RESEARCH ARTICLE

Bisindoylmaleimide I enhances osteogenic differentiation

Fangfang Zhou[1, 2], Huizhe Huang[2] ✉, Long Zhang[1] ✉

[1] Department of Molecular Cell Biology and Centre for Biomedical Genetics, Leiden University Medical Center, Leiden, The Netherlands
[2] Faculty of Basic Medical Sciences, Chongqing Medical University Medical College, Chongqing 071007, China
✉ Correspondence: L.Zhang@lumc.nl (L. Zhang), devbiology@cqmu.edu.cn (H. Huang)
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ABSTRACT

The Wnt/β-catenin and bone morphogenetic proteins (BMPs) pathways play important roles in controlling osteogenesis. Using a cell-based kinase inhibitor screening assay, we identified the compound bisindoylmaleimide I (BIM) as a potent agonist of the cytosolic β-catenin accumulation in preosteoblast cells. Through suppressing glycogen synthase kinase 3β enzyme activities, BIM upregulated β-catenin responsive transcription and extended duration of BMP initiated signal. Functional analysis revealed that BIM promoted osteoblast differentiation and bone formation. The treatment of human mesenchymal stem cells with BIM promoted osteoblastogenesis. Our findings provide a new strategy to regulate mesenchymal stem cell differentiation by integration of the cellular signaling pathways.

KEYWORDS   bisindoylmaleimide I, Wnt/β-catenin, glycogen synthase kinase 3β, bone morphogenetic protein, human mesenchymal stem cells (hMSCs), osteogenesis

INTRODUCTION

Bone differentiation is controlled by signaling networking including the bone morphogenetic proteins (BMPs)/transforming growth factor (TGF-β) and Wnt pathways. Bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, activate its receptors and initiate phosphorylation of the downstream effector proteins, known as receptor-regulated Smads, leading to signal transduction. BMPs play pivotal roles and are involved in nearly all processes associated with skeletal morphogenesis (ten Dijke, 2006).

Wnt/β-catenin signaling is another key signaling pathway required for bone formation and bone homeostasis. In the presence of Wnt ligand, frizzled (Fz) and low-density lipoprotein receptor-related protein-5 or 6 (LRP-5/6) co-receptors recruit Disheveled (Dvl) to the plasma membrane along with Axin-GSK3β, inhibiting the formation of β-catenin destruction complex, allowing β-catenin levels to increase and the activation of Wnt/β-catenin pathway (Behrens et al., 1996; Brunner et al., 1997; Zhang and Ma., 2010). Wnt/β-catenin pathway directly targets Runx2 expression and promotes mineralization during osteoblastogenesis (Gaur et al., 2005). The canonical Wnt blocks apoptosis and osteoclastogenesis by increasing the OPG/RANKL ratio (Glass et al., 2005; Holmen et al., 2005; Spencer et al., 2006). Mutations in LRP-5 profoundly affect skeletal development and result in low bone mass (Gong et al., 2001; Little et al., 2002). The Dickkopf-1 (Dkk-1) resistant LRP5V171 mutation leads to high bone density (Boyden et al., 2002). Conditional deletion of the β-catenin gene in osteoblasts leads to reduced bone mass, and osteocyte-specific β-catenin-deficient mice develop low bone mass phenotype (Glass et al., 2005; Kramer et al., 2010). Increasing evidences also indicate that autocrine Wnt signaling is required for BMP in inducing bone formation (Rawadi et al., 2003; Fujita and Janz, 2007; Qiang et al., 2008; Tang et al., 2009; Honda, et al., 2010).

As a critical kinase involved in controlling both Wnt/β-catenin and BMP signaling, glycogen synthase kinase 3β (GSK3β) serves as an effective therapeutic target for bone disease treatment. In this study, we show that through suppressing GSK3β kinase activity, bisindoylmaleimide I (BIM) promotes Wnt/β-catenin signaling and prolongs duration of BMP induced Smad pathway in preosteoblast cells. Functional read out measured it as a bone-specific alkaline phosphatase

(ALP) activator and mineralization assay uncovered it as a potent osteogenic inducer, which is further proved to regulate osteoblast differentiation from mesenchymal stem cells.

RESULTS

BIM stimulates cytosolic β-catenin accumulation in preosteoblast cells

Forming of osteoblasts contributes to bone resorption and treatment of bone diseases. We started this project with a screen to identify kinase inhibitors that could elevate cytosolic β-catenin level in preosteoblast cells. KS483 cells were treated with suboptimal doses of kinase inhibitors and then assayed for cytosolic β-catenin expression. Among all the 40 compounds tested, BIM appeared to be the most potent in triggering cytosolic β-catenin accumulation (Fig. 1). We then focused our attention on its applications in osteogenic differentiation.

BIM enhances osteogenic differentiation

It is well established that cytosolic β-catenin accumulation is a key factor that determines Wnt induced osteoblast differentiation and bone formation. We then investigated C2C12 myoblast and KS483 preosteoblast cells that were treated with BIM. As shown in Fig. 2, BIM treatment dose-dependently increased ALP activity in C2C12 cells (Fig. 2A). Quantitative real-time RT-PCR analysis indicated that osteogenic marker genes, such as *OPG* and *Runx2*, were elevated upon
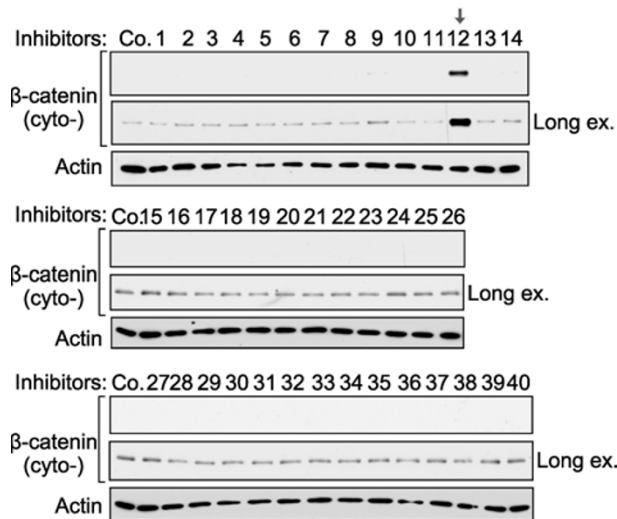

Figure 1. Identification of bisindoylmaleimide I (BIM) as a potent compound triggering cytosolic β-catenin accumulation in preosteoblast cells. KS483 cells were treated with 40 kinds of kinase inhibitors (listed in Table 1) for 1 h, cells were harvested, the membrane and cytosolic fractions were isolated, and cytosolic β-catenin and Actin were detected by western blotting. The arrow indicates BIM treatment (No.12).

Table 1 40 kinds of kinase inhibitors

|	Inhibitors	Final concentration (μmol/L)	Potential target (pathway)
Control	DMSO		-
1	SB-203580:p38	10	p38 MAPK
2	H-7:PKA, PKG, MLCK and PKC	10	PKA, PKG, MLCK and PKC
3	H-9:PKA, PKG, MLCK and PKC	10	PKA, PKG, MLCK and PKC
4	AG-494	10	EGFRK, PDGFRK
5	AG-825	10	EGFRK
6	Lavendustin A	10	EGFRK
7	RG-14620	10	EGFRK
8	Tyrphostin 23	10	EGFRK
9	AG-879	10	NGFRK
10	AG-370	10	PDGFRK
11	Tyrphostin 9	10	PDGFRK
12	Bisindoylmaleimide I	10	PKC
13	H-8	10	PKA, PKG
14	HA-1004	10	PKA, PKG
15	HDBA	10	EGFRK, CaMK II
16	KN-62	10	CamK II
17	ML-7	10	MLCK
18	N9-isopropyl-olomourcine	10	CDK
19	LFM-A13	10	BTK
20	SB-202190	10	p38 MAPK
21	PP2	10	Src-family
22	ZM 336372	10	cRAF
23	SU 4312	10	Flk-1
24	AG-1296	10	PDGFRK
25	GW 5074	10	cRAF
26	ZM 449829	10	JAK-3
27	DRB	10	CK II
28	SP600125	10	JNK
29	Y-27632	10	ROCK
30	BML-257	10	Akt
31	SC-514	10	IKK-2
32	BML-259	10	Cdk5/p25
33	HNMPA	10	IRK
34	Damnacanthal	10	p56 lck
35	Piceatannol	10	Syk
36	PP1	10	Src-family
37	AG-490	10	JAK-2
38	AG-126	10	IRAK
39	AG-370	10	PDGFRK
40	AG-879	10	NGFRK
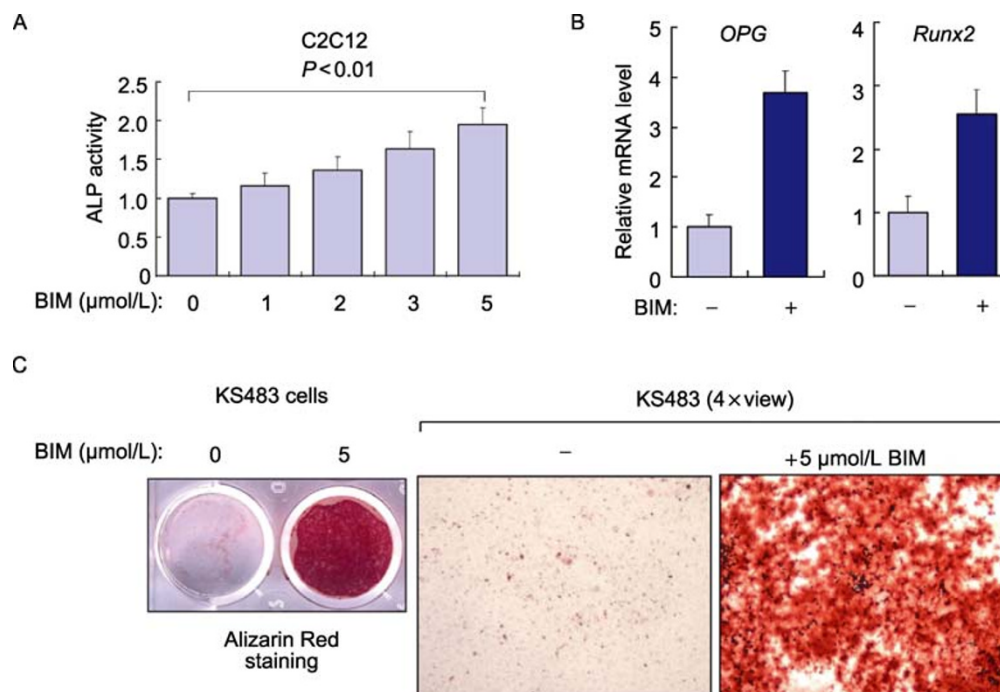
Figure 2. Bisindoylmaleimide I (BIM) enhances osteogenic differentiation. (A) C2C12 cells were treated with increasing doses of BIM for 4 days. Cells were then harvested for histochemical examination of alkaline phosphatase (ALP) activity. The histochemically stained cell material was dissolved in 50 mmol/L NaOH in ethanol, and absorbance was measured at 550 nm. Data show the mean and SD of triplicates. (B) Q-PCR analysis of BIM (5 μmol/L) treated C2C12 cells. The relative mRNA levels shown were normalized to the levels of GAPDH as internal control. For each gene the mRNA level in this control is set at "1." Values and error bars represent the mean ±SD of triplicates. (C) KS483 cells were treated with or without BIM (as indicated) in medium containing Vitamin C (50 μg/mL) and β-glycerolphosphate (5 μmol/L) for 4 days; 4 days later, the medium was replaced by normal medium containing 10% FBS, Vitamin C (50 μg/mL) and β-glycerolphosphate (5 μmol/L) for another 4 days. Cells were then fixed and stained with Alizarin Red for analysis of mineralization.

BIM treatment (Fig. 2B). And in KS483 cells, BIM significantly induced mineralization (Fig. 2C). Taken these data together, BIM may act as a potent osteogenic inducer through Wnt/β-catenin signaling pathway.

BIM activates Wnt/β-catenin signaling by suppressing GSK3β in preosteoblast cells

We next examined the underlying mechanism of BIM on canonical Wnt signaling. Reporter assay showed that the activity of Wnt-responsive TopFlash-Luc (Korinek et al., 1997) and LEF-Luc (Hsu et al., 1998) were dose-dependently increased by BIM in C2C12 and KS483 cells whereas BMP-responsive BRE-Luc (Korchynskyi and ten Dijke, 2002) was not affected (Fig. 3A–3D). The activation of Topflash-luciferase by BIM was Wnt-specific, since the control reporter Fop-flash-luciferase was not stimulated (data not shown). Furthermore, Wnt3a-induced TopFlash luciferase activity could be inhibited by DKK1, active GSK3β and dominant negative LEF-1 (dnLEF-1), whereas BIM-induced reporter activity could only be inhibited by active GSK3β and dnLEF-1, but not by

DKK1. This indicates that BIM may not play a role by stimulating Wnt secretion but act on the downstream signaling cascades (Fig. 3E and 3F). To prove this hypothesis, we treated KS483 cells with BIM and performed immunoblot analysis. Consistent with previous findings, β-catenin was accumulated in BIM treated cells. Notably, western blot analysis with phospho-specific anti-β-catenin antibodies showed that Wnt3a-CM and SB216763 inhibited the phosphorylation of β-catenin at the Ser33/37/Thr41 residues (Fig. 3G). Treatment with BIM also inhibited Ser33/37/Thr41 phosphorylation without affecting the phosphorylation of β-catenin at Ser552 and Ser675 (Fig. 3G), two previously identified phosphorylation sites by Akt and PKA respectively (Hino et al., 2005; Taurin et al., 2006; Fang et al., 2007; He et al., 2007). Additionally, phosphorylation of GSK3β at Ser9 was observed to be downregulated by BIM. These data indicate that BIM inhibits GSK3β kinase activity, and thus allows canonical Wnt activation.

BIM induces osteogenesis via Wnt/β-catenin pathway

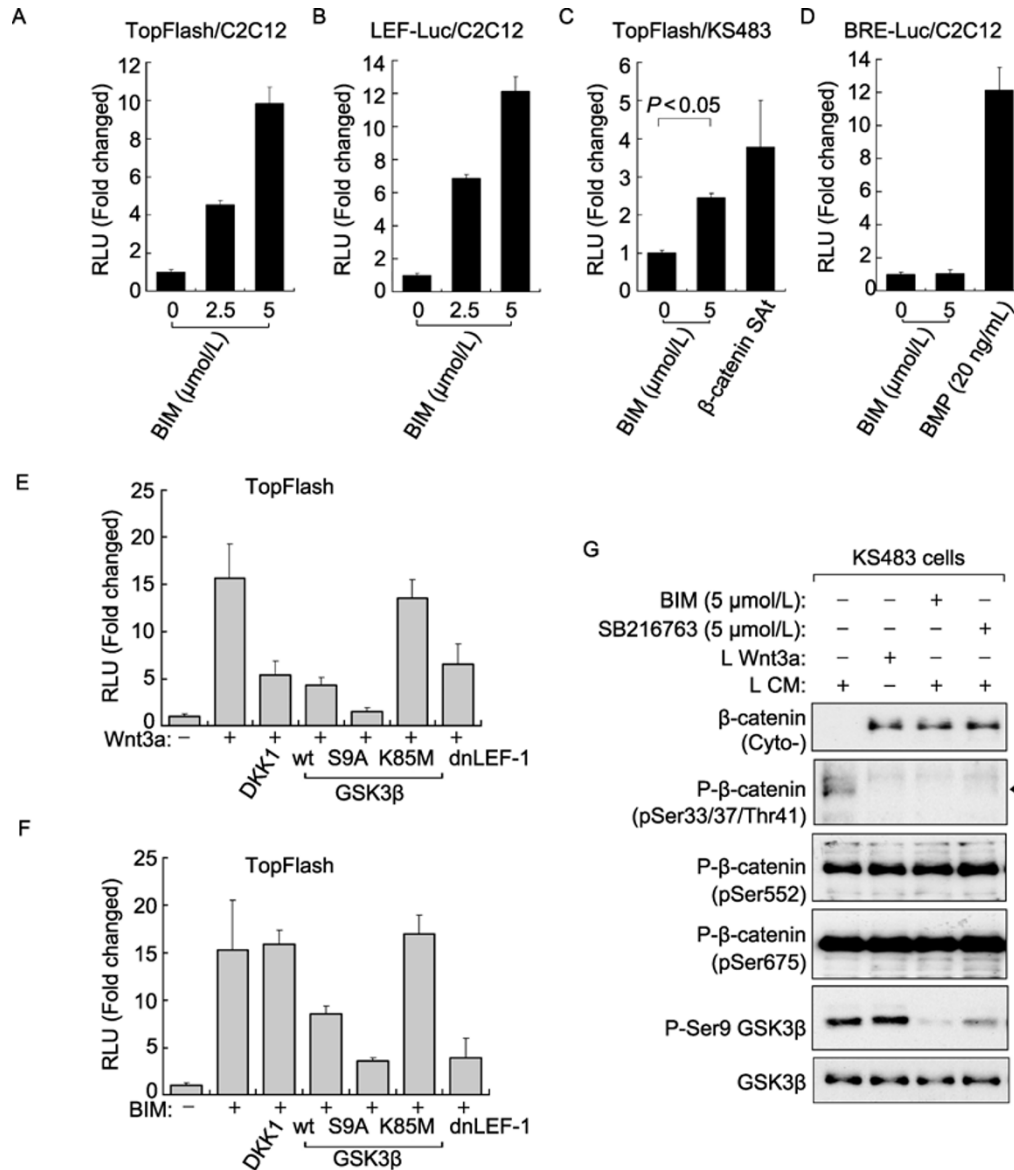We showed that BIM activates Wnt/β-catenin signaling and

Figure 3. Bisindoylmaleimide I (BIM) activates Wnt signaling. (A and B) C2C12 cells were transfected with TopFlash-Luc (A) or LEF-Luc (B) and treated with different doses of BIM as indicated for 8 h. Cells were harvested and luciferase activity was measured. (C) BIM induced TopFlash-Luc activity in KS483 cells. Constitutively active β-catenin SA mutant served as control. (D) BIM had no effect on basal level BRE-Luc reporter activity. BMP6 treatment for 8 h as control; For A–D, data are the mean ± SD. RLU, relative luciferase units. (E and F) HEK293T cells were transfected with TopFlash-Luc along with DKK1, GSK3β wt, GSK3β S9A, GSK3β K85M and dnLEF-1 as indicated. Cells were then treated with Wnt3a (E) or BIM (F) for overnight and harvested for luciferase activity analysis. (G) Immunoblot analysis of KS483 cells treated with BIM, SB216763 or L control/L Wnt3a conditioned medium. L CM, L cells control conditioned medium; L Wnt3a, L cells secreted Wnt3a conditioned medium.

promotes osteogenic differentiation. To further test whether β-catenin mediated signaling is required for BIM-induced osteogenesis, siRNAs that specifically silence β-catenin were used to serve this purpose. Firstly, BIM as well as Wnt3a stimulated Wnt reporter activity was abolished by si-β-catenin (Fig. 4A). Secondly in KS483 cells, measurement of ALP activity and Alizarin red staining analysis showed that efficient depletion of endogenous β-catenin in KS483 cells partially blocked BIM-induced ALP activity and mineralization (Fig. 4B and 4C). These data indicate that BIM induces bone forma-

tion by activating Wnt/β-catenin pathway.

BIM enhances duration of BMP-induced signaling

Although basal BMP reporter was not stimulated by BIM (Fig. 3D), we observed elevated BMP responses when BIM was co-treated with BMP ligand in cells (Fig. 5A). This suggests that BIM facilitates BMP ligand-induced signaling. As Smad1 phosphorylation in linker region by GSK3β induces its instability (Fuentealba et al., 2007), we investigated whether BIM
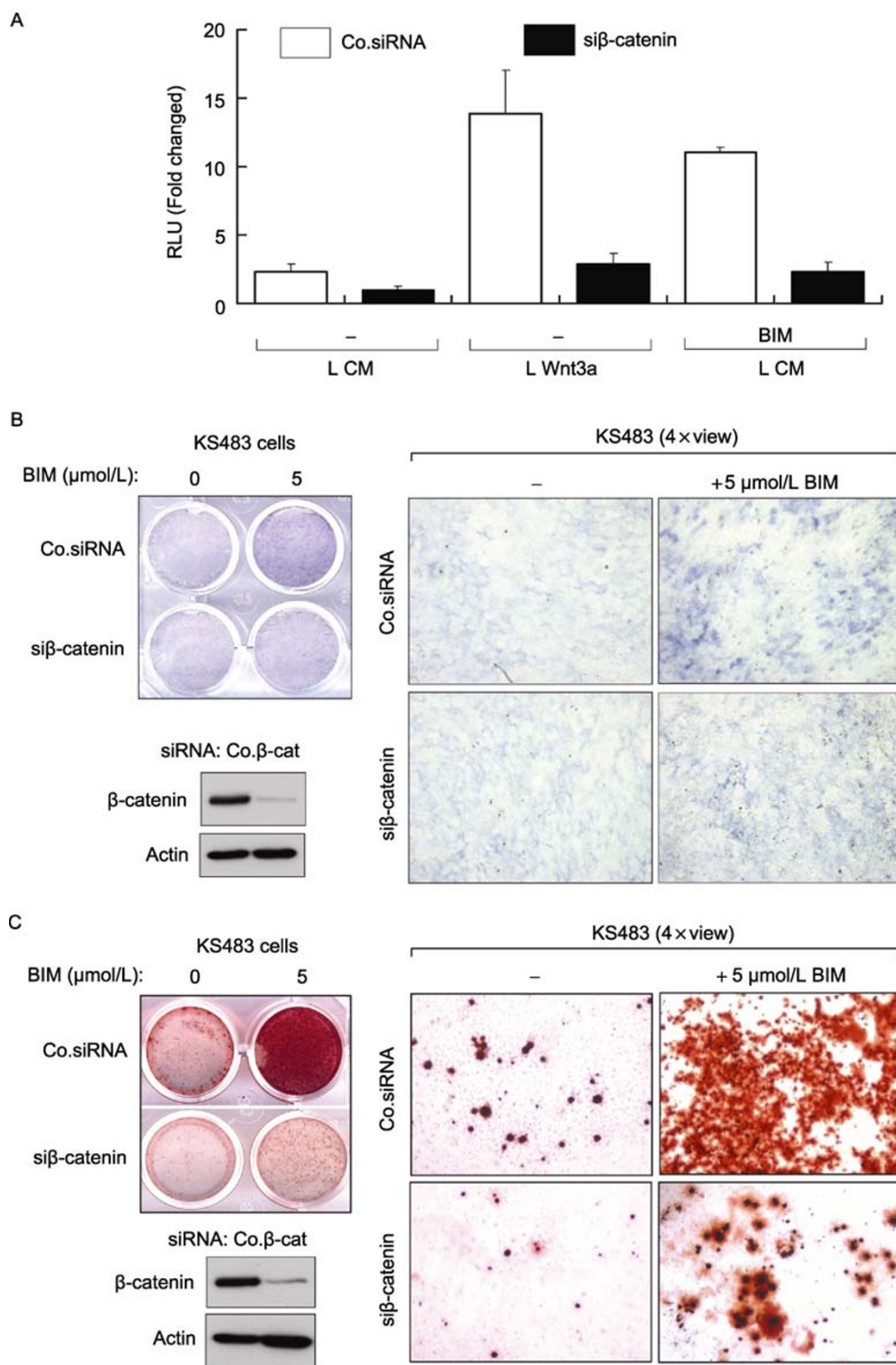
Figure 4. Bisindoylmaleimide I (BIM) promotes osteogenesis via β-catenin mediated signaling. (A) β-catenin depletion by siRNA abolished BIM-induced TopFlash-Luc activity. RLU, relative luciferase unit; L CM, L cells control conditioned medium; L Wnt3a, L cells secreted Wnt3a conditioned medium. (B) Left upper panel: β-catenin depletion inhibits BIM-induced ALP activity in KS483 cells. Left lower panel: Immunoblotting analysis showing β-catenin knockdown efficiency. Right panel: 4× view of left upper panel. (C) Left upper panel: β-catenin depletion inhibits BIM-induced mineralization in KS483 cells. Left lower panel: Immunoblotting analysis showing β-catenin knockdown efficiency. Right panel: 4× view of left upper panel. For A–C, Co.siRNA, control siRNA; siβ-catenin, β-catenin siRNA.
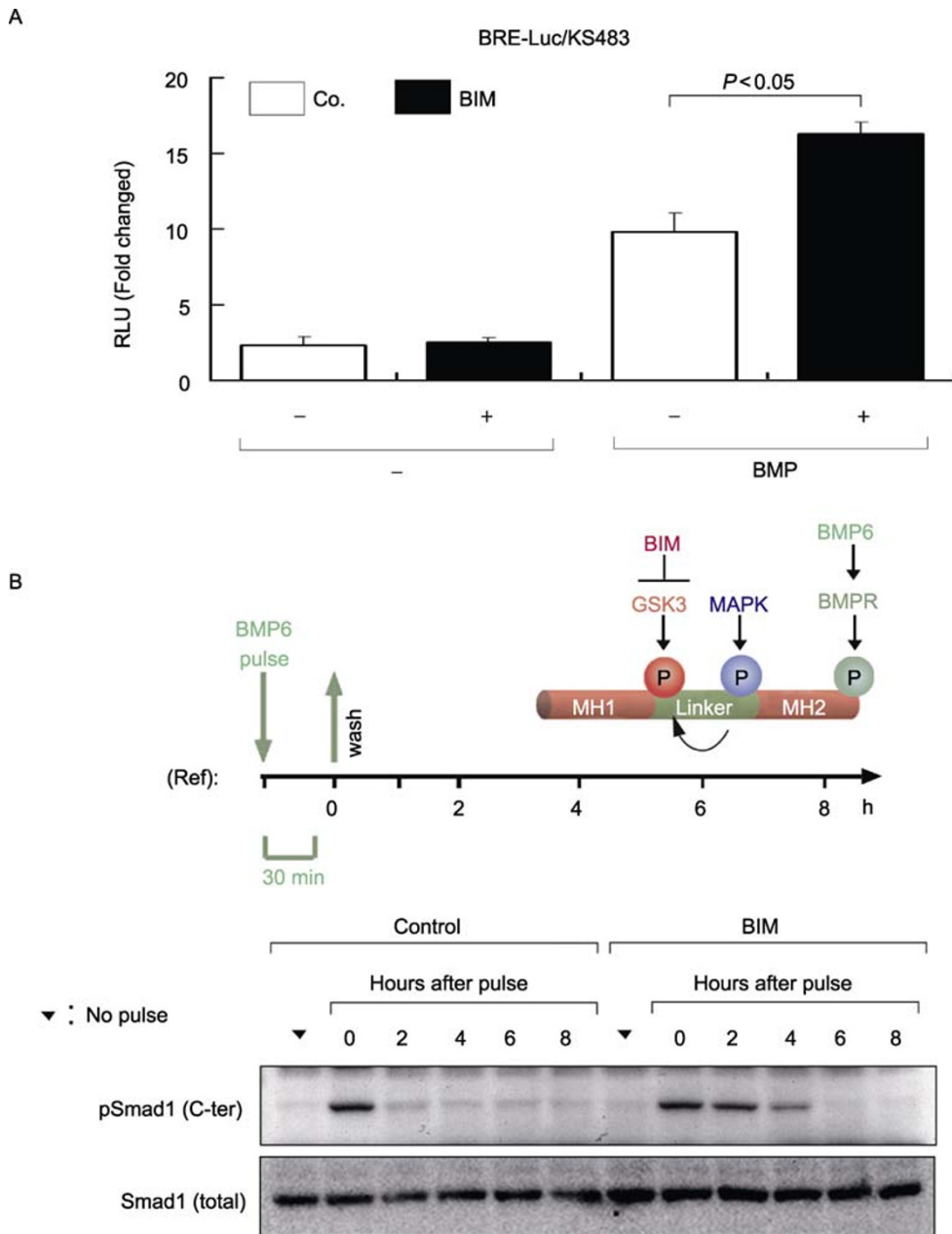
Figure 5. Bisindoylmaleimide I (BIM) elevates BMP-induced signaling. (A) BIM treatment elevates BMP-induced BRE-Luc activity in KS483 cells. RLU, relative luciferase unit; Co. control DMSO treatment. (B) Upper panel: Diagram of the model that BIM effects and the way that BMP ligand was added to the cells and then washed away. Ref: refer to (Fuentealba et al., 2007). Lower panel: Immunoblotting analysis of pSmad1 and total Smad1 level in KS483 cells after BIM and BMP treatment as indicated in upper panel.

affects BMP/Smad1 signaling as well. Immunoblot analysis showed that after BMP6 stimulation (30 min treatment and then washed away as shown in Fig. 5B top panel), the duration of BMP receptor induced C-terminal phosphorylated Smad1 was stabilized by BIM, which is consistent with the fact that BIM could suppress GSK3β kinase activity.

BIM enhances BMP-induced mesenchymal stem cell differentiation into osteoblast

Requirement for bone therapy is to increase bone formation by stimulating stem cells to commit to the osteoblast lineage, expanding the osteoprogenitor cell pool and promoting its

differentiation with a final goal of forming a mineralized bone tissue (Lian et al., 2006).

Multipotent mesenchymal stem cells are able to differentiate into osteoblasts, adipocytes, chondrocytes, and myoblasts. The differentiation of mesenchymal stem cells is regulated by interaction with specific extracellular mediators. Both Wnt/β-catenin and BMP pathways play pivotal roles in triggering the differentiation of mesenchymal stem cells to osteoblast (Takada et al., 2009; Vukicevic and Grgurevic, 2009). Therefore, we extended to examine osteoblast differentiation from human mesenchymal stem cells (hMSCs). As shown in Fig. 6A, transient BIM treatment cooperatively accelerated bone formation together with following BMP stimulation as visualized by mineralization detection (Fig. 6A). Collectively, through suppressing GSK3β kinase activity, BIM activates Wnt/β signaling and enhances BMP/Smad responses, therefore promoting osteogenesis (Fig. 6B).

DISCUSSION

Bone resorption is balanced by activated osteoclasts and bone forming osteoblasts. While targeted inhibition of osteoclasts with bisphosphonates has achieved success in preventing bone loss, searching factors to stimulate bone formation has been challenging (Rosen, 2003; Zhao and Su, 2011).

Osteogenic differentiation of mesenchymal stem cells toward osteoprogenitor and osteoblastic cells is tightly regulated by several growth and transcription factors at the molecular level, in which Wnt/β-catenin and BMP signaling is essentially involved. Therefore, searching factors/compo-

unds/drugs that can induce Wnt/β-catenin and BMP signaling would be promising to identify potential candidates of promoting bone formation. GSK3β is a central component of β-catenin destruction complex. Blocking GSK3β kinase activity prevents the phosphorylation and ubiquitination-mediated degradation of β-catenin thus leading to the accumulation of cytosolic β-catenin and subsequent activation of Wnt signaling. The intensity of BMP signals was determined by BMP receptors via Smad1 C-terminal phosphorylations. GSK3β sequentially phosphorylates Smad1 linker region, causing polyubiquitinylation of C-terminal phosphorylated-Smad1. Therefore, antagonizing GSK3β kinase activity prevents not only β-catenin degradation but also the degradation of activated Smad1, leading to the prolonged duration of BMP signals. Here in the present study, we screened up to 40 kinds of kinase inhibitors and identified BIM as an efficient inducer of Wnt/β-catenin activation by suppressing GSK3β kinase activity. Thus this study supported previous reports (Hers et al., 1999; Cho et al., 2008) and confirmed BIM as an agonist for Wnt/β-catenin cascades in osteogenic cells. We further proved its role of extending BMP signaling and explored its application as an osteogenic inducer through multiple assays. ALP activity is an early marker for bone formation; we further confirmed read out of signaling and ALP assay by analyzing calcium deposition and the formation of mineralized matrix, occurring late in differentiation.

Another aspect that deserves discussion is the role of BIM as an inhibitor for protein kinase C (Pajak et al., 2008), a multifunctional protein kinase family whose members exhibit distinct properties (Dempsey et al., 2000). It was therefore of considerable interest to identify whether the observed effects
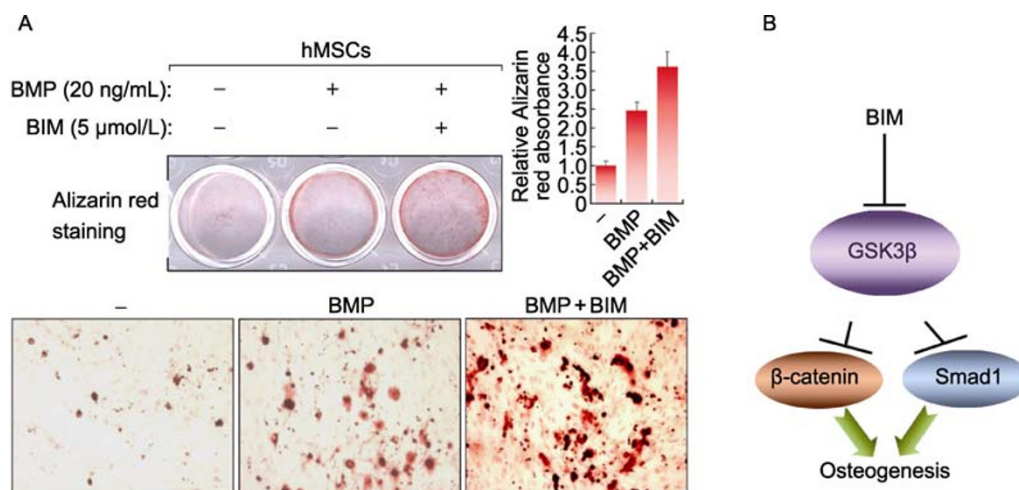

Figure 6. Bisindoylmaleimide I (BIM) enhances BMP-induced mesenchymal stem cell differentiation into osteoblast. (A) Upper left panel: hMSC cells were transiently treated with BIM for 1 h as indicated. The medium was then replaced by normal medium containing 10% FBS, Vitamin C (50 μg/mL) and β-glycerolphosphate (5 μmol/L) with or without BMP ligand for another 12 days. Cells were then harvested for Alizarin Red staining. Upper right panel: Relative Alizarin Red absorbance analysis of upper left samples. Lower panel: Amplified view of upper left panel. (B) Working model of which BIM induces osteogenesis through suppressing GSK3β activity, subsequently strengthening β-catenin- and Smad1-mediated signaling.

are related to consequences of PKC family or their individual isozyme's action. Of note, we observed a growth arrest effect in hMSCs by even suboptimal doses of BIM when it was long-time treated to cells, which was not considerably detected in preosteoblast. Whether this effect is due to suppression of PKC family members requires further investigations.

The fact that BIM enhances osteogenic differentiation emphasizes its physiological relevance of regulating Wnt/β-catenin and BMP signals as a GSK3β inhibitor. Although much more work is needed to elucidate the possible application of BIM in bone disease, the present study provides a potential therapeutic method.

MATERIALS AND METHODS

Reagents and plasmids

β-catenin antibody sample kit (#2951), GSK3β (#9315) and phospho-GSK-3â (Ser9) (#9336) antibodies were purchased from Cell Signaling Technology, Inc., USA. Actin (ab8227) was purchased from Abcam. Control and β-catenin siRNA were purchased from Thermo (Dharmacon RNAi Technologies), TopFlash-luciferase, FopFlash-luciferase, LEF-luciferase, β-catenin SA mutant, Wnt3a, GSK3β wt, S9A, K85M and dnLEF-1 constructs were as described previously (Zhang et al., 2006; Zhou et al., 2008a, 2008b, 2011a).

Cell culture

C2C12, KS483, L cells and HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone), nonessential amino acids, L-glutamine, and penicillin/streptomycin in a 5% CO_2-containing atmosphere at 37°C.

Luciferase reporter assay

Cells were transfected and lysed as described (Zhang et al., 2006) and luciferase activities were measured by a luminometer (Berthold Technologies). Reporter activity was normalized to β-Gal activity, resulting from a cotransfected internal control plasmid. Experiments were performed in triplicate.

Transfection and immunoblotting

As previously described (Nie et al., 2010; Zhang et al., 2011; Zhou et al., 2011a, 2011b), cells were transiently transfected using Lipofectamine (Invitrogen). 40 h post-transfection, cells were lysed and immunoblotting was performed with specific antibody and secondary anti-mouse or anti-rabbit antibodies that were conjugated to horseradish peroxidase (Amersham Biosciences). Proteins were visualized by chemiluminescence.

ALP staining and mineralization assays

Histochemical examination of ALP activity in cells was performed using naphtol AS-MX phosphate (Sigma) and fast blue RR salt (Sigma) (van Dinther et al., 2010). For mineralization assay cells were washed with PBS, fixed with 3.7% formaldehyde, again washed with PBS and then incubated with 2% alizarin red S solution (pH 4.2) for 2 min and washed with distilled water (van Dinther et al., 2010).

QRT-PCR (quantitative real-time-PCR)

Total RNA was isolated using NucleoSpin® RNA II kit (BIOKÉ, Netherlands) reagent. 1 μg RNA was reverse-transcribed using the RevertAid™ First Strand cDNA Synthesis Kits (Fermentas). Quantitative real-time-PCR was accomplished with SYBR Green incorporation (Applied Bioscience) using a StepOne Plus real-time PCR system (Applied Bioscience). Results were normalized to those obtained with GAPDH. Primers used for QRT-PCR were: mGAPDH forward, 5'-AACTTTGGCATTGTGGAAGG-3'; mGAPDH reverse, 5'-ACACA-TTGGGGGTAGGAACA-3'; mRunx2 forward, 5'-GAATGCTTCATT-CGCCTCAC-3'; mRunx2 reverse, 5'-GTGACCTGCAGAGATTAAC-C-3'; mOpg (osteoprotegerin) forward, 5'-CGCAAAAGTGTGGAATA-GATGTCA-3'; mOpg (osteoprotegerin) reverse, 5'-GGTAGGAACA-GCAAACCTGAAGA-3'.

Preparation of cytosolic fractions

Cytosolic and membrane fractions were prepared using the ProteoExtract kit (Calbiochem) according to the manufacturers' standard procedures.

Statistical analysis

Statistical analyses were performed with a two-tailed unpaired t test. $P < 0.05$ was considered statistically significant.

ACKNOWLEDGEMENTS

ABBREVIATIONS

ALP, alkaline phosphatase; BIM, bisindoylmaleimide I; BMPs, bone morphogenetic proteins; GSK3β, glycogen synthase kinase 3β; hMSCs, human mesenchymal stem cells; TGF-β, transforming growth factor β

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