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Inhibitory Helix-Loop-Helix Transcription Factors Id1/Id3 Promote Bone Formation In Vivo

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Abstract Bone formation is under the control of a set of transcription factors. Ids are inhibitory helix-loop-helix (HLH) transcription factors and expression of *Id* genes in osteoblasts is under the control of calciotropic agents such as BMP and vitamin D. However, the function of Ids during bone formation in vivo has not yet been elucidated. We, therefore, examined the role of Id1 and Id3 in the regulation of bone metabolism in vivo. Using wild type and Id1/Id3 heterozygous knock out mice, we analyzed calvarial bone formation in the suture by X-ray picture, proliferation, and mineralization activities of primary calvarial osteoblasts by MTT assay and alizarin red staining and onthotopic in vivo bone formation by BMP injection onto calvaria and micro CT analysis. The width of calvarial sutures was reduced by more than 50% in Id1/Id3 heterozygous knock out mice. Analyses on the cellular basis for the mechanism underlying the defects in the mutant mice revealed suppression of proliferation and mineralization in osteoblasts derived from Id1/Id3 heterozygous knock out mice. Furthermore, Id1/Id3 heterozygous knock out mice suppressed BMP-induced bone formation in vivo. These results indicated that Id1 and Id3 are positive factors to promote bone formation in vivo. J. Cell. Biochem. 93: 337–344, 2004.

Key words: inhibitor of differentiation; osteoblast; helix-loop-helix

Bone formation takes place through either membranous ossification or endochondral ossification. The process of the latter includes multiple steps of events such as mesenchymal cell condensation, chondrogenesis, and osteogenesis. During this pathway, chondrocytes become hypertrophic and then invasion of vasculatures occurs followed by osteogenesis by bone formation based on osteoblastic activity. Endochondral bone formation is observed not only during development but also during fracture healing. In such process, skeletal cells differentiate in close association with angiogenesis. Therefore, disruption in angiogenesis delays bone formation during endochondral bone formation in bone development and fracture healing [Vu et al., 1998; Gerber et al., 1999; Haigh et al., 2000; Maes et al., 2002; Yin et al., 2002]. The concerted events during the process of endochondral bone formation are under the control of various transcription factors and cytokines. However, molecules involved in these steps have not yet been fully understood.

Osteoblasts express not only positive transcription factors, but also express negative type transcription factors such as Ids [Kawaguchi et al., 1992; Ogata et al., 1993; Tamura and Noda, 1994; Beck et al., 2001]. The Id proteins belong to helix-loop-helix (HLH) protein family. They are implicated in the control of differentia-

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tion and cell-cycle progression in a variety of organisms ranging from flies to men [Norton et al., 1998; Ruzinova and Benezra, 2003]. Id proteins lack the basic domain but contain HLH motif which allows Ids to heterodimerize with positive bHLH transcription factors to prevent their binding to DNA sequence and thus, to suppress the expression of cell-type-specific genes and the cell-cycle regulatory genes [Lassar et al., 1994]. Although Ids are considered to be inhibitory molecules, their expression in osteoblasts are up-regulated by osteogenic cytokines such as BMP while it is down regulated by calciotropic hormones including $1,25(OH)_2$ vitamin D3 [Kawaguchi et al., 1992; Ogata et al., 1993; Tamura and Noda, 1994]. In vivo functions of Ids have been investigated by gene-targeting studies. Id1 knock out mice reveal no significant differences compared to wild type [Yan et al., 1997]. Id2 knock out mice exhibit reduction in lymph nodes and Payer's patches and population of natural killer (NK) cells [Yokota et al., 1999]. Id3 knock out mice show impairment in immunoglobulins production and proliferation defect in B cells [Norton et al., 1998; Pan et al., 1999].

Complete Id1 and Id3 double knock out mice are inviable due to hemorrhage throughout the ventricular system [Lyden et al., 1999]. On the other hand, mice lacking heterozygous alleles of Id1 and Id3 in any combination are indistinguishable from the wild type. However, heterozygous Id1 and Id3 deficiency reduces tumor-induced vascularization compared with wild type [Lyden et al., 1999, 2001]. In this case, heterozygous Id1 and Id3 deficiency impairs recruitment of bone marrow-derived endothelial cells and hematopoietic precursor cells to reduce angiogenesis and tumor growth [Lyden et al., 2001]. Although Ids are expressed in cultured osteoblasts, their function in bone has not been known. We examined the function of *Id* genes during bone formation in vivo in adult Id1/Id3 heterozygous knock out mice.

MATERIALS AND METHODS

Animals

Wild type mice (129Sv/C57BL6) and Id1/Id3 heterozygous knock out mice were obtained by crossing the heterozygous knock out mice. Wild type littermate mice were used as control. In total, 111 Id heterozygous knock out mice and 13 wild type mice were used. Mice were genotyped by PCR using their tail DNA. Tail DNA was prepared by digestion with 20 μg/ml proteinase K (Wako 160–14006). PCR analyses were performed using primers sets specific to the wild type and targeted alleles.

Primer sequences for Id1 were as follows; pr-22 (common oligonucleotide), 5′-cctcagcgacacaagatgcgatcg-3′; pr-k4 (wild type oligonucleotide), 5′-ggttgcttttgaacgttctgaacc-3′; pr-pgk (mutant oligonucleotide), 5′-gcacgagactagtgagacgtg-3′. PCR cycling conditions were 90°C for 30 s, 57°C for 30 s, and 65°C for 3 min, for 40 cycles. Primer sequences for Id3 were as follows; C1 (wild type oligonucleotide), 5′-gtccttctctcgggctccaggtcc-3′; Wt3 (wild type oligonucleotide), 5′-gagctcactccggaactgtatc-3′; C3 (mutant oligonucleotide), 5′-gcgtgtgctagctcttcaggccac-3′; Mut5 (mutant oligonucleotide), 5′-gcacgagactagtgagacgtgcta-3′. PCR conditions for Id3 were 95°C for 40 s, 60°C for 45 s, and 70°C for 1 min 30 s, for 39 cycles.

The amplified PCR products were analyzed on agarose gels with marker plasmid (pBR322 DNA-MSP1 Digest, NEB) to separate the wild type (1,000 bp for Id1 and 750 bp for Id3) and targeted allele (800 bp for Id1 and 300 bp for Id3) fragments (Fig. 1). All animal experiments were approved by the animal welfare committee of our institute.

X-Ray Analysis of the Suture

X-ray pictures of the calvariae removed en bloc from the skull bone of 8–9 weeks-old four wild type (Id1+/+Id3+/+) and four Id heterozygote knock out (Id1+/−Id3−/−) mice were taken at 26 kV and 1.5 mA for 2 min by using Softex system (Softex Co., Kanagawa, Japan). Measurement of the area of levels of the opening in the suture gap was conducted on the soft X-ray pictures using Luzex-F automated image analysis system (Nireco, Tokyo, Japan).

Cell Culture

Primary osteoblasts were obtained from the out growth cultures of bone fragments of calvariae taken from 8–9-weeks-old three wild type (Id1+/+Id3+/+) and three Id-mutant (Id1+/−Id3−/−) mice. The cells were cultured in α-minimum essential medium (α-MEM) supplemented with 5% fetal bovine serum. After confluence, the cells were replated at 1.5×10^4 cells/cm^2 into 96-well plates (Costar 3595). One day after plating, the cells were treated with 500 ng/ml recombinant human BMP2 or

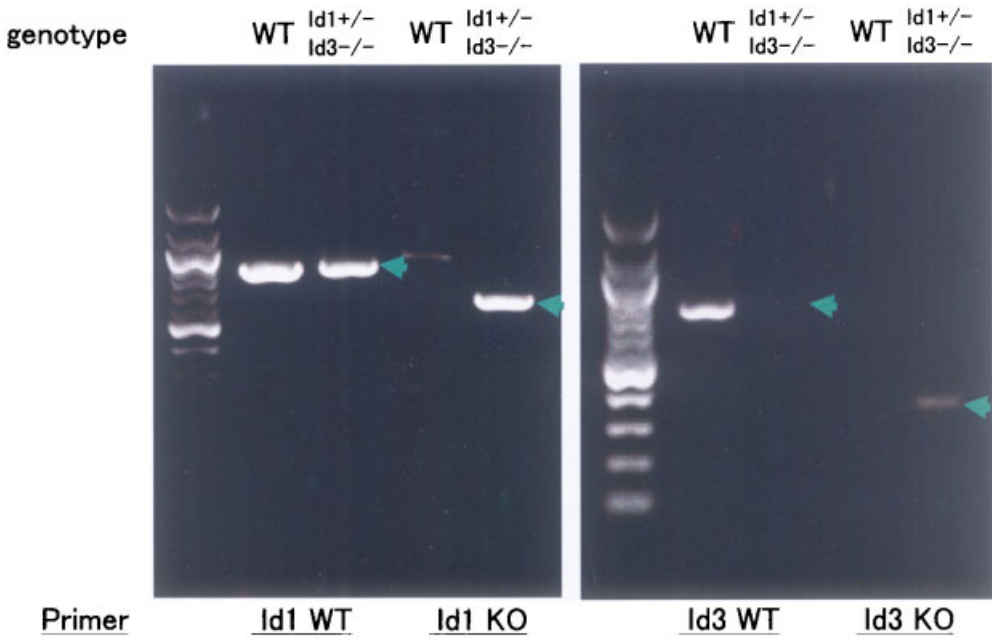

Fig. 1. PCR analyses of heterozygous Id1/Id3 double knock out mice. The amplified PCR products were analyzed on agarose gels with marker plasmid (pBR322 DNA-MSP1 Digest, NEB) to separate the wild type (WT) (1,000 bp for Id1 and 750 bp for Id3) and targeted allele (Id1+/−Id3−/−) (800 bp for Id1 and 300 bp for Id3) fragments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

vehicle for 2 days in the presence of 0.5% FBS and were subjected to 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay [Denizot and Lang, 1986]. Some of the cells were treated with 300 ng/ml recombinant human BMP2 in the presence of 5% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate for 2 weeks and were subjected to staining with 1% alizarin red. Alizarin red positive area in the wells was quantified using an image analyzer (Luzex-F, Nireco).

Semiquantative RT-PCR

Total RNA was prepared from calvarial osteoblasts according to the acid guanidium thiocyanate-phenol chloroform method [Chomczynski and Sacchi, 1987] and reverse transcribed and amplified as follows. To create first-strand cDNA, 1 μg of total RNA was used for reverse transcription. Reactions were performed in a final volume of 20 μl containing 1 μl M-MuLV reverse Transcriptase (200 U) (Gibco Life Tech, Rockville, USA), 4 μl 5× RT buffer, 1 μl dT primer (0.4 mg/ml) (Roche, Basel, Switzerland), 2 μl DTT (100 mM), 1 μl dNTPs (10 mM), 1 μl RNase inhibitor (4 U), and water. The mixture was incubated for 1 h at 37°C. The PCR condition was determined such that the band intensity should be in linear relationship to the increasing amounts of RNA and PCR cycle numbers. The bands were quantitated by densitometry using Bio-profile Version 97 (Vilber Lourmat, Cedex, France) and each value was normalized against that of GAPDH.

Primers used for RT-PCR were as follows; Id1, 5′-tacttggtctgtcggagcaa-3′ (+150 to +169) and 5′-gatcaaaccctctacccact-3 (+663 to +644) (fragment size; 495 bp); Id3, 5′-ccctctctatctctactctc-3′ (+34 to +53) and 5′-gaagagggctgggttaagat-3 (+558 to +539) (fragment size; 525 bp); osteopontin, 5′-cgacgatgatgacgatgatgat-3′ (+255 to +276) and 5′-ctggctttggaacttgcttgac-3 (+746 to + 728) (fragment size; 460 bp); osteocalcin, 5′-ctctgtctctctgacctcacag-3′ (+91 to +112) and 5′-caggtcctaaatagtgataccg-3′ (+834 to +813) (fragment size; 230 bp); p16, 5′-agtccgctgcagacagactg-3′ (+2,464 to +2,483) and 5′-cgggagaaggtagtgggtc-3′ (+7,648 to +7,629) (fragment size; 211 bp); caspase3, 5′-tcatctcgctctggtacgga-3′ (+247 to +266) and 5′-cgtacaaagaccaggaggac-3′ (+1,011 to +992) (fragment size; 765 bp); GAPDH, 5′-accacagtccatgccatcac-3′ (+566 to +585) and 5′-tccaccaccctgttgctgta-3 (+1,017 to +998) (fragment size; 452 bp).

Gene Amp PCR System 9700 (PE Applied Biosystems, Foster City, CA) was used for gene amplification. PCR amplification was performed in a final volume of 25 μl containing

0.2 μl rTaq polymerase (1 U) (Takara, Shiga, Japan), 2.5 μl 10× PCR buffer, 0.5 μl the cDNA obtained from the RT reaction, 0.5 μl of each of the specific primers (10 mM) described above, 2 μl dNTPs (2.5 mM), and 18.8 μl water. PCR cycling condition were 94°C for 40 s, 60°C for 1 min, and 72°C for 1 min. After PCR reactions, the PCR products were separated on a 1% TAE-agarose gel and stained with ethidium bromide. For the reaction of p16 primer, total RNA was reverse transcribed and amplified by using a Titan One-Tube RT-PCR kit (Boehringer Mannheim, Germany). The RT-PCR condition was employed according to the manufacturer's instructions as follows. RT reaction: 58°C for 30 min, 94°C for 2 min; first PCR: 94°C for 30 s, 58°C for 30 s, 68°C for 45 s; second PCR: 94°C for 30 s, 58°C for 30 s, 68°C for 45 + 5 s/cycle.

BMP-Induced Bone Formation In Vivo

BMP injection was conducted according to the method described previously [Noda and Camilliere, 1989]. Six-weeks-old five wild type and three Id mutant (Id1+/−Id3−/−) mice were injected with 5 μg of human recombinant BMP-2 (5 μg/20 μl) onto their calvariae every other day for 10 days and then were sacrificed on the 10th day. In these experiments, calvariae were subjected to X-ray analysis to examine the newly formed bone on the calvaria in response to BMP. The X-ray apparatus and conditions were as described above. The levels of newly formed bone were quantified using an automated image analyzer system.

Microcomputed Tomography Analysis

Calvarial bones were subjected to micro computed tomography (μCT) analysis. The new bone formation on top of the calvaria was evaluated by using the tangential sections and the heights of the new bone formed in response to BMP were evaluated [Usui et al., 2002].

Statistical Analysis

The data were expressed as mean ± SD. Statistical significance of the difference was evaluated by Mann–Whitney's *U*-test.

RESULTS

Because Id1 and Id3 are expressed in most skeletal tissues including cranial bone [Jen et al., 1996], we examined calvaria as site of bone formation in vivo. Id has been reported to be expressed by mesenchymal cells present in some distance from the osteogenic front in the suture during calvaria development [Rice et al., 2000]. In wild type mice, the calvarial sutures were clearly observed on the soft X-ray pictures. In contrast, calvarial sutures in Id heterozygous knock out mice were less obvious on the X-ray pictures (Fig. 2A). Quantification of the suture gap area revealed more than 50% reduction in the suture gap of Id heterozygous knock out mice compared to wild type (Fig. 2B). To obtain insights into the cellular basis for the mechanism of such effects of Id heterozygous knock out, we examined the differentiation in the primary calvarial osteoblasts. The cells were cultured for 2 weeks in the absence or presence of 300 ng/ml rhBMP2, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. Alizarin red staining revealed that mineralized nodules in the cultures of calvarial osteoblasts treated with rhBMP2 were less in the cells from Id heterozygous knock out mice than those in wild type mice whereas those were similar without rhBMP2 (Fig. 3A).

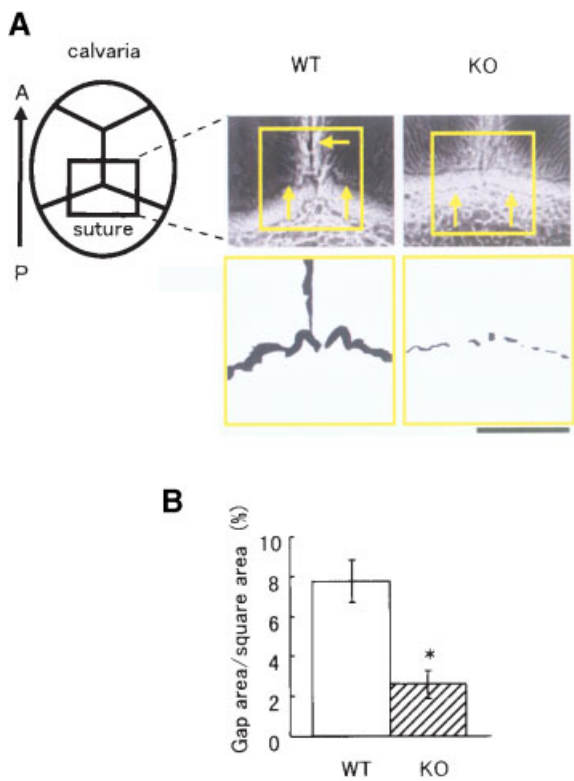

Fig. 2. Id deficiency result in narrowing in the suture gap. **A**: The lambdoid sutures (arrows) were examined by soft X-ray in 8–9 weeks old mice. **B**: Suture gap area within the lambdoid suture within the given region indicated by square in (A) was traced and quantified using an imaging analyzer. (WT: n = 4, KO: n = 4), scale bar = 1 mm, $*P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

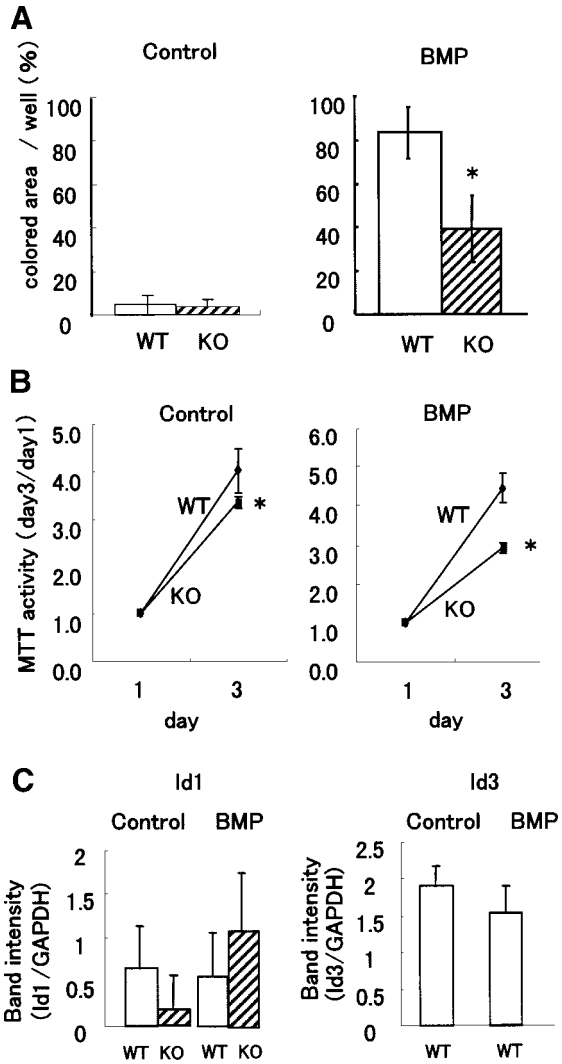

Fig. 3. Id deficiency suppresses mineralization and proliferation in osteoblasts in culture. **A**: Alizarin red staining of the primary calvarial osteoblasts. Calvaria derived osteoblasts from WT and Id-deficient (KO) mice were cultured in the presence of 300 ng/ml BMP2, ascorbic acid, and β-glycerophosphate for 2 weeks. Quantification was conducted by measuring colored area in the alizarin red stained nodules in a well (WT: n = 3, KO: n = 3; eight wells each). **B**: MTT assay of the primary cultures of calvarial osteoblasts. (WT: n = 3, KO: n = 3). Osteoblasts were obtained via outgrowth cultures of the calvaria. The cells were replated into the wells and cultured for the indicated period of time in the absence or presence of 500 ng/ml BMP2. **C**: The expression levels of Id1 and Id3 mRNA were examined based on semiquantative RT-PCR. Total RNA was extracted from primary calvarial osteoblasts cultured for 3 days with or without BMP. The mRNA levels were normalized against those of GAPDH, (WT: n = 3, KO: n = 3). $^*P < 0.05$.

Quantification of the mineralized colored area revealed more than 50% reduction in the mineralization in the cultures of calvarial cells obtained from Id heterozygous knock out mice compared to wild type (Fig. 3A: $83.5 \pm 11.8\%$ vs. $39.2 \pm 15.5\%$, $P < 0.05$). We also examined the effects of Id deficiency on the proliferation of calvaria-derived cells. Id deficiency reduced proliferation of these cells and such reduction was still observed in the presence of BMP (Fig. 3B). There were no significant difference in Id1/Id3 expression levels in the knock outs and wild type after BMP treatment (Fig. 3C).

To address the molecular basis for the Id deficiency effects on bone formation, we examined gene expression profile in the calvarial osteoblasts (Fig. 4). The expressions levels of type I collagen and alkaline phosphatase were similar in the osteoblasts from wild type and Id-deficient mice regardless of the presence or absence of BMP (data not shown). However, those of the genes encoding late osteoblastic differentiation markers, osteopontin and osteocalcin, were significantly reduced in the osteoblasts from Id-deficient mice, whereas the

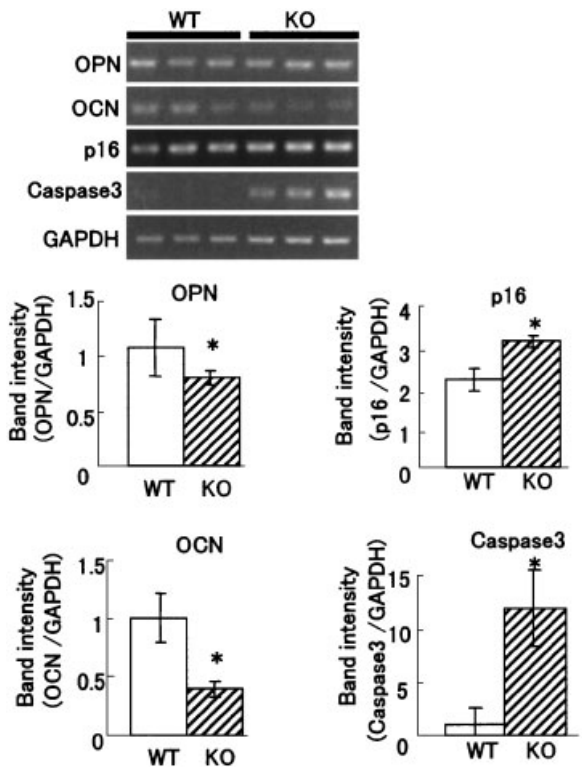

Fig. 4. Id deficiency suppressed expression of genes encoding osteoblast-phenotype-related proteins. Total RNA was extracted from primary calvarial osteoblasts cultured for 3 days and expression levels of genes encoding proteins related to osteoblastic phenotype were examined based on semiquantative RT-PCR. Quantification was conducted as described under ''Materials and Methods.'' The mRNA levels were normalized against those of GAPDH. OCN, osteocalcin; OPN, osteopontin, (WT: n = 3, KO: n = 3). $^*P < 0.05$.

expression levels of p16, a cell-cycle inhibitor, were increased by about 40% in Id heterozygous knock out coinciding with the reduction in proliferation of these osteoblastic cells. The levels of Runx2, one of the important regulators of osteocalcin and osteopontin, were similar between wild type and Id-deficient mice (data not shown).

Furthermore, expression levels of caspase 3, a hallmark of apoptosis, were enhanced by about 10-fold in Id heterozygous knock out mice suggesting Id involvement in the survival of calvarial derived cells.

Finally, we addressed the effect of Id deficiency on in vivo ectopic bone formation in response to stimulation onto the calvaria. For these experiments, recombinant human BMP2 was injected directly in vivo onto the calvaria in wild type and Id heterozygous knock out mice. Ectopic bone formation was induced by recombinant human BMP2 injection in vivo in wild type mice (Fig. 5A, middle). BMP2 injection also induced new bone formation on top of the calvaria in Id-deficient mice with similar μCT images to that observed in wild type (Fig. 5A, bottom). However, quantification of the height of the newly formed bone on the μCT pictures (Fig. 5B) as well as the area based on X-ray pictures (Fig. 5C,D) revealed reduction in Id-deficient mice. These results indicated that

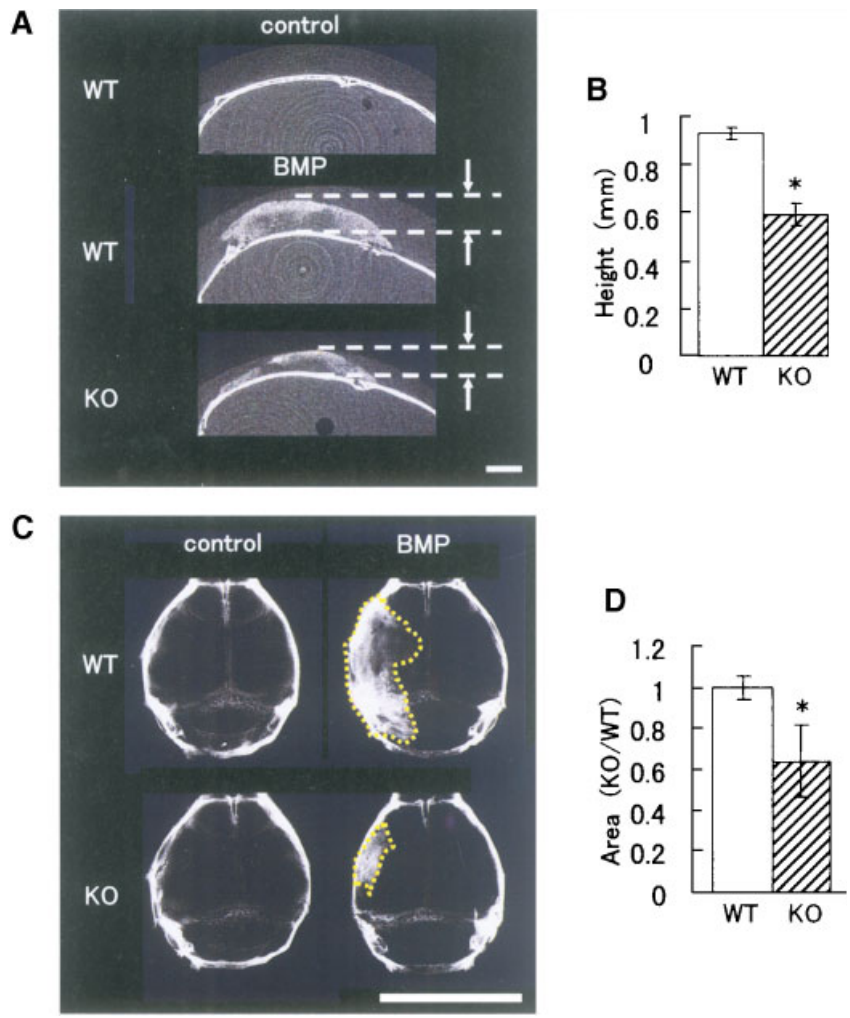

Fig. 5. Id deficiency suppresses BMP induced bone formation in vivo. 250 ng/μl BMP2 was injected onto calvariae in vivo for 10 days in 6-weeks-old WT and KO mice. **A**: Micro CT pictures of the tangential sections of newly formed bone on the calvaria. **B**: Maximum height of the BMP-induced newly formed bone measured using the micro CT pictures. **C**: X-ray pictures of the calvaria. Newly formed bone was observed as radiopaque mass on the calvaria. X-ray pictures were taken from the top of the skull of WT and KO mice. Dotted line indicates the newly formed bone after BMP injection. **D**: The area of the newly formed bone was quantified using an image analyzer. (WT: n = 5, KO: n = 3), scale bar = 1 mm, $*P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

BMP-induced bone formation in vivo is promoted by the presence of *Id* gene.

DISCUSSION

Although Ids were observed to be expressed in cultured bone cells, their function in bone in vivo was not known. We showed that Id1 and Id3 heterozygous knockout mice revealed suppression in BMP-induced new bone formation in calvaria. Our in vivo observation indicated that Ids play a positive role in in vivo bone formation. This is surprising since Id has been implicated in suppression of differentiation in muscle cells as it was observed to inhibit myoD activity in in vitro experiments [Benezra et al., 1990]. In vitro experiments using Id overexpressing cells also indicated suppression of alkaline phosphatase in osteoblasts [Glackin et al., 1992]. Our observations that Ids are required for bone formation in vivo indicated that Ids function as positive but not negative regulators with regard to bone formation in vivo. This could be at least in part due to the Ids effects on the maintenance of proliferation and survival in calvaria derived osteoblasts as we observed in the culture system.

The effects of Id deficiency on skeleton were not obvious in the heterozygous Id-knock out mice without intervention as these mice could grow normally. Id deficiency in some of the isoforms may be compensated by other Ids when the mice were not challenged. In contrast, as shown in the previous reports on Id effects on tumor-induced angiogenesis, Id deficiency effects on bone formation could be detectable when the mice were subjected to interventions such as BMP injection. This intervention possibly induces conditions where compensation may not be enough, such as rapid bone formation.

Recently, Ids were suggested to inhibit the expression of *FGFR3* gene, similarly to the action of another inhibitory HLH transcription factor, Twist [Funato et al., 2001]. Overactivation of FGFR3 signaling caused by Twist mutation results in craniosynostosis, which is characterized by premature fusion of sutures [el Ghouzzi et al., 1997]. However, the expression levels of FGFR3 were similar in the primary calvarial osteoblasts from wild type and Id-deficient mice (data not shown). Whether Id deficiency may enhance suture fusion via the activation of FGFR3 or other pathways is still to be elucidated by examining Id1 and Id3 effects during calvaria development.

Id acts as an inhibitor of muscle cell differentiation in vitro. However, both mineralization and proliferation of calvarial osteoblasts were significantly reduced in Id heterozygous knock out osteoblastic cells. Because the expression of p16 and caspase 3 genes were increased in the osteoblastic cells obtained from Id-deficient mice, osteogenic activity of these cells may be suppressed not only in terms of proliferation but also due to increase in the suppression of survival rate in the absence of Ids. Id regulates cell-cycle, senescence, and apoptosis in many cell types [Yokota and Mori, 2002]. Therefore, reduced mineralization in Id-deficient osteoblasts might be in part due to regression in development in these cells.

Our direct BMP injection experiments indicated that Id is required for BMP-induced bone formation in calvaria in vivo. Id expression was induced by BMP2 in osteoblastic MC3T3E1 cells [Ogata et al., 1993] and by BMP2 beads in developing calvarial mesenchyme [Rice et al., 2000]. These observations indicated that Ids are necessary for both embryonic and adult bone formation in vivo. Id is also induced by BMP in vessels and this Id induction is required for vascularization [Valdimarsdottir et al., 2002]. Therefore, Id could be required for concerted bone formation and angiogenesis during new bone formation induced by BMP2.

In conclusion, we identified that Ids, which have been thought to be inhibitory molecules for mesenchymal cell differentiation, are required for new bone formation in vivo.

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