Published in final edited form as:

J Bone Miner Res. 2020 June ; 35(6): 1149–1162. doi:10.1002/jbmr.3975.

β -Catenin Preserves the Stem State of Murine Bone Marrow Stromal Cells Through Activation of EZH2

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Abstract

During bone marrow stromal cell (BMSC) differentiation, both Wnt signaling and the development of a rigid cytoskeleton promote commitment to the osteoblastic over adipogenic lineage. β -catenin plays a critical role in the Wnt signaling pathway to facilitate downstream effects on gene expression. We show that β -catenin was additive with cytoskeletal signals to prevent adipogenesis, and β -catenin knockdown promoted adipogenesis even when the actin cytoskeleton was depolymerized. β -catenin also prevented osteoblast commitment in a cytoskeletal-independent manner, with β -catenin knockdown enhancing lineage commitment. Chromatin immunoprecipitation (ChIP)-sequencing demonstrated binding of β -catenin to the promoter of enhancer of zeste homolog 2 (EZH2), a key component of the polycomb repressive complex 2 (PRC2) complex that catalyzes histone methylation. Knockdown of β -catenin reduced EZH2 protein levels and decreased methylated histone 3 (H3K27me3) at osteogenic loci. Further, when EZH2 was inhibited, β -catenin's anti-differentiation effects were lost. These results indicate that regulating EZH2 activity is key to β -catenin's effects on BMSCs to preserve multipotentiality.

Keywords

ACTIN-POLYMERIZATION; ADIPOCYTE; CHIP-SEQ; H3K27ME3; OSTEOBLAST; RHOA; ROCK-INHIBITION; WNT

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Authors' roles: Conceptualization: BS, CRP, MM, AW, and JR. Methodology: BS, CRP, MM, AW, and JR. Validation: BS, ZX, CRP, and AD. Formal analysis: CRP, MM, AW, and JR. Investigation: BS, CRP, ZX, JS, and MM. Writing: BS, CRP, JS, MM, MS, AD, AW, and JR. Visualization: BS, CRP, ZX, and JR. Supervision: AW and JR. Project administration and funding acquisition: AW and JR. GU should be added to conceptualization, investigation, and writing.

Additional Supporting Information may be found in the online version of this article.

Disclosures

The authors declare no competing interests.

Introduction

The contribution of β -catenin to Wnt regulation of stem cell differentiation is a significant, but poorly understood area. Wnt promotes bone formation from mesenchymal stem cells through mechanisms that include both stem cell renewal and induction of osteoblastogenesis,⁽¹⁾ with downstream signaling that invokes protection of β -catenin from the cytoplasmic antigen-presenting cell (APC)/axin destruction complex.⁽²⁾ Although β -catenin is known to activate transcription factors of the T-cell family (TCF) target genes,⁽³⁾ its role during cell differentiation is difficult to separate from other pathways activated by Wnt. Additionally, β -catenin's effects are further confounded by the fact that for many events resulting in β -catenin activation, large changes in levels of nuclear β -catenin cannot be observed.⁽⁴⁾

Mechanical strain increases cellular β -catenin through inhibition of GSK3 β , resulting in prevention of adipocyte lineage commitment⁽⁵⁾. Some of this relies on β -catenin's binding of PPAR γ to interfere with activation of targets necessary for adipocyte formation^(6,7) but β -catenin also functions to maintain bone marrow stromal cell (BMSC) multipotentiality^(8,9) as well as to interact with the dynamic cytoskeleton^(10,11). The question remains as to whether β -catenin's anti-adipogenic effects result in the promotion of osteoblast commitment⁽¹²⁾, and whether these actions require activation of the cytoskeleton.

Contextually, BMSCs respond to their physical environment, such that a soft substrate is associated with allocation to fat lineage, and a hard substrate to osteoblast differentiation⁽¹³⁾. Such substrate recognition derives from integrin-focal adhesion constructs^(14–16). In marrow-derived BMSCs, increased cytoskeletal structure improves osteogenesis^(14,16) while cytoskeletal deficiency promotes adipogenesis.^(14,17) Dynamic mechanical stretch increases focal adhesion formation along with connecting F-actin struts,⁽¹⁷⁾ an operation that, through inhibiting GSK3 β , increases the level of β -catenin within the cell. Altogether, increasing cytoskeletal actin structure prevents BMSC adipogenesis, which may prevent β -catenin from inhibiting PPAR γ -directed adipogenesis.

With respect to effects of an accreted actin cytoskeleton directing osteogenesis, there are little data to suggest that osteogenesis results from application of dynamic strain to cells in culture, including our own work, despite clear increases in β -catenin after strain.^(7,18,19) With flow⁽²⁰⁾ and low-intensity vibration,⁽²¹⁾ some markers suggesting osteogenic lineage rise, but, compared to inhibition of adipogenesis, the rise in β -catenin is not recognized as an osteogenic stimulus. Several laboratories have provided data to suggest that mechanical challenge of BMSCs leads to signaling pathways associated with proliferation and retention of multipotentiality. Development of the actin cytoskeleton due to spreading or plating on a hard surface is associated with transfer of Yes-associated protein/transcriptional coactivator with PDZ-binding motif (YAP/TAZ) to the nucleus,⁽²²⁾ a signaling system associated with cell proliferation⁽²³⁾. The movement of YAP/TAZ from the cytoplasm might further enhance β -catenin signaling through release of retention in the APC destruction complex⁽²⁴⁾. More recently dynamic strain has been associated with increased polycomb-mediated gene silencing⁽²⁵⁾.

To understand the role of the actin cytoskeleton on BMSC osteogenic differentiation, we previously disrupted the actin cytoskeleton with cytochalasin D. Previous reports suggested that failure to form cytoplasmic structure was associated with adipogenesis^(14,26,27) However, in our cytochalasin D study we found that osteogenesis was strongly promoted in marrow-derived⁽²⁸⁾ as well as in adipose-derived BMSCs⁽²⁹⁾ Interestingly, this was associated with a decrease in the polycomb repressive complex 2 (PRC21 member, enhancer of zeste homolog 2 (EZH2)⁽²⁹⁾ EZH2 has been shown to be a critical regulator of mesenchymal lineage commitment.⁽³⁰⁾ Importantly, loss of EZH2 function enhanced osteogenic differentiation of preosteoblasts, was bone anabolic, and prevented bone loss associated with estrogen depletion^(31,32) Further, EZH2 activity has been thought to promote adipogenesis, at least when maintained during long periods in culture⁽³⁰⁾ Consistent with these studies, previous work from our laboratories revealed that EZH2 suppresses osteogenesis and promotes adipogenesis in BMSCs⁽³³⁾ Furthermore, we found that EZH2 is required for normal cell cycle progression in BMSCs and that inhibition of EZH2 blocks cell proliferation by de-repressing cell cycle inhibitors⁽³⁴⁾ Thus, self-renewal and lineage commitment of BMSCs is tightly linked to EZH2 function.

Here we set out to determine if β -catenin, separate from the Wnt pathway, and the cytoskeleton exert overlapping control of mechanisms that control BMSC differentiation. We found that the mechanism by which β -catenin inhibits adipogenesis is independent from, but additive to, the development of the actin cytoskeleton. Importantly, enhancing β -catenin levels in multipotent mesenchymal stem cells prevents both osteogenic and adipogenic lineage commitment. β -catenin's role as an inhibitor of differentiation is largely dependent on its ability to influence the activity of EZH2. This work suggests that EZH2 is a novel target of β -catenin. This interaction allows β -catenin to indirectly alter the chromatin landscape and suppress expression of lineage-specific genes.

Materials and Methods

Reagents

Fetal bovine serum was from Atlanta Biologicals (Atlanta, GA, USA}. Culture media, trypsin–EDTA reagent, and antibiotics were from Sigma-Aldrich (St. Louis, MO, USA1; LiCl, BMP-2, and Y27632 were from Fisher Scientific (Waltham, MA, USA1; lysophosphatidic acid (LPA) from Cayman Chemical (Ann Arbor, MI, USA); GSK126 from Sigma-Aldrich. Both wild-type β -catenin and β CatS33Y (Addgene, Cambridge, MA, USA; #16828 and #19286, respectively) were overexpressed by transfecting with Lipofectamine 3000 (Life Technologies, Inc., Grand Island, NY, USA; L300015).

RNA interference

The cells were transfected with siRNA (50 nm) in serum-free OptiMEM overnight before replacing the medium and adding reagents for cell treatment. siRNAs were for β Cat 5'-CCCTCAGATGGTGTCTGCCATTGTA and control (nucleotide change within same sequence) 5'-CCCAGAGGTTGTGTACCTTTCGTA. A second siRNA targeting β -catenin was also studied: 5'-GGGACGTTTACAACCGGATTGTAAT-3' and control 5'-GGGCTTGAACAGGCCGTTATCAAAT-3'.

Cells and culture conditions

Mouse BMSCs were harvested from murine marrow using a published protocol,^(35,36) representing a polyclonal BMSC line where passages 4 to 15 are utilized (IACUC approved). Some experiments were repeated in C3H10T1/2 cells and MC3T3 cells which were purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA). BMSCs were maintained in MEM containing 10% fetal bovine serum, 100 µg/mL penicillin/streptomycin. For experiments, the cells were plated at a density of 10,000 cells/cm² in six-well culture plates (Fisher Scientific) and cultured for 1 day prior to application of treatments. Adipogenic medium consisted of 0.1 µM dexamethasone, 5 µg/mL insulin, and 50µM indomethacin and osteogenic medium consisted of 50 µg/mL ascorbic acid and 10mM β-glycerophosphate.

Immunofluorescence

For microscopy, cells were seeded in U-slide four-well chamber (ibidi, Gräfelfing, Germany; cat# 80426); fixed with 4% paraformaldehyde × 10 min, permeabilized in 0.1% Triton-X 100 × 5 min, blocked with 5% goat serum × 30 min separated by 3 × 10 min PBS washes between steps. Nuclear membrane was stained by anti-lamin B1 antibody (Abcam, Cambridge, MA, USA; cat# ab16048) and visualized with Rhodamine Red-X antirabbit secondary antibody (The Jackson Laboratory, Bar Harbor, ME, USA; cat# 711-295-152). Actin stress fibers were visualized with Alexa Fluor 488 conjugated phalloidin (Invitrogen, Carlsbad, CA, USA; cat# A12379). Proliferation marker, Ki67, was stained by Alexa Fluor 488 anti-mouse Ki-67 (BioLegend, San Diego, CA, USA; cat# 652417). Nuclei were visualized by NucBlue reagent (Life Technologies; cat# R37605). Cells were imaged on an Olympus BX61 inverted microscope system using Alexa Fluor 488 Phalloidin: Semroc 3540B filters.

Cell staining

Alizarin red S (Sigma-Aldrich; kit #A5533) and von Kossa staining (Sigma-Aldrich; silver nitrate #S8157, Sodium thiosulfate #217263) performed as per the vendor's instructions to detect calcified matrix. Oil-Red O staining performed as described⁽³⁷⁾; after fixation in 2% formaldehyde, the cultures were then rinsed three times for 5 min in deionized water and cytoplasmic triglyceride droplets were stained with Oil-Red-O.

Proliferation assay

Cells were seeded in 96-well plates for 0, 1, 3, and 5 days (±siβCat (siRNA targeting b-catenin)) or for 0, 2, 4, and 6 days (±S33YβCat (mutated b-catenin)). Cell number was measured with XTT Cell Proliferation Assay Kit (ATCC; Cat# 30-1011 K).

Real-time RT-PCR

Total RNA was isolated with the RNeasy Plus mini kit (QIAGEN, Valencia, CA, USA). Reverse transcription of 1 µg of RNA in a total volume of 20 µL was performed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) prior to real-time PCR (Bio-Rad iCycler). Twenty-five-microliter (25-µL) amplification reactions contained primers (0.5µM), SYBR-green super mix (Bio-Rad Laboratories; cat# 1725125). Aliquots of

cDNA were diluted fivefold to 5000-fold to generate relative standard curves to which sample cDNA was compared. *Fabp4*, *Adipoq*, *Pparg*, *Alpl*, *Sp7*, *Runx2*, *Bglap*, and *18S* primers were as in Sen and colleagues.⁽²⁸⁾ Standards and samples were run in triplicate. PCR products were normalized to 18 S amplicons in the RT sample, and standardized on a dilution curve from RT sample.

Immunoblot

Whole-cell lysates were prepared with lysis buffer (150mM NaCl, 50mM Tris HCl, 1mM EGTA, 0.24% sodium deoxycholate, 1% Igepal, pH 7.5) containing 25mM NaF and 2mM Na₃VO₄; aprotinin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride were added prior to each lysis. Then 5 to 20 µg of fractionated or whole lysate proteins were loaded onto a 7%–10% polyacrylamide gel for chromatography and transferred to polyvinylidene difluoride membrane. After blocking, primary antibody was applied overnight at 4°C including antibodies against Pparg, Flag, and EZH2 from Cell Signaling Technology (Beverly, MA, USA; cat #2443, 8146, and 5246, respectively); Histone H3 (Sigma-Aldrich; cat# 05–928), β-catenin (Fisher Scientific; cat# PIPA516762), methylated histone (H3K27me) (Fisher Scientific; 17-622-MI), Adipoq (Fisher Scientific; cat# PA1054), Fabp4 (ProSci, Poway, CA, USA; cat# XG-6174), beta-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat# sc-23949). Secondary antibody conjugated with horseradish peroxidase was detected with ECL plus chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA). The images were acquired with a HP-Scanjet and densitometry determined using NIH ImageJ, 1.37v (Bethesda, MD, USA; <https://imagej.nih.gov/ij/>).

RNA sequencing

BMSCs were seeded at 10,000 cells/cm² and allowed to proliferate overnight. The next day, media was changed to osteogenic media and GSK126 or siRNAs were added to the cultures. Three days later, cells were lysed using TRIzol Reagent (Thermo Fisher, Waltham, MA, USA; Cat#: 15596026) and stored at –80°C until isolation. mRNA isolation was performed using the Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA, USA; Cat#: R2052) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher). Samples were assessed for RNA integrity (RIN) using the Agilent Bioanalyzer DNA 1000 chip (Invitrogen, Carlsbad, CA, USA). Only samples with RIN scores >6 and DV₂₀₀ > 50% (DV₂₀₀ is the percentage of RNA fragments that are >200 nucleotides in size) were selected for sequencing. This resulted in *n* = 3 biological replicated for each treatment group (control (CTL), EZH2 inhibitor (Ezh2i), siβCat). RNA-sequencing and subsequent primary and secondary data analysis was performed as described.^(38,39) In brief, library preparation was performed using the TruSeq RNA library preparation kit (Illumina, San Diego, CA, USA). Polyadenylated mRNAs were selected using oligo dT magnetic beads. TruSeq Kits were used for indexing to permit multiplex sample loading on the flow cells. Paired-end sequencing reads were generated on the Illumina HiSeq 2000 sequencer. Quality control for concentration and library size distribution was performed using an Agilent Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA, USA) and Qubit fluorometry (Invitrogen). Sequence alignment of reads and determination of normalized gene counts were performed using the MAP-RSeq (v.1.2.1) workflow, utilizing TopHat 2.0.6,⁽⁴⁰⁾ and

HTSeq.⁽⁴¹⁾ Normalized read counts were expressed as reads per kilobase-pair per million mapped reads (RPKM).

Gene expression analysis

Comparative analysis of RPKM data and generation of volcano plots was performed in Microsoft Excel (Microsoft Corp., Redmond, WA, USA). Venn diagram overlap analysis was performed using InteractiVenn online tool (<http://www.interactivenn.net/>).⁽⁴²⁾ Gene Ontology terms were investigated and selected using the Gene Ontology online database.^(43,44) “Nuclear Proteins” were selected from the “nucleus” GO_CC gene list (Accession: GO:0005634).

Chromatin immunoprecipitation-sequencing

Chromatin immunoprecipitation (ChIP) assay for the BMSCs treated with 50mM LiCl for 3 hours was performed as described⁽⁴⁵⁾ with antibodies to β -catenin (H-102, sc-7199; C-18, sc-1496; E-5, sc-7963; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or TCF-4 (clone 6H5-3; Millipore Corp., Billerica, MA, USA).⁽⁴⁶⁾ For ChIP-qPCR performed at the University of Wisconsin on BMSC cells treated with 50mM LiCl, 40 ng/mL Wnt3a, and 20 μ M SB415286, pull downs used the antibodies to β -catenin above and a nonspecific IgG control. Primers used for analysis were EZH2 + 2.5 kilobases (kb): F-5'-AGCACATACTCTGATGGACCTTT and R-5'-AGTCTTTATGTCAGTGTAGATGGTG; Ddit3 (C/EBP homologous protein [CHOP]) -1.5 kb: F-5'-AGTGTGCAGAGACTTGACCCGC and R-5'-ACTGCTCCTTCGCTCCAGACCTT.

ChIP assay for H3K26me3 was performed at the Mayo Clinic proteomics core facility using H3K27me3 antibody (Millipore; 17-622) and IgG control (Millipore; PP64B). For this assay, BMSCs were seeded at 10,000 cells/cm² and allowed to proliferate overnight. The next day, media was changed to osteogenic media and GSK126 or siRNAs were added to the cultures. Three days later, cells were fixed and pelleted for subsequent ChIP assay. Sequencing libraries were prepared and sequenced on the Illumina HiSeq2000 system. Subsequent bioinformatics analysis was performed using the HiChIP analysis pipeline.⁽⁴⁷⁾ Briefly, 50-base pair (bp) reads were aligned to the mm10 reference genome using the Burrows-Wheeler Aligner, and Picard was used to mark duplicates. Read pairs with one or both ends uniquely mapped were retained for further analysis. Enriched regions and peaks were identified using SICER.⁽⁴⁸⁾ ChIP tracks were viewed in the Integrated Genome Viewer (IGV) version 2.3^(49,50) on the mm10 genome build.

Statistical analysis

Results are expressed as mean $\pm$ SE. Statistical significance was evaluated by one-way ANOVA analysis of variance or *t* test as appropriate (GraphPad Prism; GraphPad Software, Inc., La Jolla, CA, USA). All experiments were replicated at least three times to assure reproducibility.

Data availability

Sequencing data from RNA sequencing and ChIP sequencing experiments can be accessed in the Gene Expression Omnibus database under accession number GSE139041.

Results

β -catenin's inhibition of adipogenesis is independent of cytoskeletal control

Preservation of cellular β -catenin, largely through Wnt signaling directed inhibition of the β -catenin destruction complex,⁽⁵¹⁾ has well-established effects to suppress adipogenic differentiation of BMSCs in vitro and in vivo.^(5,6) Both overexpression of S33Y β -catenin (β CatS33Y), a mutant of β -catenin that is resistant to proteasomal degradation⁽⁵²⁾ and wild-type β -catenin β cat in BMSCs⁽³⁵⁾ inhibited the adipogenic differentiation as shown by decreased mRNA and protein for the adipogenic proteins adiponectin and AP2, as well as master fat transcription factor Pparg protein (Fig. 1A,B). Because an increased actin cytoskeleton is also known to restrict adipocyte differentiation,^(13,15) we investigated whether β -catenin inhibition of adipogenesis involved similar signal pathways as those modulated via cytoskeleton rigidity. Diminishing the actin cytoskeleton by treating with the ROCK inhibitor Y27632 promoted adipogenic differentiation, while stimulation of actin stress fiber formation with lysophosphatidic acid (LPA) inhibited it, as expected (Fig. 1C,D). Supporting the cytoskeletal effects of these agents, both cell area and mean intensity of Alexa-phalloidin 482 F-actin stain were significantly decreased by Y27632 and increased after treatment with LPA (Fig. S1). Transfection of β CatS33Y inhibited adipogenesis induced by Y27632 (Fig. 1C), and was additive with LPA (Fig. 1D). This suggests that β -catenin inhibition of adipogenesis functions independently of information arising from the cytoskeleton.

We then knocked down β -catenin in BMSCs with siRNA⁽⁷⁾ (Fig. 2A mRNA, Fig. 2C protein). Knockdown of cellular β -catenin resulted in increased adipogenesis (Fig. 2A) and was additive to the reduced actin cytoskeleton in the presence of ROCK inhibitor (Fig. 2B). Further, loss of β -catenin promoted adipogenesis even when the actin cytoskeleton was enhanced through addition of LPA (Fig. 2C) and confirmed with the increased Oil Red O of mature adipocytes (Fig. 2D). Counts of fat-laden Oil-Red O cells were increased twofold when sipcat was present, and decreased of 70% by LPA treatment; the latter effect was abolished by the knockdown of β -catenin (Fig. S2). Overexpression of the β CatS33Y decreased Oil Red-O stain, in agreement with the protein and mRNA data (Fig. S3). Taken together, a stiff cytoskeleton is not sufficient to prevent adipogenesis when β -catenin is silenced, indicating that effects of β -catenin predominate over cytoskeletal controls of adipogenesis.

β -catenin inhibits osteogenic lineage commitment

Wnt signaling is associated with both pro-osteogenic and anti-adipogenic activity.⁽⁵³⁾ A direct role for β -catenin in regulating osteoblast development has also been proposed.^(54,55) We asked whether regulation of β -catenin in BMSCs, which are poised to enter the osteogenic lineage,⁽⁵⁶⁾ enhances this propensity at the expense of adipogenesis. β -catenin levels were augmented with the β catS33Y or knocked down with siRNA before treating cultures with osteogenic medium in the presence and absence of BMP-2.⁽⁵⁷⁾ Unexpectedly, overexpressing β -catenin inhibited bone marker expression, even in the BMP-2-treated cells (Fig. 3A), consistent with a potent anti-differentiative effect. Moreover, β -catenin knockdown increased expression of the osteogenic phenotype, enhancing the effect of

BMP2 (Fig. 3B). Staining for hydroxyapatite with either Alizarin red (top row) or Von Kossa (bottom row) confirmed that β -catenin knockdown accelerated nodule formation and interacted with BMP-2 (Fig. 3C, Fig. S4A). As well, overexpression of wild-type β -catenin also decreased the osteogenic effect of BMP-2, but to a lesser degree than that seen with overexpression of the mutant proteasome resistant S33Y β -catenin (Fig. S4B). The effect of wild-type β -catenin was expected to be less potent than that of β catS33Y vector, because the latter is resistant to proteasomal breakdown, thus increasing cellular β -catenin levels.⁽⁶⁾ These results suggest that β -catenin has a significant effect to prevent commitment to both osteogenic and adipogenic lineages.

We confirmed effects of the siRNA we have used in the past^(17,58) and in the majority of the current studies, and show in the online Supporting Information that a plural siRNA has similar effects to promote adipogenesis (Fig. S5A) and osteogenesis (Fig. S5B). These effects were consistent when studied with three further siRNAs targeting β -catenin. We also show that our standard siRNA is effective at doses of 10nM (Fig. S6).

β -catenin enhances proliferation of BMSC

Canonical Wnt/ β -catenin signaling plays a role in the maintenance of self-renewal and the undifferentiated state of stem cells,⁽⁵⁹⁾ and overexpression of the Wnt co-receptor LRP5 increases proliferation in BMSCs.⁽⁶⁰⁾ To query whether β -catenin promoted BMSC stemness, we stained for Ki-67, a nuclear protein necessary for cellular proliferation.⁽⁶⁰⁾ In BMSCs, overexpression of β CatS33Y significantly increased, while β -catenin knockdown reduced Ki-67-positive cells (Fig. 4A) and RT-PCR confirmed that β -catenin increased expression of *Mki67* (Fig. 4B). Cell proliferation showed that β catS33Y modestly enhanced while knockdown of β -catenin decreased the BMSC proliferation rate (Fig. 4C). We examined a panel of genes involved in the control of cell cycle, showing that β CatS33Y enhanced, while β -catenin knockdown decreased genes that promoted proliferation (Fig. 4D). In sum, these results suggest that β -catenin supports proliferation of BMSCs.

β -catenin restriction of BMSC lineage commitment involves EZH2

A clue to the potential mechanism by which β -catenin limits lineage commitment was that treatment of BMSCs with LiCl to inhibit GSK3 β -induced β -catenin degradation⁽¹⁰⁾ revealed that β -catenin associated with the EZH2 promoter in a LiCl-dependent manner, but not the EZH1 promoter (Fig. 5A). ChIP-Seq for β -catenin and TCF4 in the *Ezh2* and *Ezh1* loci is expanded in Fig. S7A, and for EZH1 in Fig. S7B. EZH2 has been shown to inhibit osteogenesis, promoting stemness and proliferation through genome methylation,^(31,33) leading us to speculate that effects of β -catenin might involve EZH2 activity. Indeed, overexpression of β CatS33Y increased EZH2 expression along with increased methylation of EZH2 (H3K27me3), as did LiCl treatment (Fig. 5B,C). Accompanying the increase of EZH2 expression and H3K27me3, expression of adipogenic protein expression significantly decreased in BMSCs (Fig. 5C), as well as in embryonic C3H10T1/2 pluripotent stem cells (Fig. S8). Further, knockdown of β -catenin prevented H3K27 methylation by decreasing EZH2 and reversed the pattern of the gene expression, promoting adipocytic lineage commitment (Fig. 5D).

Importantly, Wnt3a has disparate effects on EZH2 and its activity than other agents associated with β -catenin signaling. Increase of β -catenin through GSK3 β inhibition (SB415286) and LiCl show that EZH2 increases as does the level of H3K27me₃; Wnt3A does not have this effect, and may even decrease histone methylation associated with EZH2 (Fig. S9A). This is further advanced by data in MC3T3 osteoblasts, where Wnt3a *interferes* with the effects of GSK126: Fig. S9B shows that, though GSK126 inhibition of EZH2 leads to osteogenesis, Wnt3a prevents this effect. This supports that β -catenin cannot be considered sufficient to transduce Wnt effects, which thus must require stimulation of noncanonical signaling pathways.⁽⁶³⁾

We further show that agents that increase levels of cellular β -catenin cause increased association of β -catenin with the EZH2 promoter (Fig. S10A,B). To confirm the ability of the proteolysis-resistant β CatS33Y to enter the nucleus and bind cis-promoter elements, data reveals that β -catenin association with the EZH2 promoter is increased after transfection with β CatS33Y, as well as with a common target promoter for β -catenin, CHOP (Fig. S10C).

To define the role of EZH2 in mediating β -catenin's effect on stem cell lineage commitment, we treated β CatS33Y-transfected BMSCs with GSK126, an inhibitor of EZH2. When EZH2 activity was inhibited, β CatS33Y was no longer able to suppress adipocyte proteins (Fig. 5E). Densitometry for biological triplicates is shown in Fig. S11A: β CatS33Y increases H3K27me₃ levels over the control transfected cells, and GSK126 prevents this effect. The decrease in adipocyte adiponectin due to β CatS33Y is abolished in the presence of GSK126. Inactivating EZH2 had no additive effect on β CatS33Y-induced enhancement of adipogenic protein levels (Fig. 5F). Densitometry of biological triplicates again supports this finding: knockdown of β -catenin decreases H3K27 methylation and increases adiponectin expression, whereas the addition of GSK126 prevents methylation and increases protein levels of adiponectin (Fig. S11B).

In terms of osteogenesis, suppressing EZH2 activity promoted expression of osteogenic genes in BMSCs, shown by increased *Sp7*, *Bglap*, and *Alpl* (Fig. 5G). Addition of β CatS33Y to these osteogenic conditions did not further increase osteogenic commitment. Alternatively, when β -catenin was knocked down, there was further enhancement of GSK126-induced osteogenic commitment (Fig. 5H), suggesting that β -catenin has anti-lineage effects that are separable from those due to EZH2. The effects of β -catenin knockdown and EZH2 inhibition by GSK126 in BMSCs were replicated in C3H10T1/2 cells in terms of osteogenesis, where osteogenic commitment due to inhibition of EZH2 was not further enhanced by addition of β CatS33Y, and knock down of β -catenin further increased the effects of GSK126 (Fig. S12). Taken together, these results indicate that β -catenin effects to prevent BMSC lineage commitment are partially due to its stimulation of EZH2.

The effects of β -catenin knockdown and EZH2 inhibition converge on similar gene sets

To better understand the transcriptional changes leading to osteogenic lineage commitment that are associated with EZH2 inhibition (with GSK126, here "Ezh2i") or β -catenin knockdown (si β Cat), we conducted comparative analysis of the RNA-sequencing (RNAseq) datasets. First, we plotted the differentially expressed genes in both groups compared to

control (Fig. 6A). We note significant upregulation ($\log_2\text{FC} > 1$ and $p < .05$; FC = fold change) of 236 genes and significant downregulation ($\log_2\text{FC} < -1$ and $p < .05$) of 43 genes in the Ezh2i group. It is important to note the uneven distribution of upregulated versus downregulated genes (236 versus 43). This is expected because EZH2 inhibition opens chromatin and allows for increased transcription. In the si β Cat group, we note significant upregulation of 176 genes and significant downregulation of 276 genes ($\log_2\text{FC} < -1$ and $p < .05$). The ratio of upregulated to downregulated genes is inverted in this comparison (176 versus 276) because β -catenin is required for transcriptional activation of many gene targets. Our study aimed to elucidate the common mechanisms driving osteoblastic differentiation in Ezh2i and si β Cat conditions. Therefore, we focused on upregulated genes in each group because these are likely the transcripts for the upregulated effector proteins and signaling pathways. We identified 44 common genes after assessing the overlap of upregulated genes in each group (Fig. 6B). Our focus here was on transcriptional regulation of osteoblastic differentiation, so we narrowed our search to nuclear proteins. Of the 44 common genes, nine members of this list were annotated with the Gene Ontology Cellular Compartment (GO_CC) term “nucleus” (GO:0005634). Interestingly, five of the nine nuclear genes we identified were involved in osteogenic differentiation (*Cebpd*, *Dlx5*, *Hes1*, *Id4*, and *Msx1*). In Fig. S13, we performed specific RT-PCR for these five osteogenic genes in BMSCs: overexpression of β -catenin decreased four of five genes, whereas near total β -catenin knockdown caused an increase in all except for CEBPD. These results confirm that β -catenin restricts the expression of osteogenic genes.

Because EZH2 is a histone 3 lysine 27 (H3K27) methyltransferase, we assessed H3K27 trimethylation (H3K27me3) levels via ChIP followed by next generation sequencing (ChIP-seq). We assessed H3K27me3 levels upstream of the five osteoblastic genes of interest (Fig. 6C) and found that in several instances both EZH2i and si β Cat decrease H3K27me3 levels upstream of these significantly upregulated genes (ie, *Cebpd*). Together, these data suggest that a reduction in H3K27me3 resulting from EZH2i or si β Cat induces expression of osteoblast-specific genes and drives differentiation.

Finally, ChIP-seq data for H3K27me3 marks and RNA-seq mRNA expression data from BMSCs treated with the EZH2 inhibitor GSK126 or siRNA for β -catenin were examined for both adipogenic and proliferation-related genes (Fig. S14). The results show that the adipogenic and cell cycle-related genes we selected are not heavily marked by H3K27me3 modifications and that the effects of GSK126 on H3K27me3 deposition are variable. The latter indicates that changes in H3K27me3 levels for some genes are indirect (eg, by effects on H3K27me3 demethylases) or involve EZH1 rather than EZH2. Regardless of whether changes occur in H3K27me3 on these genes, we found that GSK126 strongly enhances expression of mature fat markers (eg, *Plin1* and *Adipoq*), while only marginally modulating proliferation markers. In contrast, siRNA for β -catenin enhanced neither adipogenic or proliferation markers. Collectively, the ChIP-seq and RNA-seq results suggest that classical adipogenic genes we selected for our analysis are not directly regulated by EZH2 or β -catenin related mechanisms during adipogenic differentiation.

Discussion

Although β -catenin is known to drive the growth of stem⁽⁶³⁾ and cancer cells⁽⁵⁹⁾ as the canonical messenger of the Wnt pathway, it has also been linked to differentiation pathways.^(55,64) Indeed, Wnts are potent stimulators of osteoblastogenesis.⁽⁶⁵⁾ This effect of Wnt has been thought to arise as a byproduct of β -catenin's effect to constrain adipocyte lineage commitment of BMSCs,⁽⁵³⁾ essentially protecting the multipotential nature of these cells to respond to osteoblastic signals.⁽⁹⁾ Here we show that along with limiting adipocyte lineage, pure β -catenin signaling is not osteogenic, but in fact anti-osteogenic. These anti-differentiative and pro-proliferative effects of β -catenin predominate over signals conveyed through the cytoskeleton that regulate differentiation. Further, we show for the first time that the effect of β -catenin to restrain lineage commitment utilizes the downstream effects of a previously unrecognized target of β -catenin, EZH2, the functional enzymatic component of PRC2.

Reinforcing cytoplasmic actin structure has similar effects as does β -catenin to prevent adipocyte lineage commitment.^(14,66) This is typified by the effect of mechanical stretch, which protects β -catenin from proteasomal degradation by inhibiting GSK3 β similar to Wnt,⁽⁷⁾ to also generate a (small GTPase RhoA-dependent cytoskeleton.⁽¹⁷⁾ Concurrent effects on β -catenin and the actin cytoskeleton are also seen with the use of LPA, which, through its G protein-coupled receptors stimulates RhoA⁽⁶⁷⁾ and also activates phosphorylation of GSK3 β and release of β -catenin from the APC destruction complex.⁽⁶⁸⁾ In dissecting the influences of the actin cytoskeleton and β -catenin, we have shown here that β -catenin is additive but not dependent on building the actin cytoskeleton: β -catenin had anti-differentiative effects even when inhibitors of RhoA were present. Further, β -catenin also interferes with osteoblastic lineage commitment stimulated by either osteogenic medium or by the addition of BMP2. In turn, knockdown of β -catenin enhanced both osteogenic and adipogenic differentiation. In both marrow-derived MSC, and in C3H10T1/2 cells, β -catenin instead functions to promote proliferation with upregulation of positive cell cycle genes.

Once inside the nucleus, β -catenin can heterodimerize with lymphoid enhancer-binding factor/T cell factor to induce gene expression.⁽³⁾ β -catenin can also prevent PPAR γ activation of targets necessary for adipocyte formation by binding PPAR γ .⁽⁶⁹⁾ Although these functions are relevant to stem cell differentiation, the majority of the β -catenin anti-lineage commitment effect appeared to depend on its ability to modulate both the level and activity of EZH2. The observation that β -catenin was associated with the EZH2 promoter in LiCl-treated cells, combined with previous data showing that Ezh2 inhibits osteogenesis,⁽³¹⁾ led to our finding that EZH2 is key to the ability of β -catenin to block differentiation. In this system, the loss of EZH2 activity itself promoted osteogenic differentiation as expected, but importantly led to a near ablation of β -catenin's effect to prevent lineage commitment.

Several prior studies have suggested that EZH2 might enhance the effects of β -catenin. In breast cancer cells, EZH2 and β -catenin augmented the effects of the other in terms of stimulating proliferation genes and it was suggested that this involved a direct interaction between the proteins.⁽⁷⁰⁾ Moreover, EZH2-overexpressing breast cells accumulated β -catenin in the nuclei, supporting a link between EZH2 and β -catenin activation.⁽⁷¹⁾ Our data

carries this further, suggesting that β -catenin directly increases EZH2 levels and activity, perhaps setting up a positive autocrine loop that promotes stem cell expansion.

It might be surmised that a role of canonical β -catenin signaling in the bone forming effects of Wnt, which promotes bone mass in animals and humans,^(65,72) is misplaced—indeed, nuanced studies cast doubt. For instance, β -catenin signaling may promote proliferation but reduce osteogenic differentiation of stem cells under inflammatory conditions.⁽⁷³⁾ Other reports showed that deletion of β -catenin in early osteogenic precursors actually increased osteoblast number and enhanced bone formation activity⁽⁵⁴⁾ and that expression of constitutively activated β -catenin in osteoblasts supported proliferation but impaired terminal osteoblast differentiation, subsequently resulting in decreased bone strength.⁽⁷⁴⁾ Instead, to induce osteoblast differentiation and function, Wnt ligands may depend on noncanonical signaling such as the Wnt-planar cell polarity pathway (Wnt-PCP pathway) and the Wnt-calcium pathway (Wnt-Ca²⁺ pathway). In the Wnt-PCP pathway, *Wnt5a* regulates limb morphogenesis⁽⁷⁵⁾ and osteoblastogenesis.⁽⁷⁶⁾ Wnt-LRP5 signaling induces mTORC2-AKT signaling activity and triggers glycolytic enzymes in bone cells to promote bone formation.⁽⁷⁷⁾

To gain a full appreciation of how Wnt signaling and β -catenin control the self-renewing stem cell state versus mesenchymal lineage commitment, it would be informative to perform a comprehensive analysis of the epigenome of BMSCs under different biological conditions. These studies would need to dissect the epigenetic effects of β -catenin responsive to and independent of Wnt signaling, as well as consider the effects of canonical (β -catenin-dependent) and noncanonical (β -catenin-independent) Wnt signaling under conditions that maintain the undifferentiated, osteogenic, or adipogenic cell fates. Beyond direct effects of β -catenin binding to its target promoters, these studies would also need to examine primary histone modifications that control the opening of heterochromatin into transcriptionally poised or active states, including marks linked to tissue-specific super-enhancers (eg, H3K4me1, H3K27ac1. Although these studies are warranted, they are considered beyond the scope of the present work.

It is most likely that a balance of signals must be reached that involve both renewal and proliferation of stem cells, dependent on β -catenin as demonstrated by our data showing a positive relationship of β -catenin to expression of Ki-67, and other contextual signals that support lineage commitment⁽⁷⁸⁾ β -catenin's role in proliferation is well accepted in cancer cell proliferation.⁽²⁾ Similarly, EZH2 has been studied in cancer progression, because it is not only involved in regulation of cell cycle progression but aberrantly overexpressed in various malignant tumors. The dys-regulation of EZH2 accelerates cell proliferation⁽⁷⁹⁾ and promotes tumor growth.^(80,81) Further, there is evidence that EZH2 and β -catenin together have at least additive effects on tumor progression. Twelve identified Wnt/ β -catenin signal antagonists are repressed by EZH2 to promote β -catenin-dependent carcinogenesis⁽⁸²⁾ It is perhaps not surprising that β -catenin and EZH2 interact to promote growth.

As to our finding of a negative role for β -catenin in differentiation, EZH2 also prevents osteoblast differentiation of BMSCs.^(31,83) Activation of cyclin-dependent kinase phosphorylates EZH2 at Thr-487, inhibiting activity, and results in BMSC osteoblast

differentiation.⁽⁸⁴⁾ Further there are multiple targets for EZH2 in BMSCs that target differentiation,⁽⁸⁵⁾ and through repression of Wnt genes facilitates adipogenesis.⁽⁸⁶⁾ To further understand the intersection of β -catenin and potential downstream reliance on EZH2 in preventing lineage commitment, we sought to identify common gene targets. To obtain clear unbiased results, rather than overexpressing β -catenin and EZH2 (which would depend on degree of overexpression), we conducted RNA-seq on BMSCs where β -catenin was knocked down and *Ezh2* was inhibited. We found 44 common genes to be significantly upregulated in both inhibitory conditions. These 44 genes accounted for 33% of all genes elevated in response to β -catenin knockdown. This gene group appears to be shared in promotion of osteoblastic lineage that is generated in both conditions. To understand transcriptional regulation dictating osteoblast differentiation, we filtered genes encoding nuclear proteins: we found that five of the nine nuclear proteins were directly related to RUNX2, a key regulator of osteoblast differentiation and skeletal development. These include *Dlx5*, *Hes1*, *Msx1*, *Cebpd*, and *Id4*, all directly or indirectly associated with activation of bone-specific genes.^(87–91) Indeed, ChIP-seq showed that both β -catenin knockdown and EZH2 inhibition, alone, lowered H3K27me3 levels at these osteoblastogenic gene loci. Lowered H3K27me3, through decreased condensation of specific gene structures, would likely allow for their elevated expression.

In summary, our results indicate a novel mechanism by which β -catenin protects the multipotency of BMSCs in bone. This involves β -catenin's direct binding of the EZH2 promoter to regulate EZH2 expression and activity, indicating the signaling axes by which β -catenin controls lineage are more complicated than previously thought.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the NIH (National Institute of Arthritis and Musculoskeletal and Skin Diseases [NIAMS] AR066616 to JR and AR049069 to AW); (National Institute of General Medical Sciences [NIGMS] P20 GM109095 and P20 GM103408 to GU).

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Fig. 1. β -catenin inhibition of adipogenesis is independent of cytoskeleton. (A, B) After transfection with pcDNA3-S33Y β -catenin (Addgene #19286) or human β -catenin pcDNA3 (Addgene #16828) plasmids for 24 hours, the cells were cultured in A medium for 3 days. Adipogenic markers were tested by PCR (left panel) or Western blot (right panel). After S33Y β -catenin overexpression, the cells were treated with (C) Y27632 (10 μ M) or (D) LPA (30 μ M), then cultured in A medium for 3 days. Adipogenic markers were tested by PCR or Western blot; or the cells were stained with phalloidin (green). Scale bars = 25 μ m. Graph bars show mean $\pm$ SE, * p < .05, ** p < .01.

Fig. 2. β-catenin effects predominate over cytoskeleton in control of adipogenesis. (A) After transfection with siRNA for β-catenin for 24 hours, cells were cultured in A medium for 3 days. Adipogenic markers were tested by PCR (left panel) or Western blot (right panel). After knocking down β-catenin (RT-PCR shown), cells were treated with (B) Y27632 (10μM) or (C) LPA (30μM) before culture in A medium for 3 days. Adipogenic markers were tested by PCR or Western blot; or (D) cells were stained with Oil-Red O. Graph bars show mean ± SE, **p* < .05, ***p* < .01.

Fig. 3. β -catenin inhibits osteogenic differentiation. After transfection with (A) pcDNA3-S33Y β -catenin or (B) siRNA for β -catenin for 24 hours, cells were cultured in O-medium $\pm$ BMP-2 for 4 days. Osteogenic markers were tested by PCR. (C) Cells were stained with Alizarin red (upper panel) or Von Kossa (lower panel), arrows show nodules, with insets of higher magnification ($\times 40$). Graph bars show mean $\pm$ SE.

Fig. 4.

β-catenin enhances proliferation state of BMSCs. After transfection with pcDNA3-S33Y β-catenin or siRNA for β-catenin for 24 hours, cells were cultured in MEM medium for 3 days. (A) Cell number positive for Ki-67 is shown after staining for Ki67 and LaminB1 (nucleus). (B) PCR for Ki-67. (C) After transfection with pcDNA3-S33Y β-catenin or siRNA for β-catenin for 24 hours, cells cultured in O medium were counted at 0, 2, 4, and 6 days for S33Y-β-catenin overexpression; or at 0, 1, 3, and 5 days for β-catenin knockdown cells. (D) RT-PCR for a panel of proliferation genes in cells treated with empty vector,

S33yβcat, or siCTL versus siβ-catenin for 6 days; experiment repeated 2 times. Graph bars show mean ± SE, * $p < .05$, ** $p < .01$.

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Fig. 5.

β -catenin effects on BMSC differentiation involve EZH2. (A) ChIP-seq for association of β -catenin or TCF4 to promoters of *Ezh2* or *Ezh1*. Overlaid ChIP-seq tracks are displayed as either vehicle (yellow) or LiCl-treated (blue). Overlapping track data appear as green. Genomic location and scale are indicated (top) and maximum height of tag sequence density for each data track is indicated on the y-axis (top left each track, normalized to input and 10^7 tags). Gene transcriptional direction is indicated by an arrow and exons by boxes. (B) BMSCs cultured in MEM $\pm$ LiCl for 48 hours; WB shows EZH2, methylated H3K27, and

H3. BMSCs were cultured in A medium for 3 days and were probed for adipocyte markers after overexpression (*C*) or knockdown of β -catenin (*D*). (*E*) BMSCs were cultured in A medium and treated with GSK126 for 3 days (Ezh2i); WB as shown in cells treated with EV or S33Y β cat and for (*F*) with β -catenin knockdown; see also Fig. S3. (*G*) BMSCs were cultured in O medium and then treated with GSK126 for 4 days; RT-PCR for osteogenic proteins are shown in response to S33Y β cat overexpression, or in (*H*) β -catenin knockdown. Graph bars show mean $\pm$ SE, * p < .05, ** p < .01. EV = empty vector; WB = western blot.

Fig. 6. EZH2i and siβCat alter transcriptional networks and H3K27me3. (A) Volcano plots showing differentially expressed genes in Ezh2i (left) and siβCat (right) versus siCTL (red = $\log_2FC < -1$ and $p < .05$; blue = $1 > \log_2FC > -1$ and $p < .05$; green = $\log_2FC > 1$ and $p < .05$; gray = $p > .05$). (B) Venn diagram of common significantly upregulated genes in Ezh2i and siβCat treatments. (C) “Nuclear proteins” selected from list of 44 common genes using GO_CC term “nucleus.” mRNA expression (RPKM) of nuclear genes commonly upregulated in EZH2i and siβCat and H3K27me3 ChIP-seq tracks upstream of the TSS. TSS = transcription start site.