International Journal of Biochemistry and Cell Biology 116 (2019) 105614

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

BMP9 prevents induction of osteopontin in JNK-inactivated osteoblasts via Hey1-Id4 interaction

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ARTICLE INFO

Keywords:
BMP9
Osteopontin
Osteocalcin
Osteoblast
Differentiation
Id4
Hey1

ABSTRACT

Osteopontin (OPN) is an osteoblast-derived secretory protein that plays a role in bone remodeling, osteoblast responsiveness, and inflammation. We recently found that osteoblast differentiation is type-specific, with conditions of JNK inactivation inducing osteoblasts that preferentially express OPN (OPN-type). Since OPN-type osteoblasts highly express osteogenesis-inhibiting proteins and Rankl, an important inducer of osteoclastogenesis, an increased appearance of OPN-type osteoblasts may be associated with inefficient and poor-quality bone regeneration. However, whether specific osteogenic inducers can modulate OPN-type osteoblast differentiation is completely unknown. Here, we demonstrate that bone morphogenic protein 9 (BMP9) prevents induction of OPN-type osteoblast differentiation under conditions of JNK inhibition. Although JNK inactivation suppressed both BMP2- and BMP9-induced matrix mineralization and osteocalcin expression, the expression of Rankl and specific cytokines such as Gpha2, Esm1, and Sfrp1 under similar conditions was increased in all cells except those treated with BMP9. Increased expression of Id4, a critical transcriptional regulator of OPN-type osteoblast differentiation, was similarly prevented only in BMP9-treated cells. We also found that BMP9 specifically induces the expression of Hey1, a bHLH transcriptional repressor, and that Id4 inhibits the suppressive effects of Hey1 on Opn promoter activity by forming Id4-Hey1 complexes in osteoblasts. Using site-direct mutagenesis, ChIP, and immunoprecipitation, we elucidated that BMP9-induced overexpression of Hey1 can overcome the effects of Id4 and suppress OPN expression. We further found that p38 activation and JNK inactivation are involved in BMP9-induced Hey1 expression. Collectively, these data suggest that BMP9 is a unique osteogenic inducer that regulates OPN-type osteoblast differentiation.

1. Introduction

Osteoblasts play a central role in bone metabolism by producing various bone matrix proteins, regulating matrix mineralization, and controlling osteoclastic activity (Staines et al., 2012). Secretory amounts and patterns of osteoblast-derived proteins such as

Abbreviations: OPN, osteopontin; OCN, osteocalcin; JNK, p54/p46 c-jun N-terminal kinase; Gpha2, glycoprotein hormone alpha 2; Esm1, endothelial cell-specific molecule 1; Rankl, receptor activator of nuclear factor kappa-β ligand; BMP, bone morphogenic protein; Sfrp1, secreted frizzled-related protein 1; Id4, inhibitor of DNA binding 4; Hey1, hairy/enhancer-of-split related with YRPW motif protein 1; DOX, doxycycline

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https://doi.org/10.1016/j.biocel.2019.105614
Received 17 April 2019; Received in revised form 9 August 2019; Accepted 16 September 2019
Available online 21 September 2019
1357-2725/

osteopontin (OPN) and osteocalcin (OCN) characterize osteoblast function. OPN is an osteoblast-derived secretory protein that modulates the bone remodeling process, chemotaxis of inflammatory cells, and osteoblast responsiveness to extracellular stimuli (Kahles et al., 2014; Kusuyama et al., 2017).

We recently found that p54/p46 c-jun N-terminal kinase (JNK) inactivation influences differentiating osteoblasts toward a high OPN, low OCN expression phenotype (OPN-type) (Kusuyama et al., 2019a). OPN-type osteoblasts express specific cytokines such as glycoprotein hormone alpha 2 (Gpha2) and endothelial cell-specific molecule 1 (Esm1). Receptor activator of nuclear factor kappa-β ligand (Rankl), a critical inducer of osteoclastogenesis (Okamoto et al., 2017), as well as inhibitory cytokines of bone matrix formation such as secreted frizzled-related protein 1 (Sfrp1) (Gaur et al., 2009), are also highly expressed in OPN-type osteoblasts. This secretory profile of OPN-type osteoblasts raises the possibility that increased differentiation of these osteoblasts may activate osteoclastic bone resorption but not fully induce new bone formation in the bone remodeling process.

Bone morphogenic protein 9 (BMP9) has been characterized as the most osteogenic BMP, showing more bone-regenerative potential than BMP2 (Kusuyama et al., 2019b; Lamplot et al., 2013). BMP9 also acts as a crucial modulator of angiogenesis (Hu et al., 2013), and its osteogenic properties are highly upregulated by mechanical stimulation. Different signaling pathways have been indicated for BMP9 and BMP2-induced osteoblastic differentiation (Drevelle et al., 2013; Eiraku et al., 2019; Lauzon et al., 2016). These reports raise the possibility that BMP9 does not induce OPN-type osteoblast differentiation via its specific signaling pathway. If treatment with BMP9 can modify the ratio of OPN-type and other-type osteoblastic differentiation, it can potentially be utilized to generate more efficient and high-quality bone regeneration.

In this study, we explored whether BMP9 treatment affects the differentiation of OPN-type osteoblasts under JNK-inactivated conditions. We found that OPN-type osteoblast differentiation, characterized by high expression of OPN, inhibitor of DNA binding 4 (Id4), Esm1, and Gpha1, was prevented by BMP9 treatment in primary osteoblasts. Furthermore, we found that increased expression of hairy/enhancer-of-split related with YRPW motif protein 1 (Hey1), a bHLH transcriptional repressor (Fischer and Gessler, 2007), preserves bone regenerative capacity in BMP9-treated cells by overcoming the inhibitory effects of Id4 on Hey1 function. These data indicate that BMP9 is a unique osteogenic promoter that regulates the multi-directional differentiation of osteoblasts.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant mouse BMP2 was purchased from Aviscera Bioscience (Santa Clara, CA). Recombinant mouse BMP9 was purchased from WAKO (Osaka, Japan). SP600125, a JNK-specific inhibitor, was purchased from LC Laboratories (Woburn, MA). U0126, an ERK-specific inhibitor, SB203080, a p38-specific inhibitor, and LY3039478, a Notch-specific inhibitor, were purchased from Funakoshi (Tokyo, Japan). Antibodies against OPN, Id4, Hey1, Hey2, HeyL and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against OCN were purchased from Abcam (Cambridge, UK). Mouse Hey1 recombinant protein was obtained from Mybiosource (San Diego, CA).

2.2. Cell culture

Primary osteoblasts were isolated from newborn C57BL/6 mouse calvariae (Japan Clea, Tokyo, Japan) as previously described (Kusuyama et al., 2017). Osteogenic differentiation was induced by the addition of 10 ng/ml BMP2 or BMP9 and 5 mM β-glycerophosphate to Eagle's α-minimum essential medium (WAKO) containing 10% FBS, 50 units/ml penicillin, and 50 mg/ml streptomycin.

2.3. Alizarin red S staining

Matrix mineralization was visualized as previously described (Kusuyama et al., 2014).

2.4. Western blot analysis

Cells were lysed in cell lysis buffer. Immunoblotting was performed as previously described (Kusuyama et al., 2014).

2.5. Quantitative polymerase chain reaction (qPCR) analysis

Total RNA collection and real-time PCR were conducted as previously described (Kusuyama et al., 2016). The primer sequences used for the qPCR analysis are as follows: 5′-CGG ACG AGA ATG GAA ACT TGA-3′ and 5′-GAT GCC TCT CCG TCT TTT CC-3′ for Hey1, 5′-TGA AGA TGC TCC AGG CTA CAG G-3′ and 5′−CCT TCC ACT GAG CTT AGG TAC C-3′ for Hey2, and 5′-CTG GAG AAA GCT GAG GTC TTG C-3′ and 5′-ACC TCA GTG AGG CAT TCC CGA A-3′ for HeyL. The other sequences used have been previously described (Kusuyama et al., 2019a; Nakao et al., 2014).

2.6. Generation of Opn promoter constructs, site-directed mutagenesis, and promoter assay

Genomic DNA constructs containing 1100 base pairs of the 5′-upstream region of the putative transcriptional initiation site for the mouse *Opn* gene were cloned by PCR from C57BL/6 genomic DNA using the following primer pair: GGT ACC TGC TGA AGA TTT CTG TAA TAG (sense) and GCT AGC TGC TGG CTC AGA CCT CCC (antisense). PCR products were ligated into pCR-Blunt II-TOPO vectors (Life Technologies, Carlsbad, CA), and the vectors were subsequently digested by KpnI and NheI restriction enzymes. The resulting DNA fragments were subcloned into a luciferase reporter vector, pNL2.1 (Promega, San Luis Obispo, CA). To generate site-directed mutagenesis of the putative Hey1 binding sites in the *Opn* gene, mutated DNA fragments were constructed by PCR using the following primer pairs: CCC AAG GT*C ACA*<u>*aa*T GGC</u> ACT GCT TA and TAA GCA GTG CCA TTT GTG ACC TTG GG, CTA CCC GGC CCA TTT GCT CCT ACA CT and AGT GTA GG<u>A GCA</u> <u>*aa*T GGG</u> CCG GGT AG (the putative Hey1 binding sites are underlined; -959 to -950, -109 to -100 and altered bases are given in italicized lowercase letters). The resultant plasmids were stably co-transfected with G418-resistant plasmids into MC3T3-E1 cells by Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions, and selection was performed using 1.0 mg/ml G418. As a negative control cell line, pNL2.1 vectors were stably transfected into MC3T3-E1 cells. After G418 selection, three individual cell lines were isolated for each plasmid, and the lines were then induced to differentiate in osteogenic differentiation medium for 6 days. Relative luciferase activities were measured by the Nano-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions.

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously (Kusuyama et al., 2016). The *Opn* promoter-specific primers used are as follows: CAT TGC AAA ATG TGA AGT GC (sense) and GAT AGA GCC CGC CTA AGC AA (antisense).

2.8. Tet-on and Tet-on 3 G inducible expression system

Establishment of the MC3T3-E1 Tet-On Id4 cells used has previously been described (Kusuyama et al., 2019a). pCMV-Tet3 G vectors (Clontech, Mountain View, CA) were stably transfected into MC3T3-E1 cells

by Lipofectamine 3000 according to the manufacturer's instructions, followed by selection with 500 mg/ml G418. Coding cDNAs for mouse Hey1 were amplified by PCR with specific primers using total RNA from C57BL/6 mouse primary osteoblasts as the template. After confirmation of the DNA sequences, these cDNAs were cloned into pTRE3 G vectors (Clontech Laboratories, CA, USA), producing inducible expression plasmids termed pTRE3 G-Hey1. pTRE3 G-Hey1 plasmids and linear hygromycin markers (Clontech) were stably transfected into the MC3T3-E1 pCMV-Tet3 G cell line by Lipofectamine 3000. After selection with 0.15 mg/ml hygromycin B, the isolated resistant clones were tested for inducible protein expression by the inserted cDNAs with 2 μg/ml doxycycline (DOX). Three separate cell lines with adequate inducible protein expression were analyzed for each construct.

2.9. Immunoprecipitation

The immunoprecipitation assay was performed using Hey1 antibody as described previously (Kusuyama et al., 2017).

2.10. Statistical analyses

Statistical analyses were performed using Student's *t*-test or ANOVA. When ANOVA indicated a significant difference, specific differences were identified with Tukey and Bonferroni post hoc analyses ($*p < 0.01$). All data are expressed as the mean ± SEM. Results are representative examples of at least three independent experiments.

3. Results

3.1. BMP9 prevents the appearance of OPN-type osteoblasts during osteogenic differentiation under conditions of JNK inhibition

Our previous studies showed that JNK inhibitor treatment highly suppresses osteogenic differentiation in the presence of ascorbic acid, BMP2, and fibroblast growth factor 2 (FGF2) (Kusuyama et al., 2019a). Accordingly, we analyzed the effects of JNK inhibition on BMP9-induced osteogenesis. Treatment with SP600125, a JNK-specific inhibitor, robustly suppressed matrix mineralization (Fig. 1A) and OCN protein expression (Fig. 1B) in both BMP2 and BMP9-treated primary osteoblasts. Consistent with our previous findings, OPN expression was robustly upregulated in osteoblasts by treatment with JNK inhibitor during BMP2-induced osteogenic differentiation. However, JNK inactivation did not induce OPN in BMP9-induced differentiated osteoblasts. We also analyzed the mRNA expression of characteristic genes of OPN-type osteoblasts (Kusuyama et al., 2019a) and found that the expression of *Id4*, *Esm1*, *Gpha1*, *Rankl*, and *Sfrp1* was not increased by JNK inactivation in BMP9-treated osteoblasts (Fig. 1C). These results suggest that BMP-9 is a unique osteogenic inducer that prevents OPN-type osteoblast differentiation under conditions of JNK inactivation.

3.2. BMP9 promotes Hey1 expression through p38 activation and JNK inactivation

The repressive role of Id4 in mRNA transcription such as androgen receptor (AR) (Komaragiri et al., 2016) and vascular endothelial growth factor A (VEGFA) (Pruszko et al., 2017), depends on various basic helix-loop-helix (bHLH) binding partners (Patel et al., 2015). Our previous study showed that Id4 overexpression promotes OPN expression in osteoblasts (Kusuyama et al., 2019a), suggesting that Id4 interacts with transcriptional repressors such as Hey1, Hey2, and HeyL (Weber et al., 2014) to promote *Opn* expression. Accordingly, we hypothesized that BMP2 and BMP9 may differentially affect the expression of Hey family proteins during osteogenic differentiation. We found that Hey1 and Hey2 mRNA expression increases in osteoblasts following BMP2 or BMP9-induced osteogenic differentiation, whereas HeyL expression was not affected (Fig. 2A). Interestingly, JNK inhibitor treatment promoted Hey1 expression only in BMP-9-treated cells. Consistent with these results, JNK inhibition suppressed Id4 and promoted Hey1 protein expression (Fig. 2B). We also explored the involvement of proteins of the BMP9 signaling pathway, including ERK (Ye et al., 2014), p38 (Zhu et al., 2018), and Notch (Cao et al., 2017), in Hey1 expression. Treatment of MC3T3-E1 cells with SB203580, a p38-specific inhibitor,

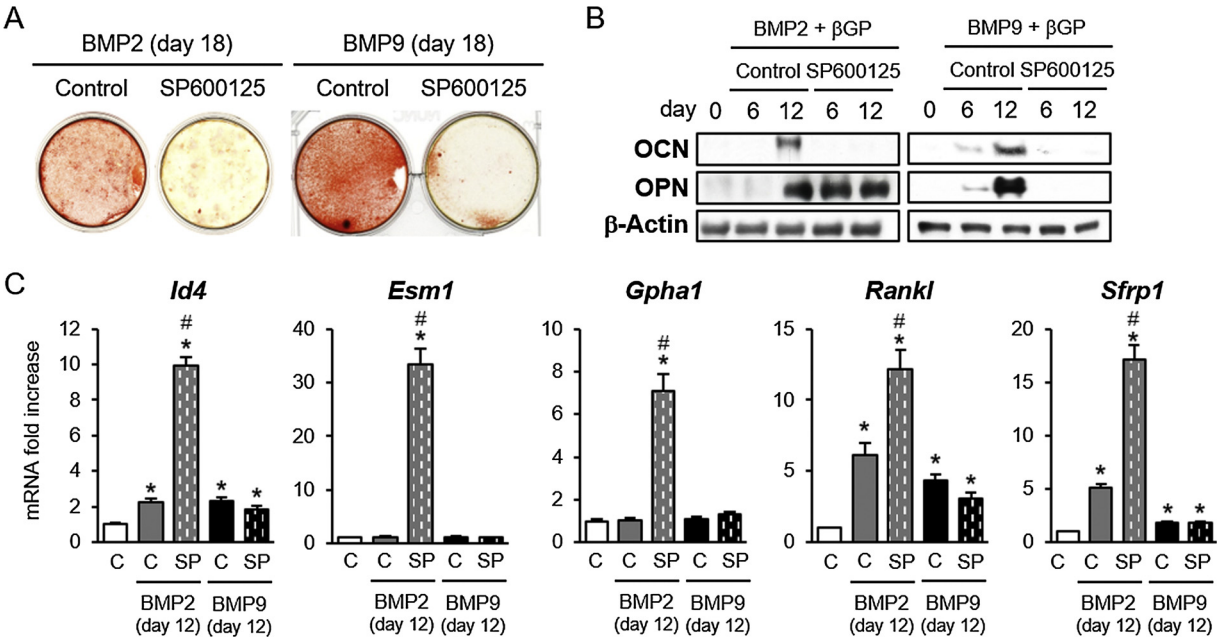

Fig. 1. BMP9 stimulation does not induce OPN-type osteoblasts. A–C. Primary osteoblasts were differentiated by the addition of 10 ng/ml recombinant BMP2 or 10 ng/ml recombinant BMP9 and 5 mM β-glycerophosphate to the culture medium with or without 5 μM of the JNK-specific inhibitor SP600125 for the indicated days. Cells were stained with Alizarin Red S for the detection of matrix mineralization (A). Cell lysates were collected and analyzed by the indicated antibodies (B). Gene expression was analyzed by real-time PCR. Relative mRNA expression levels are shown as normalized to *Rpl13a*. Error bars represent ± 1 s.d. (*P < 0.01; vs Control, #P < 0.01; BMP2 C vs BMP2 SP). (C) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

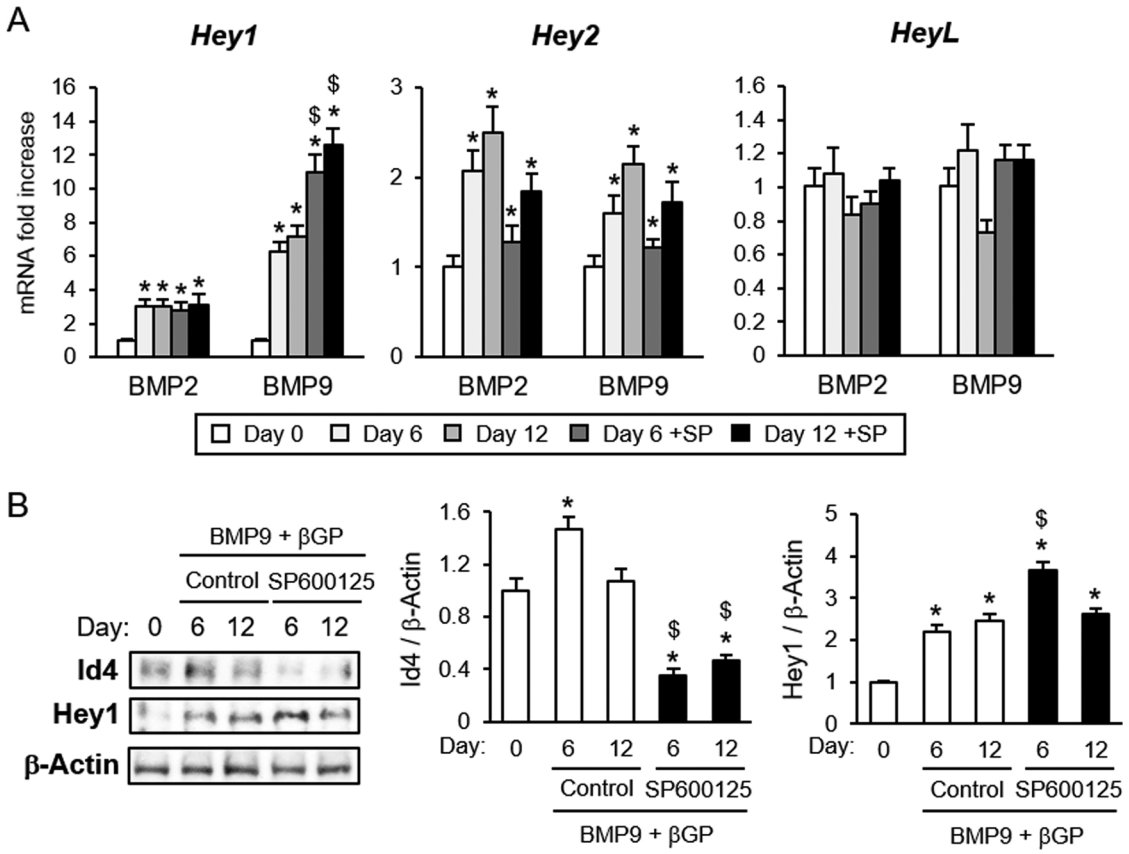

Fig. 2. BMP9 treatment induces Hey1 expression in both JNK-activated and JNK-inactivated osteoblasts. A & B. Primary osteoblasts were differentiated by the addition of 10 ng/ml recombinant BMP2 or 10 ng/ml recombinant BMP9 and 5 mM β-glycerophosphate to the culture medium with or without 5 μM of the JNK-specific inhibitor SP600125 for the indicated days. Gene expression was analyzed by real-time PCR. Relative mRNA expression levels are shown as normalized to *Rpl13a*. (*P < 0.01; vs Day 0, $P < 0.01; vs BMP9 Day 6) (A). Cell lysates were collected and analyzed by the indicated antibodies. Band intensities were calculated by ImageJ. Error bars represent ± 1 s.d. (*P < 0.01; vs Day 0, $P < 0.01; vs Control Day 6) (B).

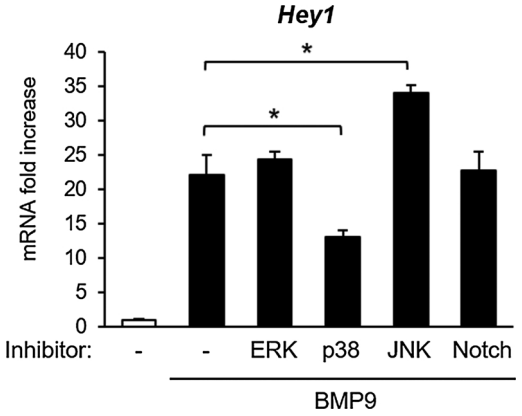

Fig. 3. BMP9 regulates Hey1 expression through p38 and JNK. A. MC3T3-E1 cells were treated with 10 μM U0126, an ERK-specific inhibitor; 10 μM SB203580, a p38-specific inhibitor; 10 μM SP600125, a JNK-specific inhibitor; or 1 μM LY3039478, a Notch-specific inhibitor, for 1 h. After the treatment, cells were stimulated with 50 ng/ml of BMP9 for 1 h. Gene expression was analyzed by real-time PCR. Relative mRNA expression levels are shown as normalized to *Rpl13a* (A). Error bars represent ± 1 s.d. ($p^* < 0.01$).

significantly suppressed BMP9-induced Hey1 mRNA expression (Fig. 3). Inhibition of ERK and Notch, on the other hand, had no effect on Hey1 induction. These results suggest that Hey1 has a distinct role in BMP9-induced osteogenic differentiation.

3.3. Induction of Hey1 by BMP9 prevents OPN expression in osteoblasts

In order to investigate a possible Id4-Hey1-mediated mechanism of *Opn* transcriptional activation, we first used JASPAR (http://jaspar.genereg.net/) to search the 1100 base pair upstream region of the mouse *Opn* gene for putative Hey1 binding sites. We found two potential binding sites for Hey1 located at the -959 to -950 and -109 to -100 upstream regions. These putative Hey1 binding elements were mutated by site-directed mutagenesis in wild-type *Opn* promoter constructs. We then stably transfected MC3T3-E1 cells with wild type (control) or mutated (mutation A: -959 to -950, mutation B: -109 to -100, or mutation A & B) reporter plasmids containing 1000 base pair lengths of the 5′-upstream region of the mouse *Opn* gene. These cells were induced to differentiate for 6 days, and the luciferase reporter activities were measured. We found that mutation of the -959 to -950 putative Hey1 binding site significantly increased reporter activity during osteogenesis (Fig. 4A). However, mutation of the -109 to -100 site did not affect luciferase activity. These findings suggest that OPN induction during osteogenic differentiation is presumably regulated by the Hey1 binding site located -959 to -950 base pairs upstream of the mouse *Opn* gene. In order to confirm that Hey1 interacts with the predicted binding sequence in the endogenous *Opn* gene, we performed ChIP in MC3T3-E1 Tet-On Id4 cells using an antibody specific for Hey1. The presence of the Hey1 binding sequence in the immunoprecipitated chromatin was analyzed by qPCR using a pair of primers (-1030/-860) that targeted the Hey1 binding region. The ChIP results showed Hey1 binding to the putative site in the 5′-upstream region of the mouse *Opn* gene in differentiated osteoblasts (Fig. 4B). These results indicate that the Hey1 binding site in the 5′ upstream region of the mouse *Opn* gene

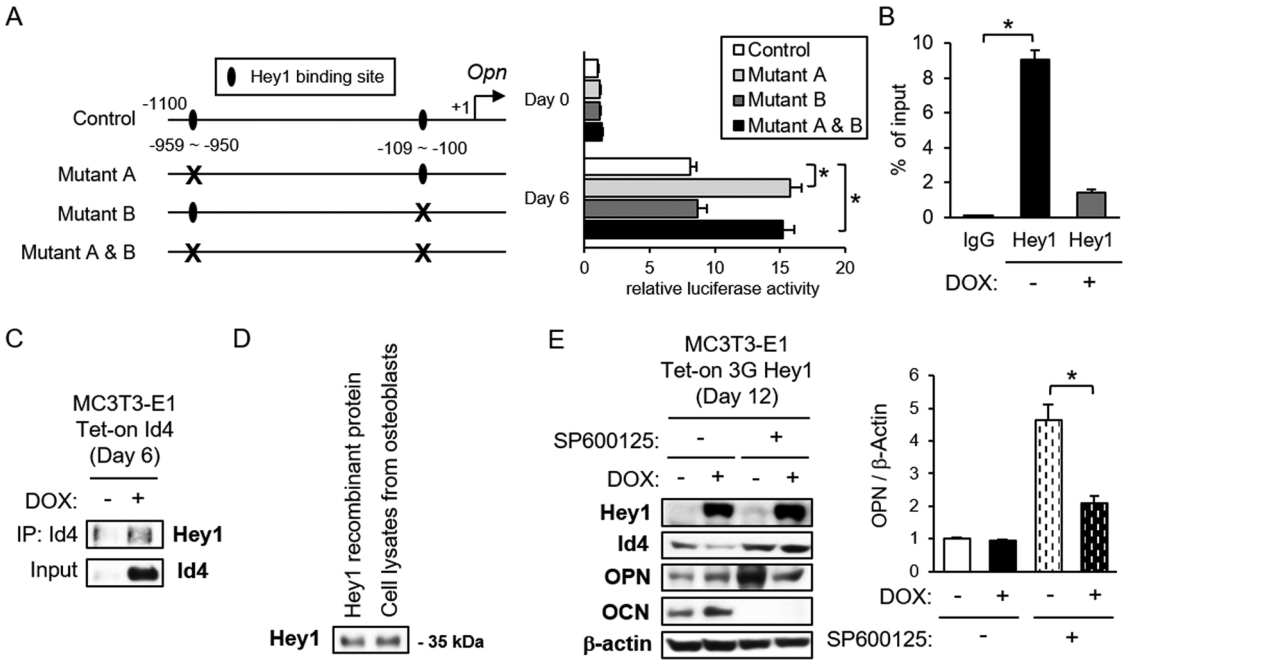

Fig. 4. OPN expression is regulated by a Hey1-Id4 axis. A. MC3T3-E1 cells were stably transfected with pNL2.1-*Opn* wild-type control or mutated reporter plasmids. The cells were induced to differentiate for 6 days. Cytoplasmic lysates were analyzed for luciferase activity in triplicate. Error bars represent ± 1 s.d. ($p^* < 0.01$). Three independent cell clones were isolated and analyzed for each reporter plasmid to ensure reproducible results. The results from a representative cell clone are shown for each construct. B. MC3T3-E1 Tet-On Id4 cells were cultured in osteogenic differentiation medium with or without 2 μg/ml of DOX for 6 days. Chromatins were extracted and immunoprecipitated with an antibody against Hey1. qPCR analyses of the immunoprecipitated DNA were carried out. C. MC3T3-E1 Tet-On Id4 cells were differentiated as in (B). Immunoprecipitation was performed with anti-Id4 antibody. The precipitates were separated by SDS-PAGE followed by detection using the indicated antibodies. D. Mouse Hey1 recombinant protein and cell lysates from differentiated (day 6) primary osteoblasts were analyzed by Hey1 antibody. E. MC3T3-E1 Tet-On 3 G Hey1 cells were differentiated as in (B). Cell lysates were collected and analyzed by the indicated antibodies. Band intensities were calculated by ImageJ. Error bars represent ± 1 s.d. ($p^* < 0.01$).

located between -1030 and -860 is functionally involved in *Opn* promoter activation. Using immunoprecipitation, we also demonstrated a functional interaction between Id4 and Hey1. Co-precipitation of Id4 and Hey1 was detected in Id4-overexpressed cells (Fig. 4C). However, molecular sizes of cell lysates from differentiated primary osteoblasts was similar to recombinant mouse Hey1 protein (Fig. 4D). Moreover, Hey1 overexpression partially blocked JNK inactivation-induced OPN hyperexpression in osteoblasts induced to differentiate by BMP2 (Fig. 4E). Collectively, these results suggest that Id4 inhibits the suppressive effects of Hey1 on *Opn* promoter activity by binding with Hey1. Induction of Hey1 by BMP9 seems to prevent the appearance of OPN-type osteoblasts.

4. Discussion

By using primary osteoblasts derived from mouse calvariae and the pre-osteoblastic cell line MC3T3-E1, we show that BMP9 does not induce OPN-type osteoblast differentiation under conditions of JNK inactivation, unlike other inducers of osteoblast differentiation (Fig. 1). We also demonstrate that BMP9 specifically induces Hey1 expression under both JNK-activated and JNK-inactivated conditions (Fig. 2). These results raise the possibility that BMP9 treatment may have beneficial effects on osteoblast differentiation by inhibiting the appearance of OPN-type osteoblasts.

We recently showed that Id4 is a master transcriptional regulator of OPN-type osteoblast differentiation (Kusuyama et al., 2019a). In this regard, our current study demonstrates that distinct differences exist between BMP9- and BMP2-induced osteogenic differentiation, with BMP9 stimulation causing low expression of Id4 and high expression of Hey1 in differentiating osteoblasts (Fig. 2). Consistent with our results, a previous study has reported that BMP9 treatment upregulates Hey1 expression in mesenchymal stem cells (Sharff et al., 2009). The authors show that silencing Hey1 expression diminishes BMP9-induced osteogenic differentiation both in vitro and in vivo, indicating that Hey1 partly mediates the effects of BMP9 on osteoblasts. Several studies have also reported different signaling pathways for BMP9 and BMP2, leading to distinct phenotypic differences between osteoblasts induced to differentiate by each BMP. For example, different kinetics of Smad activation (Drevelle et al., 2013) and Akt phosphorylation (Lauzon et al., 2016) were observed between BMP9- and BMP2-treated MC3T3-E1 cells. Previous studies have additionally reported that JNK activation is an essential component of the signaling pathway controlling BMP9-induced osteogenic differentiation (Wang et al., 2017; Zhao et al., 2013). Since JNK inactivity is a strong inducer of OPN-type osteoblast differentiation, the potent activation of JNK by BMP9 might suppress the induction of OPN-type osteoblasts. We recently reported that BMP9-induced JNK phosphorylation was stronger and faster than BMP2 in osteoblasts (Eiraku et al., 2019). On the other hand, phosphorylation of Smad1/5 was similarly induced by BMP9 and BMP2. However, JNK inhibitor treatment upregulated BMP9-induced Hey1 expression (Fig. 3), indicating that JNK activity does not directly control the Hey1-mediated regulation of osteoblast differentiation. On the other hand, p38 inhibition suppressed Hey1 expression in BMP9-treated osteoblasts (Fig. 3). This data corresponds with another recent study showing that p38 is critically involved in the BMP9-induced osteogenic differentiation of mesenchymal stem cells (Zhu et al., 2018). Thus, it seems that an antagonistic signaling pathway involving JNK and p38 regulates the effects of BMP9 on Id4 and Hey1 expression. Further studies are needed to elucidate the specific signaling pathway of BMP9-induced osteogenic differentiation.

Id4 acts as a transcriptional repressor but lacks DNA binding activity (Patel et al., 2015). One of our novel findings in this study is that Hey1 binds to Id4 to cooperatively regulate OPN expression (Fig. 4). Hey1 was reported to promote (Sharff et al., 2009) or inhibit (Zamurovic

et al., 2004) osteogenic differentiation through interaction with runt-related transcription factor 2 (Runx2), a master transcription factor regulating osteoblast differentiation. In addition, both Hey1-deficient and Hey1-overexpressing mice show modest osteopenia (Salie et al., 2010). These studies suggest that Hey1 controls osteogenesis by interacting with other sequence-specific transcriptional regulators during osteogenic differentiation. Hey1 has also been reported to directly act on the basic transcriptional machinery to repress promoters (Fischer and Gessler, 2007). However, our immunoprecipitation assay did not show a large increase in molecular weight for interacting Hey1 compared to non-interacting Hey1 (Fig. 4D). These results collectively suggest that Id4-Hey1 complexes are directly involved in the regulation of OPN-type osteoblast differentiation.

We previously showed that Id4 overexpression or knockdown influences the expression of Gpha2 and Esm1 (Kusuyama et al., 2019a). We also demonstrated that Gpha2 and Esm1 expression is increased in OPN-type osteoblasts. Therefore, it is possible that Id4 regulates a broad range of OPN-type osteoblast-specific gene expression. A previous study showed that Id4 associates with other Hey family proteins (Tokuzawa et al., 2010). For example, Id4 was found to release hairy and enhancer of split-1 (Hes1) from Hes1-Hey2 complexes, as well as to increase the stability and transcriptional activity of Runx2 (Tokuzawa et al., 2010). Our current study mechanistically shows that BMP9-induced Hey1 upregulation overcomes the inhibitory effects of Id4 on Hey1-mediated inhibition of *Opn* expression (Fig. 4). Thus, it is presumed that Hey1 saturation prevents Id4-Hey1 complex formation from inducing OPN expression in BMP9- and JNK inhibitor-treated osteoblasts. Furthermore, complexes of Id4-Hey family proteins might affect osteoblast differentiation in multiple ways.

In summary, we have demonstrated that BMP9 treatment prevents the differentiation of OPN-type osteoblasts under conditions of JNK inactivation. Specifically, we found that BMP9 promotes Hey1 expression, which in turn masks Id4 function in OPN-type osteoblast differentiation. Since OPN enhances bone resorption and inflammation and decreases the functional response of osteoblasts, clinical BMP9 treatment has the potential to inhibit the appearance of OPN-type osteoblasts and, therefore, to promote bone formation. This study also provides novel insights into the role that interactions between Id4 and the Hey family of proteins play in bone metabolic disorders and potential bone regeneration therapies.

Author contributions

JK performed most of the experiments, interpreted the results and prepared the manuscript. CS performed the experiments shown in Fig. 3. TN and KN provided technical assistance for analysis and interpretation of the data shown in Fig. 2. KB, TO and MSA provided technical help on the experiments shown in Figs. 1,2, & 4 and also assisted with data interpretation. KS and IS provided technical help on the experiments shown in Fig. 1. TM directed and supervised the project.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgements

We thank Ms. Yoko Amita and Ms. Yuka Kajiya for the secretarial assistance and Brent G Albertson and Nathan S Makarewicz for English proofreading. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japanese Association for Oral Biology Grant-in-Aid for Young Scientists, the Kanzawa Medical Research Foundation, the Nakatomi Foundation, and the Japan Foundation for Aging and Health.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.105614.

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