Inhibition of the epigenetic suppressor EZH2 primes osteogenic differentiation mediated by BMP2

Amel Dudakovic, Rebekah M. Samsonraj, Christopher R. Paradise, Catalina Galeano-Garces, Merel O. Mol, Daniela Galeano-Garces, Pengfei Zan, M. Lizeth Galvan, Mario Hevesi, Oksana Pichurin, Roman Thaler, Dana L. Begun, Peter Kloen, Marcel Karperien, A. Noelle Larson, Jennifer J. Westendorf, Simon M. Cool, Andre J. van Wijnen

1Department of Orthopedic Surgery, Mayo Clinic, Rochester, MN, USA
2Department of Biochemistry & Molecular Biology, Mayo Clinic, Rochester, MN, USA
3Mayo Clinic Graduate School of Biomedical Sciences, Mayo Clinic, Rochester, MN, USA
4Center for Regenerative Medicine, Mayo Clinic, Rochester, MN, USA
5Department of Orthopedic Surgery, Amsterdam University Medical Center, Amsterdam, the Netherlands
6Department of Orthopedic Surgery, Second Affiliated Hospital of Zhejiang University, School of Medicine, Hangzhou, China
7Department of Orthopedic Surgery, Shanghai Tenth People’s Hospital Affiliated to Tongji University, School of Medicine, Shanghai, China
8Department of Developmental BioEngineering, University of Twente, Enschede, the Netherlands
9Glycotherapeutics Group, Institute of Medical Biology, Agency for Science, Technology and Research (A*STAR), Singapore
10Department of Orthopaedic Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Running title: Synergy between BMP2 and Ezh2 inhibition

Keywords: BMP2; osteoblast; mesenchymal stem cell; epigenetics; methyltransferase; histone methylation; bone development; osteogenesis; enhancer of zeste homolog; Ezh2; chromatin regulation

ABSTRACT

Bone-stimulatory therapeutics include bone morphogenetic proteins (e.g. BMP2), parathyroid hormone, and antibody-based suppression of WNT antagonists. Inhibition of the epigenetic enzyme Enhancer of Zeste Homolog 2 (EZH2) is both bone-anabolic and osteo-protective. EZH2 inhibition stimulates key components of bone-stimulatory signaling pathways, including the BMP2 signaling cascade. Because of high costs and adverse effects associated with BMP2 use, here we investigated whether BMP2 dosing can be reduced by co-treatment with EZH2 inhibitors. Co-administration of BMP2 with the EZH2 inhibitor GSK126 enhanced differentiation of murine (MC3T3) osteoblasts, reflected by increased alkaline phosphatase activity, alizarin red staining, and expression of bone-related marker genes (e.g. Bglap and Phospho1).

Strikingly, co-treatment with BMP2 (10 ng/ml) and GSK126 (5 μM) was synergistic and was as effective as 50 ng/ml BMP2 at inducing MC3T3 osteoblastogenesis. Similarly, the BMP2–GSK126 co-treatment stimulated osteogenic differentiation of human bone marrow–derived mesenchymal stem/stromal cells, reflected by induction of key osteogenic markers (e.g. Osterix/SP7 and IBSP). A combination of BMP2 (300 ng local) and GSK126 (5 μg local and 5 days of 50 mg/kg systemic) yielded more consistent bone healing than single treatments with either compound in a mouse calvarial critical-sized defect model according to results from μCT, histomorphometry, and surgical grading of qualitative X-rays. We conclude that EZH2 inhibition facilitates BMP2-mediated induction of osteogenic differentiation of progenitor cells and maturation of committed osteoblasts. We propose that epigenetic
Synergy between BMP2 and Ezh2 inhibition

priming, coupled with bone anabolic agents, enhances osteogenesis and could be leveraged in therapeutic strategies to improve bone mass.

Bone morphogenetic proteins (BMPs) are protein ligands of the transforming growth factor superfamily of morphogens and growth factors that control many biological processes, including osteoblast differentiation and bone regeneration (1,2). BMP2 is the first osteogenic member of this superfamily (3,4), and one of the most widely studied and clinically relevant members of this protein family. BMP2 and BMP7 are potent inducers of bone formation in vitro and function by activating canonical BMP signaling via binding to type I and II BMP receptors (1,5,6). Activated BMP receptors then phosphorylate and activate Smad proteins (e.g., Smad1, 5, and 8), which in turn complex with a co-Smad (Smad4). These Smad complexes then translocate into the nucleus to induce transcriptional changes within activated cells (7). Runt-related transcription factor 2 (Runx2), the homeodomain transcription factor Dlx5, and the Zn-finger protein Osterix/Sp7 are key genes induced by canonical BMP signaling (8-14). Transcriptional induction of Runx2 results in the activation of the osteogenic cascade in progenitor cells to stimulate osteoblast differentiation and bone formation (15).

Fracture healing is a regenerative process that recapitulates many of the events that occur during fetal stages of skeletal developmental (16,17). Progenitor cells differentiate directly into osteoblasts during intramembranous bone repair while a cartilaginous callus precedes bone formation during endochondral bone repair. Osteogenic pathways, including BMP signaling, are critical for proper healing through both intramembranous and endochondral mechanisms (18). While normal fracture healing results in complete bone restoration, 5% to 10% of all fractures do not heal properly (19-21) resulting in 100,000 nonunions each year in the United States (22). Delayed fracture healing can result in increased time lost from work and medical costs, chronic pain, opioid use, and disability. BMP proteins are induced during native fracture repair (23,24) and their administration alone or in combination with carrier materials (e.g., collagen sponge) has been shown to promote healing in fracture and critical-sized defect animal models (25-28). Following promising outcomes in clinical trials (29-31), BMP2 is in current clinical use for orthopedic indications including tibial fracture healing and high risk spine fusion (32). Despite its success, the clinical applications of high-concentrations of BMP2 are limited due to high cost (33) and detrimental side-effects such as heterotopic ossification, osteolysis, and airway obstruction (2,34). Thus, there is a need for safe augmentation of fracture healing and bone fusion in surgeries where there is a high risk of nonunion.

Epigenetic mechanisms are critical regulators of skeletal development and osteoblast differentiation (35-37). Enhancer of zeste homolog 2 (Ezh2), the catalytic sub-unit of the polycomb-repressive complex 2 (PRC2), catalyzes mono-, di- and tri-methylation of lysine 27 of histone H3 (H3K27me1, H3K27me2 and H3K27me3) (38,39). An alternative PRC2 complex in which enhancer of zeste homolog 1 (Ezh1) serves as the catalytic sub-unit possesses the same enzymatic activity but appears to have a more restricted biological role (38,40,41). The enzymatic activity of the PRC2 complex and accumulation of the H3K27me3 mark is associated with chromatin condensation and gene suppression (38). While Ezh2 is essential for proper skeletal patterning and bone formation (42-48), reducing the H3K27 methyltransferase activity of Ezh2 enhances osteogenic lineage commitment and osteoblast differentiation in vitro, as well as bone formation in vivo (44,49-56). Mechanistically, Ezh2 loss results in a reduction in stem cell numbers and promotes expression of established bone related genes, stimulates expression of genes involved in the activation of ligand-dependent signaling pathways (e.g., WNT, PTH, and BMP2 pathways), and enhances Smad1/5 phosphorylation (BMP2 signaling) in differentiating osteoblasts (44,47,54,57). In a reciprocal manner, forced expression of Ezh2 inhibits in vitro and in vivo osteogenesis, highlighting the importance of Ezh2 in the osteogenic fate of stem cells (51).

Ezh2 inhibition may enhance bone-anabolic pathways in osteoblasts and be leveraged in clinical practice to promote biological bone repair. Because Ezh2 inhibitors such as GSK126 and EPZ-6438 have been assessed in clinical trials (58,59), it may be feasible to combine these agents with BMP2 to enhance bone healing and reduce cost and side-effects associated with BMP2.
Therefore, in this study we assessed whether combining BMP2 treatment with Ezh2 inhibition would maximize osteogenic differentiation. We show that co-treatment of BMP2 and the Ezh2 inhibitor GSK126 promotes osteoblast differentiation in vitro and supports intramembranous bone healing in a critical-sized calvarial defect model in vivo.

Results

**Ezh2 inhibition synergizes with BMP2 to induce osteogenic differentiation of MC3T3 pre-osteoblasts** – Our group and others have shown that loss of Ezh2 function stimulates in vitro osteogenesis, enhances in vivo bone formation, and prevents bone loss associated with estrogen depletion (44,51,54-56). Additional analyses demonstrated that Ezh2 inhibition stimulates paracrine signaling, including activation of BMP signaling, enhanced expression of Wnt ligands, and up-regulation of the Pth1r receptor (54). To understand the interplay between osteoblast stimulatory effects of Ezh2 inhibition and the BMP signaling pathway, we assessed the impact of GSK126, a specific Ezh2 inhibitor, in combination with recombinant BMP2 during osteogenic differentiation of MC3T3 pre-osteoblasts (Fig. 1). Differentiating MC3T3 cells were treated with an established concentration of GSK126 (5µM) (54) and varying concentrations of BMP2 (Fig. 1A). As demonstrated previously (44,54), GSK126 enhances expression of osteoblast-related genes (osteocalcin/Bglap and phosphoethanolamine/phosphocholine phosphatase1/Phospho1) (Fig. 1B and Suppl. Fig. 1). Similarly, BMP2 treatment also stimulates expression of these genes in a dose-dependent manner. Even the lowest concentration of BMP2 (2.5 ng/ml) enhances the expression of osteogenic genes. Maximal osteogenic gene expression is reached with 50 ng/ml of BMP2 in MC3T3 cells. In this assay, 5µM GSK126 is equivalent to 5 ng/ml BMP2 when comparing expression of Bglap and Phospho1 (compare blue dashed line with red line). Interestingly, when compared to BMP2 administration, the combination of 5µM GSK126 with various BMP2 concentrations further stimulates osteogenic gene expression. It is noteworthy that low concentrations of BMP2 (2.5-10 ng/ml) in combination with GSK126 achieve expression levels similar to high BMP2 concentrations (compare red line to green line). Importantly, the combination of GSK126 and BMP2 exceeds the maximal effects of BMP2 alone on osteogenic gene expression. The effects of combinatory treatments are significantly different when compared to BMP2 treatment alone (red vs green line). As a further illustration of co-stimulatory effects, we compared gene expression at individual BMP2 concentrations (Fig 1C). While GSK126 and BMP2 enhance osteogenic gene expression, the dual administration of these pro-osteogenic factors synergistically activates Bglap and Phospho1 expression.

To further characterize this biological interaction between BMP2 and Ezh2 inhibition, GSK126 (5 µM) was combined with low (10 ng/ml) and high (50 ng/ml) concentrations of BMP2 in differentiating MC3T3 cells (Fig. 2A). As anticipated, both Ezh2 inhibition and BMP2 treatment stimulates expression of the key osteogenic transcription factor (osterix/Sp7) and extra-cellular matrix genes (Bglap and bone sialoprotein/Ibsp) (Fig. 2B). Pairwise comparisons of each treatment group using two-way analysis of variation (ANOVA) demonstrates significantly different gene expression profiles throughout the differentiation time course for all three genes (Sp7, Bglap, and Ibsp) (Suppl. Table 1). Of note, the combination of GSK126 and BMP2 (at both low and high concentrations) significantly enhances the effects of either agent alone. This is especially evident with the combination of GSK126 and low BMP2 concentration (compare dashed green line to blue and dashed red lines). Expression profiles of Sp7, Bglap, and Ibsp for vehicle-treated samples are shown as individual line graphs (Fig. 2C), demonstrating that differentiation of MC3T3 cells enhances expression of osteogenic genes in the absence of BMP2 and GSK126. However, because the effects of BMP2 and GSK126 are rather dramatic, vehicle-treatment under standard differentiation conditions in osteogenic media (black line) result in a far more modest up-regulation of osteogenic genes that is difficult to appreciate on a log-scale. The synergistic effects of GSK126 and low BMP2 are evident when assessing Bglap and Ibsp expression on a single representative time-point (day 6) during osteogenic differentiation (Fig. 2D). The gene expression data are corroborated by results obtained using assays detecting alkaline
phosphatase activity (Fig. 2E) and alizarin red staining (Fig. 2F), which are established histochemical assays for osteoblast differentiation. While minimal alizarin red staining is observed in low BMP2 and GSK126 treatment groups at this early stage of osteoblast differentiation, robust mineral deposition is observed with co-administration of GSK126 and low BMP2 on day 20 of osteoblastogenesis. Hence, our results demonstrate that inhibition of Ezh2 synergizes with BMP2 to stimulate differentiation of MC3T3 pre-osteoblasts.

Differential gene expression changes with Ezh2 inhibition and BMP2 administration - To assess the mechanisms by which GSK126 and BMP2 co-stimulate osteoblast differentiation, RNA from differentiating MC3T3 cells (described in Fig. 2) was subjected to RNA-seq analysis (Fig. 3). Unbiased principal component analysis (PCA) (60) of robustly expressed genes (RPKM > 0.3 in at least one group per time point, n = 11,974 genes on day 1, n = 12,307 genes on day 6) reveals BMP2- and GSK126-specific clustering of gene expression profiles on day 1 (Fig. 3A) and day 6 (Fig. 3B). It is noteworthy that GSK126 plus BMP2 combination clusters differently when compared to each compound alone, suggesting distinct effects of these agents on gene expression in differentiating osteoblasts. Based on these findings, we set out to identify genes that are differentially expressed on day 1 and day 6 across the six different treatment groups (Vehicle, 10 ng/ml BMP2, 50 ng/ml BMP2, 5µM GSK126, 10 ng/ml BMP2 + 5µM GSK126, and 50 ng/ml BMP2 + 5µM GSK126). For this analysis, we selected genes that were detected by RNA-seq (average RPKM >0.1 across six samples at each-time point) and were differentially expressed with a fold change greater than two (FC > 2) between any of the six treatment groups at each time-point. This bioinformatic analysis revealed 1,263 differentially expressed genes on day 1 (Fig. 3C) and 3,712 differentially expressed genes on day 6 (Fig. 3D) between the six treatment groups during MC3T3 differentiation. Interestingly, hierarchical clustering of differentially expressed genes reveals a grouping between low BMP2 concentration plus GSK126 with high concentration BMP2 alone or combination with GSK126. A clear hierarchical clustering separation occurs between these three highly-osteogenic treatment groups when compared with the other three treatment groups with less osteogenic potential (vehicle, 10 ng/ml BMP2, and 5µM GSK126). This global expression analysis demonstrates that BMP2 and GSK126 each induce different gene expression programs in differentiating MC3T3 cells. In addition, it appears that GSK126 enhances expression of BMP2-responsive genes, while this compound may also indirectly inhibit genes suppressed by BMP2 during osteoblast differentiation.

Co-stimulation of bone-related genes by concurrent administration of BMP2 and GSK126 – To identify genes that are synergistically activated by BMP2 and GSK126, we selected for highly expressed genes (RPKM > 0.3) that are induced by BMP2/GSK126 combination when compared to vehicle (FC > 2 on day one, FC > 4 on day four) and are also robustly up-regulated in the BMP2/GSK126 combination when compared to sole BMP2 and GSK126 treatments (FC>1.4 on day one, FC > 2 on day four). These analyses reveal twenty three co-activated genes with low BMP2 and GSK126 combination, while high BMP2 and GSK126 combination results in eighteen co-stimulated genes on day one of osteogenic differentiation (Suppl. Table 2 and Fig. 4A). Several of these genes (e.g., Dlx3, Ibsp, Wif1, Igfbp5, Hey1, and Nog) are implicated with skeletal formation. Nine of these genes are commonly up-regulated between low BMP2 plus GSK126 and high BMP2 plus GSK126 combinations. Both low and high concentrations of BMP2 in combination with GSK126 result in synergistic activation of seventy eight and eighty two genes, respectively, of which forty seven are common between the two combination groups on day 6 of osteoblast differentiation (Suppl. Table 3 and Fig. 4B). Due to the significant overlap between the two drug combinations on day six, additional bio-informatics analysis was performed on the commonly up-regulated genes (n = 47). DAVID 6.8 enrichment score analysis reveals several biological processes that are represented by these commonly up-regulated genes (Fig. 4C). Interestingly, two out of ten most highly enriched categories (green bars, ‘biomineralization’ and ‘ossification’) are directly related to bone formation processes. To investigate potential protein-protein networks within the enriched gene set interactions, these forty seven genes were assessed by STRING analysis (Fig. 4D). This
Synergy between BMP2 and Ezh2 inhibition

analysis reveals a major protein-protein interaction network termed ‘biomineral tissue development’ (red circles), which includes several well-characterized osteoblast/osteocyte-related genes (e.g., Ibsp, Phex, Bglap, Dmp1, and Spp1). In sum, these transcriptome data demonstrate that the combination of BMP2 and GSK126 results in co-stimulatory up-regulation of genes that are involved in osteoblast-related processes.

Similar to co-stimulated genes, bioinformatic analysis reveals gene sets that are co-suppressed by the combination of BMP2 and GSK126 (Suppl. Fig. 2 and Suppl. Tables 2 and 3). Combination of either low BMP2 with GSK126 or high BMP2 with GSK126 results in the co-suppression of genes on day one (Suppl. Fig. 2A) and day six (Suppl. Fig. 2B) of osteogenic differentiation. However, there is no overlap on day one while only nineteen genes overlap on day six when low and high BMP2 concentrations are combined with GSK126. Because of few overlapping genes (n = 19), DAVID 6.8 enrichment score analysis (Suppl. Fig. 2C) and STRING analysis (Suppl. Fig. 2D) on day six was performed on all co-suppressed genes (n = 101). Interestingly, enrichment score analysis reveals a robust suppression of genes related to cell division, including enriched categories such as ‘cell cycle’, ‘chromosome’, and ‘microtubule’. In support, protein-protein network interaction by STRING reveals an interaction network termed ‘cell division’ (blue circles). Thus, these data demonstrate that, in addition to enhancing expression of osteogenic genes, the combination of BMP2 and GSK126 suppresses cell division in MC3T3 cells to generate a post-proliferative state that supports differentiation.

To assess whether gene expression changes directly correlate with changes in H3K27me3 upon Ezh2 inhibition, ChIP-Seq analysis was performed on MC3T3 cells treated with vehicle and 5µM GSK126 for twenty four hours. Our analysis is focused on up-regulated genes since the loss of H3K27me3 is associated with gene activation. While our initial analysis demonstrated an overall reduction in H3K27me3, including in regulatory regions of key osteogenic loci (54), our current analyses focused on assessing H3K27me3 status of genes that are activated by dual administration of BMP2 and GSK126 (see Fig. 4 B-D). These analyses reveal that twenty four hour treatment with 5µM GSK126 reduces H3K27me3 levels at genomic regions of genes that are up-regulated by prolonged (six days) GSK126 and BMP2 treatment during osteogenic differentiation (Fig. 4E). Because significant up-regulation of key osteogenic/osteocytic markers (e.g., Phex, Bglap, Dmp1, and Spp1) occurs by dual administration of BMP2 and GSK126, we assessed H3K27me3 levels at genomic regions of these bone related genes. As illustrated by the Dmp1 locus (Fig. 4F), administration of GSK126 did not significantly alter H3K27me3 levels of mature osteoblast/osteocyte markers in MC3T3 cells. Because of the low number of loci that are activated by dual BMP2 and GSK126 treatment on day one (i.e., low BMP2 plus GSK126 and high BMP2 plus GSK126), it is not informative to perform general epigenomic profiling by averaging H3K27me3 marks across gene bodies. Yet, examination of loci-specific assessment reveals a significant reduction in H3K27me3 at genomic regions spanning the Dlx3 gene (Fig. 4F), a key osteogenic transcription factor that is up-regulated by the dual administration of GSK126 and BMP2 (Fig. 4A) and whose function is required for early stages of osteogenic differentiation (61). These results collectively indicate that activation of late osteoblast markers by dual administration of BMP2 and GSK126 may not be caused by changes in H3K27me3 at these loci, but rather may change Ezh2 mediated methylation events in the regulatory regions of osteogenic factors that are activated during earlier stages of osteoblast differentiation.

BMP2 and Ezh2 inhibition coordinately activate osteogenic lineage-commitment in human bone marrow-derived MSCs (hBMSCs)–

To assess BMP2 and GSK126 interaction during osteogenic differentiation in an uncommitted non-immortalized cell culture model, we utilized hBMSCs derived from commercially purchased bone marrow (62) (Fig. 5). As with pre-committed MC3T3 osteoblasts, hBMSCs were treated with BMP2 and GSK126 during the first six days of osteogenic differentiation (Fig. 5A). Similar to results with MC3T3 osteoblasts and human adipose-derived MSCs (hAMSCs) (44,54), preventing the formation of new H3K27me3 marks by GSK126 reduces total H3K27me3 marks in a concentration-dependent manner in hBMSCs.
Synergy between BMP2 and Ezh2 inhibition

(Fig. 5B). However, unlike in MC3T3 and hAMSCs, inhibition of H3K27me3 formation by GSK126 does not completely eliminate H3K27me3 in hBMSCs. The latter finding suggests that attrition of H3K27me3 marks due to histone demethylation in hBMSCs proceeds slower than in MC3T3s and hAMSCs. Also, no appreciable effects of GSK126 are observed on EZH2, Histone 3 (H3), and GAPDH protein levels, except for the highest concentration (10µM). As anticipated, BMP2 treatment increases osteogenic gene expression in a concentration-dependent manner (Fig. 5C). For comparison, expression of the housekeeping gene AKT1 is not altered with BMP2 and GSK126 administration relative to GAPDH. We note that GSK126 treatment does not have a significant impact on the expression of bone related genes during osteogenic differentiation of hBMSCs, presumably in part because H3K27me3 levels are less acutely downregulated upon Ezh2 inhibition. Similar to MC3T3 osteoblasts, the addition of GSK126 increases the osteogenic effects of BMP2 treatment on gene expression in hBMSCs. Interestingly, co-stimulation is observed for GSK126 and high BMP2 concentrations on day 6 (top graphs), but the combination of GSK126 with a low BMP2 dose shows strongest co-stimulation on day 13 (bottom graphs). These findings demonstrate that BMP2 and GSK126 co-induce osteogenic lineage commitment of hBMSCs, complementing the co-stimulatory effects observed during osteoblast maturation of MC3T3 cells.

Co-administration of BMP2 and GSK126 results in more consistent bone healing – Encouraged by our in vitro results, we performed initial feasibility studies to address whether BMP2 and GSK126 are able to co-stimulate bone formation in vivo in a critical-sized calvarial defect mouse model (Fig. 6) (63). The defect was created in the left parietal bone using a 2.5 mm dental trephine attached to a low-speed dental drill controlled by a manual foot pedal. The experiment was performed in adult (twelve week old) C57BL/6J male mice, which were sacrificed and assessed four weeks after surgery (sixteen weeks of age). Vehicle, BMP2 (0.3µg), GSK126 (5µg), and the BMP2/GSK126 combination were administrated at the defect site at time of surgery. Mice also received (intraperitoneal injections) 50 mg/kg GSK126 or vehicle (DMSO) one day before surgery, on the day of surgery, and three consecutive days after surgery (54). X-ray analysis demonstrates minimal healing in the vehicle and GSK126-treated mice (Fig. 6A and Suppl. Fig. 3). While robust healing is observed in some BMP2 treated animals, several calvarial defects did not heal properly within this group. Except for one exception, all mice exhibit robust calvarial healing with dual administration of BMP2 and GSK126. To quantify the healing process, micro-computed tomography (µCT) (Fig. 6B&D) and histomorphometric (Fig. 6 C&E) analyses of the calvarial defects were performed. These studies revealed significant differences between various treatment groups when assessing for bone mineral density (BMD) and bone volume fraction (BV/TV; bone volume-to-total volume ratio), including a robust difference between vehicle treated mice and mice treated with the combination of both BMP2 and GSK126. Interestingly, µCT and histomorphometry reveal more consistent healing in the combination group when compared to the BMP2 group (note spread of red vs green data points).

As an additional quantification method, two orthopedic surgeons were tasked to blindly grade the healing process by scoring X-ray images of the calvarial defects (Fig. 7 and Suppl. Fig. 3). We utilized a modified version of the method described by Spicer and colleagues (64). In this study (scale 0 to 5), a score of 0 indicates no bone formation while a score of 5 represents a completely healed defect (see methods section for additional information) (Fig. 7A). In addition, the surgeons were also asked to rank order the calvaria defects (n=22) from worst (score of 1) to best (score of 22) healed (Fig. 7B). Robust healing scores are evident in the BMP2 and combination groups (Fig. 7A). BMP2 is significantly different when compared to vehicle treatment when assessed by healing score and rank. Interestingly, the combination treatment of BMP2 and GSK126 is significantly different when compared to vehicle and GSK126 treatment as quantified by both methods. To assess the potential for observer bias, we examined the healing score and rank between the two surgeons. The inter-observer correlation was excellent between both orthopedic surgeons, with a spearman’s rho (ρ) of 0.93 for healing grade and 0.91 for overall rank in terms of healing (Fig.
Synergy between BMP2 and Ezh2 inhibition

7C). Taken together, the µCT and histomorphometry results, as well as the surgeon-defined healing score and rank indicates that BMP2 and the combination of both BMP2 and GSK126 mediate robust bone healing. However, the healing scores revealed a more consistent healing in the combination group when compared to BMP2 alone.

To address potential differences in healing consistencies between BMP2 and BMP2/GSK126 combination, additional statistical analysis was performed to assess for variance. In regards to the distribution of the data points, the point estimate of the variance for BMP2 versus the combination group favors the combination group as more consistent (lower estimated variance) in every comparison made (BMD, BV/TV (µCT), BV/TV (histomorphometry), healing score, and healing rank (see Figs. 6 and 7). The statistical odds that five out of five comparisons of variance estimates favor the same group (BMP2 plus GSK126) is unlikely to be driven by chance alone (p = 0.01). The distribution of data for BMD, as presented in Fig. 6B, reveals a variance of 18,861 for the BMP2 group and 2,633 for the combination group (7-fold less variance, p = 0.047). Similarly, the variance of BMP2 BV/TV via µCT is 0.026, as compared to 0.006 for the combination group, representing over 4-fold less variance for the combination group (p = 0.13). For histomorphometric evaluation, the variance of BMP2 scoring is 2.25 compared to 0.5 for the combination group (p = 0.11). Finally, when assessing healing rank (Fig. 7B), the variance for BMP2 is 42.3, as compared to the combination group variation of 3.8, or 10-fold less (p = 0.02). In sum, our statistical assessment for variance indicates that the combination group yields more consistent healing when compared to BMP2 treatment alone.

Taken together, our feasibility studies to examine the effects of co-treating a bone defect with both BMP2 and GSK126 are encouraging. These initial in vivo studies revealed that both drugs are compatible: at a minimum, the results show that GSK126 does not antagonize the osteogenic effects of BMP2, and in a more optimistic interpretation may reduce individual variation in healing outcomes (e.g., perhaps by accelerating the bone repair process).

Discussion

We and others have demonstrated that Ezh2 is required for skeletal development and recognized the concept that inhibition of this epigenetic enzyme can enhance bone formation in vitro and in vivo (42-56). The established evidence suggests that the pro-osteogenic effects of Ezh2 inhibition arise from de-repression of osteogenic genes (e.g., Sp7) as well as stimulation of osteogenic pathways through up-regulation of ligands (e.g., Wnt10b), receptors (Pth1r), and post-translation modification (e.g., Smad1/5 phosphorylation) (39,54). Based on this evidence, we hypothesized that inhibition of Ezh2 may be combined with established bone-anabolic therapeutics to stimulate osteogenesis and bone formation. Combining Ezh2 inhibition with BMP2 treatment is especially attractive as lowering concentration of this ligand could enhance its therapeutic potential, lower its side-effects, and may reduce costs associated with its use (23,33,34). Consequently, this study assessed the effects of combining BMP2 and GSK126 (Ezh2 inhibitor) on osteogenesis in vitro and bone healing in vivo.

Our current studies confirm the previously established pro-osteogenic effects of Ezh2 inhibition (GSK126) in MC3T3 cells (54). While Ezh2 inhibition has also been shown to stimulate osteogenic commitment of human adipose derived MSCs (hAMSCs) (44), we did no observe an enhancement in the expression of osteogenic genes in hBMSCs treated with GSK126. These divergent effects could be attributed to the varying levels and stability of H3K27me3 levels upon Ezh2 inhibition. Consistent with this reasoning, while H3K27me3 is completely eliminated by 2µM GSK126 in hAMSCs (44), a significant amount of H3K27me3 is observed when hBMSCs are treated with 10µM GSK126 (present study). The different sensitivities to Ezh2 inhibition may be due to varying pharmacodynamics and pharmacokinetics (e.g., cellular import and export of GSK126, and drug metabolism), as well as molecular differences in Ezh2 regulation (e.g., Ezh2 protein turnover) in different cell types. It is also possible that different expression patterns or activities of the other H3K27 methyltransferase (Ezh1) and opposing H3K27 demethylases (Jhd1, Kdm6a, and Kdm6b) may contribute to varying cellular responses to Ezh2 inhibition (i.e., GSK126) (38-40,65).
The pro-osteogenic effects of BMP2 were established about three decades ago (3,4,66-68). As anticipated, BMP2 stimulates osteogenic differentiation of MC3T3s and hBMSCs in a concentration-dependent manner. Previous studies have suggested that modifying the DNA and histone protein epigenomes may enhance BMP2-induced osteogenesis (50,69-72). We show here that combination of BMP2 and Ezh2 inhibition (GSK126) results in strong co-stimulatory enhancement of MC3T3 and hBMSC differentiation. Importantly, we establish that dual GSK126 and BMP2 treatment results in enhanced expression of key osteoblastic/osteocytic genes (e.g., Dmp1) that is accompanied by a reduction in genes related to cell division. These findings are in line with studies showing that BMP2 enhances osteogenesis while also suppressing cell proliferation in differentiating cells (73,74). Similarly, inactivation of Ezh2 is associated with enhanced osteogenic differentiation and cell cycle alterations (44,47,54). Interestingly, while GSK126 reduces overall H3K27me3 at genomic regions of genes activated by the combination of BMP2 and GSK126, our analysis did not reveal significant alterations in histone methylation of late osteogenic markers such as Dmp1, suggesting that activation of more proximal events linked to key osteogenic transcription factors (e.g., reduction of H3K27me3 at the Dlx3 locus) may support the pronounced co-stimulated activation of late phenotypic markers of osteogenic differentiation.

One key question is how synergism between GSK126 and BMP2 signaling during osteoblast differentiation is achieved. This question requires consideration of temporal and causative perspectives at both molecular and cellular levels. Pharmacological doses of GSK126 (0.2-2.0 µM) quantitatively reduce deposition of H3K27me3 marks in MSCs and osteoblasts within 6 hours (44,54). Within several days, the loss of gene-suppressive H3K27me3 marks results in the activation of quiescence related genes to stop proliferation and promotes osteogenic differentiation by stimulation of PTH, WNT and BMP signaling pathways (44,47,54). Yet, mature osteoblast-specific genes (e.g., Bglap/osteocalcin) are not maximally expressed until at least 10 days after initiation of osteoblast differentiation (54). Hence, GSK126 dependent demethylation is a mechanistic event that occurs very proximal to osteoblast maturation.

Exogenous administration of BMP2 rapidly induces the serine/threonine kinase activity of BMP receptors (e.g., Bmpr1a, Bmpr2) that phosphorylate Smad1-Smad5, while phospho-Smad1/5 then interacts with Runx2 and induces expression of the Bglap/osteocalcin which is directly controlled by at least three Runx2 binding sites (1,75-78). Thus, BMP2 signaling directly connects the bone morphogenic activity of BMP2 to the transcriptional activity of a bone-specific master regulator via protein phosphorylation. Therefore, biological synergy between Ezh2 inhibition and BMP2 is not mediated at the molecular level, but must be understood at the level of cellular pathways.

While BMP receptor dependent protein kinase activity at the cell surface cannot alter the Ezh2-dependent methylation of H3K27me3 in the nucleus, our previous data have shown that inhibition of H3K27me3 enhances the expression of genes that activate the endogenous BMP2-BMP receptor-Smad1/5 axis (54). Consistent with this model, we find that Ezh2 inhibition by GSK126 in MC3T3 osteoblasts decreases H3K27me3 at the Dlx3 locus and increases expression of Dlx3; Dlx3 is closely related to Dlx5 and both represent osteogenic and BMP2 responsive homeodomain transcription factors. Because Dlx3/5 proteins act initially upstream of RUNX2 (11,12), and because Runx2 is upstream of the BMP2 responsive Sp7/Osterix protein (11,79), t is apparent that GSK126 accelerates early stages of osteoblast differentiation by direct effects on Dlx3 and indirectly on the molecular interplay between Dlx3, Runx2 and Sp7.

BMP2 signaling during osteoblast differentiation is thought to activate several positive and negative feedback loops to ensure orderly progression of osteoblast differentiation. For example, activation of endogenous production of BMP2 proteins by osteoblasts is expected to ensure sustained paracrine signaling of this potent osteogenic morphogen (70,80,81). This study shows that GSK126 reduces the deposition of epigenetic marks that normally suppress expression of BMP2 responsive osteogenic genes such as Dlx3. Hence, GSK126 pre-conditions and facilitates the BMP2 response by reducing epigenetic barriers in chromatin. This mechanism
Synergy between BMP2 and Ezh2 inhibition is known as ‘epigenetic priming’ and has been previously described in the cancer field to clarify the enhanced efficacy of anti-cancer drugs in the presence of epigenetic inhibitors (37,82,83). The general significance of our findings is that it extends the concept of epigenetic priming as a cancer therapy into a viable strategy for promotion of osteoblast differentiation in bone regenerative medicine.

As part of ongoing studies aimed at developing strategies to promote bone formation and healing, our groups recently developed a mouse calvarial critical-sized defect model (63). We applied this model in our studies as an illustration of the translational potential of our work. As anticipated, local administration of BMP2 resulted in robust healing four weeks after defect formation. Our current studies confirm the healing potential of local administration of BMP2, but administration of GSK126 (local plus global) did not quantitatively improve the healing process. The in vivo results were less impressive than our in vitro findings, because combination of BMP2 and GSK126 did not result in statistically significant differences in healing compared to BMP2 alone. However, GSK126 certainly did not antagonize the osteogenic effects of BMP2 and co-treatment appeared to be trending towards modestly improved healing. The encouraging finding of these studies is that the combination regimen resulted in more consistent healing compared to BMP2 treatment alone, as measured by analytical techniques (µCT and histomorphometry) and subjective measurements by orthopedic surgeons (healing score and rank).

Combination treatment of GSK126 and BMP2 yields more consistent calvarial bone healing compared to BMP2 alone based on statistical assessment of variance. Our bone healing scores were based on endpoint analysis, which shows significant variation in the extent of bone healing. The latter is attributable to individual differences in the rate of bone formation in mice. While GSK126 cannot quantitatively increase bone healing beyond completion (i.e., 100%), it can temporally accelerate healing, which is expected to decrease individual variation in endpoint healing scores. Hence, the observed reduction in variance for the combination of GSK126 and BMP2 is not inconsistent with improved healing relative to BMP2 alone.

While in vitro experimentation allowed for titration of BMP2 and GSK126 to establish a synergistic interaction, our in vivo studies were limited to previously established delivery methods and dosing regimens for these pro-osteogenic agents. One of the limitations of our study is the timing of BMP2 and GSK126 delivery, which may have reduced overall efficacy of the co-treatment. BMP2 was delivered locally while GSK126 was delivered locally and systemically (initial five days). Fine tuning GSK126 administration may result in more pronounced and increasingly titratable combinatorial effects. Loss of Ezh2 activity has been shown to induce cell cycle alteration in primary mesenchymal stem cells, and balancing the desired effects (i.e., enhanced osteogenesis) (44,54) with potential side effects (i.e., cell cycle arrest) (47) may be required to best harness the beneficial effects of Ezh2 inhibition. Future studies on the optimization of dosing for both BMP2 and Ezh2 may consider several routes of deliveries and assess whether it is realistic to have both treatments be given at the same times, which would ultimately be preferable from a surgical standpoint. Another limitation of our in vivo study is the administration of 300ng BMP2, a dose which was shown by our group to stimulate calvarial defect healing in mice (63). This BMP2 dosing regimen is similar to other calvarial defect healing mouse studies (84-86). Yet, this dose was optimized to support efficient bone healing with BMP2 and hence would not be the most effective method for examining the efficacy of BMP2 in the presence of GSK126, which ideally would be performed with suboptimal doses. Of interest, while our study was in progress, Reyes and colleagues demonstrated that BMP2-induced healing can be enhanced by MMP10 co-administration at four weeks but not at eight weeks post-surgery (86). Thus, future studies on the impact of Ezh2 inhibition on BMP2-mediated calvarial healing should examine earlier stages of the bone healing process and consider lower doses of BMP2.

The key finding of this study is that coinubation of BMP2 with an epigenetic drug (the Ezh2 inhibitor GSK126) primes osteoblast differentiation in culture. Our present study demonstrates that short-term application of
Synergy between BMP2 and Ezh2 inhibition

GSK126 is sufficient to greatly enhance osteogenic effects of BMP2 in vitro in MC3T3s and hBMSCs. Thus, it may be possible to condition or program MSCs with BMP2 and epigenetic drugs such as GSK126 ex vivo before implantation into bone defects to enhance the desired bone-anabolic effects. This approach may be more appropriate in the case of Ezh2 inhibitors as the osteogenic effects can be maximized and the undesired effects virtually eliminated as the recipient (study animal or human) would not come in contact with drug but rather epigenetically modified cells. Indeed, studies have shown that engineered hAMSCs over-expressing BMP2 and miR-148b (miRs/microRNAs are a form of epigenetic regulation) result in robust calvarial defect healing in immunocompromised mice (87,88). Mechanistically, it was concluded that miR-148b enhanced and prolonged BMP2 expression to stimulate osteogenesis both in vitro and in vivo.

Recent studies on Ezh2 and its inhibitors have suggested that it may be possible to manipulate H3K27 methylation to stimulate osteogenesis and bone formation. While previous evidence demonstrated bone anabolic and osteoprotective effects of Ezh2 inhibitors in mouse models of osteoporosis (54-56), our current evidence suggests that Ezh2 inhibition is not sufficient to enhance the bone healing process in vivo. However, our studies suggest that Ezh2 inhibition may provide the basis for new strategies for epigenetic priming to enhance the osteogenic effects of BMP2 in vitro and perhaps in vivo.

**Experimental Procedures**

**MC3T3 Cell Culture** - MC3T3 sc4 murine calvarial osteoblasts (89) were purchased from American Type Culture Collection and maintained in α-minimal essential medium without ascorbic acid (Gibco) containing 10% fetal bovine serum (Atlanta Biologicals), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco).

**hBMSC Cell Culture** - hBMSCs were isolated from bone marrow mononuclear cells purchased from Lonza (cat.no. 2M-12SC) by plastic adherence. Briefly, frozen bone marrow mononuclear cells were thawed in maintenance media comprising of advanced minimum essential medium (Gibco) containing 10% fetal bovine serum, 2 mM L-glutamine (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin. Cell suspensions were centrifuged at 200g for 15 min for three cycles, cell pellets were re-suspended in maintenance media and at 30,000 cells/cm². Cells were allowed to adhere for 7 days following which non-adherent cells in the media were removed by aspiration. Fresh media was added and replaced every 3-4 days. Adherent cells were allowed to form colonies and expand for 3-5 weeks after when colonies were harvested and re-plated at 5,000 cells/cm². Cells from passage 4-6 were used for all in vitro and in vivo experiments.

**Osteogenic Differentiation** - For osteogenic differentiation, MC3T3s and hBMSCs were seeded in respective maintenance medium at a density of 10,000 cells/cm². Next day, osteogenic medium supplemented with vehicle, GSK126 (Xcess Biociences Inc.), recombinant human/mouse/rat BMP2 (R&D Systems; 355-BM), and combination of GSK126/BMP2 was added to the cells. Osteogenic medium for MC3T3 cells consisted of 50μg/ml ascorbic acid (Sigma) and 4mM β-glycerol phosphate (Sigma). Osteogenic medium for hBMSCs contained 50μg/ml ascorbic acid, 8mM β-glycerol phosphate, and 10⁻⁸ M dexamethasone (Sigma). Three days later, old medium was replaced with a fresh batch of osteogenic medium supplemented with vehicle, GSK126, BMP2, and combination of GSK126/BMP2. On day six of differentiation, fresh osteogenic medium without supplements was added and replenished every two to three days.

**Real-time reverse transcriptase PCR (RT-qPCR)** - Total RNA was isolated using the Directzol™ RNA kit (Zymo Research) and quantified using the NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific). RNA was then reverse transcribed into cDNA by the SuperScript III First-Strand Synthesis System (Invitrogen). Transcrip levels were then measured using the 2ΔΔCt method and normalized Gapdh (set at 100), a housekeeping gene. Gene-specific primers are shown (Suppl. Table 4).

**Western Blotting** - Cell lysis and western blotting was performed as described previously (44,47,48,54). 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), and H3K27me3 (1:5,000; 17-622; Millipore).
Alkaline Phosphatase Activity Assay - On day six of osteogenic differentiation, media was removed and cells were washed one time with PBS. Tris-EDTA buffer (0.1X) was then added to the wells to completely cover the cells. The plate was then stored at -80°C for at least two hours and then thawed back to room temperature. 500µl of para-nitrophenylphosphate solution (2.5mg 4-nitrophenylphosphate disodium salt hexahydrate (Sigma) per 1 ml of buffer [0.1M diethanolamine, 150mM NaCl, 2mM MgCl2]) was added to each well. The plate was incubated for 30 minutes (time may vary) at room temperature before measuring absorbance at 405nm using the SpectraMAX Plus spectrophotometer. Values were fit to a standard curve prepared using reconstituted alkaline phosphatase enzyme (Roche) to determine relative enzymatic activity.

Alizarin Red Staining - Cells were fixed in 10% neutral buffered formalin and stained with 2% Alizarin Red (Sigma) to visualize calcium deposition. Absorption of Alizarin Red stain was quantified with ImageJ software (90).

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.

Calvarial defect healing model – Assessment of calvarial healing was performed in twelve week old male C57BL/6J mice purchased from Jackson Laboratories. This critical-sized calvarial defect model was recently described in great detail (63). Briefly, a 2.5 mm dental trephine attached to a low-speed (~1500 rpm) dental drill controlled by a manual foot pedal was used to generate a critical-sized defect in the left parietal bone. A fibrin clot was prepared using the TISSEEL kit (Baxter) for drug delivery. Vehicle, BMP2 (0.3µg), GSK126 (5µg), and BMP2/GSK126 combination was prepared at a final volume of 6µl, mixed with 6µl of thrombin solution, and then combined with 6µl of sealer protein to form the fibrin clot. The prepared biomaterial was placed into the defect site. In addition to local administration, 50 mg/kg GSK126 and vehicle (DMSO) were also administered by intraperitoneal injections (20% Captisol adjusted to pH 4-4.5 with 1N acetic acid) one day before surgery, on the day of surgery, and three days after surgery as described (54). Mice were euthanized four weeks after surgery. Skulls were harvested, fixed in 10% neutral buffered formalin (NBF) for 48 hours, and stored in 70% ethanol. Skulls were assessed by µCT and histomorphometry.

µCT analysis - Calvarial defects were scanned using a Scanco vivaCT40 system (Scanco) at 70-kV, 114-µA with an integration time of 221 ms for a 10.5 isometric voxel size. 3D renderings were created using Microview (Parallax). Regions of interest were hand traced to isolate healing bone from native calvarial bone. A software-specific threshold of 220 (which corresponds to 2500 HU) was used to define mineralized tissue. Standard morphometric parameters were measured using Scanco software.

X-ray analysis - After µCT analysis, skulls were dissected to remove all bone structures that are located underneath calvarial bones. The removal of all these bones allowed for a clear visualization of the defects by x-ray analysis. X-rays of calvarial defects were taken utilizing the same settings (Faxitron, LX60).

Histomorphometry - Following µCT and x-ray assessment, histomorphometry was performed as previously described (47,54,63). Briefly, skulls were embedded in methyl methacrylate resin and sectioned using a rotary retraction microtome to generate 5µm thick coronal sections. The resulting sections (widest point of the defect) were then stained by Gomori’s trichrome (91). Quantitative histomorphometry was performed utilizing Bioquant Osteo Image Analysis Software (Bioquant) to establish the percentage of bone volume (BV/TV). A box of 3.45mm² (2.3mm by 1.5mm) was used for data quantification.

Healing score and rank analysis - Healing score was assessed as described by Spicer et al. with a modification (64). Spicer and colleagues utilized a scale that is based on a score between 0 and 4 (0 = no bone formation within defect, 1 = few bony spicules dispersed through defect, 2 = bony bridging only at defect borders, 3 = bony bridging over partial length of defect, and 4 = bony bridging entire span of defect at longest.
point). We added an additional point to the scale (5 = completely closed defect) to account for completely healed calvarial defects as measured by x-ray analysis. In addition to the scoring, we also ranked the healing calvarial defects (0-22, 0 = worst healed, 22 = best healed). The scoring and ranking was blindly performed by two orthopedic surgeons. Their scores were then averaged and graphed.

High Throughput RNA Sequencing and Bioinformatic Analysis – RNA from differentiating MC3T3 cells treated with vehicle, GSK126, BMP2, and GSK126/BMP2 combination (see Fig. 2A) were assessed by RNA-seq analysis as previously reported (44,47,48,54,92,93). For each condition and time point, equal amount of RNA from three biological replicates were pooled and then subjected to RNA-seq analysis. Gene expression is expressed in reads/kilobase pair/million mapped reads (RPKM). RNA-seq data were deposited in the Gene Expression Omnibus of the National Center for Biotechnology Information (GSE135984). Venn Diagrams were generated using Venny 2.1 online tool (BioinfoGP). Principal Component Analysis (PCA) was performed using ClustVis online tool (60). Functional annotation clustering of differentially expressed genes was performed using DAVID Bioinformatic Resources 6.8 database (DAVID 6.8) (94,95). Hierarchical clustering was performed using Morpheus matrix visualization and analysis software after a Log2 adjustment was made for each gene row (Broad Institute). Protein-protein interaction networks were generated using STRING Database version 10.5 (96).

ChIP-Seq analysis – H3K27Me3 status at BMP2 and GSK126 activated genes was assessed using publically available ChIP-sequencing data sets (GSE83506) (54). In brief, ChIP-Seq assay was performed 24 hours after administration of vehicle (DMSO) or 5µM GSK126 to MC3T3 cells cultured in osteogenic media (50 µg/ml ascorbic acid and 4mM beta glyceral phosphate) (54). Read coverage over the mm10 genome was determined using the deepTools2 bamCoverage package (97). H3K27Me3 intensity was then assessed at specific genomic regions for the 113 genes identified as upregulated at day 6 after BMP2 and GSK126 treatment using the deepTools2 computeMatrix package (97). Bin size was set to 1000 bases and coverage was assessed 30kb upstream of the TSS and 30KB downstream of the TES. Resulting values were visualized using GraphPad Prism 8.2.0. (San Diego, California USA, www.graphpad.com).

Statistics – For in vitro studies, data are shown as mean ± standard deviation and statistical analysis was performed with unpaired Student's t-test or analysis of variance (ANOVA). When the overall ANOVA F-test was significant, subsequent pairwise comparisons were performed using two-way ANOVA. For in vivo studies, the data are summarized using means and standard deviations unless otherwise noted. Each calvarial defect (mouse) is represented by a data point within the figures. The in vivo study outcomes were compared between the 4 experimental groups (vehicle, BMP2, GSK126, GSK126 + BMP2) using one-factor ANOVA. Separate analyses were performed for each outcome. When the overall ANOVA F-test was significant, pairwise comparisons were performed between the experimental groups using the Ryan-Einot-Gabriel-Welsch multiple comparisons test in order to maintain the overall type-I error rate at α = 0.05. Significance is noted in the figures, when applicable (*, p < 0.05; **, p < 0.01; and ***, p < 0.001).

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: ANL receives research support from Zimmer Biomet, Medtronic, and is an unpaid consultant for Globus.

Data availability
RNA-Seq (GSE135984) and ChIP-Seq (GSE83506) data have been deposited in the Gene Expression Omnibus of the National Center for Biotechnology Information. All other data are contained within the manuscript.
Author contributions
AD and AJvW designed the study. AD, RMS, CRP, CGG, MM, DGG, PZ, MLG, MH, DLG, OP, and RT performed the biological experiments. AD, PK, MK, ANL, JJW, SMC and AJvW provided interpretation of results and guidance of studies. AD and AJvW wrote the paper with comments from all authors.

Acknowledgements
This work was supported, in whole or in part, by National Institutes of Health grants R01 AR049069 (AvW) and a Career Development Award in Orthopedics Research (AD). We also appreciate the generous philanthropic support of William H. and Karen J. Eby, and the charitable foundation in their names.

We thank current and past members of our laboratories, including Scott Riester, Farzaneh Khani, Leila Bagheri, Sofia Jerez, Margarita Carrasco Jeldres, Eric Lewallen, and Emily Camilleri for stimulating discussions. We acknowledge the support of Asha Nair from the Bioinformatics Core, Dirk Larson from Biomedical Statistics and Informatics, Medical Genome Facility, as well as Biomaterials Characterization and Quantitative Histomorphometry Core Facility. We thank William Shaughnessy for providing administrative supervision and guidance to Daniela Galeano-Garces.

Abbreviations
The abbreviations used are: BMP, bone morphogenetic protein; PTH, parathyroid hormone; Ezh2, Enhancer of zeste homolog 2; Runx2, Runt-related transcription factor 2; Dlx5, distal-less homeobox 5; PRC2, polycomb-repressive complex 2; Ezh1, enhancer of zeste homolog 1; Bglap, osteocalcin; Phospho1, phosphoethanolamine / phosphocholine phosphatase1; Ibisp, bone sialoprotein, hBMSCs, human bone-marrow derived mesenchymal stem/stromal cells; Sp7/Osx, Osterix; µCT, microcomputed tomography; RPKM, Reads Per Kilobase of transcript per Million mapped reads, FC, fold change; Dlx3, distal-less homeobox 5; Wif1, WNT Inhibitory Factor 1; Igfbp5, Insulin Like Growth Factor Binding Protein 5, Hey1, Hes Related Family BHLH Transcription Factor With YRPW Motif 1; Nog, Noggin; DAVID, The Database for Annotation, Visualization and Integrated Discovery; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins, Phex, Phosphate Regulating Endopeptidase Homolog X-Linked, Dmp1, Dentin matrix protein 1; Spp1, Secreted Phosphoprotein 1; hAMSCs, human adipose-derived mesenchymal/stromal cells; Ak1, RAC-alpha serine/threonine-protein kinase; Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; BMS, bone mineral density; BV/TV, bone volume fraction, bone volume-to-total volume ratio; Jhdm1d, Jumonji C domain containing histone demethylase 1 homolog D; Kdm6a, Lysine-specific demethylase 6a; Kdm6b, Lysine-specific demethylase 6b; Pth1r, Parathyroid hormone/parathyroid hormone-related peptide receptor; miR, microRNA; RT-qPCR, real-time polymerase chain reaction; H3, histone 3, PBS, phosphate buffered saline; EDTA, Ethylenediaminetetraacetic acid; RPM, rotations per minute, NBF, neutral buffered formalin; ANOVA, Analysis of variance
Synergy between BMP2 and Ezh2 inhibition

References

1. Chen, D., Zhao, M., and Mundy, G. R. (2004) Bone morphogenetic proteins. *Growth factors* (Chur, Switzerland) **22**, 233-241

2. Russow, G., Jahn, D., Appelt, J., Mardian, S., Tsitsilonis, S., and Keller, J. (2018) Anabolic Therapies in Osteoporosis and Bone Regeneration. *Int J Mol Sci* **20**

3. Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. A. (1988) Novel regulators of bone formation: molecular clones and activities. *Science* **242**, 1528-1534

4. Sampath, T. K., Coughlin, J. E., Whetstone, R. M., Banach, D., Corbett, C., Ridge, R. J., Ozkaynak, E., Oppermann, H., and Rueger, D. C. (1990) Bovine osteogenic protein is composed of dimers of OP-1 and BMP-2A, two members of the transforming growth factor-beta superfamily. *J Biol Chem* **265**, 13198-13205

5. Shi, Y., and Massague, J. (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700

6. Katagiri, T., and Watabe, T. (2016) Bone Morphogenetic Proteins. *Cold Spring Harb Perspect Biol* **8**

7. ten Dijke, P., Korchnytskyi, O., Valdimarsdottir, G., and Goumans, M. J. (2003) Controlling cell fate by bone morphogenetic protein receptors. *Mol Cell Endocrinol* **211**, 105-113

8. Lee, K. S., Kim, H. J., Li, Q. L., Chi, X. Z., Ueta, C., Komori, T., Wozney, J. M., Kim, E. G., Choi, J. Y., Ryoo, H. M., and Bae, S. C. (2000) Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol Cell Biol* **20**, 8783-8792

9. Takazawa, Y., Tsuji, K., Nifui, A., Kurosawa, H., Ito, Y., and Noda, M. (2000) An osteogenesis-related transcription factor, core-binding factor A1, is constitutively expressed in the chondrocytic cell line TC6, and its expression is upregulated by bone morphogenetic protein-2 during development of the osteoblast phenotype. *Endocrinology* **142**, 4026-4039

10. Banerjee, C., Javed, A., Choi, J. Y., Green, J., Rosen, V., van Wijnen, A. J., Stein, J. L., Lian, J. B., and Stein, G. S. (2001) Differential regulation of the two principal Runx2/Cbfa1 n-terminal isoforms in response to bone morphogenetic protein-2 during osteoblast differentiation: involvement in regulation of osteocalcin gene expression. *Mol Endocrinol* **15**, 1681-1694

11. Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R., and de Crombrugghe, B. (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108**, 17-29

12. Komori, T. (2019) Regulation of Proliferation, Differentiation and Functions of Osteoblasts by Runx2. *Int J Mol Sci* **20**

13. Einhorn, T. A., and Gerstenfeld, L. C. (2015) Fracture healing: mechanisms and interventions. *Nature reviews. Rheumatology* **11**, 45-54
Synergy between BMP2 and Ezh2 inhibition

17. Shapiro, F. (2008) Bone development and its relation to fracture repair. The role of mesenchymal osteoblasts and surface osteoblasts. *European cells & materials* **15**, 53-76

18. Runyan, C. M., and Gabrick, K. S. (2017) Biology of Bone Formation, Fracture Healing, and Distraction Osteogenesis. *The Journal of craniofacial surgery* **28**, 1380-1389

19. Tzioupis, C., and Giannoudis, P. V. (2007) Prevalence of long-bone non-unions. *Injury* **38 Suppl 2**, S3-9

20. Giannoudis, P. V., Jones, E., and Einhorn, T. A. (2011) Fracture healing and bone repair. *Injury* **42**, 549-550

21. Zura, R., Xiong, Z., Einhorn, T., Watson, J. T., Ostrum, R. F., Prayson, M. J., Della Rocca, G. J., Mehta, S., McKinley, T., Wang, Z., and Steen, R. G. (2016) Epidemiology of Fracture Nonunion in 18 Human Bones. *JAMA surgery* **151**, e162775

22. Hak, D. J., Fitzpatrick, D., Bishop, J. A., Marsh, J. L., Tilp, S., Schnettler, R., Simpson, H., and Alt, V. (2014) Delayed union and nonunions: epidemiology, clinical issues, and financial aspects. *Injury* **45 Suppl 2**, S3-7

23. Bostrom, M. P., Lane, J. M., Berberian, W. S., Missri, A. A., Tomin, E., Weiland, A., Doty, S. B., Glaser, D., and Rosen, V. M. (1995) Immunolocalization and expression of bone morphogenetic proteins 2 and 4 in fracture healing. *J Orthop Res* **13**, 357-367

24. Onishi, T., Ishidou, Y., Nagamine, T., Yone, K., Imamura, T., Kato, M., Sampath, T. K., ten Dijke, P., and Sakou, T. (1998) Distinct and overlapping patterns of localization of bone morphogenetic protein (BMP) family members and a BMP type II receptor during fracture healing in rats. *Bone* **22**, 605-612

25. Sciadini, M. F., and Johnson, K. D. (2000) Evaluation of recombinant human bone morphogenetic protein-2 as a bone-graft substitute in a canine segmental defect model. *J Orthop Res* **18**, 289-302

26. Bouxsein, M. L., Turek, T. J., Blake, C. A., D’Augusta, D., Li, X., Stevens, M., Seeherman, H. J., and Wozney, J. M. (2001) Recombinant human bone morphogenetic protein-2 accelerates healing in a rabbit ulnar osteotomy model. *J Bone Joint Surg Am* **83**, 1219-1230

27. Luppen, C. A., Blake, C. A., Ammirati, K. M., Stevens, M. L., Seeherman, H. J., Wozney, J. M., and Bouxsein, M. L. (2002) Recombinant human bone morphogenetic protein-2 enhances osteotomy healing in glucocorticoid-treated rabbits. *J Bone Miner Res* **17**, 301-310

28. Einhorn, T. A., Majeska, R. J., Mohaideen, A., Kagel, E. M., Bouxsein, M. L., Turek, T. J., and Wozney, J. M. (2003) A single percutaneous injection of recombinant human bone morphogenetic protein-2 accelerates fracture repair. *J Bone Joint Surg Am* **85**, 1425-1435

29. Jones, A. L., Bucholz, R. W., Bosse, M. J., Mirza, S. K., Lyon, T. R., Webb, L. X., Pollak, A. N., Golden, J. D., and Valentin-Opran, A. (2006) Recombinant human BMP-2 and allograft compared with autogenous bone graft for reconstruction of diaphyseal tibial fractures with cortical defects. A randomized, controlled trial. *J Bone Joint Surg Am* **88**, 1431-1441

30. Swiontkowski, M. F., Aro, H. T., Donell, S., Esterhai, J. L., Goulet, J., Jones, A., Kregor, P. J., Nordsetten, L., Paiement, G., and Patel, A. (2006) Recombinant human bone morphogenetic protein-2 in open tibial fractures. A subgroup analysis of data combined from two prospective randomized studies. *J Bone Joint Surg Am* **88**, 1258-1265

31. Baskin, D. S., Ryan, P., Sonntag, V., Westmark, R., and Widmayer, M. A. (2003) A prospective, randomized, controlled cervical fusion study using recombinant human bone morphogenetic protein-2 with the CORNERSTONE-SR allograft ring and the ATLANTIS anterior cervical plate. *Spine (Phila Pa 1976)* **28**, 1219-1224; discussion 1225
Synergy between BMP2 and Ezh2 inhibition

32. Ong, K. L., Villarraga, M. L., Lau, E., Carreon, L. Y., Kurtz, S. M., and Glassman, S. D. (2010) Off-label use of bone morphogenetic proteins in the United States using administrative data. *Spine (Phila Pa 1976)* **35**, 1794-1800

33. Garrison, K. R., Donell, S., Ryder, J., Shemilt, I., Mugford, M., Harvey, I., and Song, F. (2007) Clinical effectiveness and cost-effectiveness of bone morphogenetic proteins in the non-healing of fractures and spinal fusion: a systematic review. *Health technology assessment (Winchester, England)* **11**, 1-150, iii-iv

34. James, A. W., LaChaud, G., Shen, J., Asatrian, G., Nguyen, V., Zhang, X., Ting, K., and Soo, C. (2016) A Review of the Clinical Side Effects of Bone Morphogenetic Protein-2. *Tissue Eng Part B Rev* **22**, 284-297

35. Gordon, J. A., Stein, J. L., Westendorf, J. J., and van Wijnen, A. J. (2015) Chromatin modifiers and histone modifications in bone formation, regeneration, and therapeutic intervention for bone-related disease. *Bone* **81**, 739-745

36. Pike, J. W., Meyer, M. B., St John, H. C., and Benkusky, N. A. (2015) Epigenetic histone modifications and master regulators as determinants of context dependent nuclear receptor activity in bone cells. *Bone* **81**, 757-764

37. van Wijnen, A. J., and Westendorf, J. J. (2019) Epigenetics as a New Frontier in Orthopedic Regenerative Medicine and Oncology. *J Orthop Res* **37**, 1465-1474

38. Margueron, R., and Reinberg, D. (2011) The Polycomb complex PRC2 and its mark in life. *Nature* **469**, 343-349

39. Dudakovic, A., and van Wijnen, A. J. (2017) Epigenetic Control of Osteoblast Differentiation by Enhancer of Zeste Homolog 2 (EZH2). *Current Molecular Biology Reports* **3**, 94-106

40. Margueron, R., Li, G., Sarma, K., Blais, A., Zavadil, J., Woodcock, C. L., Dynlacht, B. D., and Reinberg, D. (2008) Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. *Mol Cell* **32**, 503-518

41. Marchesi, I., Giordano, A., and Bagella, L. (2014) Roles of enhancer of zeste homolog 2: from skeletal muscle differentiation to rhabdomyosarcoma carcinogenesis. *Cell Cycle* **13**, 516-527

42. Wyngaarden, L. A., Delgado-Olguin, P., Su, I. H., Bruneau, B. G., and Hopyan, S. (2011) Ezh2 regulates anteroposterior axis specification and proximodistal axis elongation in the developing limb. *Development* **138**, 3759-3767

43. Schwarz, D., Varum, S., Zemke, M., Scholer, A., Baggioni, A., Draganova, K., Koseki, H., Schubeler, D., and Sommer, L. (2014) Ezh2 is required for neural crest-derived cartilage and bone formation. *Development* **141**, 867-877

44. Dudakovic, A., Camilleri, E. T., Xu, F., Riester, S. M., McGee-Lawrence, M. E., Bradley, E. W., Paradise, C. R., Lewallen, E. A., Thaler, R., Deyle, D. R., Larson, A. N., Lewallen, D. G., Dietz, A. B., Stein, G. S., Montecino, M. A., Westendorf, J. J., and van Wijnen, A. J. (2015) Epigenetic Control of Skeletal Development by the Histone Methyltransferase Ezh2. *J Biol Chem* **290**, 27604-27617

45. Hemming, S., Cakouros, D., Codrington, J., Vandyke, K., Arthur, A., Zannettino, A., and Gronthos, S. (2017) EZH2 deletion in early mesenchyme compromises postnatal bone microarchitecture and structural integrity and accelerates remodeling. *Faseb J* **31**, 1011-1027

46. Lui, J. C., Garrison, P., Nguyen, Q., Ad, M., Keembiyehetty, C., Chen, W., Jee, Y. H., Landman, E., Nilsson, O., Barnes, K. M., and Baron, J. (2016) EZH1 and EZH2 promote skeletal growth by repressing inhibitors of chondrocyte proliferation and hypertrophy. *Nat Commun* **7**, 13685

47. Dudakovic, A., Camilleri, E. T., Paradise, C. R., Samsonraj, R. M., Gluscevic, M., Paggi, C. A., Begun, D. L., Khani, F., Pichurin, O., Ahmed, F. S., Elsayed, R., Elsalanty, M., McGee-Lawrence, M. E., Karperien, M., Riester, S. M., Thaler, R., Westendorf, J. J., and
van Wijnen, A. J. (2018) Enhancer of zeste homolog 2 (Ezh2) controls bone formation and cell cycle progression during osteogenesis in mice. *J Biol Chem* **293**, 12894-12907

48. Camilleri, E. T., Dudakovic, A., Riester, S. M., Galeano-Garcés, C., Paradise, C. R., Bradley, E. W., McGee-Lawrence, M. E., Im, H. J., Karperien, M., Krych, A. J., Westendorf, J. J., Larson, A. N., and van Wijnen, A. J. (2018) Loss of histone methyltransferase Ezh2 stimulates an osteogenic transcriptional program in chondrocytes but does not affect cartilage development. *J Biol Chem* **293**, 19001-19011

49. Wei, Y., Chen, Y. H., Li, L. Y., Lang, J., Yeh, S. P., Shi, B., Yang, C. C., Yang, J. Y., Lin, C. Y., Lai, C. C., and Hung, M. C. (2011) CDK1-dependent phosphorylation of EZH2 suppresses methylation of H3K27 and promotes osteogenic differentiation of human mesenchymal stem cells. *Nat Cell Biol* **13**, 87-94

50. Wang, H., Meng, Y., Cui, Q., Qin, F., Yang, H., Chen, Y., Cheng, Y., Shi, J., and Guo, Y. (2016) MiR-101 Targets the EZH2/Wnt/beta-Catenin the Pathway to Promote the Osteogenic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells. *Sci Rep* **6**, 36988

51. Hemming, S., Cakouros, D., Isenmann, S., Cooper, L., Menicanin, D., Zannettino, A., and Gronthos, S. (2014) EZH2 and KDM6A act as an epigenetic switch to regulate mesenchymal stem cell lineage specification. *Stem Cells* **32**, 802-815

52. Zhu, X. X., Yan, Y. W., Chen, D., Ai, C. Z., Lu, X., Xu, S. S., Jiang, S., Zhong, G. S., Chen, D. B., and Jiang, Y. Z. (2016) Long non-coding RNA HoxA-AS3 interacts with EZH2 to regulate lineage commitment of mesenchymal stem cells. *Oncotarget* **7**, 63561-63570

53. Chen, Y. H., Chung, C. C., Liu, Y. C., Yeh, S. P., Hsu, J. L., Hung, M. C., Su, H. L., and Li, L. Y. (2016) Enhancer of Zeste Homolog 2 and Histone Deacetylase 9c Regulate Age-Dependent Mesenchymal Stem Cell Differentiation into Osteoblasts and Adipocytes. *Stem Cells* **34**, 2183-2193

54. Dudakovic, A., Camilleri, E. T., Riester, S. M., Paradise, C. R., Gluscevic, M., O’Toole, T. M., Thaler, R., Evans, J. M., Yan, H., Subramaniam, M., Hawse, J. R., Stein, G. S., Montecino, M. A., McGee-Lawrence, M. E., Westendorf, J. J., and van Wijnen, A. J. (2016) Enhancer of Zeste Homolog 2 Inhibition Stimulates Bone Formation and Mitigates Bone Loss Caused by Ovariectomy in Skeletally Mature Mice. *J Biol Chem* **291**, 24594-24606

55. Jing, H., Liao, L., An, Y., Su, X., Liu, S., Shuai, Y., Zhang, X., and Jin, Y. (2016) Suppression of EZH2 Prevents the Shift of Osteoporotic MSC Fate to Adipocyte and Enhances Bone Formation During Osteoporosis. *Mol Ther* **24**, 217-229

56. Fang, C., Qiao, Y., Mun, S. H., Lee, M. J., Murata, K., Bae, S., Zhao, B., Park-Min, K. H., and Ivashkiv, L. B. (2016) Cutting Edge: EZH2 Promotes Osteoclastogenesis by Epigenetic Silencing of the Negative Regulator IRF8. *J Immunol* **196**, 4452-4456

57. Woodhouse, S., Pugazhendhi, D., Brien, P., and Pell, J. M. (2013) Ezh2 maintains a key phase of muscle satellite cell expansion but does not regulate terminal differentiation. *J Cell Sci* **126**, 565-579

58. Kim, K. H., and Roberts, C. W. (2016) Targeting EZH2 in cancer. *Nat Med* **22**, 128-134

59. Comet, I., Riising, E. M., Leblanc, B., and Helin, K. (2016) Maintaining cell identity: PRC2-mediated regulation of transcription and cancer. *Nat Rev Cancer* **16**, 803-810

60. Metsalu, T., and Vilo, J. (2015) ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res* **43**, W566-570

61. Hassan, M. Q., Javed, A., Morasso, M. I., Karlin, J., Montecino, M., van Wijnen, A. J., Stein, G. S., Stein, J. L., and Lian, J. B. (2004) Dlx3 transcriptional regulation of osteoblast differentiation: temporal recruitment of Msx2, Dlx3, and Dlx5 homeodomain proteins to chromatin of the osteocalcin gene. *Mol Cell Biol* **24**, 9248-9261
Synergy between BMP2 and Ezh2 inhibition

62. Samsonraj, R. M., Paradise, C. R., Dudakovic, A., Sen, B., Nair, A. A., Dietz, A. B., Deyle, D. R., Cool, S. M., Rubin, J., and van Wijnen, A. J. (2018) Validation of Osteogenic Properties of Cytochalasin D by High-Resolution RNA-Sequencing in Mesenchymal Stem Cells Derived from Bone Marrow and Adipose Tissues. Stem Cells Dev 27, 1136-1145

63. Samsonraj, R. M., Dudakovic, A., Zan, P., Pichurin, O., Cool, S. M., and van Wijnen, A. J. (2017) A Versatile Protocol for Studying Calvarial Bone Defect Healing in a Mouse Model. Tissue Eng Part C Methods 23, 686-693

64. Spicer, P. P., Kretlow, J. D., Young, S., Jansen, J. A., Kasper, F. K., and Mikos, A. G. (2012) Evaluation of bone regeneration using the rat critical size calvarial defect. Nat Protoc 7, 1918-1929

65. Ge, K. (2012) Epigenetic regulation of adipogenesis by histone methylation. Biochim Biophys Acta 7, 727-732

66. Wang, E. A., Rosen, V., Cordes, P., Hewick, R. M., Kriz, M. J., Luxenberg, D. P., Sibley, B. S., and Wozney, J. M. (1988) Purification and characterization of other distinct bone-inducing factors. Proc Natl Acad Sci U S A 85, 9484-9488

67. Katagiri, T., Yamaguchi, A., Ikeda, T., Yoshiki, S., Wozney, J. M., Rosen, V., Wang, E. A., Tanaka, H., Omura, S., and Suda, T. (1990) The non-osteogenic mouse pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. Biochem Biophys Res Commun 172, 295-299

68. Yamaguchi, A., Katagiri, T., Ikeda, T., Wozney, J. M., Rosen, V., Wang, E. A., Kahn, A. J., Suda, T., and Yoshiki, S. (1991) Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. J Cell Biol 113, 681-687

69. Lee, Z. H., Kim, H. J., and Ryoo, H. M. (2015) A Novel Osteogenic Activity of Suberoylanilide Hydroxamic Acid is Synergized by BMP-2. Journal of bone metabolism 22, 51-56

70. Cho, Y. D., Yoon, W. J., Kim, W. J., Woo, K. M., Baek, J. H., Lee, G., Ku, Y., van Wijnen, A. J., and Ryoo, H. M. (2014) Epigenetic modifications and canonical wingless/int-1 class (WNT) signaling enable trans-differentiation of nonosteogenic cells into osteoblasts. J Biol Chem 289, 20120-20128

71. Gao, S., Wang, Z., Wang, W., Hu, X., Chen, P., Li, J., Feng, X., Wong, J., and Du, J. X. (2017) The lysine methyltransferase SMYD2 methylates the kinase domain of type II receptor BMPR2 and stimulates bone morphogenetic protein signaling. J Biol Chem 292, 12702-12712

72. Sun, J., Ermann, J., Niu, N., Yan, G., Yang, Y., Shi, Y., and Zou, W. (2018) Histone demethylase LSD1 regulates bone mass by controlling WNT7B and BMP2 signaling in osteoblasts. Bone research 6, 14

73. Hay, E., Hott, M., Graulet, A. M., Lomri, A., and Marie, P. J. (1999) Effects of bone morphogenetic protein-2 on human neonatal calvaria cell differentiation. J Cell Biochem 72, 81-93

74. Huang, W., Rudkin, G. H., Carlsten, B., Ishida, K., Ghasri, P., Anvar, B., Yamaguchi, D. T., and Miller, T. A. (2002) Overexpression of BMP-2 modulates morphology, growth, and gene expression in osteoblastic cells. Exp Cell Res 274, 226-234

75. Phimphilai, M., Zhao, Z., Boules, H., Roca, H., and Franceschi, R. T. (2006) BMP signaling is required for RUNX2-dependent induction of the osteoblast phenotype. J Bone Miner Res 21, 637-646

76. Javed, A., Afzal, F., Bae, J. S., Gutierrez, S., Zaidi, K., Pratap, J., van Wijnen, A. J., Stein, J. L., Stein, G. S., and Lian, J. B. (2009) Specific residues of RUNX2 are obligatory for formation of BMP2-induced RUNX2-SMAD complex to promote osteoblast differentiation. Cells, tissues, organs 189, 133-137
77. Lee, M. H., Kim, Y. J., Kim, H. J., Park, H. D., Kang, A. R., Kyung, H. M., Sung, J. H., Wozney, J. M., Kim, H. J., and Ryoo, H. M. (2003) BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. *J Biol Chem* **278**, 34387-34394

78. Javed, A., Gutierrez, S., Montecino, M., van Wijnen, A. J., Stein, J. L., Stein, G. S., and Lian, J. B. (1999) Multiple Cbfa/AML sites in the rat osteocalcin promoter are required for basal and vitamin D-responsive transcription and contribute to chromatin organization. *Mol Cell Biol* **19**, 7491-7500

79. Matsubara, T., Kida, K., Yamaguchi, A., Hata, K., Ichida, F., Meguro, H., Aburatani, H., Nishimura, R., and Yoneda, T. (2008) BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. *J Biol Chem* **283**, 29119-29125

80. Cho, Y. D., Kim, W. J., Yoon, W. J., Woo, K. M., Baek, J. H., Lee, G., Kim, G. S., and Ryoo, H. M. (2012) Wnt3a stimulates Mepe, matrix extracellular phosphoglycoprotein, expression directly by the activation of the canonical Wnt signaling pathway and indirectly through the stimulation of autocrine Bmp-2 expression. *J Cell Physiol* **227**, 2287-2296

81. Lu, Z., Wang, G., Dunstan, C. R., and Zreiqat, H. (2012) Short-term exposure to tumor necrosis factor-alpha enables human osteoblasts to direct adipose tissue-derived mesenchymal stem cells into osteogenic differentiation. *Stem Cells Dev* **21**, 2420-2429

82. Scandura, J. M., Roboz, G. J., Moh, M., Morawa, E., Brenet, F., Bose, J. R., Villegas, L., Gergis, U. S., Mayer, S. A., Ippoliti, C. M., Curcio, T. J., Ritchie, E. K., and Feldman, E. J. (2011) Phase 1 study of epigenetic priming with decitabine prior to standard induction chemotherapy for patients with AML. *Blood* **118**, 1472-1480

83. Vicente-Duenas, C., Hauer, J., Cobaleda, C., Borkhardt, A., and Sanchez-Garcia, I. (2018) Epigenetic Priming in Cancer Initiation. *Trends in cancer* **4**, 408-417

84. Auh, Q. S., Park, K. R., Yun, H. M., Lim, H. C., Kim, G. H., Lee, D. S., Kim, Y. C., Oh, H., and Kim, E. C. (2016) Sulfuretin promotes osteoblastic differentiation in primary cultured osteoblasts and in vivo bone healing. *Oncotarget* **7**, 78320-78330

85. Herberg, S., Aguilar-Perez, A., Howie, R. N., Kondrikova, G., Periyasamy-Thanavanan, S., Elsalanty, M. E., Shi, X., Hill, W. D., and Cray, J. J. (2017) SDF-1beta synergizes with BMP-2 to augment cell-mediated healing of critical-sized mouse calvarial defects. *J Tissue Eng Regen Med* **11**, 1806-1819

86. Reyes, R., Rodriguez, J. A., Orbe, J., Arnau, M. R., Evora, C., and Delgado, A. (2018) Combined sustained release of BMP2 and MMP10 accelerates bone formation and mineralization of calvaria critical size defect in mice. *Drug delivery* **25**, 750-756

87. Liao, Y. H., Chang, Y. H., Sung, L. Y., Li, K. C., Yeh, C. L., Yen, T. C., Hwang, S. M., Lin, K. J., and Hu, Y. C. (2014) Osteogenic differentiation of adipose-derived stem cells and calvarial defect repair using baculovirus-mediated co-expression of BMP-2 and miR-148b. *Biomaterials* **35**, 4901-4910

88. Li, K. C., Lo, S. C., Sung, L. Y., Liao, Y. H., Chang, Y. H., and Hu, Y. C. (2017) Improved calvarial bone repair by hASCs engineered with Cre/loxP-based baculovirus conferring prolonged BMP-2 and MiR-148b co-expression. *J Tissue Eng Regen Med* **11**, 3068-3077

89. Wang, D., Christensen, K., Chawla, K., Xiao, G., Krebsbach, P. H., and Franceschi, R. T. (1999) Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. *J Bone Miner Res* **14**, 893-903

90. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**, 671-675
91. Villanueva, A. R., and Mehr, L. A. (1977) Modifications of the Goldner and Gomori one-step trichrome stains for plastic-embedded thin sections of bone. *The American journal of medical technology* **43**, 536-538

92. Dudakovic, A., Camilleri, E., Riester, S. M., Lewallen, E. A., Kvasha, S., Chen, X., Radel, D. J., Anderson, J. M., Nair, A. A., Evans, J. M., Krych, A. J., Smith, J., Deyle, D. R., Stein, J. L., Stein, G. S., Im, H. J., Cool, S. M., Westendorf, J. J., Kakar, S., Dietz, A. B., and van Wijnen, A. J. (2014) High-resolution molecular validation of self-renewal and spontaneous differentiation in clinical-grade adipose-tissue derived human mesenchymal stem cells. *J Cell Biochem* **115**, 1816-1828

93. Dudakovic, A., Gluscevic, M., Paradise, C. R., Dudakovic, H., Khani, F., Thaler, R., Ahmed, F. S., Li, X., Dietz, A. B., Stein, G. S., Montecino, M. A., Deyle, D. R., Westendorf, J. J., and van Wijnen, A. J. (2017) Profiling of human epigenetic regulators using a semi-automated real-time qPCR platform validated by next generation sequencing. *Gene* **609**, 28-37

94. Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* **37**, 1-13

95. Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**, 44-57

96. Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M., Doncheva, N. T., Morris, J. H., Bork, P., Jensen, L. J., and Mering, C. V. (2019) STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res* **47**, D607-d613

97. Ramirez, F., Ryan, D. P., Gruning, B., Bhardwaj, V., Kilpert, F., Richter, A. S., Heyne, S., Dundar, F., and Manke, T. (2016) deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic Acids Res* **44**, W160-165
Figure 1. GSK126 potentiates BMP2-induced expression of osteogenic genes in MC3T3 cells. Experimental set-up for BMP2 and GSK126 treatment and osteogenic differentiation of MC3T3 pre-osteoblasts (a). GSK126 and BMP2 were administered on days zero and three of differentiation (cells were exposed to compounds for six days). RNA was isolated on day nine of osteogenic differentiation. Expression (RT-qPCR) of two established osteogenic markers, Bglap and Phospho1, on day nine of differentiation (n = 3) (b). Differentiating MC3T3 cells were treated with vehicle (black dashed line), 5µM GSK126 (blue dashed line), varying concentrations of BMP2 (2.5 to 100 ng/ml) in the absence (red solid line) or presence (green solid line) of 5µM GSK126. Expression of Bglap and Phospho1 upon 5µM GSK126 administration alone or in combination with and 25ng/ml and 5ng/ml BMP2, respectively (n = 3). * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

Figure 2. BMP2 and GSK126 synergistically activate osteogenic differentiation of MC3T3 cells. Experimental protocol illustrating the treatment, differentiation, and analysis of MC3T3 cells. Expression (RT-qPCR) of osteogenic markers (Sp7, Bglap, and Ibsp) at several time points (n = 3) (b). Expression (RT-qPCR) of osteogenic markers in the vehicle treated group (n = 3) (c). Expression (RT-qPCR) of Bglap and Ibsp on day six (n = 3) (d). Alkaline phosphatase activity assay on day six (n = 3) (e). Alizarin red staining (left) and quantification (right) on day twenty (n = 3) (f). * = p < 0.05, ** = p < 0.01, *** = p < 0.001, a vs b = p < 0.001.

Figure 3. Differential gene expression changes upon BMP2 and GSK126 treatment in MC3T3 cells. RNA derived from differentiating MC3T3 cells treated with vehicle, GSK126, BMP2, and GSK126/BMP2 combination was assessed by mRNA-seq analysis (see Fig. 2a, day one and day six samples). Unbiased PCA on day one (a) and day six (b) of osteogenic differentiation (RPKM > 0.3 in at least one group for each time point). The analysis included 11,974 (day one) and 12,307 (day six) genes. Hierarchical clustering of differentially expressed genes on day one (c) and day six (d) of osteogenic differentiation. The analysis included genes that were detected by mRNA-seq technology (average RPKM >0.1 across six samples at each time-point) and were differentially expressed (fold change (FC) > 2 between any of the six treatment groups at each time-point). The resulting hierarchical clustering is made up of 1,263 (day one) and 3,712 (day six) genes.

Figure 4. Synergistic activation of osteogenic genes by dual administration of BMP2 and GSK126 in MC3T3 cells. To identify genes that are synergistically activated by BMP2 and GSK126 administration, we selected for genes that are highly expressed (RPKM > 0.3), are highly induced by BMP2/GSK126 combination when compared to vehicle treatment (FC > 2 on D1, FC > 4 on D6), and are enhanced by BMP2/GSK126 combination treatment when compared to individual BMP2 and GSK126 treatments (FC > 2 on D1, FC > 2 on D6). Venn diagram analysis showing genes that are synergistically activated when low (10 ng/ml) and high (50 ng/ml) BMP2 concentrations are combined with GSK126 (5µM) on day one (A) and day six (B) of osteogenic differentiation. Gene list and expression profiles are shown in supplementary materials (Suppl. Tables 2 & 3). DAVID 6.8 enrichment score analysis (C) and STRING analysis (D) of synergistic genes commonly up-regulated in low BMP2 plus GSK126 and high BMP2 plus GSK126 treatment (n = 47) on day six of osteogenic differentiation. H3K27me3 enrichment analysis by ChIP-Seq of all genes that are up-regulated by low and high BMP2 plus GSK126 combination (n = 113, see panel B) in vehicle and 5µM GSK126 treated (24 hours) MC3T3 cells (E). ChIP-Seq track examples for Dmp1 and Dlx3 of input and H3K27me3 pull-downs in vehicle (V) and 5µM GSK126 (G) treated MC3T3 cells (F).

Figure 5. GSK126 potentiates BMP2-induced expression of osteogenic genes in hBMSC. Experimental set-up for BMP2 and GSK126 treatment and osteogenic differentiation of hBMSCs (A). Western blotting analysis of differentiating hBMSCs upon Ezh2 inhibition (B). Cells were treated with vehicle and varying GSK126 concentrations (0.6 to 10µM). Protein was harvested and assessed on day
three of osteogenic differentiation. RT-qPCR analysis of osteogenic markers (SP7 and IBSP) and AKT1 (housekeeping gene) during osteogenic commitment of hBMSCs (n = 3, a vs b = p < 0.001, c vs d = p < 0.05) (C). Gene expression analysis was performed on days six and thirteen of osteogenic differentiation.

**Figure 6. BMP2 and GSK126 combination results in more consistent bone healing.** Critical-sized calvarial defect model (left parietal bone) was created and treated (vehicle, BMP2, GSK126, and combination of BMP2 and GSK126) as described in methods. Relevant tissues (skulls/calvaria) were harvested and processed four weeks (28 days) after the surgery. Representative x-rays of four treatment groups (A). X-rays of all defects are shown in Suppl. Fig 3. µCT (B) and histomorphometric (C) quantification of the calvarial defect model (n = 4 to 7, mean ± STD, ** = p < 0.01, *** = p < 0.001). Each point on the graph represents an individual mouse. Defect margins were identified and native bone was excluded from the quantification. BMD = bone mineral density, BV/TV = bone volume-to-total volume ratio (i.e., bone volume fraction). Examples of µCT scans (D) and histologic coronal staining (E) of calvarial defects for each treatment group.

**Figure 7. Healing consistency within the BMP2 and GSK126 group is supported by orthopedic surgeon assessment.** Defect x-rays were scored blindly based on healing score (0-no healing to 5-completely closed) (A) and healing rank (1-worst healed to 22-best healed) (B) by two orthopedic surgeons (* = p < 0.05). The scores are averages of the scores and ranks given by the two orthopedic surgeons. Detailed methodology is described in the methods section. Correlation graph confirming consistent defect ranking by the two orthopedic surgeons (C).
Synergy between BMP2 and Ezh2 inhibition

Figure 1
Synergy between BMP2 and Ezh2 inhibition

Figure 2
Synergy between BMP2 and Ezh2 inhibition

Figure 3

A. PCA Plot Day 1

B. PCA Plot Day 6

C. 1263 differentially expressed genes on Day 1 (FC > 2)

D. 3712 differentially expressed genes on Day 6 (FC > 2)
Figure 4

A. Genes up-regulated on day 1

B. Genes up-regulated on day 6

C. Up-regulated Genes on Day 6 (Common in Both Combinations, n = 47)

D. Up-regulated Genes on Day 6 (Common in Both Combinations, n = 47)

E. H3K27Me3 Enrichment Score

F. Heatmaps showing gene expression levels and H3K27Me3 enrichment for different conditions.
Synergy between BMP2 and Ezh2 inhibition

Figure 5

A

Osteogenic Differentiation (AA, βGP, and Dex)

D0  D3  D6  D13

* = GSK126 and BMP2 addition

B

GSK126 (μM)

Veh  0.6  1.3  2.5  5  10

EZH2  100

H3K27me3  15

H3  15

GAPDH  37

C

SP7 (D6)

IBSP (D6)

AKT1 (D6)

SP7 (D13)

IBSP (D13)

AKT1 (D13)

GSK126 (μM): 0  0  0  5  5  5

BMP2 (ng/ml): 0  10  50  0  10  50

Norm. Exp. ± STD

a  b  c  d
Synergy between BMP2 and Ezh2 inhibition

Figure 6

A

Vehicle | BMP2 | GSK126 | Combo

5 mm

B

BMD (μCT) | BV/TV (μCT)

*** | ***

BMD (g/cm³) | BV/TV

*** | ***

Vehicle | BMP2 | GSK126 | Combo

C

BV/TV (Histomorphometry)

**

Vehicle | BMP2 | GSK126 | Combo

D

Vehicle | BMP2 | GSK126 | Combo

1 mm

E

Vehicle | BMP2 | GSK126 | Combo

1 mm
Figure 7

A. Healing Score

B. Healing Rank

C. Ranking Correlation

\[ p = 0.91 \]  
\[ p < 0.001 \]
Inhibition of the epigenetic suppressor EZH2 primes osteogenic differentiation mediated by BMP2

Amel Dudakovic, Rebekah M. Samsonraj, Christopher R. Paradise, Catalina Galeano-Garces, Merel O Mol, Daniela Galeano-Garces, Pengfei Zan, M Lizeth Galvan, Mario Hevesi, Oksana Pichurin, Roman Thaler, Dana L. Begun, Peter Kloen, Marcel Karperien, A. Noelle Larson, Jennifer J. Westendorf, Simon M. Cool and Andre J. van Wijnen

J. Biol. Chem. published online April 24, 2020

Access the most updated version of this article at doi: 10.1074/jbc.RA119.011685

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts