Enhancer of zeste homolog 2 (Ezh2) controls bone formation and cell cycle progression during osteogenesis in mice

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Running title: Ezh2 function in proliferating osteoprogenitor cells

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SUMMARY

Epigenetic mechanisms control skeletal development and osteoblast differentiation. Pharmacological inhibition of the histone 3 Lys-27 (H3K27) methyltransferase enhancer of zeste homolog 2 (Ezh2) in wildtype mice enhances osteogenesis and stimulates bone formation. However, conditional genetic loss of Ezh2 early in the mesenchymal lineage (i.e. through excision via Prrx1 promoter–driven Cre) causes skeletal abnormalities due to patterning defects. Here, we addressed the key question whether Ezh2 controls osteoblastogenesis at later developmental stages beyond patterning. We show that Ezh2 loss in committed pre-osteoblasts by Cre expression via the Osterix/Sp7 promoter (Ezh2 cKO) yields phenotypically normal mice. The Ezh2-cKO mice have normal skull bones, clavicles and long bones, but exhibit increased bone marrow adiposity, and reduced male body weight. Remarkably, in vivo Ezh2 loss results in a low trabecular bone phenotype in young mice as measured by microCT and histomorphometry. Thus, Ezh2 affects bone formation stage-dependently. We further show that Ezh2 loss in bone marrow–derived mesenchymal cells suppresses osteogenic differentiation and impedes cell cycle progression as reflected by decreased metabolic activity, reduced cell numbers, and changes in cell cycle distribution and in expression of cell cycle markers. RNA-Seq analysis of Ezh2 cKO calvaria revealed that the cyclin-dependent kinase inhibitor Cdkn2a is the most prominent cell cycle target of Ezh2. Hence, genetic loss of Ezh2 in mouse pre-osteoblasts inhibits osteogenesis in part by inducing cell cycle changes. Our results suggest that Ezh2 serves a bifunctional role during bone formation by suppressing osteogenic lineage commitment while simultaneously facilitating proliferative expansion of osteoprogenitor cells.

Mineralization of fetal tissues to form a bony skeleton is restricted to the later stages of gestation and expression of bone specific genes remains silenced during the earlier stages of
embryogenesis. Silencing of genes and their regulatory regions is mediated by the tightly controlled organization of promoters and enhancers into nucleosomes, the fundamental units of chromatin (1,2). Condensation of chromosomal loci in heterochromatin silences gene expression, and occurs concomitant with post-translational modifications of histone N-terminals. One of the major mechanisms for heterochromatin formation is through tri-methylation of histone 3 at lysine 27 (H3K27me3) (3). The polycomb-repressive complex 2 (PRC2), which contains three structural proteins and the catalytic subunit enhancer of zeste homolog 2 (Ezh2), catalyzes the mono-, di- and tri-methylation of H3K27. Enhancer of zeste homolog 1 (Ezh1) can also serve as the catalytic subunit within the PRC2 complex and may also suppress gene expression, albeit by a different mechanisms (3-5). Methylation of H3K27me3 is reversible by three histone demethylases, Jhdm1d, Kdm6a, and Kdm6b (6). Hence, deposition and retention of H3K27me3 marks reflects the dynamic balance in the activities of opposing enzymes.

Epigenetic mechanisms play a critical role during skeletal development and osteoblast differentiation (2,7-13). In addition, the epigenomic landscape of differentiating osteoblasts has been documented in considerable detail (14-17). Several recent studies from our group have demonstrated that Ezh2 and other epigenetic regulators play a critical role during mesenchymal lineage commitment and osteoblast differentiation (18-25). It has been established that Ezh2 expression is significantly down-regulated during osteogenic commitment of human adipose-derived stem cells (AMSCs) (26,27). Furthermore, loss or inhibition of Ezh2 was shown to enhance osteogenic and inhibit adipogenic differentiation of AMSCs (26), human bone marrow-derived mesenchymal stem cells (BMSCs) (28), as well as MC3T3 mouse pre-osteoblasts (29). Mechanically, Ezh2 suppresses the expression of osteogenic genes and ligand-dependent signaling pathways (e.g., WNT, PTH, and BMP2) to favor adipogenic differentiation (26,28-32). Importantly, three studies have shown that inhibition of Ezh2 prevents bone loss that is associated with estrogen depletion in vivo (29,33,34). In addition, one of these studies also reported bone anabolic effects of Ezh2 in mice (29).

With the exception of Ezh1, mice completely lacking expression of any of the PRC2 components are not viable (18). Thus, studies have assessed tissue-restricted genetic loss of Ezh2 in the mesoderm (35), neural crest (36), mesenchymal stem cells (26,35,37), and chondrocytes (38). Loss of Ezh2 in the mesoderm and neural crest causes embryonic lethality, while loss of this epigenetic enzyme in the mesenchyme produces viable mice which have several skeletal abnormalities including craniosynostosis, clindactyly, shortened limbs, and reduced bone volume. The loss of Ezh2 in cartilage does not result in a significant phenotype, but dual loss of Ezh1 and Ezh2 severely impairs skeletal development (38). A similar phenotype was observed with the loss of Eed, a structural component of the PRC2 complex (39), suggesting that both Ezh1 and Ezh2 require the PRC2 complex for their function.

In this study, we assessed the role of Ezh2 in osteoblast differentiation and function. We show that conditional Ezh2 loss in osteoblast precursors permits normal patterning and developmental, but shows a remarkable trabecular bone phenotype reflected by reduced bone volume in young animals. Mechanistic assessment of this phenotype indicates that loss of bone is due to cytostatic effects on osteoprogenitor cells. The latter finding indicates that beyond the known biological role of Ezh2 in suppressing osteogenic differentiation, it has a novel positive function in early stages of bone formation by supporting pre-osteoblast proliferation.

RESULTS

Loss of Ezh2 in bone results in phenotypically normal mice – Our laboratory and others have demonstrated that conditional loss of Ezh2 in the mesenchyme (Ezh2 cKO<sup>osx</sup>) results in several developmental defects (e.g., shorted limbs, craniosynostosis, clindactyly) (26,37). A subset of these phenotypic changes are easily understood as skeletal patterning defects that may result from the well-known activation of the Prrx1 driver in the limb bud stage (40). However, to gain insight into the specific role of Ezh2 in the osteoblast-lineage and bone, Ezh2<sup>flex/flox</sup> mice were bred with Osx-Cre mice (41) to generate control (CON, Ezh2<sup>wt/wt</sup>; Osx-Cre), Ezh2<sup>flex/flox</sup> mice and conditional knockout animals (Ezh2<sup>cKO<sub>osx</sub></sup>, Ezh2<sup>flex/flox</sup>; Osx-Cre). Loss of Ezh2 in osteoblasts results in animals that are

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indistinguishable from CON mice (Fig. 1a). In addition, x-ray analysis revealed normal skeletal formation in Ezh2 cKO\textsuperscript{ox} animals (Fig. 1b). Weight assessment of female mice at 3, 8 and 12 weeks of age did not reveal any differences between CON and Ezh2 cKO\textsuperscript{ox} animals (Fig. 1c). However, Ezh2 cKO\textsuperscript{ox} male animals have slightly lower total body mass compared to CON mice at the earlier time-points (3 and 8 weeks of age). Micro–computed tomography (microCT, μCT) analysis of male and female mice did not reveal any changes in the structure of the skulls (Fig. 1d, e) or abnormalities of the clavicles (Fig. 1f). Thus, with the exception of minor changes in weights of young male mice, the loss of Ezh2 in osteoblasts (in \textit{Ezh2\textsuperscript{lox/lox}}: Osx-Cre mice) results in phenotypically normal mice.

**Bone-specific loss of Ezh2 results in a low trabecular bone phenotype in young animals** - To assess the effects of Ezh2 loss on bone quality, μCT analysis was performed near proximal tibial epiphysis (trabecular bone) and tibial mid-shaft (cortical bone) of a fraction of male mice exhibiting similar weights at several stages of life (3, 8, and 12 weeks of age). Assessment of cortical bone demonstrates that there are no major differences between Ezh2 cKO\textsuperscript{ox} and CON mice (Suppl. Table 1). A minor reduction in cortical porosity (Ct. Po.) is observed at eight weeks in Ezh2 cKO\textsuperscript{ox} animals, which is statistically significant with a moderate level of confidence (p<0.05). Analysis of trabecular bone reveals several quantitatively modest changes in bone parameters at three weeks of age (Suppl. Table 2 and Figure 2a). A reduction in trabecular bone volume (BV/TV), trabecular number (Tb.N.), and connectivity density (ConnD), is observed in Ezh2 cKO mice. While the reduction in these bone parameters is numerically modest, it is significant at an adequate confidence level (p<0.05). We also observe an apparent increase in trabecular spacing (Tb.Sp.) upon bone-specific loss of Ezh2, but this trend is not statistically significant (p>0.05). Interestingly, no significant differences in trabecular bone parameters are observed at eight (Suppl. Table 2 and Fig. 2b) and twelve (Suppl. Table 2 and Fig. 2c) weeks of age between CON and Ezh2 cKO\textsuperscript{ox} animals. Histomorphological analysis was performed on femora derived from three week old male mice (Fig. 3). Von Kossa-McNeal (a) and Goldner’s Trichrome (b) staining of the distal femora demonstrate that the growth plate and cartilage morphologies are similar between CON and Ezh2 cKO animals. However, Goldner’s Trichrome staining of the femora midshafts reveals that cKO animals exhibit enhanced bone marrow adiposity (c). Similar to μCT analysis at three weeks of age, histomorphometric analysis of distal femora shows a reduction in trabecular bone volume (BV/TV) with the loss of Ezh2 in osteoblasts (p<0.05) (d). The change in bone volume is accompanied by a reduction in osteoblasts surface per bone surface (Ob.S/BS), osteoclast surface per bone surface (Oc.S/BS), as well as number of osteoclast per bone perimeter (N.Oc/B.Pm)(p<0.05 in all cases). Although we observed an apparent decrease in the osteoblasts surface per bone surface in adult cKO mice, these numerical changes do not reach significance (p>0.05) (Suppl. Fig. 1). Yet, reductions in osteoclast surface per bone surface are evident remained lower in Ezh2 cKO mice at twelve weeks of age (p<0.05). Thus, while the deletion of Ezh2 in osteoblasts does not alter growth plate and cartilage formation, Ezh2 loss leads to increased bone marrow adiposity and yields a mild low trabecular bone phenotype in young animals. The latter finding indicates that Ezh2 function in osteoblasts is required for formation or maintenance of trabecular bone.

**Cre-specific recombination of the Ezh2 conditional allele results in the loss of Ezh2 protein in primary calvarial osteoblasts** – The SET domain of Ezh2 possesses H3K27 methyltransferase activity and its loss renders Ezh2 inactive. In order to assess the efficiency of the Cre-recombination of the Ezh2 conditional allele (42) in osteoblasts, primary calvarial osteoblasts derived from Ezh2\textsuperscript{floxed/floxed} were infected with adenovirus expressing GFP (Ad-GFP) or an adenovirus expressing GFP and Cre recombinase (Ad-GFP-Cre) (Fig. 4a). The administration of Ad-GFP to these cells is expected to generate wild-type Ezh2, while the addition of Ad-GFP-Cre is predicted to result in truncated Ezh2 lacking the SET domain (Fig. 4b). As anticipated, administration of both adenoviruses results in the expression of GFP, while Cre recombinase expression is only detected in the cells infected with Ad-GFP-Cre (Fig. 4c). To detect the mutant Ezh2 allele, a primer pair that spans across exons 15 and 20 was used (Fig. 4b). The addition of Ad-GFP does not induce gene rearrangement, but addition of Ad-GFP-Cre results in the formation of
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the mutant Ezh2 allele (Fig. 4c). Furthermore, we observe a reduction in the expression of the SET domain and total Ezh2 mRNA levels. The addition of Ad-GFP-Cre results in the loss of Ezh2 protein levels as detected by Western blotting four days after infection (Fig. 4d). Collectively, these results demonstrate that Cre-specific recombination of the Ezh2 locus results in fusion of exons 15 and 20 at the mRNA level and the loss of Ezh2 protein in primary osteoprogenitor cells.

Loss of functional Ezh2 inhibits osteoblast differentiation of bone-marrow derived mesenchymal stem cells - To assess a direct effect of Ezh2 loss on progenitor cells, bone-marrow derived mesenchymal stem cells (BMSCs) were isolated from 6 to 8 week old male and female CON and Ezh2 cKO<sup>lox</sup> mice and differentiated into the osteogenic lineage (Fig. 5). RT-qPCR analysis demonstrates that expression of osteogenic genes (e.g., osterix/Sp7, osteocalcin/Bglap, alkaline phosphatase/Alpl, and bone sialoprotein/Isbp) is suppressed in male and female Ezh2 cKO<sup>lox</sup> BMSCs undergoing osteogenic differentiation (Fig. 5a). In support of RT-qPCR analysis, alizarin red staining of BMSCs cultures on day seventeen of osteogenic differentiation demonstrates a significant reduction in mineral deposition with Ezh2 loss (Fig. 5b and c). Similar to alizarin red staining, a reduction in alkaline phosphatase staining is observed with BMSCs derived from Ezh2 cKO<sup>lox</sup> animals (Suppl. Fig. 2). Western blot analysis reveals that Ezh2 expression is reduced, while H3K27me3 is increased during osteogenic commitment of mouse-derived BMSCs (Fig. 5d). A reduction in Ezh2 and H3K27me3 is observed (see days 3 and 7) in BMSCs derived from Ezh2 cKO<sup>lox</sup> animals. As a loading control, the levels of histone H3 remain stable during the differentiation time-course. Hoechst staining, which is a proxy for the number of cells in each treatment condition, reveals that Ezh2 loss results in the reduction of DNA content at day seven (Fig. 5e). These results show that Ezh2 is only actively expressed during early proliferative stages of BMSC differentiation and that H3K27me3 levels are controlled by another enzyme (e.g., Ezh1) at later maturation stages of osteogenic differentiation in BMSCs. Furthermore, the loss of osteogenic potential of BMSCs upon genetic Ezh2 loss demonstrates that Ezh2 performs a critical and previously unrecognized positive function during proliferative expansion of BMSCs as they gradually become committed to Osx-positive osteoprogenitor cells within the population.

It is plausible that loss of Ezh2 in Osx-positive osteoblasts in vivo may affect regulatory interplay with BMSCs in situ within the bone environment. Hence, the BMSCs isolated from Osx-Cre positive mice may differ from naïve BMSCs developing in the bone micro-niche of wild type mice. Therefore, we assessed BMSCs differentiation after acute deletion of Ezh2 in vitro utilizing Ad-GFP and Ad-GFP-Cre virus particles on BMSCs from Ezh2<sup>lox/lox</sup> animals that are otherwise genetically and phenotypically normal (Fig. 6a). Corroborating our data obtained with primary calvarial osteoblasts (Fig. 4), the addition of Ad-GFP-Cre resulted in the expression of Cre recombinase and the truncated version of Ezh2 (Ezh2<sup>mut</sup>) (Fig. 6b). In addition, a reduction in wild type mRNAs that encompass the Ezh2 SET domain (Ezh2<sup>SET</sup>) and total Ezh2 mRNA is observed. Acute loss of Ezh2 results in a small, but significant, reduction in cell viability as measured by MTS activity ten days after addition of virus particles (Fig. 6c). Although not significant, DNA content as measured by Hoechst staining is also reduced as a result of Ezh2 loss in BMSCs (Fig. 6d). Acute deletion of Ezh2 reduces the expression of several osteogenic markers (e.g., Bglap, Isbp, Alpl, osteomodulin/Omd) at day 14 after osteogenic induction (Fig. 6e). In support of mRNA reduction of key osteogenic genes, a significant reduction in alizarin staining of these primary cell cultures is observed (Fig. 6f and g). Western blotting analysis demonstrates a loss of Ezh2 protein and a reduction in H3K27me3 with the addition of Ad-GFP-Cre (Fig. 6h). Consistent with the loss of the exon encoding the SET domain upon Cre excision, a faster migrating Ezh2 protein band is observed after the recombination event reflecting the truncated short-lived residual Ezh2 protein that lacks methyl transferase activity. Taken together, our data show that Ezh2 protein expression is lost during both in vivo excision (Ezh2 cKO<sup>lox</sup>) and ex vivo excision of Ezh2 (Ad-GFP-Cre with Ezh2<sup>lox/lox</sup> BMSCs). Our results indicate that loss of Ezh2 protein reduces cellular H3K27me3 levels, which in principle is permissive for osteogenic differentiation, but may simultaneously inhibit osteogenic differentiation of primary mouse BMSC populations by reducing the number of osteogenic progenitor cells.
finding corroborates the emerging idea that Ezh2 performs an essential cellular function during osteoprogenitor proliferation, while suppressing the bone cell maturation program.

**Acute loss of Ezh2 impacts cell cycle progression in mouse BMSCs** – Because loss of Ezh2 reduces cell number (as assessed by DNA content) and metabolic activity (as monitored by MTS assays), we examined the contribution of Ezh2 to cell cycle progression of BMSCs derived from wild type (C57BL/6) or Ezh2\(^{\text{loxP/loxP}}\) mice (Fig. 7a). The infection of these BMSCs with Ad-GFP-Cre virus results in expression of GFP in both female and male BMSCs (Fig. 7b), which results in the expected recombination of Ezh2 locus in Ezh2\(^{\text{loxP/loxP}}\) BMSCs (data not shown). RT-qPCR analysis shows stable expression of the housekeeping gene Eukaryotic Elongation Factor 1a1 (Eef1a1) when normalized to that of the housekeeping gene Gapdh at day 6 after infection (Fig. 7c). However, loss of Ezh2 suppresses the expression of two proliferation markers, Mki67 and Ccnb2, that encode respectively the Ki67 antigen and Cyclin B2. Furthermore, the two principal mRNA products (p16 and p19) of the Cdkn2a locus, which are critical inhibitors of cell cycle progression, are significantly up-regulated with Ezh2 loss. A similar trend is also observed with Cdkn1a (p21), another cell cycle inhibitor. Flow cytometric analysis shows that loss of Ezh2 alters the distribution of cells in the different phases of the cell cycle (Fig. 7d). In both male and female BMSCs, the loss of Ezh2 significantly shifts the cells from S/G2 phases into the G0/G1 phases of the cell cycle, suggesting that cell cycle progression of BMSCs is impeded in the G0 or G1 phase when Ezh2 activity is eliminated. Because male and female cells were processed independently, these technical considerations limit direct comparison of cell cycle distributions between the two sexes. In support of these findings, MTS activity assays show reduced metabolic activity on days 6 and 11 with Ezh2 loss (Fig. 7e). No significant changes are observed day after plating (day 0) and three days after the addition of the virus to the cell populations. Thus, Ezh2 loss in BMSCs reduces the number of cells by attenuating G0 or G1 cell cycle progression in BMSCs.

**Altered expression of cell cycle genes in mouse calvaria lacking Ezh2 expression** – To investigate the mechanism by which Ezh2 controls cell cycle progression, we examined in vivo gene expression by RNA-seq analysis of mRNAs derived from calvaria of CON and Ezh2 cKO pups (Fig. 8). Consistent with genomic truncation of the Ezh2 locus through Cre excision over loxP sites, RNAseq analysis of mRNA collected from Ezh2 cKO calvaria exhibits a marked reduction in the number of reads mapped to exons 16 to 19 (Fig. 8a, b). Bio-informatic analysis of genes with robust average expression (>0.1 RPKM) demonstrates that ~500 genes are up-regulated and ~200 genes are down-regulated upon loss of Ezh2 (fold change > 1.4 and p-value < 0.05) (Fig. 8c). Similar to observations in primary BMSCs cultures, the cell cycle inhibitory gene Cdkn2a is one of the most prominently up-regulated genes in Ezh2 cKO calvaria (Fig. 8c, d). Of the ~700 differentially expressed genes, about 8.5% (n=61) associate with the Gene Ontology Biological Process (GO_BP) term ‘cell cycle’ (Fig. 8e). These RNA-seq results corroborate findings obtained with ex vivo cultures of BMSCs, and establish that genetic loss of Ezh2 perturbs in vivo expression of cell cycle regulatory genes in mouse calvaria.

**DISCUSSION**

Our group has pursued multiple strategies for understanding the cell autonomous effects of Ezh2 in mesenchymal stromal cells and committed osteoblasts within bone tissue (reviewed in (18)). We have previously shown that genetic inactivation of Ezh2 in calvarial bones (Ezh2 cKO\(^{\text{Prrx1\_Cre}}\), Ezh2\(^{\text{loxP/loxP}}\): Prrx1-Cre) enhances expression of osteogenic genes. These mice exhibited several major phenotypic changes including a domed head and premature suture fusion (i.e., craniosynostosis) (26), a phenotype that could originate from any developmental stage when the Prrx1 promoter first begins to express Cre. This permanent genetic deletion of Ezh2 at an early developmental stage limited our interpretation. However, these studies did not permit a direct assessment of the acute cell autonomous mechanistic effects of Ezh2 deletion in osteoblasts during normal skeletal patterning. Our present study assessed the genetic role of Ezh2 in the osteoblastic lineage and demonstrates that Ezh2 is necessary for early proliferative stages of osteoblastogenesis. This function complements its known activity as a suppressor of osteoblast maturation.
Conditional deletion of Ezh2 in osteoblasts in vivo (using the Osx/Cre driver) results in mice with normal skeletal patterning. Female animals do not exhibit any obvious anatomical changes, while male cKO mice weigh less than CON animals at 3 and 8 weeks of age. μCT reconstructions indicate that skull structure, calvaria suture formation and clavicle formation are within the normal range of variation in mice lacking Ezh2 in the osteoblast lineage. Interestingly, μCT analysis reveals a mild low trabecular bone mass phenotype at three weeks of age that resolves at eight weeks. These findings are supported by histomorphometric analysis at three weeks of age. Interestingly, a reduction in osteoblasts is accompanied by a reduction in osteoclasts at three weeks of age, suggesting that bone formation and resorption remain coupled with osteoblastic loss of Ezh2. This coupling effect is also observed at twelve weeks of age, where the number of osteoclasts is significantly different between control and Ezh2 cKO animals. Histologic assessment reveals normal growth plate and cartilage development, but shows enhanced formation of cells positive for lipid droplets in the bone marrow cavity of cKO animals. Loss of Ezh2 in mouse BMSCs reduces osteogenic differentiation ex vivo, in part because of negative effects on cell cycle progression that occur concomitant with up-regulation of cyclin-dependent kinase inhibitors (e.g., CDKN2A/p16<sup>INK4A</sup>-p19<sup>ARF</sup>) and down-regulation of genes supporting mitotic division (e.g., cyclin B2). Consistent with these results, changes in the expression of cell cycle genes are also observed in Ezh2 cKO calvaria. Of interest, BMSCs from six to eight week old Ezh2 cKO animals exhibit a reduction in osteogenic differentiation, but the bone parameters resolve at this age in vivo. This suggests that Ezh2 is required for the commitment of progenitor cells into the osteogenic lineage, while coupling between osteoblast and osteoclast restores net trabecular bone formation in vivo. We note that the contribution of Ezh2 to cell cycle progression has been well-documented (43-45) for non-osseous cell types and that small-molecule inhibitors of Ezh2 are used clinically to inhibit cancer cell proliferation (46,47). Taken together, our study indicates that Ezh2 controls osteoblastic differentiation by both supporting cell cycle progression of osteoblast progenitor cells and suppressing maturation of precursor cells that are committed to the osteoblast lineage.

The gain in our understanding that Ezh2 is a bifunctional regulator of osteoblastogenesis clarifies several other previous results. For example, previous studies have shown that mesenchymal loss of Ezh2 results in several skeletal abnormalities (26,37), and these apparent patterning defects can now be interpreted as resulting from a reduction in the expansion of committed osteoblast progenitors when Ezh2 is inactivated in Ezh2 cKO mice that express the Prrx1-Cre driver. The in vivo importance of Ezh2 in osteoprogenitor cells during early stages of bone formation in skeletally immature mice is corroborated by our observation that Ezh2 cKO that express the Osx-Cre driver have a transient low trabecular bone volume phenotype in young adult mice. Thus, genetic inactivation of Ezh2 in early stages of skeletogenesis generally has negative biological effects. However, Ezh2 inhibition also has clear bone stimulatory potential from the perspectives of both bone tissue-engineering and bone anabolic therapy. For example, transient loss, in marked contrast to irreversible loss of Ezh2, using of Ezh2 function (knock-down or small-molecule inhibitors) in cultured mesenchymal cells stimulates osteogenic differentiation in vitro, while Ezh2 inhibition also enhances in vivo bone formation and prevents bone-loss associated with estrogen depletion (26,28,29,33,34).

Our previous work has indicated that Ezh2 loss in the mesenchymal compartment yields senescence-like phenotype, characterized by enhanced expression of Cdkn2a, a well-characterized cell cycle and senescence marker, as well as enhanced bone-marrow lipid droplet formation (26). Deletion of Ezh2 co-regulators, Hdac3 and Zfp521, in osteoblast progenitors produces similar marrow phenotypes (48-50). Our current study also shows that Ezh2 loss results in bone-marrow adiposity and enhanced expression of Cdkn2a. It is plausible that absence of Ezh2 could potentially activate an adipogenic program that causes accumulation of fat droplets in cells that are pre-committed to the osteogenic lineage. Indeed, fate-mapping studies have shown that Osx-Cre positive cells can contain lipid droplets (51,52). These data suggest that, similar to Hdac3 (53), Ezh2 could potentially regulate lipid storage in pre-osteoblasts. It is noteworthy that the expression of
Cdken2a is more robust in primary calvaria derived from animal that lack Ezh2 expression in the mesenchyme (26) when compared to animals that lack expression of this epigenetic enzyme in pre-osteoblasts (current study). Our current and past findings are also supported by a recent study which shows that the loss of Ezh2 in Nestin-expressing cells, a population of mesenchymal stem cells, induces a senescence phenotype in mice (54).

Recent studies have demonstrated that Ezh2 possesses methyltransferase independent function (55-57). Therefore, we considered that Ezh2 inactivation by elimination of the SET domain in principle still could result in a truncated Ezh2 protein that may retain some of its biochemical functions. We consider it less likely that this mutant protein is rapidly degraded in mesenchymal cells. Permanent Ezh2 loss by genetic inactivation is expected to destabilize the PRC2 complex resulting in weakening of chromatin structure and inducing gene expression changes indirectly. Long-term loss during development could potentially provoke compensatory mechanisms to ensure skeletal formation that proceeds through less efficient mechanisms.

Our group and others have previously presented solid data in support of the concept that Ezh2 inhibition has bone stimulatory properties (26,28,29,33,34). The present work indicates that inhibitory strategies could also provoke negative effects on bone, especially during initial stages of bone growth. Notwithstanding this limitation in these negative biological effects during maturation of the mammalian skeleton, it may still remain realistic to leverage Ezh2 inhibition for short-term, local applications in skeletally mature mammalian species and in bone tissue engineering to accelerate maturation of committed osteoblasts. Therapeutic strategies utilizing Ezh2 inhibitors could potentially be employed to enhance spinal fusion, stimulate orthopedic implant grafting, and heal non-union fractures. However, Ezh2 inhibition is clearly not beneficial for adolescent or young adult mice, nor does it appear to be suitable for long-term-applications (i.e., chronic conditions such as osteoporosis). Our current data indicate that undesired effects of Ezh2 suppression, including cell cycle impediment and senescence, may counter the beneficial effects (i.e., enhanced expression of osteogenic genes) in bone stimulatory strategies.

**EXPERIMENTAL PROCEDURES**

**Calvarial Osteoblast Culture** – Calvaria (parietal and frontal bones) were dissected from 2-3 days old Ezh2lox/lox pups and digested three times (20 min, 1 hr, 1 hr) in collagenase digestion medium (αMEM, 2mg/ml Collagenase type II, and 0.005% trypsin) at 37°C (58). The cells from the third digest and the remaining calvarial bones were plated and maintained in αMEM supplemented with 10% FBS, 1% antibiotic and antimycotic, and 1% non-essential amino acids. Passage two cells were plated (16k/cm²) and infected with adenovirus expressing GFP (Ad-GFP) or GFP and Cre recombinase (Ad-GFP-Cre) (Vector Biolabs).

**Bone Marrow Mesenchymal Stem Cell Cultures** - Bone marrow mesenchymal stem cells (BMSCs) were isolated from wild type C57Bl/6 mice, Ezh2lox/lox animals, or Ezh2 CON or Ezh2 cKOlox mice as described (59). Briefly, bone marrow was flushed from femurs and tibias of 6-8 week old mice, which is an age that yields a robust and consistent number of BMSCs. Cells were immediately seeded for experiments in 6- and 12-well plates (1 million cells per cm²) or cultured in T175 flasks for four to six days before seeding the adherent cell population in 6- and 12-well plates (20,000 cells per cm²). BMSCs were maintained in αMEM supplemented with 20% FBS, 1% antibiotic and antimycotic, and 1% non-essential amino acids. BMSCs were selected by their ability to adhere to the culture plates (at least three days) before exposing to Ezh2 inhibitor, adenovirus, and differentiation medium. Osteogenic differentiation was accomplished by the addition of 50μg/ml ascorbic acid, 10mM beta glyceral phosphosphate, and 10⁻⁸ M dexamethasone in the maintenance medium. Media were changed every three days.

**MTS Activity Assay** – MTS activity was assayed according to the manufacturer’s protocol (Promega). Absorbance was measured at 490nm using a SpectraMAX Plus spectrophotometer (Molecular Devices).

**Hoechst staining** - Media was aspirated from wells and cells were washed 1X with PBS. After aspiration of PBS, 500ul of Hoechst 33258 (Sigma 94403) (0.5ul/ml in PBS) was added to each well. Plates were left to incubate in the dark at room temperature for 20 minutes. After
incubation, fluorescence intensity was measured at 340nm excitation wavelength and 460nm emission wavelength using an F200 Infinite Pro (Tecan) plate reader. Measurements were fit to a standard DNA curve to determine relative DNA content.

Real-time reverse transcriptase PCR (RT-qPCR) - RNA was isolated using the Direct-zol™ RNA kit (Zymo Research). RNA was then reverse transcribed into cDNA by the SuperScript III First-Strand Synthesis System (Invitrogen). Transcript levels were measured using the 2\(^{-\Delta \Delta CT}\) method and normalized to the housekeeping gene Gapdh (set at 100). Gene specific primers are shown (Suppl. Table 3).

Western Blotting - Cell lysis and western blotting was performed as described previously (26,29). Proteins were visualized using the ECL Prime detection kit. Primary antibodies used were: Actin (1:10,000; sc-1616; Santa Cruz), H3 (1:10,000; 05-928; Millipore), H3K27me3 (1:5,000; 17-622; Millipore), Ezh2 (1:10,000; 5246; Cell Signaling), and Tubulin (1:10,000; E7; University of Iowa Hybridoma Bank).

Alkaline Phosphatase and Alizarin Red Staining – for alkaline phosphatase activity, cell cultures were fixed in 10% neutral buffered formalin and stained with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium to monitor the enzymatic activity of alkaline phosphatase (Promega). For Alizarin red staining, cells were also fixed in 10% neutral buffered formalin and stained with 2% Alizarin Red to visualize calcium deposition. Absorption of Alizarin Red stain was quantified with ImageJ software (60).

Animal welfare - Animal studies were conducted according to guidelines provided by the National Institutes of Health and the Institute of Laboratory Animal Resources, National Research Council. The Mayo Clinic Institutional Animal Care and Use Committee approved all animal studies. Animals were housed in an accredited facility under a 12-hour light/dark cycle and provided water and food (PicoLab Rodent Diet 20, LabDiet) ad libitum.

Deletion of function Ezh2 in osteoblasts - Mice containing a conditional Ezh2\(^{fl/fl}\) allele (42) flanking the SET domain were obtained from a Mutant Mouse Regional Resource Center (B6;129P2-Ezh2tm1Tara/Mmnc University of North Carolina, Chapel Hill). Ezh2 function was conditionally deleted in osteoblasts by these mice with animals that express Cre recombinase under the control of the Sp7/Osx promoter (41). Our studies characterized control (CON, Ezh2\(^{wt/wt}\); Osx-Cre+) and conditional knockout (Ezh2 cKO\(^{osx}\), Ezh2\(^{fl/oosf}\); Osx-Cre+) animals. The following primers were used for genotyping: Ezh2 (For: TGTCAATGTTGCTGAGTATTCTAC, Rev: GGAACCTCGATAATGTGAAC; Cre (For: TCCAATTTAAGGACCCAGCACA, Rev: CCTGATCTGGCAATTGCTGTA).

Micro-computed tomography analysis – Right tibias were harvested from 3, 8, and 12 week old wildtype and Ezh2 cKO\(^{osx}\) mice. Soft tissue surrounding the bone was carefully removed and samples were fixed in 10% neutral buffered formalin (NBF) for 48 hours followed by storage in 70% ethanol. Tibias were scanned with an ex vivo \(\mu\)CT system (Skyscan 1174; Skyscan) equipped with a 50-kV, 800-\(\mu\)A X-ray tube and a 1.3-megapixel charge-coupled device coupled to a scintillator. Samples were maintained in a moist environment during scanning to prevent dehydration and scanned in air using 8 \(\mu\)m (3 and 8 weeks old) or 10 \(\mu\)m (12 weeks old) isotropic voxels with an integration time of 400 ms. The region of analysis selected was between 13% and 16% of bone length (3 week old mice) or between 12% and 15% of bone length (8 and 12 week old mice) relative to the proximal tibial epiphysis for trabecular bone analysis. Mid-shafts of the tibias were assessed for the cortical parameters. Reconstructions were performed in NRecon software (Skyscan), and subjected to morphometric analyses using CTAn software (Skyscan). A minimum greyscale threshold of 60 (3 weeks old) or 70 (8 and 12 weeks old) was used.

Skulls were harvested from 3 week old wildtype and Ezh2 cKO\(^{osx}\) mice. Samples were fixed in 10% neutral buffered formalin (NBF) for 48 hours followed by storage in 70% ethanol. Skulls were scanned using a Skyscan vivaCT40 system (Scanco) at 70-kV, 114-\(\mu\)A with an integration time of 221 ms for a 10.5 isometric voxel size. 3D renderings were created using Microview (Parallax).

Histology and histomorphometric analysis – Right femurs were harvested from 3 week old wildtype and Ezh2 cKO\(^{osx}\) animals. Soft tissue surrounding the bone was carefully removed and samples were fixed in 10% neutral buffered formalin for 48 hours followed by storage in 70% ethanol. Samples were then subjected to dehydration and scanned in air using 8 \(\mu\)m (3 and 8 weeks old) or 10 \(\mu\)m (12 weeks old) isotropic voxels with an integration time of 400 ms. The region of analysis selected was between 13% and 16% of bone length (3 week old mice) or between 12% and 15% of bone length (8 and 12 week old mice) relative to the proximal tibial epiphysis for trabecular bone analysis. Mid-shafts of the tibias were assessed for the cortical parameters. Reconstructions were performed in NRecon software (Skyscan), and subjected to morphometric analyses using CTAn software (Skyscan). A minimum greyscale threshold of 60 (3 weeks old) or 70 (8 and 12 weeks old) was used.

Skulls were harvested from 3 week old wildtype and Ezh2 cKO\(^{osx}\) mice. Samples were fixed in 10% neutral buffered formalin (NBF) for 48 hours followed by storage in 70% ethanol. Skulls were scanned using a Skyscan vivaCT40 system (Scanco) at 70-kV, 114-\(\mu\)A with an integration time of 221 ms for a 10.5 isometric voxel size. 3D renderings were created using Microview (Parallax).
ethanol. Un-decalcified femurs were embedded in methyl methacrylate resin and sectioned using a rotary retracting microtome. Sections of 5 µm thickness were stained by Gomori’s trichrome method as previously described (61). Calcified bone was visualized in green and osteoid in red. Quantitative histomorphometry was performed utilizing OsteoMeasure™ to estimate the percentage of bone volume (BV/TV, %), osteoblast surface (Ob.s), osteoid surface, number of osteoblasts (N.Ob.) and osteoclasts (N.Oc.) per bone parameter (B.Pm). In addition, trabecular thickness (Tb.Th, µm) was also derived from trabecular parameters. Six fields of view were measured scanning a total area of 2.1 mm² at a distance of approximately 1 mm away from the growth plate. All measurements were made in the trabecular region. Cortical bone was marked as non-tissue and was not included in the measurements.

Flow cytometric analysis – Cells were harvested by trypsin, washed with PBS, and fixed in 70% ethanol overnight at 4°C. Cell were then washed two times with PBS and stained with 0.5 mL FxCycle propidium iodide/RNase staining solution (Molecular Probes) for 30 minutes at room temperature in the dark. Cell cycle distribution (>10,000 events) was collected on the FACSCanto system (BD Biosciences) and the data analyzed utilizing FACSDiva 8.0.1 software (BD Biosciences).

High Throughput RNA Sequencing and Bioinformatic Analysis of Primary Calvaria – Calvaria were dissected out from 2 to 3 day old pups, washed in PBS, digested for 20 minutes in collagenase digestion medium (αMEM, 2mg/ml Collagenase type II, and 0.005% trypsin) at 37°C, washed in PBS, and snap frozen with liquid nitrogen and stored at -80°C. RNA was then isolated utilizing Ultra Turrax T25 (IKA) and Direct-zol™ RNA kit (Zymo Research). High throughput read mapping and bioinformatic analyses for RNA-seq were performed as previously reported (26,27,29,62). Gene expression is expressed in reads/kilobase pair/million mapped reads. RNA-seq data were deposited in the Gene Expression Omnibus of the National Institute for Biotechnology Information (GSE111245). Gene tracks were viewed using Interactive Genome Viewer (IGV) 2.3.98 with the mm9 build loaded (63,64). Cell cycle-related genes (n = 1742) have been obtained from the gene ontology consortium (65,66).

Ex vivo assays – For assays that utilized primary calvarial osteoblasts and BMSCs, cells from several animals (typically two to five) were pooled and then plated out as three biological replicates. These assays were repeated at least three times. Representative experiments are shown.

Statistics – For ex vivo studies, data are shown as mean ± standard deviation and statistical analysis was performed with unpaired Student's t-test. For in vivo studies, results are depicted by scatter plots that contain mean ± standard error with each dot representing one mouse. To assess for bone parameters (µCT and histomorphometry), six to ten animals derived from at least three independent litters were used in these studies. Statistical analysis was performed using Student's unpaired t-test or Tukey post-hoc tests for multiple comparisons using GraphPad Prism-7 software. Significance is noted in the figures, when applicable (*: p<0.05).
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CONFLICT OF INTEREST
The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Dr. Karperien is founder and shareholder of Hy2Care B.V.

AUTHOR CONTRIBUTIONS
AD and AJvW designed the study. AD, ETC, CRP, RMS, MG, CAP, DLB, FK, OP, FSA and RE performed the biological experiments. AD, ME, MEML, MK, SMR, RT, JJW, and AJvW provided interpretation of results and guidance of studies. AD and AJvW wrote the paper with comments from all authors.
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FIGURE LEGENDS

Figure 1. Bone-specific loss of Ezh2 bypasses developmental defects. Images (four weeks, female) (a), x-ray analysis (four weeks, female) (b), and weight assessment (c) of CON and Ezh2 cKO\textsuperscript{lox/lox} animals. μCT analysis of skulls (d, e) and clavicles (f) of CON and Ezh2 cKO\textsuperscript{lox/lox} animals (three weeks, male).

Figure 2. Osteoblastic loss of Ezh2 induces a low trabecular mass phenotype in young animals. Weights of animals assessed as well as tibial trabecular bone parameters assessed by μCT of CON and Ezh2 cKO\textsuperscript{lox/lox} male mice at three (a), eight (b), and twelve (c) weeks age. Structural parameters measured by μCT include trabecular bone volume fraction (BV/TV, %), trabecular number (Tb.N., mm\textsuperscript{-1}), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), and connectivity density (ConnD, 1/mm\textsuperscript{3}).

Figure 3. Osteoblast-specific loss of Ezh2 results in osteoblast and osteoclast alterations in young mice. Von Kossa-McNeal (a) and Golder’s Trichrome (b) staining of distal femurs (three week old, male). Goldner’s Trichrome staining of femur mid-shafts (three week old, male) (c). Static histomorphometry analysis of femurs derived from three week old male CON and Ezh2 cKO\textsuperscript{lox/lox} animals (d). Histomorphometric parameters measured includes trabecular bone volume fraction (BV/TV, %), osteoblast surface (Ob.S/BS, %), osteoclast surface (Oc.S/BS, %), trabecular thickness (Tb.Th, μm), number of osteoblasts per bone perimeter (N.Ob./B.Pm., mm\textsuperscript{-1}, mm\textsuperscript{2}), and number of osteoclasts per bone perimeter (N.Oc./B.Pm., mm\textsuperscript{-1}, mm\textsuperscript{2}).

Figure 4. Cre expression in Ezh2\textsuperscript{lox/lox} calvarial osteoblasts results in the loss of Ezh2 protein. Illustration of the experimental protocol for Ad-GFP/Ad-GFP-Cre infection and osteogenic differentiation of primary calvarial osteoblasts derived from 2-3 day old Ezh2\textsuperscript{lox/lox} animals (a). Schematic diagram of the Ezh2\textsuperscript{lox/lox} allele showing the recombination event and highlighting the use RT-qPCR primers to detect wildtype, mutated (truncated), and SET domain of Ezh2 (b). Expression of GFP, Cre, Ezh2\textsuperscript{mut}, Ezh2\textsuperscript{SET}, and total Ezh2 mRNA levels four days after the addition of Ad-GFP and Ad-GFP-Cre (n = 3) (c). Western blotting analysis four days after the infection of cells (d).

Figure 5. Genetic loss of Ezh2 inhibits osteogenic differentiation of BMSCs. BMSCs were isolated from male and female 6-8 week old CON and Ezh2 cKO\textsuperscript{lox/lox} mice and differentiated into the osteogenic lineage as described in methods. RT-qPCR analysis of osteogenic markers during osteogenic commitment of BMSCs (n = 3) (a). Alizarin red staining (b) and quantification (c) of BMSCs differentiated for seventeen days (n = 3). Hoechst staining (DNA content) of BMSCs differentiated for seven days (n = 3) (e). Western blotting analysis of Ezh2 and H3K27me3 levels in BMSCs (male) undergoing osteogenic differentiation (d).

Figure 6. Acute loss of Ezh2 inhibits osteogenic differentiation of BMSCs. BMSCs were isolated from 7 week old Ezh2\textsuperscript{lox/lox} male mice, infected with Ad-GFP or Ad-GFP-Cre, and differentiated into the osteogenic lineage (a). Expression of Cre, Ezh2\textsuperscript{mut}, Ezh2\textsuperscript{SET}, and total Ezh2 mRNA levels on day four of osteogenic differentiation (n = 3) (b). MTS toxicity assay (n = 3) (c) and Hoechst staining (DNA content) (n = 3) (d) on day seven of differentiation. RT-qPCR analysis of osteogenic markers on day fourteen of differentiation (n = 3) (e). Alizarin red staining (f) and quantification (n = 3) (g) day fourteen of differentiation. Western blotting analysis of Ezh2 and H3K27me3 on day four of osteogenic differentiation (h).

Figure 7. Acute loss of Ezh2 disrupts the cell cycle in BMSCs. BMSCs were isolated from 6-8 week old C57/B6 CON and Ezh2\textsuperscript{lox/lox} male and female mice, infected with Ad-GFP-Cre, and differentiated into the osteogenic lineage (a). Microscopy images on day three of GFP expression (green) in male and female BMSCs infected with Ad-GFP-Cre (b). RT-qPCR analysis (day six) of Eef1a1 and several cell
Ezh2 function in proliferating osteoprogenitor cells

cycle markers in BMSCs (male, n = 3) (c). Cell cycle analysis (day six) of male and female BMSCs (n = 3) (d). Letters (a and b) denote significance between columns within each of the two figure panels. MTS activity assay of BMSCs at various time points after infection and induction of differentiation (female, n = 3) (e).

**Figure 8. Changes in expression of cell cycle genes with Ezh2 loss in mouse calvaria.** Calvaria were isolated from 2 to 3 day old CON and cKO female pups and RNASeq analysis was performed (n = 3). Genome browser view (IGV) of Ezh2 exons 4-20 (a). Exons 1-3 were omitted due to space constrain (a very long intron between exons three and four). A closer IGV view of exons 14-20 (b). The SET domain is located within exons 16-19 of Ezh2. Gene expression changes with the loss of Ezh2 in mouse calvaria based on fold change (FC) > 1.4 and p-value < 0.05 (e). All genes with an average expression > 0.1 RPKM were included in the analysis. Significantly up-regulated (red, n = 506) and down-regulated (blue, n = 209) genes are highlighted. Expression of Cdkn2a in CON and cKO calvaria (d). Venn diagram analysis showing that cell cycle-related genes exhibit altered expression with the loss of Ezh2 in calvaria (e).
Figure 1

Ezh2 function in proliferating osteoprogenitor cells
Figure 2

(a) Ezh2 function in proliferating osteoprogenitor cells. 

(b) BV/TV (%) distribution.

(c) Tb.N. (1/mm) distribution.

(d) Tb.Th. (mm) distribution.

(e) Tb.Sp. (mm) distribution.

(f) Conn.D. (mm²) distribution.

(g) CON vs. cKO comparison at different time points: 3 weeks, 8 weeks, and 12 weeks.
Figure 3

a) von Kossa-McNeal (Distal Femur)

b) Goldner's Trichrome (Distal Femur)

c) Goldner's Trichrome (Femur Midshaft)

d) Quantitative analysis of bone formation and remodeling:

- BV/TV (%)
- Ob./BS (%)
- Oc./BS (%)
- Tb.Th. (μm)
- N.Ob./B.Pm. (mm⁻²)
- N.Oc./B.Pm. (mm⁻²)

Significance indicated by asterisks (*).
Ezh2 function in proliferating osteoprogenitor cells

Figure 4

(a) Schematic representation of the experimental setup.
(b) Diagram illustrating the expression and Cre-mediated deletion of the Ezh2 allele.
(c) Bar graphs showing normalized expression levels of GFP, Cre, and Ezh2 mutants.
(d) Western blot analysis of Ezh2 and Tubulin expression levels.
Figure 5

(a) Graphs showing the expression levels of Sp7, Bglap, Alpl, and Ibsp in both male and female BMSCs. The graphs illustrate the normalized expression levels across different differentiation days (Diff. Day) for each genotype (CON and cKO).

(b) Images of Alizarin Red staining for both male and female BMSCs, comparing the area (%) and DNA content (µg/µl) between CON and cKO genotypes.

(c) Graph showing the area (%) of Alizarin Red staining for both male and female BMSCs.

(d) Western blot images for Ezh2, H3K27me3, and H3, comparing the expression levels between D3, D7, and D14 for both CON and cKO genotypes.

(e) Graph showing the DNA content (µg/µl) for both male and female BMSCs.
Figure 6

(a) Schematic representation of osteo differentiation stages.

(b) Normalized expression of Cre, Ezh2mut, Ezh2SET, and Ezh2.

(c) Absorbance ± STD (480nm) for Ad-GFP and Ad-GFP-Cre.

(d) DNA ± STD (μg/ml) for Ad-GFP and Ad-GFP-Cre.

(e) Normalized expression of Alpl, Bglap, Il1b, and Omd.

(f) Image of cell cultures.

(g) Percentage area ± STD for Ad-GFP and Ad-GFP-Cre.

(h) Western blot analysis for Ezh2, H3K27me3, and H3.
Ezh2 function in proliferating osteoprogenitor cells

Figure 7

(a) Osteo. Differentiation

(b) WT F/F

(c) Eef1a1 Mki67 Ccnb2 Cdkn2a (p16)

(d) % Cells

(e) Norm. MTS Activity
Ezh2 function in proliferating osteoprogenitor cells

Figure 8

(a) CON
(b) cKO

Chr6: Ezh2 (exons 4-20)

LoxP sites within Ezh2 locus

(c) CON vs cKO

Log2(FC) vs Average RPKM

506 Down in cKO animals (FC > 1.4, p < 0.05)
209 Down in cKO animals (FC > 1.4, p < 0.05)

(d) Cdkn2a

RPKM

0 0.5 1.0 1.5 2.0 2.5

1 2 3 4 5 6

CON cKO

(e) up in cKO animals (506) down in cKO animals (209)
483 0 171
23 0 38
1681

cell cycle (1742)
Enhancer of zeste homolog 2 (Ezh2) controls bone formation and cell cycle progression during osteogenesis in mice

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