/fl mice by detecting SA-β-Gal activity. Common characteristics of senescent cells are irreversible growth arrest, the development of a unique secretome, termed the senescence-associated secretory phenotype (SASP), and the resistance to apoptosis (Farr and Almeida, 2018). Consistent with the importance of the p53/p21 and p16/Rb tumor-suppressor pathways in inducing cellular senescence (Moussavi-Harami et al., 2004), we detected a significant increase of p21 and p16INK4a expression in Sod2-deficient osteoblasts, suggesting that ROS induced growth arrest of these cells is dependent on the activation of both these tumor-suppressor pathways. Accordingly, we also detected more p16INK4a positively stained osteoblasts in situ. Despite the increased oxidative stress level in the bone tissue of Runx2CreSod2fl/fl mice, apoptosis was not increased in osteoblast lineage cells in these mice, as could be expected due to the resistance of senescent cells to apoptosis (Farr and Almeida, 2018).
Chronic inflammation, which is associated with aging and thus also referred to as inflamm-aging (Xia et al., 2016), plays a crucial role in age-related bone loss through the actions of proinflammatory cytokines (Pietschmann et al., 2016). Elevated oxidative stress, particularly mtROS, is discussed to be involved in inducing inflammatory cytokines via activation of nuclear factor (NF)κB. Various studies have shown that several SASP cytokines, which are involved in the regulation of bone turnover, are elevated during the aging process. IL-6 is an important example, because it increases steadily with aging. Moreover, IL-6 is a potent promoter of osteoclast differentiation and activation, thus supporting bone resorption (Jilka et al., 1992). TNF-α stimulates bone resorption and inhibits new bone formation (Nanes, 2003). Accordingly, we found a higher expression of both, IL-6 and TNF-α, in osteoblasts enclosing the bone trabeculae of Runx2CreSod2^{fl/fl} mice as well as in isolated mesenchymal progenitor cells. These findings are strongly compatible with the impact of inflammatory cytokines on the development of osteoporosis (Pietschmann et al., 2016).

The redox transcription factors forkhead box, class O (FoxO) counteract ROS production by upregulating antioxidant enzymes, including catalase and SOD2, maintaining bone cell function and preserving skeletal homeostasis (Klotz et al., 2015; Teixeira et al., 2010). In aging mice, FoxO-target gene expression increases in bone, accompanied by an elevation in oxidative stress marker expression (Almeida et al., 2007). In agreement with this, we detected increased FoxO1 expression, an early molecular regulator of osteoblast differentiation (Teixeira et al., 2010), particularly in osteoblasts lining the bone trabeculae near the growth plate of Runx2CreSod2^{fl/fl} mice. This implies that FoxO levels enhanced in response to excessive ROS, possibly not only to maintain the osteoblast phenotype, but also to prevent subsequent senescence (Rached et al., 2010).
In conclusion, we demonstrated that mice with an osteoblast lineage-specific Sod2 deficiency display a low bone mass phenotype due to impaired osteoblast proliferation and differentiation, osteoblast senescence, accumulation of adipocytes in the bone marrow and increased osteoclast activity, suggesting that mitochondrial redox balance in osteoblast lineage cells is indispensable for skeletal homeostasis and may be an attractive target for therapeutic intervention of age-related bone loss. As osteoblast senescence has not been observed in ovariectomy-induced mice (Farr et al., 2019), our model might open up new possibilities to study this important cellular mechanism of aging. In that regard, application of anti-oxidative or senolytic drugs might be a promising strategy to reduce the number of senescent and thus dysfunctional cells in osteoporotic bone, which might prevent aggravation of the disease, on one hand, and improve fracture healing, on the other hand. In fact, a senolytic co-treatment using dasatinib and the antioxidant quercetin, was previously described to restore the osteogenic capacity of senescent bone marrow mesenchymal stem cells as demonstrated in enhanced bone repair in aged mice (Zhou et al., 2021). Taken together, the Runx2CreSod2fl/fl mice represent a suitable model to further investigate bone repair, novel treatment strategies and underlying pathomechanisms in the context of age-related osteoporosis.

Material and Methods

Animals

Sod2fl/fl (C57BL/6 background) and Runx2-Cre transgenic animals were described previously (Rauch et al., 2010; Strassburger et al., 2005; Treiber et al., 2011). Runx2-Cre were initially generated on an FVB/N background and backcrossed for at least 10 generations to C57BL/6. Genotyping was performed using primers (5´-CCA GGA AGA CTG CCA GAA GG -3´, 5´-TGG CTT GCA GGT ACA GGA G -3´, 5´-GGA
detecting a 780-bp wildtype sequence and a 600-bp transgenic Runx2-Cre sequence. Using primers (5′-GAG GGG CAT CTA GTG GAG AAG-3′, 5′-AGG AAA GTC ACC TCC ACA CAC AG-3′) an 800-bp wildtype allele and a 1081-bp Sod2<sup>fl/fl</sup> allele were detected. All mice were housed at two to five per cage under a 12 h/12 h dark/light cycle, and food and water were supplied ad libitum. Runx2CreSod2<sup>fl/fl</sup> mice were mated with Sod2<sup>fl/fl</sup> mice. Organ removal was performed from Runx2CreSod2<sup>fl/fl</sup> mice and the littermate control mice. Organ removal was approved by the local ethics committee (Regierungspräsidium Tübingen, o.135-4) and was performed in accordance with the national and international regulations for the care and use of laboratory animals. 12- and 52-week-old female and male mice were used in the study.

**Micro-computed tomography (µCT)**

µCT analysis was performed as described previously (Kemmler et al., 2015) To investigate the bone phenotype of mice, right femurs were fixed in 4% formalin and lumbar vertebrae (L3) were dehydrated in 80% ethanol. Femurs and lumbar vertebrae were scanned using a µCT device (Skyscan 1172, Kontich, Belgium) set at a resolution of 8 µm, a maximum voltage of 50 kV and a power of 200 mA. Reconstruction and analysis were performed using software (NRecon, Data viewer and CT analyzer) from Skycan. Two defined hydroxyapatite (HA) phantoms (250 and 750 mg/cm<sup>3</sup>), scanned together with the bones in each scan, were used to determine BMD. According to the guidelines of the American Society for Bone and Mineral Research (ASBMR), two global thresholds (394 and 642 mg HA/cm<sup>3</sup>) were used to distinguish between mineralized and non-mineralized tissue, respectively. The regions of interest (ROI) were defined: the area between 240 µm and 960 µm from the proximal end of the growth plate of the femur and the entire trabecular area of the
vertebral body for the analysis of trabecular bones; the region from the border of the trochanter to the following 2000 μm for the assessment of the cortical bone. TMD, bone volume per tissue volume ratio (BV/TV), Tb.N, Tb.Sp and Tb.Th and Ct.Th were selected as the common parameters for bone evaluation (Bouxsein et al., 2010; Kovtun et al., 2017).

**Histomorphometric analysis**

Undecalcified sections were stained for the analysis of tissue composition. Methyl methacrylate-embedded sections were immersed in 2-methoxyethyl acetate and a decreasing ethanol series. Plump cuboidal blue cells located on the surface of bone were identified as osteoblasts by toluidine-blue staining. Cells on the bone surface stained red with two or more nuclei were considered to be osteoclasts by TRAP staining. Adipocyte number was determined after staining with hematoxylin and eosin. Osteoblast number per bone perimeter (N.Ob/B.Pm, mm⁻¹), osteoblast surface per bone surface (Ob.S/BS, %), osteoclast number per bone perimeter (N.Oc/B.Pm, mm⁻¹) and osteoclast surface per bone surface (Oc.S/BS, %) were measured using Osteomeasure software (Osteometrics, Atlanta, USA). The area at a distance of 200 μm from the growth plate was determined as the region of interest under the light microscope at a 20-fold magnification.

**Immunohistochemistry**

Paraffin-embedded sections were prepared as previously described (Haffner-Luntzer et al., 2016). To perform antigen retrieval, slides with sections were immersed in 10 mM citrate buffer (pH 6.0) in a 95°C water bath for 20 min. The sections were incubated with primary antibodies (rabbit polyclonal anti-mouse p16INK4a antibody, Abcam, Cambridge, UK; rabbit monoclonal anti-mouse FoxO1 antibody, Cell
Signaling, Danvers, MA, USA; rabbit polyclonal anti-mouse TNF-α antibody, Abcam; rabbit polyclonal anti-mouse IL-6 antibody, Bioss, Woburn, Massachusetts, USA) and with the secondary goat anti-rabbit antibody, followed by incubation with avidin-biotin-complex and NovaRed substrate (Vector, Peterborough, UK) or with secondary goat anti-rabbit Alexa Flour 594 (Thermo Fisher Scientific). Control sections were treated in parallel and incubated with the isotype control antibody (Jackson Immunoresearch, UK). For nuclei staining, hematoxylin (Waldeck, Münster, Germany) was used. Before analyzing sections microscopically, the slides were mounted with Vitro-Clud (Langenbrinck, Emmendingen, Germany) or Fluoromount (Sigma-Aldrich, Taufkirchen, Germany).

**Cryosectioning of undecalcified bone**

Mice tibia immediately after isolation were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Germany) and frozen in liquid nitrogen. Bones were cut and collected as 10 μm-thick sections on adhesive cryofilm (Section-lab Co, Hiroshima, Japan). The cryosections were used for the senescence-associated beta-galactosidase (SA-β-Gal) activity assay.

**Cell isolation and culture**

Osteoblasts were generated from long bones of mice as described previously (Rapp et al., 2018). Following digestion with 300 U/mL collagenase type IV (Sigma-Aldrich) in α-MEM (Biochrom, Berlin, Germany) for 2 h with shaking at 37°C and under 5% CO₂ in an incubator, the bones were placed in six-well plates in α-MEM supplemented with 10% fetal bovine serum superior (Biochrom), 1% penicillin/streptomycin, 1% L-glutamine, 0.5% amphotericin B (Thermo Fisher Scientific) in a 37°C and 5% CO₂ incubator. Mesenchymal progenitor cells (MSCs)
were isolated from the long bones of mice as described previously (Rapp et al., 2018). Bone marrow cells were seeded at a density of $5.5 \times 10^7$ cells/cm$^2$ in expansion medium (MesenCult™ Expansion Kit, Mouse, Stemcell Technologies, Vancouver, Canada). According to the manufacturer’s instructions, the MSCs were cultured with additional MesenPure™ at 37°C and under an atmosphere of 6.0% O$_2$ and 8.5% CO$_2$. Osteoblasts and MSCs in passages 3–5 were used for further experiments.

**Dihydroethidium (DHE) staining**

Cells were seeded into 24-well-plates at a density of 20,000 cells/well and incubated for 24 h. Following rinsing with phosphate-buffered saline (PBS), the cells were incubated with DHE (Sigma Aldrich) diluted in PBS (10 μM) for 30 min at 37°C under 8.5% CO$_2$. The samples were washed with PBS, visualized and fluorescence images captured using a microscope with Ex/Em 488/574–595 nm (red) and Ex/Em 352/461 nm (blue) (Nijmeh et al., 2010). DHE staining was quantified in cultured osteoblasts by using ImageJ software (NIH, Bethesda, USA, version 2.0.0) and calculating the corrected total fluorescence (CTCF).

**TUNEL assay**

The TUNEL assay was performed as described in the manufacturer’s protocol (Biozol, Eching, Germany). Rehydrated paraffin-embedded sections were washed with PBS and immersed with 20 mg/mL proteinase K for 30 min at 37°C. To perform the TUNEL assay, sections were permeabilized with TUNEL reaction solution for 2 h in a humid chamber at 37°C in the dark.
**Gene expression analysis**

RNA was harvested using RLT buffer (Qiagen, Hilden, Germany) containing 1% β-mercaptoethanol (Sigma Aldrich) from mesenchymal progenitor cells and osteoblasts after cultivation in proliferation medium for 3 d (d 0) followed by cultivation in osteogenic differentiation for an additional 10 d (d10) and 14 d (d 14), respectively. RNA isolation was performed according to the instructions of RNeasy mini kit (Qiagen). Total RNA was diluted in RNase-free water, and the RNA concentration was determined by spectrophotometry. A total of 1 μg total RNA in RNase-free water was used to generate cDNA in a total volume of 20 μl using Omniscript Reverse Transcriptase (Qiagen). qRT-PCR was performed using the SensiFast SYBR HiROX One Step Kit according to the manufacturer’s protocol (Stratagene, Amsterdam, Netherlands) and analyzed by QuantStudio 3 (Thermo Fisher Scientific). Primer pairs for the amplification of Alpl (5’-GCT GAT CAT TCC CAC GTT TT-3’, 5’-GAG CCA GAC CAA AGA TGG AG-3’), Runx2 (5’-CCA CCA CTC ACT ACC ACA CG-3’, 5’-CAC TCT GGC TTT GGG AAG AG-3’), Pparg (5’-CGT GAA GCC CAT CGA GGA CAT-3’, 5’-GGG TGG TTC AGC TTG AGC TGC AG-3’), RANKL (5’-ATG AAA CAT GGG GAA GC-3’, 5’-CTT GGG ATT TTG ATG CTG GT-3’), Opg (5’-CTT CCT GGG GAA GC-3’, 5’-GGT TCT CAC CCA CCA CCC TTA GG-3’), p53 (5’-GGG TGG TTG ATG TCC CTC T-3’, 5’-GTC TTG ATG TCC CTC T-3’), p21 (5’-CCT CCC AAG ATA GCC GAG TT-3’, 5’-AGA CGA CAC AGG TGA GGA AG-3’), p16INK4a (5’-CTT CCT GGG GAA GC-3’, 5’-GGT TCT CAC CCA CCA CCC TTA GG-3’), Sod2 (5’-AAC TCA GGT CGC TCT TCA GC-3’, 5’-GCT TCT TCC CCA CCC TTA GG-3’), Cebpa (5’-CGT CGT CCT CCC AGA GGA CCA ATT A-3’, CAC CCT TGG ACA ACT AGG GGA GAG G-3’), aP2 (5’-GTG ACA AGC TGG TGG TGG AAT-3’, 5’-CAT CCA GGC CTC TTC CTT TGG-3’), Plin1 (5’-TAC CTA GCT GCT TTC TCG GTG-3’, 5’-CAC AGG CAG CTG AAC TC-3’), Tnfa (5’-GGC
CAC CAC GCT CTT CTG TCT ACT-3’, 5’-TGA TCT GAG TGT GAG GGT CTG GGC-3’) and Il6 (5’-TCC TTC CTA CCC CAA TTT CC-3’, 5’-GCC ACT CCT TCT GTG ACT CC-3’) were used. The mRNA expression was calculated by the term PCR efficiency $^{\Delta Ct \text{ (gene of interest)}}$. The PCR efficiencies from the different primer pairs were calculated using the software LinRegPCR (Ramakers et al., 2003). Beta-2-microglobulin ($B2m$) was selected as the housekeeping gene.

**Western blot analysis**

10 µg of protein lysate from osteoblasts was separated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (BioRad, Hercules, USA). The membranes were incubated with antibodies rabbit monoclonal anti-mouse FoxO1 antibody (Cell Signaling) and rabbit monoclonal anti-mouse GAPDH antibody (Cell Signaling) overnight at 4°C. Protein bands were visualized as described previously (Liedert et al., 2011).

**BrdU assay and cytological staining**

The proliferation assay was performed using the BrdU Cell Proliferation Assay Kit (Cell Signaling) according to the manufacturer’s instructions. Osteoblasts were seeded into 96-well plates at 2000 cells/well and were incubated with BrdU reagent for 24 h.

AP staining was performed to detect AP activity using an alkaline phosphatase kit (Sigma Aldrich). Following incubation with osteogenic differentiation medium (DMEM/F12 medium supplemented with 10% fetal calf serum, 0.1 µM dexamethasone, 10 mM β-glycerophosphate disodium, 0.2 mM ascorbate-2-phosphate) for 14 d, cells were fixed with citrate-acetone-formaldehyde fixing solution for 2 min. The AP staining solution was prepared immediately prior to staining. For
the staining process, the cells were incubated with AP staining solution, and the plates were kept in the dark for 15 min. The cells were washed with Aqua dest. and images were captured using a digital camera.

Alizarin red and von-Kossa staining were used to analyze matrix mineralization of differentiated osteoblasts. Cells were fixed with 4% formalin solution for 15 min followed by incubation with 0.1% alizarin red staining solution (Sigma Aldrich) for 30 min. For von-Kossa staining, the cells were incubated with 5% silver nitrate solution for 60 min at room temperature (in the dark). Following rinsing with Aqua dest, the cells were incubated with 1% pyrogallol solution for 5 min followed by incubation with 5% sodium thiosulfate solution for 5 min. Adipocytes were detected using 0.2% Oil-red O staining solution after cultivation for 3 d in adipogenic differentiation medium (α-MEM supplemented with 1 μM dexamethasone, 0.1 mM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 μg/ml insulin) and successive cultivation in proliferation medium for 7 d.

**Senescence-associated beta-galactosidase (SA-β-Gal) activity assay**

According to the manufacturer’s protocol (Sigma Aldrich), osteoblasts were seeded into six-well-plates at a density of 40,000 cells/well and fixed with fixation buffer for 7 min. The cells were immersed in PBS and incubated with staining mixture solution at 37°C without CO₂ overnight. The senescent cell ratio was assessed as the ratio of the total number of stained (blue) cells to the total number of cells counted (≥1000) in one well.

Bone cryosections were prepared to perform SA-β-Gal staining. Following washing with PBS, the bone cryosections were fixed with fixation buffer for 7 min and incubated with staining mixture solution at 37°C overnight. The slides were dried and
mounted with fluoromount™ aqueous mounting medium before capturing images using a microscope.

**Data analysis**

Data are presented as means ± SD. IBM SPSS statistics version 24 software and Graphpad Prism version 6.07 software were used to analyze data. Following evaluation for normality by Shapiro-Wilk test, data were analyzed by t-test, one-way ANOVA with Fisher’s LSD post hoc test and two-way ANOVA with Sidak multiple comparison test. *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001 and were defined as different significance levels.

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**Conflict of Interest:** The authors declare no conflict of interest.

**References**


Fig. 1. Bone mass loss in osteoblast lineage-specific Sod2-deficient female mice aged 12 and 52 weeks. (A) μCT-based quantification of the trabecular bone volume per tissue volume ratio (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp) in the distal femora of Runx2CreSod2fl/fl mice (◼) compared to Sod2fl/fl mice (●). (B) μCT-based quantification of the TMD and cortical thickness (C.Th) of the femora of Runx2CreSod2fl/fl mice (◼) compared to Sod2fl/fl mice (●). (C) Representative 2D μCT images of the femora. (D) Histomorphometric quantification of BV/TV, Tb.N, Tb.Th, and Tb.Sp in the vertebral bodies of Runx2CreSod2fl/fl mice (◼) compared to Sod2fl/fl mice (●) determined by μCT. (E) Representative histological images of vertebral body sections after von Kossa staining and representative 2D μCT images of vertebral bodies. Bars represent mean ±SD (n=6-7/group). Asterisks indicate statistically significant differences between the groups (* <0.05, ** <0.01, *** <0.001, **** <0.001)
Fig. 2. Bone mass loss is caused by a decreased number of osteoblasts and an increased number of osteoclasts in osteoblast lineage-specific Sod2-deficient mice aged 12 and 52 weeks. (A) Representative images of femur cross-sections stained with toluidine blue from Sod^{2−/−} mice and Runx2CreSod{2−/−} mice. Histomorphometric analysis of osteoblast number per bone perimeter (N.Ob/B.Pm) and osteoblast surface per bone surface (Ob.S/BS) in the femora of Runx2CreSod{2−/−} mice (◼) compared to Sod^{2−/−} mice (●). Red arrows indicate osteoblasts (scale bar 50 μm). (B) Representative images of femur cross-sections stained for tartrate-resistant acid phosphatase (TRAP) from Sod^{2−/−} mice and Runx2CreSod{2−/−} mice. Histomorphometric analysis of osteoclast number per bone
perimeter (N.Oc/B.Pm) and osteoclast surface per bone surface (Oc.S/BS) in the femora of Runx2CreSod2\textsuperscript{fl/fl} mice (□) compared to Sod2\textsuperscript{fl/fl} mice (●). Red arrows indicate osteoclasts (scale bar 50 μm). (C) Representative images of femur cross-sections stained with hematoxylin and eosin (HE). Quantification of adipocytes identified as ghost-like cells (scale bar 200 μm). Bars represent mean ±SD (n=6-7/group, scale bar 50 μm). Asterisks indicate statistically significant differences between the two groups (* <0.05, ** <0.001)
Fig 3. Sod2 deficiency in the osteoblast lineage leads to higher ROS generation and impaired osteoblast proliferation and impaired mesenchymal osteoprogenitor function with increased adipogenic marker expression. (A) Detection of ROS generation in osteoblasts from Sod2\(^{+/+}\) mice and Runx2CreSod2\(^{+/+}\) mice \textit{in vitro} by dihydroethidium (DHE) assay and quantification by calculating the corrected total cell fluorescence (CTCF) of DHE-positive cells. (B) QRT-PCR analysis of Sod2 expression in osteoblasts from Sod2\(^{+/+}\) mice (●) and Runx2CreSod2\(^{+/+}\) mice (◼). (C) Quantification of osteoblast proliferation from Sod2\(^{+/+}\) mice (●) and Runx2CreSod2\(^{+/+}\) mice (◼) by BrdU assay. (D) QRT-PCR analysis of Runx2, Alpl and PPar\(\gamma\) expression in mesenchymal osteoprogenitor cells from Sod2\(^{+/+}\) mice (●) and Runx2CreSod2\(^{+/+}\) mice (◼) on day 0 (third day after seeding) and additionally after 10 days (day 10) of cultivation in differentiation medium. (E)
QRT-PCR analysis of aP2, Cebpα and Plin1 expression in mesenchymal osteoprogenitor cells from Sod2<sup>fl/fl</sup> mice (●) and Runx2CreSod2<sup>fl/fl</sup> mice (◼) on day 0 (third day after seeding) and additionally after 10 days (day 10) of cultivation in osteogenic differentiation medium. Bars represent mean ±SD (n=3-7/group, scale bar 200 μm). Asterisks indicate statistically significant differences between the groups (* <0.05, ** <0.01, *** <0.001, **** <0.0001).
Fig 4. Sod2 deficiency in the osteoblast lineage leads to impaired osteoblast function and mesenchymal osteoprogenitor differentiation.

(A) QRT-PCR analysis of *Rankl* and *Opg* expression and *Rankl/Opg* ratio in osteoblasts from *Sod2*^{fl/fl} mice and *Runx2CreSod2*^{fl/fl} mice after cultivation in differentiation medium for 14 days in osteogenic differentiation medium. (B) Representative images of osteoblast cultures from *Sod2*^{fl/fl} mice and *Runx2CreSod2*^{fl/fl} mice on day 14 of cultivation in osteogenic differentiation medium and alkaline phosphatase (AP), von Kossa and alizarin red staining. Representative images of mesenchymal osteoprogenitor cell cultures from *Sod2*^{fl/fl} mice and *Runx2CreSod2*^{fl/fl} mice after Oil-red O staining after cultivation in adipogenic...
differentiation medium for 3 days and (C) quantification by calculating the integrated
optical density (IOD). Bars represent mean ±SD (n=3-8/group). Asterisks indicate
statistically significant differences between the two groups (* <0.05, ** <0.01)
Fig. 5. **Sod2** deficiency in osteoblast lineage cells leads to senescence *in vitro* and *in vivo*. (A) Representative images of senescent osteoblasts from *Sod2*<sup>fl/fl</sup> mice and Runx2CreSod2<sup>fl/fl</sup> mice detected *in situ* (tibia cryosections) by histochemical staining of senescence-associated β-galactosidase (SA-β-Gal) activity (blue) and quantification by calculating the integrated optical density (IOD) (B) Detection and quantification of senescent osteoblasts from *Sod2*<sup>fl/fl</sup> mice (●) and Runx2CreSod2<sup>fl/fl</sup> mice (◼) *in vitro* based on cytochemical staining of SA-β-Gal activity (blue). (C) QRT-PCR analysis of senescence-associated tumor suppressor markers *p53*, *p21* and *p16<sup>INK4a</sup>* expression in osteoblasts from *Sod2*<sup>fl/fl</sup> mice (●) and Runx2CreSod2<sup>fl/fl</sup> mice.
(D) Representative immunofluorescence images of femur cross-sections stained for p16\textsuperscript{INK4a} (arrows indicate p16\textsuperscript{INK4a}-positive cells) and number of p16\textsuperscript{INK4a}-positive cells in femur cross-sections from Sod2\textsuperscript{fl/fl} mice (●) and Runx2CreSod2\textsuperscript{fl/fl} mice (□). Bars represent mean ±SD (n=4-9/group, scale bar 200 μm). Asterisks indicate statistically significant differences between the two groups (* <0.05, ** <0.01, *** <0.001)
Fig. 6. Sod2 deficiency in osteoblast lineage cells does not increase osteoblast apoptosis but leads to increased expression of senescence-associated secretory-phenotype (SASP) markers and FoxO1. (A) Representative images of femur cross-sections from Sod2^{fl/fl} mice and Runx2CreSod2^{fl/fl} mice after TUNEL assay to detect apoptosis. (B) Representative images of femur cross-sections from male Sod2^{fl/fl} mice and Runx2CreSod2^{fl/fl} mice after immunostaining for SASP factors IL-6 and TNF-α and redox-regulated transcription FoxO1 and (C) quantification by calculating the integrated optical density (IOD). (n=3/group, scale bar 200 μm)
Fig. 7. Increased expression of senescence-associated secretory-phenotype (SASP) markers \( \text{Tnf} \alpha \), \( \text{Il6} \) and FoxO1 in mesenchymal osteoprogenitor cells with \( \text{Sod2} \) deficiency. (A) QRT-PCR analysis of senescence-associated markers \( \text{Tnf} \alpha \) and \( \text{Il6} \) expression in mesenchymal osteoprogenitor cells after osteogenic differentiation from \( \text{Sod2}^{fl/fl} \) mice (●) and \( \text{Runx2CreSod2}^{fl/fl} \) mice (◼) \textit{in vitro}. (B) Western blot analysis of FoxO1 expression in osteoblasts from \( \text{Sod2}^{fl/fl} \) mice (●) and \( \text{Runx2CreSod2}^{fl/fl} \) mice (◼) and quantification by calculating the integrated optical density (IOD). Bars represent mean ±SD (n=6/group). Asterisks indicate statistically significant differences between the groups (\( ** < 0.01, *** < 0.001, **** < 0.0001 \)).
Fig. S1. Bone mass loss in osteoblast lineage-specific Sod2-deficient male mice aged 12 and 52 weeks.

(A) μCT-based quantification of the trabecular bone volume per tissue volume ratio (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp) in the distal femora of male Runx2CreSod2fl/fl mice (◼) compared to male Sod2fl/fl mice (●). (B) μCT-based quantification of the tissue mineral density (TMD) and cortical thickness (C.Th) of the femora of male Runx2CreSod2fl/fl mice (◼) compared to male Sod2fl/fl mice (●). (C) Histomorphometric quantification of BV/TV, Tb.N, Tb.Th, and Tb.Sp in the vertebral bodies of male Runx2CreSod2fl/fl mice (◼) compared to male Sod2fl/fl mice (●) determined by μCT. Bars represent mean ±SD (n=4-10/group). Asterisks indicate statistically significant differences between the groups (* <0.05, ** <0.01, *** <0.001, **** <0.0001).
Fig. S2. Decreased growth plate thickness in female mice with osteoblast lineage-specific Sod2 deficiency. Representative images of femur cross-sections stained with toluidine blue showing the growth plate from female Sod2^fl/fl^ mice and Runx2CreSod2^fl/fl^ mice. Quantification of growth plate thickness in femur cross-sections from Sod2^fl/fl^ mice (●) and Runx2CreSod2^fl/fl^ mice (◼) Bars represent mean ±SD (n=4/group). Asterisks indicate statistically significant differences between the groups (* <0.05).