



Rapid Osteogenic Enhancement of Stem Cells in Human Bone Marrow Using a Glycogen-Synthase-Kinase-3-Beta Inhibitor Improves Osteogenic Efficacy In Vitro and In Vivo

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ABSTRACT

Non-union defects of bone are a major problem in orthopedics, especially for patients with a low healing capacity. Fixation devices and osteoconductive materials are used to provide a stable environment for osteogenesis and an osteogenic component such as autologous human bone marrow (hBM) is then used, but robust bone formation is contingent on the healing capacity of the patients. A safe and rapid procedure for improvement of the osteoanabolic properties of hBM is, therefore, sought after in the field of orthopedics, especially if it can be performed within the temporal limitations of the surgical procedure, with minimal manipulation, and at point-of-care. One way to achieve this goal is to stimulate canonical Wingless (cWnt) signaling in bone marrow-resident human mesenchymal stem cells (hMSCs), the presumptive precursors of osteoblasts in bone marrow. Herein, we report that the effects of cWnt stimulation can be achieved by transient (1–2 hours) exposure of osteoprogenitors to the GSK3 β -inhibitor (2',Z,3'E)-6-bromindirubin-3'-oxime (BIO) at a concentration of 800 nM. Very-rapid-exposure-to-BIO (VRE-BIO) on either hMSCs or whole hBM resulted in the long-term establishment of an osteogenic phenotype associated with accelerated alkaline phosphatase activity and enhanced transcription of the master regulator of osteogenesis, Runx2. When VRE-BIO treated hBM was tested in a rat spinal fusion model, VRE-BIO caused the formation of a denser, stiffer, fusion mass as compared with vehicle treated hBM. Collectively, these data indicate that the VRE-BIO procedure may represent a rapid, safe, and point-of-care strategy for the osteogenic enhancement of autologous hBM for use in clinical orthopedic procedures. *STEM CELLS TRANSLATIONAL MEDICINE* 2018;7:342–353

SIGNIFICANCE STATEMENT

Non-union defects of bone are a major problem in orthopedics, especially for patients with a low healing capacity. Autologous human bone marrow (hBM) is often used as a source for osteogenic stem cells, but efficacy is contingent on the healing capacity of the patients. Herein, this study shows that exposure of hBM to a glycogen-synthase-kinase inhibitor for a very short time, initiates an irreversible cascade that increases the osteogenic capacity of resident stem cells. Importantly, the BIO-treated hBM has the capacity to generate stronger and more compact fusion masses when tested in a rodent spine fusion model. This approach could represent a feasible and cost-effective strategy for functional enhancement of autologous hBM for orthopedic applications.

INTRODUCTION

Non-union defects of bone are a major problem in orthopedics, especially for those with a low capacity for healing. Of the 13 million yearly fractures that occur in the U.S., about 10% fail to repair and up to 25% of spinal fusion procedures fail [1–3]. Fixation devices are frequently used to restore the proper alignment and load bearing capacity of bone immediately, and osteoconductive materials such as synthetic or processed bone

fillers are used in an attempt to bridge large bone deficits and provide a solid substrate for osteoprogenitor cell migration, angiogenesis, and callus formation [4]. This is usually accompanied by an osteoinductive component such as recombinant bone morphogenic proteins (BMPs) and/or autologous cell preparations [5]. Live, autologous bone grafts are frequently used for this purpose, and they are highly effective, but the approach is associated with donor site morbidity and the volume of available graft material is limited [6].

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Autologous human bone marrow (hBM) concentrate administered with an appropriate scaffold represents a promising alternative to bone graft and can be prepared in relatively large quantities [7]. However, when compared with bone graft, hBM contains a minute proportion of osteogenic and angiogenic progenitors and osteoanabolic potential can be limited [8]. Success is also contingent on the healing capacity of the patients' donated tissue which can be significantly affected by age, disease, and the use of tobacco or alcohol [9–11]. A safe and rapid procedure for improvement of the osteoanabolic properties of hBM is, therefore, highly sought after in the field of orthopedics, especially if it can be performed within the temporal limitations of the surgical procedure, with minimal manipulation of the specimen, and at point-of-care. One way to achieve this goal is to stimulate canonical Wntless (cWnt) signaling in bone marrow-resident human mesenchymal stem cells (hMSCs), the presumptive precursors of osteoblasts in bone marrow [12–16]. In this pathway, extracellular cWnt ligands bind to the transmembrane receptor frizzled (Frz) and the co-receptor lipoprotein-related protein 5 and 6 (LRP-5/6) on the surface of the target cell. Activation of Frz recruits the cytoplasmic bridging molecule, disheveled (Dsh), so as to inhibit the action of glycogen-synthase-kinase-3-beta (GSK3 β). Inhibition of GSK3 β decreases phosphorylation of β -catenin, preventing its degradation by the ubiquitin-mediated pathway. The stabilized β -catenin acts on the nucleus by activating T-cell factor (TCF) mediated transcription of target genes [17–19] that elicit a variety of effects including induction of the early stages of osteogenic differentiation [15, 20–23]. In vitro studies have demonstrated that cWnt signal transduction from the membrane to the nucleus occurs very rapidly in the order of minutes and hours [24–27] suggesting that the initial stages of cWnt signaling could be achieved over a duration compatible with standard orthopedic procedures, thus raising the attractive notion of a point-of-care procedure for osteogenic enhancement of autologous bone marrow. Janeczek et al. took the first steps developing this approach by demonstrating that a rapid (24 hours) and transient exposure of human bone marrow to Wnt3A resulted in the generation of an osteogenically enhanced sub-population of STRO-1^{high}/GlycophorinA^{neg} skeletal stem cells [28]. Herein, we report that the effects of cWnt stimulation can be achieved by transient exposure of subcultured hMSCs to the GSK3 β -inhibitor (2',3',5'-tri-O-acetyl-6-bromoindirubin-3'-oxime (BIO) [29, 30] for 1–2 hours followed by withdrawal of BIO and washing. In addition, very-rapid-exposure-to-BIO (VRE-BIO) on either hMSCs or whole unfractionated hBM resulted in the long-term establishment of a persistent osteogenic phenotype associated with accelerated alkaline phosphatase (ALP) activity and enhanced transcription of the master regulator of osteogenesis, Runx2. When VRE-BIO treated hBM was tested for efficacy in a rat posterolateral spinal fusion model, VRE-BIO treatment caused the formation of a denser, more interconnected bone matrix that was more resistant to deformation as compared with vehicle-treated hBM. Collectively, these data indicate that the VRE-BIO procedure may represent a rapid, safe, and point-of-care strategy for the osteogenic enhancement of autologous hBM for use in clinical orthopedic procedures.

MATERIALS AND METHODS

Detailed methods are provided in Supporting Information.

Human MSCs

Bone marrow (2–5 ml) was collected with informed consent from donors undergoing spine fusion procedures at Baylor Scott and White Hospital (Temple, TX) in accordance with an institutionally approved protocol. Human MSCs were cultured by plastic adherence from the nucleated fraction of bone marrow as described [31].

Human BM

The mononuclear fraction of hBM was prepared by Ficoll density gradient centrifugation and frozen at -180°C in 5×10^7 -cell aliquots containing α -MEM, 50% (vol/vol) FCS and 5% dimethyl-sulfoxide, and 20 units per ml DNase I (Sigma).

VRE-BIO Procedure

Plastic adherent hMSCs were cultured to 50%–60% confluence (5,000–10,000 cells per cm^2) in complete culture media (CCM) and hBM (1×10^7 cells) was suspended in CCM at 1×10^6 cells ml^{-1} . One thousand-fold stocks of BIO (Sigma, St. Louis, MO) were prepared in dimethyl-sulfoxide (DMSO) and added to media at the desired working concentration.

Immunocytochemistry for β -Catenin

Human MSCs were seeded at 1,000 cells per cm^2 in 4 cm^2 chamber slides (Nunc Lab-Tek II, Thermo Scientific, Waltham, MA). When the hMSCs established a monolayer of about 60% confluence, the cultures were subjected to VRE-BIO procedures. Slides were then processed and visualized as previously described [14].

Immunoblotting

Triton-insoluble/soluble subcellular fractions were prepared using a previously described protocol [32] with modifications [14]. Samples were immunoblotted as previously described [33]. Chemiluminescence was detected using a Versadoc Imaging System and analyzed using QuantityOne software (BioRad, Hercules, CA).

β -Catenin Enzyme Linked Immunosorbent Assay

One million hMSCs at a density of approximately 8,000 cells per cm^2 were incubated in CCM containing BIO or vehicle for 1 hour. Cultures were washed in phosphate buffered saline (PBS) and recovered by trypsinization. After centrifugation and further washing in PBS, pellets were lysed in 200 μl PBS containing 1% (vol/vol) Nonidet P-40 (Sigma), 5 mM EDTA, 150 mM NaCl, and protease inhibitor cocktail (Roche, Basel, Switzerland) for 30 minutes at 4°C . Lysates were subjected to centrifugation at 2,000g followed by protein quantification using a standard Bradford assay (BioRad). Concentrations were between 0.8 and 1.6 mg/ml, 100 μl of the sample was used per well of an enzyme linked immunosorbent assay (ELISA) plate (soluble beta catenin assay, R&D Systems, Minneapolis, MN).

Quantitative RT-PCR

Total RNA was extracted from 1×10^6 hMSCs or 1×10^7 bone marrow cells using a total RNA isolation kit (High Pure, Roche). Yields were in the range of 500–800 μg per sample for hMSCs and 100–500 μg per sample for bone marrow. The 260/280 nm ratios ranged between 1.7 and 1.9. For quantitative RT-PCR, 1 μg of total RNA was used to synthesize cDNA (Superscript III cDNA kit, Invitrogen, Carlsbad, CA). One half- μg of cDNA was amplified in a 25 μl reaction containing SYBR-green PCR master mix (Fast SYBR Green, Applied Biosystems Invitrogen) on a C1000 thermocycler

fitted with a real-time module (CFX96, Biorad). Expression data were calculated using the $2^{-\Delta\Delta CT}$ method using human GAPDH as a reference [34, 35]. Experimental variation was quantified by comparing the mean fold change between controls with each separate control sample, thereby defining the range of variation for a fold-change measurement of 1. Amplimers were as follows; GAPDH for: ctctctgctcctctgttcgac, GAPDH rev: tgagcgtatggctcgct [36]. Runx2 for: gcaaggttcaacgatctgaga, Runx2 rev: ttcccagggtccatctactg [37]. Osx for: gtgggcagctagaagggagt, Osx rev: aattagggcagctgcagga [37]. All annealing temperatures were set to 60°C.

ALP Colorimetric Assay

Human MSCs were plated in 12-well plates at 100 cells per cm^2 and cultured with CCM until reaching about 8,000 cells per cm^2 . VRE-BIO was performed on the monolayers followed by washing in PBS and replacement with osteogenic base media (OBM) consisting of CCM containing 50 $\mu\text{g}/\text{ml}$ ascorbic acid and 5 mM β -glycerol phosphate. For assay of adherent cells from hBM, 1×10^7 cells were subjected to VRE-BIO followed by washing in PBS and plating in 4 cm^2 tissue culture wells in the presence of CCM. After 24 hours, the nonadherent cells were washed away and media was replaced with OBM. Media was changed every 2 days for 8 days following measurement of ALP activity as previously described [38]. The rates were normalized against cell number and statistically analyzed using one-way analysis of variance (ANOVA) and Dunnett's post-test for multiple comparisons with control.

Osteoprotegerin ELISA

Osteoprotegerin ELISA was performed according to the manufacturer's instructions (R&D Systems) on 1-day (hMSC monolayers) or 2-day (hBM-derived monolayers) conditioned media diluted at 1 in 10 with phosphate buffered saline containing 0.1% (vol/vol) Tween 20.

Quantification of Cells

Cells in monolayers were enumerated using the CyQuantGR fluorescent nucleic acid labeling system (Invitrogen) using a previously described extended processing protocol to counteract the effects of high extracellular matrix concentrations [39].

Posterolateral Lumbar Fusion Model in Rats

All procedures were performed in accordance with an approved animal use protocol from the Baylor Scott & White Animal Care and Use Committee. Ten 6-week-old (approximately 135 g) female athymic nude rats (Hsd:RH-Foxn^{tmu}) ($n = 5$ per group) were acquired from Harlan Laboratories (Indianapolis, IN). A bilateral intertransverse process fusion at L5-L6 was performed on them as previously described [40], but with some modifications [41].

Manual Palpation

After 8 weeks, rats were humanely euthanized under deep anesthesia. The spines (L3 to sacral vertebrae, with ilium) were carefully dissected with associated muscle tissue and placed in cold PBS. The explanted spines were manually tested for intersegmental motion by the three blinded observers in accordance with a standardized experimental protocol [42]. Due to the potential subjectivity of the manual fusion assessments, inter-observer variation was evaluated statistically by Kappa test [43–47]. In all cases, Kappa criteria (>0.9) were satisfied and fusion and palpation data were analyzed for significance using a Fisher exact test (summing small p -values method). Fusion was further confirmed for each

specimen. Palpation results were then compared with visual interpretation of micro-computed tomographic scans to further confirm fusion. Complete correlation between scans and palpation results were observed.

Micro-Computed Tomography

Spines were subjected to tomographic scanning using a Skyscan 1174 high resolution specimen imager (Bruker, Kontich, Belgium). Images were scanned at 26 W, 661 μA , 39 kV with 33 $\mu\text{m}/\text{pixel}$ resolution. Axial images were generated using NRecon software (Bruker, Billerica, MA), with thresholding set to 500–10,000 Hounsfield Units. In most cases, it was impossible to distinguish the new bone from the mature transverse processes because outgrowths were completely contiguous with original bone. We therefore used previously described methods that compared total volumes of fused vertebrae [41]. For histomorphometric analysis of fusion beds, specimens were scanned at 18 $\mu\text{m}/\text{pixel}$ resolution with image smoothing and ring artifact compensation set to maximum. To maximize resolution, 10 images were averaged per degree for 360°. Axial images were generated with thresholding set to 300–3,200 Hounsfield Units. A region of interest consisting of one hundred axial images taken from the left fusion bed, in line with the central 1.8 mm of the L5 vertebra, was selected and subjected to a comprehensive 3D histomorphometric analysis. Values from the BIO-treated and control groups were analyzed statistically by Student's t test and p -values $<.05$ were considered statistically significant. Three-dimensional images were rendered by CTvol software (Bruker).

Uniaxial Compression Testing

Excised spines were cleared of connective tissue by proteinase K digestion and fine dissection and allowed to dry in a desiccated environment for 15 hours at 21°C–25°C. Using a 22 mm diameter, 0.12 mm thick diamond coated rotary blade (Strauss Diamond, Palm Coast FL) fitted to a dental drill, a 4 mm-thick axial slice was prepared that included the fusion mass at the intervertebral space between the L5 and L6 vertebrae. Slices were immobilized with the cut surface of the fusion beds exposed. Following a previous experimental procedure [48], an ADMET eXpert 7600 single column testing system equipped with a 10 lb transducer and 1 mm indentation probe was used to apply a preload of 0.1N to immobilize and maintain indenter contact with the specimen. A constant indentation rate was then applied (1 mm minute^{-1}) and mean force/displacement measured so as to calculate the modulus (kPa) from the linear elastic region (0.05–0.2 strain).

Statistics

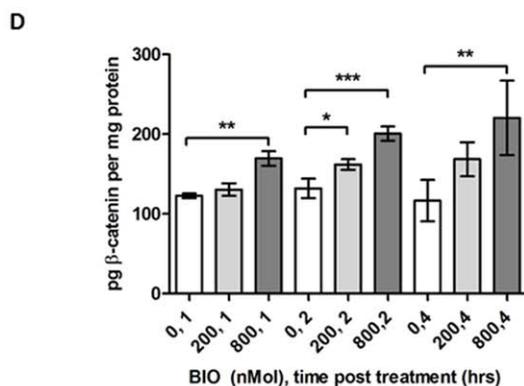
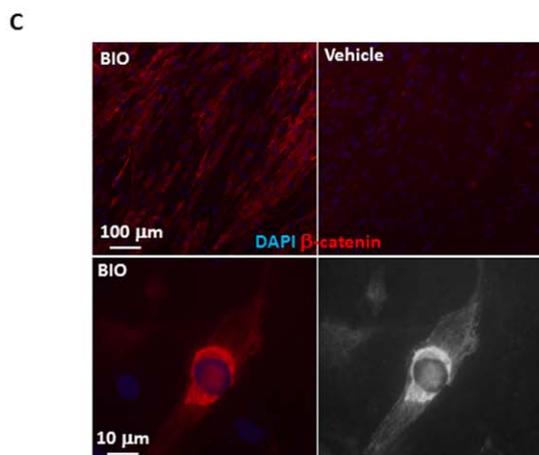
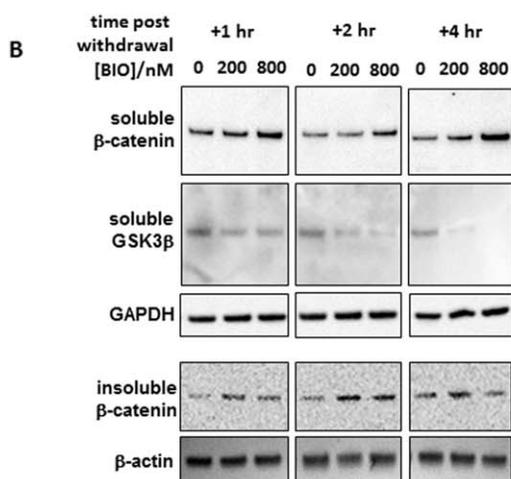
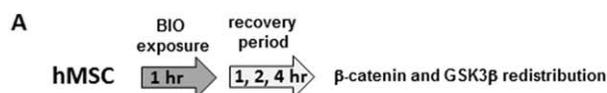
Parametric data were analyzed by one-way ANOVA with Tukey's or Dunnett's post-test where indicated. Binary (fusion) data were tested by 2-tailed Fisher Exact test. For ANOVA and Fisher tests, a p -value of $<.05$ was considered statistically significant. Testing of inter-observer agreement was performed with the Cohen's Kappa test.

RESULTS

Rapid and Transient Exposure of hMSCs to BIO Stimulates Endogenous Markers of cWnt Signaling

We aimed to develop a clinical point-of-care protocol for the stimulation of bone marrow osteoprogenitor activity based on rapid acceleration of cWnt signaling. During the assays, exposure conditions were limited to a maximum of 4 hours, a duration

compatible with the viability of hBM aspirates [49] and also orthopedic surgical procedures. To avoid the need for fresh human BM preparations, we performed initial experiments with monolayers of hMSCs. To determine whether BIO had the capacity to stimulate cWnt signaling after short exposure times, we screened BIO stimulation conditions with concentrations ranging between 100 and 800 nM BIO and durations between 30 minutes and 4 hours.



We selected this range of concentrations based on previous studies on the effects of BIO on hMSCs [16, 38, 50]. After withdrawal of BIO and replacement of standard media, we subjected the hMSCs to various recovery times (1–4 hours) and measured β -catenin and GSK3 β levels in detergent-soluble (cytosolic) and insoluble subcellular fractions. The detergent-insoluble fraction contained nuclei, cytoskeleton, and various other membranous structures [14]. We reasoned that upregulated cWnt signaling would result in nuclear accumulation of β -catenin and depletion of cytosolic GSK3 β over a relatively short time course [24, 26, 27]. From these assays, we ascertained that 1 hour of BIO exposure at a concentration between 200 and 800 nM consistently upregulated levels of β -catenin in soluble fractions while downregulating the presence of soluble GSK3 β (Fig. 1A, 1B). This effect was concentration dependent and sustained for the duration of the assay (4 hours). Levels of β -catenin in detergent insoluble fractions followed a similar pattern, but tended to diminish at higher BIO concentrations after 4 hours (Fig. 1B); the reason for these reduced levels are unclear, but potent feedback mechanisms are a likely explanation for this observation. Upregulated β -catenin levels in hMSCs after VRE-BIO treatment could be confirmed by immunocytochemistry (Fig. 1C top left and right) in the majority of cells treated. High power imaging also demonstrated a nuclear/perinuclear distribution of β -catenin in those cells that responded to the BIO but cells with very low β -catenin immunoreactivity were evident in BIO treated cultures, indicating that VRE-BIO treatment affects a subpopulation of cells within cultures of hMSCs. Upregulation of β -catenin levels in VRE-BIO treated hMSC cultures could also be demonstrated when cell lysates were subjected to ELISA assays but the relative increases were surprisingly modest when compared with the immunofluorescence data (Fig. 1D). This discrepancy is probably attributed to the tendency of immunocytochemistry to detect insoluble targets whereas ELISA is better suited to measuring soluble targets. Collectively, these data indicate that 1 hour of transient BIO exposure modulates the subcellular distribution and steady-state levels of GSK3 β and β -catenin in a manner indicative of accelerated cWnt signaling.

Rapid and Transient Exposure of hMSCs to BIO Stimulates Early Markers of Osteogenic Differentiation

We next questioned whether VRE-BIO exposure could stimulate osteogenic differentiation in hMSCs. For this purpose, hMSCs were transiently exposed to BIO for 1 hour and exposed to osteogenic differentiation media for up to 8 days (Fig. 2A). Following

Figure 1. VRE-BIO exposure on hMSCs causes downregulation of cytosolic GSK3 β and upregulation of β -catenin. **(A):** Experimental scheme. **(B):** Immunoblot assays for β -catenin and GSK3 β on soluble and insoluble fractions of BIO-treated hMSCs at various time points during the recovery period. **(C):** Immunocytochemistry for β -catenin on monolayers of hMSCs treated for 2 hours with 800 nM BIO followed a 2-hour recovery period (top left) as compared with a vehicle treated control (top right). High power image of BIO-treated hMSC with peri-nuclear accumulation of β -catenin (bottom left) with traces of immunoreactive material in the nucleus (bottom right). Red color indicates β -catenin immunoreactivity and blue indicated nuclear staining with DAPI. **(D):** Enzyme linked immunosorbent assay measurement of β -catenin levels in hMSC extracts treated with BIO. Statistics are ANOVA with Dunnett's post-test (*, $p < .05$; **, $p < .01$; ***, $p < .005$). Abbreviations: BIO, GSK3 β -inhibitor (2',3'E)-6-bromoindirubin-3'-oxime; DAPI, 4',6-diamidino-2-phenylindole; GSK3 β , glycogen-synthase-kinase-3-beta; hMSCs, human mesenchymal stem cells.

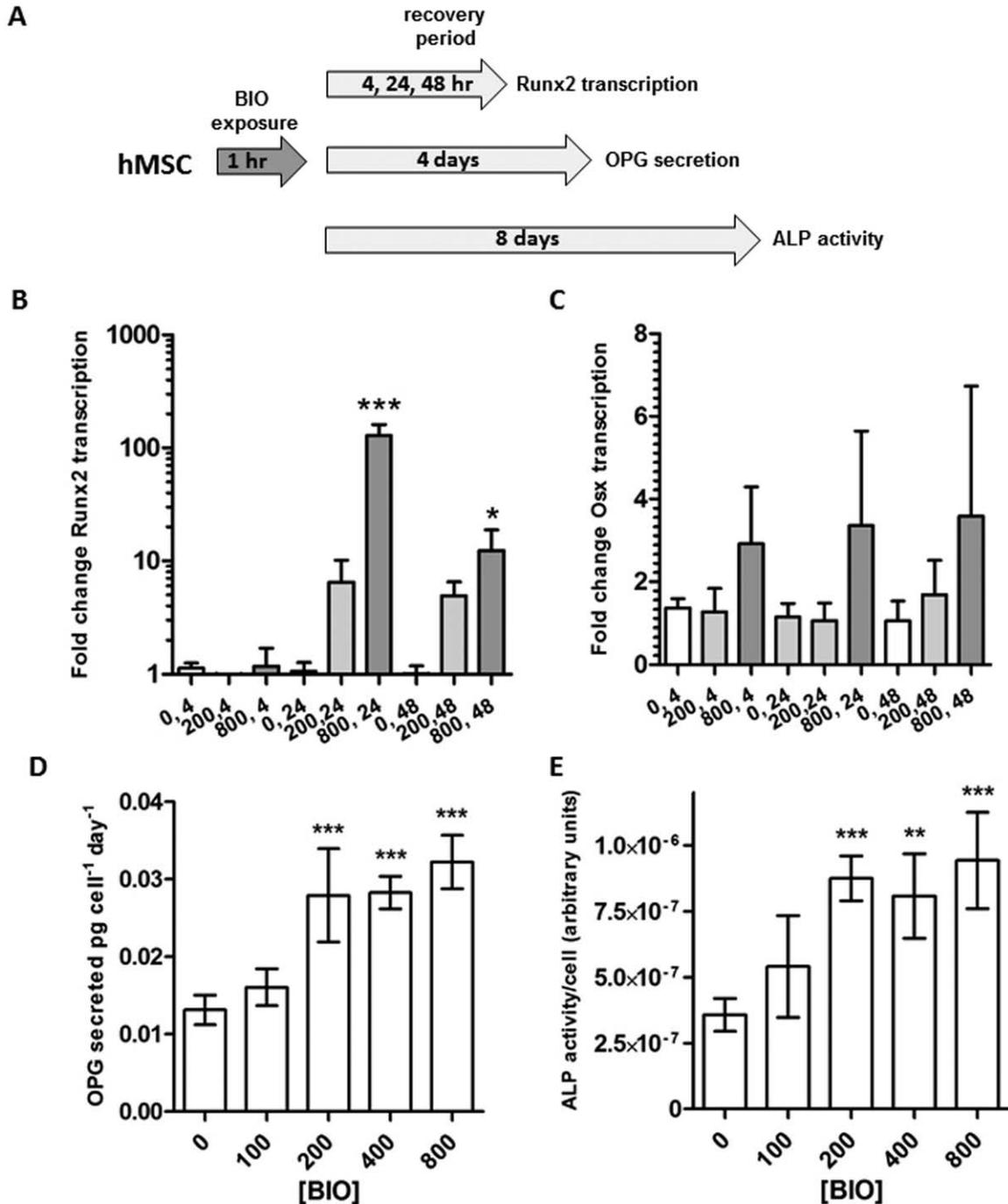


Figure 2. VRE-BIO on hMSCs upregulates ALP activity, OPG secretion, and Runx2 transcription. **(A):** Experimental scheme. **(B):** Runx2 transcription in monolayers of hMSCs subjected to VRE-BIO treatment (0, 200, or 800 nM BIO) followed by 4, 24, and 48 hours in the presence of osteogenic stimulus. Fold-change transcription is normalized to vehicle only treatment after a 4-hour recovery period. **(C):** Oxs transcription in monolayers of hMSCs subjected to VRE-BIO treatment (conditions as B). **(D):** OPG secretion by monolayers of hMSCs subjected to VRE-BIO treatment followed by 4 days in the presence of osteogenic stimulus. Activity is normalized to cell number. **(E):** ALP activity on monolayers of hMSCs subjected to VRE-BIO treatment followed by 8 days in the presence of osteogenic stimulus. Activity is normalized to cell number and media conditioning time. For all Panels, statistics are ANOVA with Dunnett's post-test (*, $p < .05$; **, $p < .01$; ***, $p < .005$) with comparison against 0 nM BIO. Abbreviations: ALP, alkaline phosphatase; BIO, GSK3 β -inhibitor (2',3',5'-bromoindirubin-3'-oxime); hMSCs, human mesenchymal stem cells; OPG, osteoprotegerin.

treatment, transcription of the master regulator of osteogenic differentiation, runt-related transcription factor 2 (Runx2) was significantly upregulated after 800 nM BIO exposure followed by 24 hours of recovery (Fig. 2B). In contrast, transcription of osterix,

another key regulator of osteogenic differentiation was not significantly affected by VRE-BIO. After a 4-day recovery period in osteogenic conditions, secretion of the osteogenic protein ligand osteoprotegerin (OPG) was measured by ELISA, and we observed

that pre-exposure to 200–800 nM BIO for 1 hour resulted in enhanced OPG output (Fig. 2D). A similar observation was made for the osteogenic marker ALP after 8 days when enzyme activity was measured on intact monolayers by colorimetric assay (Fig. 2E).

Together, these data demonstrate that VRE-BIO treatment has the potential to enhance osteogenic responses by hMSCs when also subjected to standard osteogenic culture conditions.

Rapid and Transient Exposure of Whole BM to BIO Stimulates Early Markers of Osteogenic Differentiation

We then tested the effects of VRE-BIO treatment on whole BM *in vitro*. Aliquots of 1×10^7 hBM cells were incubated for 1 hour in the presence of 200 nM or 800 nM BIO and subjected to a recovery period of up to 8 days in osteogenic media (Fig. 3A). After 24 and 48 hours of recovery under suspension conditions, qRT-PCR demonstrated that Runx2 transcription was upregulated as compared with untreated controls, but to a lesser degree than observed with monolayer hMSCs (Fig. 3B). In some experiments, 1×10^7 cells were plated in a 4 cm² tissue culture well after VRE-BIO treatment, and adherent cells were allowed to attach under standard culture conditions for 24 hours. Cultures were then washed to remove suspension cells, and allowed to expand and differentiate in the presence of osteogenic media for 8 days (Fig. 3A). Compared with untreated controls, VRE-BIO at a concentration of 800 nM resulted in enhanced OPG secretion and ALP activity by the plastic adherent, hMSC-containing, component of the bone marrow (Fig. 3C, 3D). VRE-BIO did not appear to affect cell yields, with monolayers reaching about 60,000 cells per well in each case. Assuming a fibroblastic colony forming unit frequency of about 3×10^{-5} [51], this suggests an average doubling time of approximately 25 hours. Trials ($n = 30$) on hBM confirmed that 1 hour of exposure to BIO had no detrimental effect on the viability of hMSC-containing colony forming units (CFU).

These results demonstrate that 800 nM VRE-BIO treatment for 1 hour on unfractionated hBM results in upregulated transcription of the osteogenic master-regulator Runx2, and sustained osteogenic enhancement of the plastic adherent, hMSC-containing subpopulation.

VRE-BIO-Treated hBM Generates More Compact De Novo Bone with Greater Trabecular Connectivity and Increased Stiffness When Tested in an Experimental Rodent Posterolateral Lumbar Fusion Model

To examine whether VRE-BIO treatment improved the osteogenic capacity of hBM *in vivo*, we used a spinal fusion model in immune compromised rats. For this purpose, hBM was subjected to VRE-BIO for 1 hour with 800 nM BIO because these conditions resulted in the most robust upregulation of osteogenic parameters tested. The hBM was then combined with a radiolucent scaffold we developed consisting of porcine gelatin foam with hMSC-derived extracellular matrix and positioned over decorticated the posterolateral processes of the L4-L6 vertebrae followed by an 8 week (period of healing [41, 52]) (Fig. 4A). In a previous study, we demonstrated that the ECM scaffold exhibits no inherent healing capacity without supplementation with an appropriate cell source [41]. When compared with untreated hBM, VRE-BIO treated hBM did not affect fusion frequency (5/5 fused in each case) or the volume of the fusion masses (Fig. 4B). When fusion masses were subjected to high resolution scanning, classical histomorphometric parameters such as trabecular spacing, thickness, and number were also unaffected (data not shown). However, we did observe

that *de novo* bone generated by VRE-BIO treated hBM had a significantly lower surface: volume ratio (Fig. 4C), fractal dimension (Fig. 4D), and trabecular pattern factor (Fig. 4E). Reduced values for these parameters tend to describe a denser, less diffuse bone structure with greater interconnectivity [53–56]. Qualitatively, this appeared to be the case upon inspection of 3D-reconstructed high-resolution scans of fusion masses (Fig. 4G). Histological examination of the fusion masses confirmed the μ CT data, demonstrating a greater level of *de novo* bone with greater connectivity in BIO-treated fusion masses as compared with controls (Fig. 4H). The histological staining also indicated that the *de novo* bone in BIO-treated masses consisted of a greater degree of blue-stained mature bone (Fig. 4H, below) rather than the predominance of immature osteoid present in control specimens (Fig. 4H, above). BIO-treated fusion masses also exhibited a greater degree of bone marrow cellularity as compared with controls. When the fusion masses were subjected to biomechanical indentation testing, the BIO-treated fusion masses withstood significantly higher compressive force than the controls, further supporting the observation that the BIO-treated fusion masses were mechanically stronger and probably denser than control samples (Fig. 4F).

DISCUSSION

GSK3 β antagonists are thought to modulate cWnt signaling by preventing the formation of a functional β -catenin destruction complex. The increased level of free intracellular β -catenin activates the osteogenic differentiation of some types of adult stem/progenitor cells [23]. In a previous study, we observed that long-term incubation of confluent hMSCs for up to 8 days in the presence of BIO resulted in upregulation endogenous markers of cWnt signaling, thereby enhancing markers of osteogenic activity *in vitro* in a manner very similar to the results reported herein [38]. Long-term BIO-treated hMSCs were viable after 8 days, at the time of harvest, but when implanted into calvarial lesions generated in nude mice, the cells did not exhibit osteogenic efficacy, nor did they engraft. These observations were deemed BIO-dependent because hMSC-treatment conditions that did not involve BIO exhibited osteogenic efficacy and long-term engraftment. It is likely that a combination of sustained cWnt signaling and the potential cross-reactivity of BIO with several cyclin dependent kinases [30] could have contributed to the failure of the hMSCs. Because the transduction of cWnt signals from the receptor to the nucleus is reported to be in the order of minutes [24], we postulated that a cellular response could be detected after exposure to a cWnt agonist for hours rather than days. This is attractive for cell-based therapies because newly recovered autologous cell preparations could be osteogenically enhanced at the point of care for durations compatible with surgical procedures. This dismisses the need for a local clinical good manufacturing practice (cGMP) culture facility and the need for a dedicated visit by the patient to provide hBM. If the activating agent is washed from the cell preparations prior to administration, it also minimizes exposure to potent bioactive agents. Initial studies performed by Janeczek et al. [28] demonstrated the feasibility of this approach by exposing hBM to Wnt3a ligand for 24 hours prior to assays of clonogenicity and osteogenesis. Wnt stimulation for 24 hours resulted in elevated presence of cWnt signaling markers in the STRO-1 positive component of hBM that harbors the hMSCs thought to contribute to osteogenesis. Wnt3a exposure also

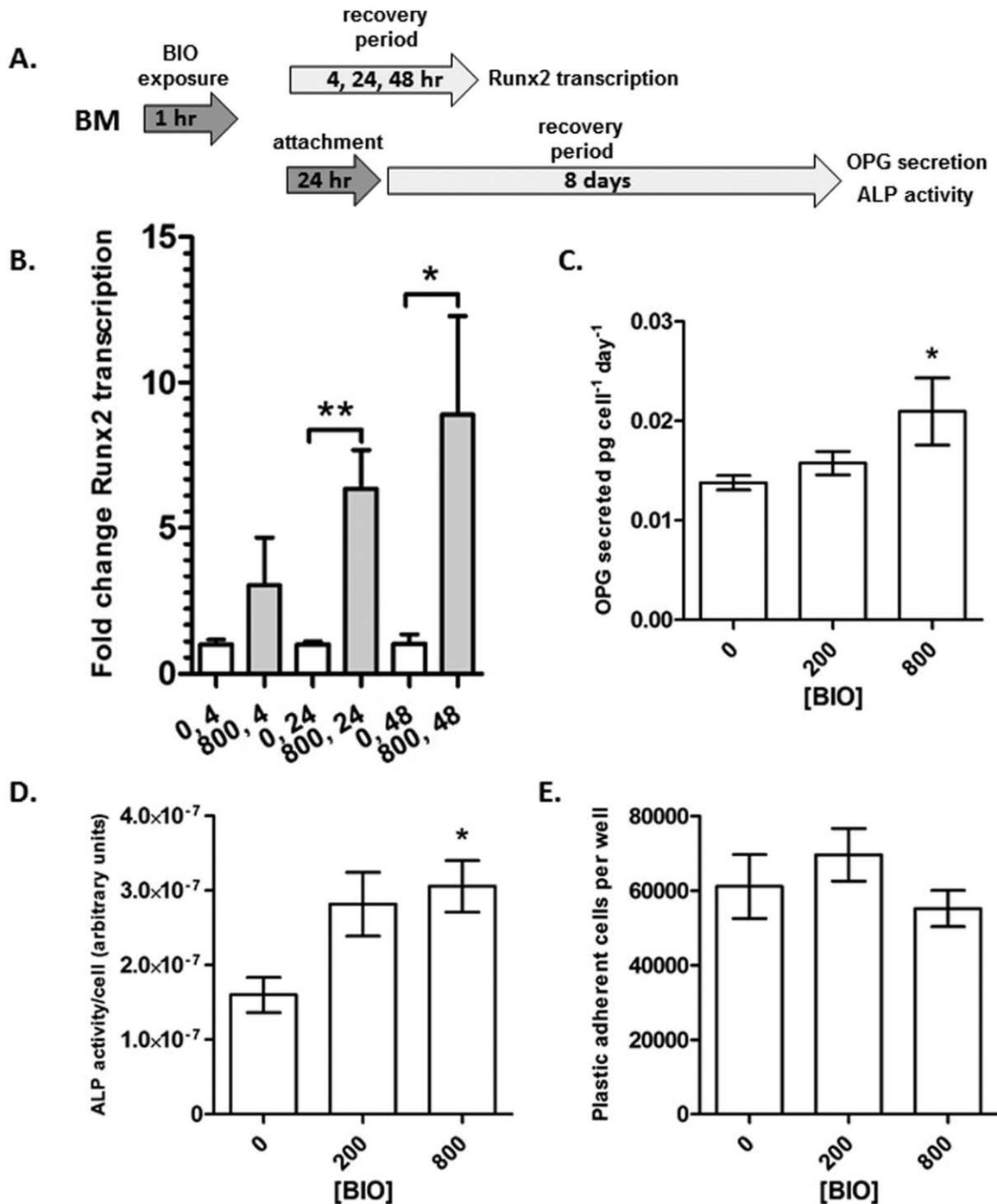


Figure 3. VRE-BIO exposure on human BM upregulates overall Runx2 transcription, ALP activity and OPG secretion in resident plastic adherent human mesenchymal stem cells (hMSCs). **(A):** Experimental scheme. **(B):** Runx2 transcription in suspended BM subjected to VRE-BIO treatment followed by 4, 24, and 48 hours in the presence of osteogenic stimulus. Fold-change transcription is normalized to vehicle only treatment after a 4-hour recovery period. **(C):** OPG secretion by monolayers of hMSCs subjected to VRE-BIO treatment followed by a 24-hours attachment period, then 4 days in the presence of osteogenic stimulus. Activity is normalized to cell number and media conditioning time. **(D):** ALP activity on monolayers of hMSCs subjected to VRE-BIO treatment followed by a 24-hours attachment period, then 8 days in the presence of osteogenic stimulus. Activity is normalized to cell number. **(E):** The number of plastic adherent cells present after 24-hours attachment period and 8 days of osteogenic stimulus. For (B), statistics are Student's *t* test on arcsine transformed data (*, $p < .05$; **, $p < .01$). For (C, D, E), statistics are ANOVA with Dunnett's post-test (*, $p < .05$) with comparison against 0 nM BIO. Abbreviations: ALP, alkaline phosphatase; BIO, GSK3 β -inhibitor (2',2',3',E)-6-bromoindirubin-3'-oxime; BM, bone marrow; OPG, osteoprotegerin.

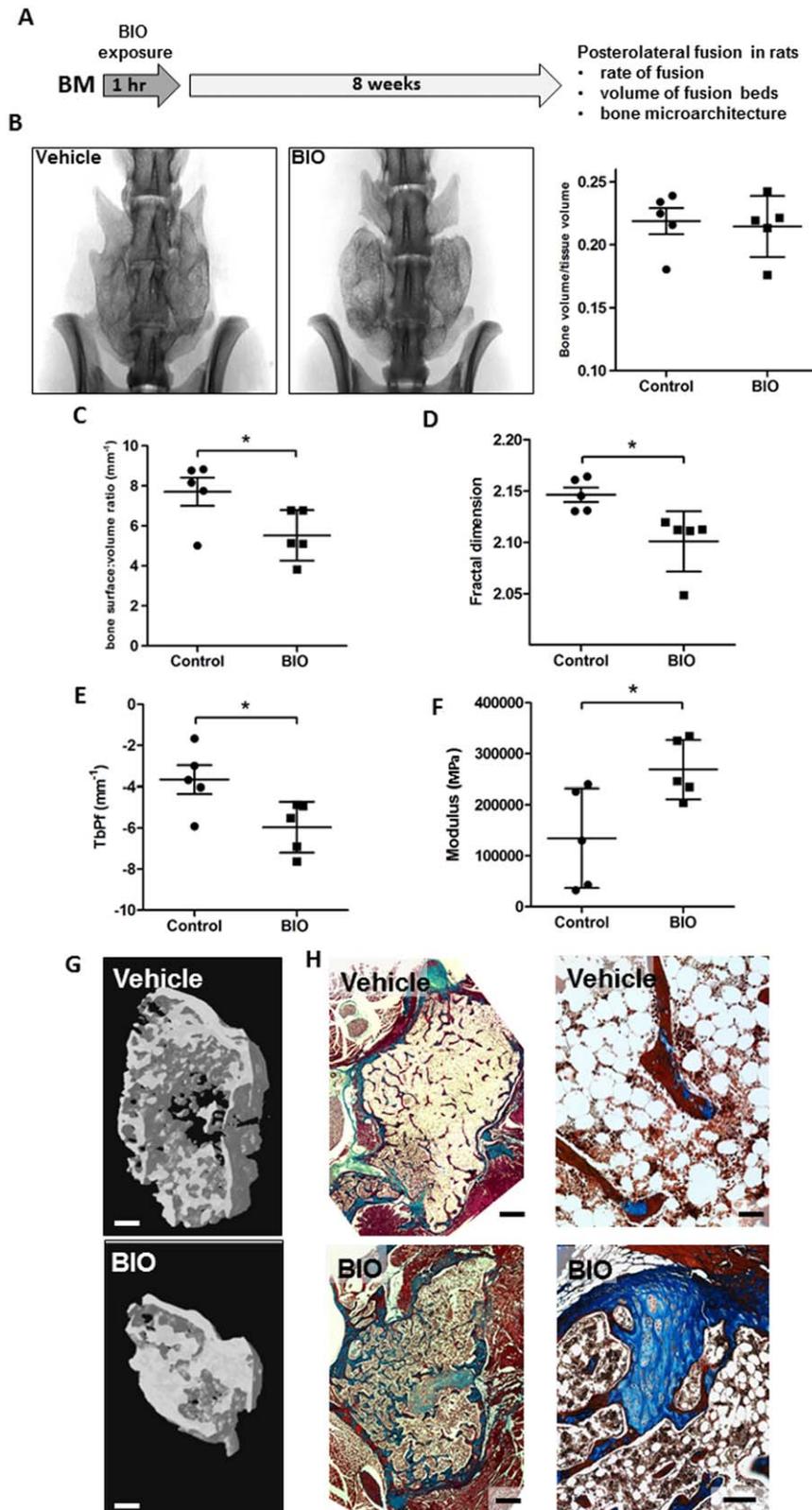
upregulated the frequency of STRO-1 cells, increased the formation of osteogenic single cell derived colonies, and enhanced in vitro osteogenic potential. The authors attributed their results to

the Wnt-mediated expansion of a STRO-1 positive/glycophorin A negative osteogenic progenitor sub-population in hBM. Interestingly, and in agreement with our previous observations with BIO

and hMSCs, long-term (14 days) exposure of hBM cultures to Wnt3a inhibited osteogenesis.

Herein, we used BIO to enhance cWnt signaling through inhibition of GSK3 β rather than by directly agonizing the Frz and LRP

receptors with Wnt ligands. The rationale for this approach was based on the superior availability of small molecule GSK3 β inhibitors and the relative ease of clinical translation as compared with recombinant Wnt ligands. In initial experiments, we exposed



semiconfluent, culture-expanded, plastic adherent hMSCs to various concentrations of BIO for short durations ranging from 1 to 4 hours. It is generally accepted that plastic adherent hMSCs contain STRO-1 positive osteogenic progenitors, and the use of hMSCs minimized the need for extensive volumes of donated hBM. Under these conditions, it was found that a single hour of exposure to 200–800 nM BIO caused a dose-dependent upregulation of cytosolic and insoluble β -catenin and reduced the detection of cytosolic GSK3 β that was detectable 4 hours after withdrawal of BIO. The observed accumulation and distribution of β -catenin and GSK3 β is typical of conditions where cWnt signaling is amplified in hMSCs [38] and in agreement with the rapid kinetics predicted by simulations [25] and experimental studies on cultured cells [24, 26, 27]. As predicted by previously published observations [57], the rapid cytosolic and nuclear accumulation of β -catenin was sufficient to upregulate transcription of the master osteogenic regulator Runx2 [58] that was maximally detected 24 hours after transient incubation with 800 nM BIO with a slight decline after 48 hours. Runx2 upregulation was also observed at the lower dose of 200 nM BIO, but was not deemed statistically significant based on the criteria set for the study. Likewise, the osteogenic transcription factor Osterix [59] appeared to be transcriptionally upregulated with 800 nM BIO, but inter-assay variation was high. We have previously reported that osteogenic hMSCs upregulate secretion of the osteogenic ligand OPG after about 4 days of osteogenic stimulus followed by maximal activity of ALP 2–6 days thereafter [39]. When pre-established cultures of hMSCs were transiently treated with 200–800 nM BIO for 1 hour, we observed that OPG and ALP was upregulated at day 4 and 8 respectively when compared with untreated controls. We also observed that hMSCs recovered from hBM by plastic adherence exhibited increased OPG secretion and ALP activity if the hBM was exposed to 800 nM BIO for 8 hours. Collectively, these data indicate that transient BIO exposure activates elements of the cWnt pathway that initiates the early stages of osteogenic differentiation. The induced osteogenic phenotype persists for days after cessation of the original BIO stimulus, a phenomenon that could be explained by several previously reported mechanisms. One possible cause for the longevity of transient BIO activation could arise from reported cooperativity between the BMP/SMAD and cWnt axes and the establishment of feed-forward signaling loop between the two pathways resulting in Runx2 synthesis [12, 60]. Once initiated, Runx2 signaling can persist for long periods or even become irreversible, causing accumulation of epigenetic modifications that are retained post-mitotically [47–49]. This signaling is further strengthened through the action of BIO on GSK3 β , which has the capacity to attenuate Runx2 by direct phosphorylation [46]. The current literature and the work herein therefore support a model where transient cWnt activation “locks in” the osteogenic

phenotype of hMSCs and their daughter cells through a mechanism involving a BMP/SMAD/cWnt feed-forward loop, increased stability of existing Runx2, and epigenetic stimulation of Runx2 expression. Of interest was the observation that while cWnt upregulation could be observed in the presence of standard complete media, upregulation of later factors such as OPG and ALP, required the presence of OBM. The specific reason for this requirement is unclear, but it appears that the persistence of the sustained osteogenic phenotype is dependent the presence of basal osteogenic factors such as a source of ascorbate and phosphate commonly found in healing bone tissue.

To test the potential efficacy of the VRE-BIO approach in vivo, hBM from a single human donor was incubated in media containing 800 nM BIO for 1 hour during preparation of a posterolateral fusion bed in nude rats. The 800-nM concentration was chosen because it was well tolerated by the cells and resulted in robust effects on the osteogenic parameters tested. The hBM was washed in PBS followed by mixing with a previously described scaffold generated from the gelatin foam coated with extracellular matrix derived from cultured hMSCs [41]. Controls received no BIO but were treated in exactly the same way. After 8 weeks of healing, we observed that fusion frequency was equally high in both groups when assayed by manual palpation, the standard approach of subjectively moving the vertebral joint to assess motion in all directions. When carefully performed by trained, blinded observers, and statistically analyzed for interobserver variation, this method is currently regarded as the most reliable method for assessment of fusion when combined with an appropriate radiographic technique such as computational tomography [42, 61]. Computational and biomechanical [62] approaches have been proposed to assess fusion [63], but while these protocols promise to reduce subjectivity in the evaluation of fusion, they require specialized equipment and expertise and their advantages over manual palpation have not been well-characterized to date. While the rate of fusion and also the volume of bone generated was comparable, the fusion beds in the VRE-BIO subjects exhibited key differences when compared with untreated controls. We found that while the control fusion beds exhibited a more hollow, trabecular conformation bounded by a thin outer layer of bone, the VRE-BIO bone was more compact. These differences could be detected qualitatively by 3D renderings of μ CT scans and histology, but also by measurable parameters of compactness, trabecularity, and interconnectivity such as surface:volume ratio, fractal dimension, and trabecular pattern factor. When the fusion beds were excised and subjected to indentation testing, the VRE-BIO specimens were harder to deform, exhibiting increased stiffness. According to the indentation analysis, the stiffness of bone in the fusion mass was increased by approximately twofold, from 134.5 MPa to 270 MPa. These values are relatively low as compared

Figure 4. VRE-BIO exposure on hBM improves histomorphometric parameters of de novo bone when cells are administered in a preclinical model of posterolateral lumbar spine fusion. **(A):** Experimental scheme. **(B):** Scans of representative specimens demonstrating the presence of fusion masses between L4, 5 and 6 in both control and BIO-treated groups (left) volumetric measurements of fusion beds demonstrate that bone formed in each case is equivalent between control and BIO-treated groups (right). **(C):** Bone surface to volume ratio in fusion masses is lower in BIO-treated group suggesting a more compact conformation. **(D):** Fractal dimension calculations on fusion masses demonstrate a denser conformation in BIO-treated specimens. **(E):** Trabecular pattern factor measurements demonstrate bone in BIO-treated fusion masses exhibit greater connectivity than controls. **(F):** Compression testing demonstrates that BIO-treated fusion masses are stiffer than control masses. **(G):** Three dimensional renderings of representative regions of interest indicate a denser, more interconnected bone conformation in BIO-treated fusion masses as compared with controls, but equivalent overall bone volume. Bar = 1 mm. **(H):** Micrographs of Masson's trichrome stained axial sections of fusion masses at low (left) and high (right) power. BIO-treated fusion masses appear to harbor a greater level of mature bone and increased cellularity. For low power, bar = 0.75 mm, for high power, bar = 100 μ m and 125 μ m for *vehicle* and *BIO*, respectively. Statistics for (B–F) calculated with Student's *t* test (*, *p* < .05). Abbreviations: BIO, GSK3 β -inhibitor (2',3',5'-6-bromindirubin-3'-oxime); BM, bone marrow; Tbpf, trabecular bone pattern factor.

with studies on intact vertebrae from healthy and osteoporotic rats (200–900 MPa) [64], but the *de novo* bone in the fusion beds had not undergone the successive rounds of remodeling that strengthens newly formed bone. Nevertheless, a twofold increase in biomechanical stiffness is likely to contribute to the durability of the fusion [65] and reduce the probability of hardware loosening. This would be especially advantageous in cases where bone healing could be compromised by age, disease and/or tobacco use [66, 67].

The enhanced *in vitro* osteogenic activity of hMSCs grown out of BIO-treated hBM samples suggests that BIO improves osteogenic capabilities of hBM by acting in part on hMSCs, but this does not necessarily exclude the possibility that BIO acts on other cell types within hBM. For example, while the frequency of osteoclasts was not measured in this study, the predicted increase of BMP, cWnt ligand, and OPG caused by BIO could theoretically result in their reduced numbers and an overall reduction in bone resorption [68]. Closely related to osteoclasts, macrophages can constitute between 2.5% and 25% of the nucleated component of hBM depending on donor, physiological status and macrophage phenotype [69–71]. Macrophages have the capacity to polarize into the inflammatory M1 subclass [71] or a more immunomodulatory M2 [70] subclass with regenerative and remodeling capacity [72]. Interestingly, cWnt signaling has been recently shown to drive macrophages to the M2 subtype [73], and bioactive factors secreted by M2 macrophages have osteogenic effects on osteoprogenitors [74, 75]. In a recent study, BIO has also been shown to stimulate the proliferation of mandibular chondrocytes *in vivo* [76].

While hMSCs do indeed represent a minute subpopulation of the total cellular component of hBM (< 0.1%), the resident hMSCs in the administered hBM could proliferate to physiologically significant numbers during the experiment, and extremely rapid proliferation of mesenchymal cells have been observed in the early stages of bone healing [77]. In this study, we observed extremely rapid outgrowth of hMSCs from BIO-treated hBM with an average doubling time of about 25 hours. With an average colony forming unit frequency of 1 per 3×10^5 bone marrow cells, and assuming 50% viability, this could result in 7×10^8 cells per fusion mass over 4 weeks based on the dose of hBM cells administered. Given the average volume of a fibroblast ($2,000 \mu\text{m}^3$), this could generate a condensed cell mass of $1,000 \text{mm}^3$ which could account for the fusion masses observed.

The data presented herein suggest that VRE-BIO methodology is likely to be useful for the improvement of recovered hBM used at point of care for orthopedic procedures, but it may also be of use for stimulation of osteogenic potential in expanded hMSCs too. *In vivo* experiments are certainly warranted to test this approach, but strategies that use cultured hMSCs are subject to several future challenges before implementation in the clinic. Significant milestones include optimization of protocols for careful characterization, addressing FDA concerns related to manipulated cell sources as well as the costs and logistical challenges associated with hMSC expansion in the cGMP setting. The direct

treatment of whole, uncultured hBM, therefore, represents a more rapid path to clinical translation.

CONCLUSION

The data presented here suggest that transient exposure of hBM to 800 nM GSK3 β inhibitor, BIO for 1 hour, has a stimulatory effect on the osteoprogenitor cells therein. When BIO-treated hBM is used in a rodent model of spine fusion, the resultant fusion mass is more compact and stiffer than controls that received untreated hBM. BIO is a member of the tyrian purple indirubin family of molecules which have a long record of safety in humans [78–80] and have a high oral LD₅₀ in rats, canines and mice [50, 81]. The exposure conditions for the VRE-BIO method are temporally compatible with the majority of surgical procedures and could be performed readily at the point of care with minimal additional training. We therefore propose that the VRE-BIO method could represent a feasible and cost-effective strategy for functional enhancement of autologous hBM for orthopedic applications.

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AUTHOR CONTRIBUTIONS

B.C.: Collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. S.Z.: Collection and/or assembly of data, data analysis and interpretation, administrative support, final approval of manuscript. U.K.: Conception and design, collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. C.C.: Conception and design, provision of study material or patients, financial support final approval of manuscript. L.C.: Collection and/or assembly of data, administrative support, final approval of manuscript. A.G.: Conception and design, collection and/or assembly of data, data analysis and interpretation, financial support, final approval of manuscript. C.G.: Conception and design, collection and/or assembly of data, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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