

A microRNA regulatory mechanism of osteoblast differentiation

Hiroyuki Inose^a, Hiroki Ochi^b, Ayako Kimura^{a,c}, Koji Fujita^{a,c}, Ren Xu^a, Shingo Sato^a, Makiko Iwasaki^a, Satoko Sunamura^d, Yasuhiro Takeuchi^e, Seiji Fukumoto^f, Kuniaki Saito^g, Takashi Nakamura^h, Haruhiko Siomi^g, Hiroshi Ito^d, Yoshiyasu Arai^a, Ken-ichi Shinomiya^{a,c}, and Shu Takeda^{a,d,1}

^aDepartment of Orthopedics, Graduate School, ^cGlobal Center of Excellence Program, and ^bDepartment of Developmental and Regenerative Biology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan; ^bDepartment of Veterinary Science, Faculty of Veterinary Medicine, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino-shi, Tokyo 180-8602, Japan; ^eToranomon Hospital Endocrine Center, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan; ^fDivision of Nephrology and Endocrinology, Department of Internal Medicine, University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan; and ^dSection of Nephrology, Endocrinology and Metabolism, Department of Internal Medicine and ^gDepartment of Molecular Biology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Edited by Clifford J. Tabin, Harvard Medical School, Boston, MA, and approved October 13, 2009 (received for review August 17, 2009)

Growing evidence shows that microRNAs (miRNAs) regulate various developmental and homeostatic events in vertebrates and invertebrates. Osteoblast differentiation is a key step in proper skeletal development and acquisition of bone mass; however, the physiological role of non-coding small RNAs, especially miRNAs, in osteoblast differentiation remains elusive. Here, through comprehensive analysis of miRNAs expression during osteoblast differentiation, we show that miR-206, previously viewed as a muscle-specific miRNA, is a key regulator of this process. miR-206 was expressed in osteoblasts, and its expression decreased over the course of osteoblast differentiation. Overexpression of miR-206 in osteoblasts inhibited their differentiation, and conversely, knock-down of miR-206 expression promoted osteoblast differentiation. In silico analysis and molecular experiments revealed connexin 43 (Cx43), a major gap junction protein in osteoblasts, as a target of miR-206, and restoration of Cx43 expression in miR-206-expressing osteoblasts rescued them from the inhibitory effect of miR-206 on osteoblast differentiation. Finally, transgenic mice expressing miR-206 in osteoblasts developed a low bone mass phenotype due to impaired osteoblast differentiation. Our data show that miRNA is a regulator of osteoblast differentiation.

Connexin43 | miR-206 |

The osteoblast, a cell type of a mesenchymal origin, plays a major role in skeletal development and bone formation (1, 2). Understanding the regulatory mechanism of osteoblast differentiation is a prerequisite for developing strategies to treat bone loss diseases such as osteoporosis (3–5). In the last two decades, progress in molecular and genetic research has uncovered various regulatory processes of osteoblast differentiation (1, 2, 4). Central to this regulation are transcription factors; Runx2, Osterix, and β -catenin are, to date, the transcription factors known to be essential for osteoblast differentiation (2). In addition, while some transcription factors, including C/EBP β , Smad1, and Smad5, bind to Runx2 and enhance its transcriptional activity, others, such as Twist, inhibit Runx2 transcriptional activity (6). However, given the fact that the number of coding genes in vertebrates and invertebrates (which lack a skeleton) is comparable (7), there must be additional mechanisms for controlling skeletal development other than transcriptional regulation of gene expression.

Recently, miRNAs have emerged as important regulators in various developmental, physiological, and pathological conditions such as tumorigenesis, viral infection, and cell differentiation and function (8–10). miRNAs are single-stranded small RNA molecules that are approximately 21 or 22 nucleotides long (9). They do not encode protein; instead, they regulate the level of other proteins by decreasing messenger RNA (mRNA) levels or inhibiting translation by binding the 3'UTR of the target mRNA (8). Surprisingly, non-coding RNA accounts for 98% of

all genomic output in humans (11), and it has been proposed that the proportion of non-coding RNA to protein-coding RNA is correlated with developmental complexity (12).

Previous reports implicated miRNAs in the differentiation of osteoclasts and osteoblasts (13–21). However, their importance in the regulation of osteoblast differentiation *in vivo*, if any, remains to be established. Here we show that one particular miRNA, miR-206, is expressed in the osteoblastic cell lineage and that its expression gradually decreases in parallel with osteoblast differentiation. Interestingly, modulating miR-206 expression in osteoblasts markedly affects their differentiation potential in part by altering the accumulation of connexin 43 (Cx43). Finally, osteoblast-specific expression of miR-206 *in vivo* leads to severe bone loss due to impairment of osteoblast differentiation. Thus, this study reveals a physiological regulatory mechanism of osteoblast differentiation mediated by miRNA.

Results

Identification of miRNAs Whose Expression Varies During Osteoblast Differentiation. To study the potential involvement of miRNAs in osteoblast differentiation, we first attempted to identify miRNAs that are expressed in the osteoblastic cell lineage, particularly miRNAs whose expression is altered during osteoblast differentiation. To that end, we treated multipotent C2C12 mesenchymal progenitor cells with recombinant BMP-2 for 2 days, an established model for studying osteoblast differentiation (22). We then comprehensively analyzed the expression of miRNAs before and after BMP-2 treatment using a microarray that detects all known miRNAs (23). miR-133a was downregulated by BMP-2 treatment (Fig. 1A and Figs. S1 and S2A) as previously reported (13), suggesting that the experiment was properly conducted. Osteoblasts express many miRNAs, and most of the miRNAs (36%) were downregulated by BMP-2 treatment, while only 4% of them were upregulated (Fig. 1A and Fig. S1). Of these, we were interested in miR-206 because its expression was most significantly downregulated during osteoblast differentiation (Fig. 1A). As miR-206 was originally shown to be expressed exclusively in skeletal muscle and heart (24–28), we first verified its expression in the osteoblastic lineage using primary mouse osteoblasts and bones by four different experiments. First, as

Author contributions: Y.T., S.F., T.N., H.S., H. Ito, Y.A., K. Shinomiya, and S.T. designed research; H. Inose, H.O., A.K., K.F., R.X., S. Sato, M.I., S. Sunamura, and K. Saito performed research; H. Inose and S.T. analyzed data; and S.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹To whom correspondence should be addressed. E-mail: shu-tyk@umin.ac.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/090931106/DCSupplemental.

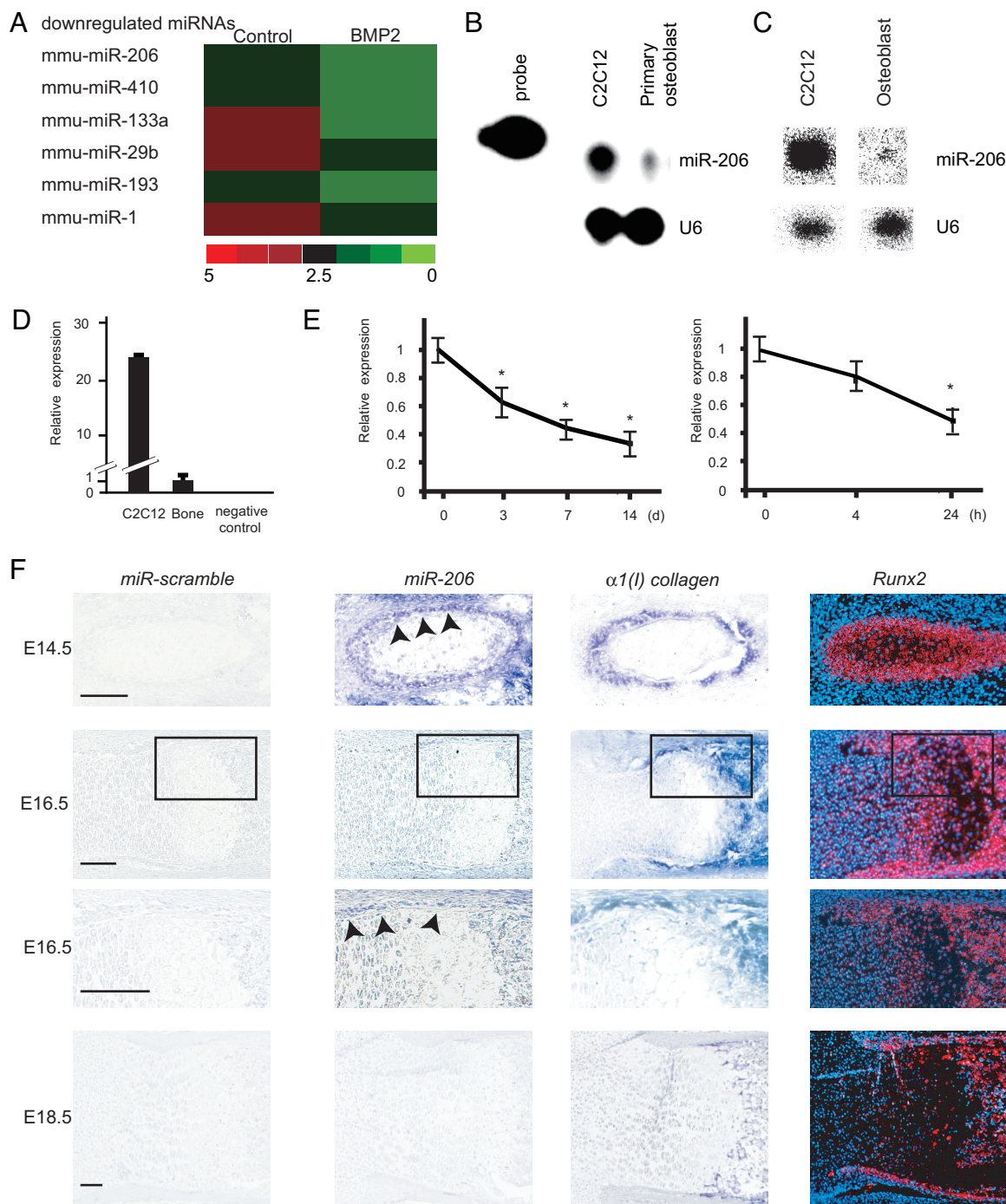


Fig. 1. Expression of miR-206 during osteoblast differentiation. (A) miRNA array expression data from C2C12 cells cultured in growth medium (Control) or in differentiation medium containing BMP-2. Red denotes high expression and green denotes low expression relative to the median; only the representative miRNA nodes that were significantly downregulated in the differentiation medium are shown. (B–D) Expression of miR-206 in osteoblasts: RNase protection assay (B), Northern blot analysis (C), and quantitative RT-PCR analysis (D) to detect miR-206 expression in osteoblasts. Total RNA was isolated from primary mouse osteoblasts, mouse femur or C2C12 cells. U6 RNA was used as a loading control. Note the distinct expression of miR-206 in primary osteoblasts and bone (B–D). (E) Change in miR-206 expression during osteoblast differentiation: quantitative RT-PCR analysis. Mouse primary osteoblasts were treated in differentiation medium (Left) or with the addition of BMP-2 (Right) for each indicated length of time. Note the significant decrease in parallel with the progression of osteoblast differentiation. *, $P < 0.05$ vs. 0 time point, $n = 6$. (F) In situ hybridization analysis of miR-206 expression in mouse embryos: Rib (E14.5, Top) and femur (E16.5, middle and E18.5, Bottom) cryosections. Bottom panel of E16.5 embryo shows the higher magnification of the black rectangular region in the top panel. Adjacent sections were hybridized with *scramble-miRNA* (left), *miR-206* (Middle Left), $\alpha 1(I)$ collagen (Middle Right), and *Runx2* (Right) probes. Note the miR-206 expression in perichondrium osteoblastic cells at E14.5 (arrowheads) and in cells of bone collar at E16.5 (arrowheads). (Scale bar, 100 μm .)

shown by an RNase protection assay, miR-206 was clearly expressed in primary osteoblasts (Fig. 1B). Second, by Northern blot analysis, which is almost 10 times less sensitive than an

RNase protection assay (29, 30), miR-206 was also shown to be expressed in primary osteoblasts (Fig. 1C). Third, by real-time PCR analysis specific to miR-206, we observed miR-206 expres-

sion both in femur and primary osteoblasts (Fig. 1*D* and *E*), and interestingly, miR-206 expression gradually decreased during the course of osteoblast differentiation (Fig. 1*E*). Fourth and most importantly, to investigate the dynamic pattern of miR-206 expression in bone, we performed *in situ* hybridization analysis using DIG-labeled probes. At E14.5, miR-206 was expressed in muscle and perichondrium osteoblastic cells, whose identity was verified by the coexpression of $\alpha 1(I)$ collagen and *Runx2*, markers for osteoblasts (Fig. 1*F*). At E16.5, miR-206 was still expressed in the cells of the bone collar, although the expression was decreased compared to the expression at E14.5 (Fig. 1*F*). At E18.5, miR-206 expression in bone was close to background level (Fig. 1*F*). The gradual decrease of miR-206 expression during skeletogenesis *in vivo* is consistent with its gradual decrease of expression during the course of *in vitro* osteoblast differentiation. To further confirm *in vivo* expression of miR-206 in osteoblasts, we also performed double staining for *in situ* hybridization to detect miR-206 and immunohistochemistry to detect *Runx2*. High resolution confocal microscopic analysis revealed that miR-206 colocalize with *Runx2* in osteoblast (Fig. S3). Taken together, these four independent experiments confirmed that miR-206 is expressed in the osteoblastic cell lineage.

miR-206 Regulates Osteoblast Differentiation. The decrease in the expression of miR-206 during osteoblast differentiation prompted us to test if miR-206 inhibits osteoblast differentiation. To this end, we infected a vector expressing both miR-206 and a blasticidin-resistance gene into C2C12 cells and isolated five stable blasticidin-resistant clones that also express miR-206 to determine if continuous expression of miR-206 affected their ability to differentiate into osteoblasts. As controls, we also infected either an empty vector or a miR-133-expressing vector. C2C12 cells expressing empty vector differentiated normally into the osteoblastic lineage upon BMP-2 treatment. In contrast, osteoblastic differentiation of C2C12 cells expressing miR-133 was significantly impaired (Fig. S2*B*), as previously reported. Importantly, none of the five clones expressing miR-206 differentiated into the osteoblastic lineage, as shown by the lack of induction of alkaline phosphatase activity (Fig. 2*A*). To rule out the possibility that stable expression of miR-206 altered the properties of C2C12 cells, we also transiently transfected a miR-206-expressing vector into C2C12 cells. In this transient DNA transfection assay, miR-206 also repressed osteoblastic differentiation (Fig. 2*B*). To test if miR-206 regulates osteoblast differentiation in a physiological manner, we next used primary mouse osteoblasts because C2C12 is a myogenic cell line. The results showed that continuous expression of miR-206 significantly inhibited osteoblast differentiation as demonstrated by the decrease in alkaline phosphatase activity and *bglap* expression (Fig. 2*C* and Fig. S4). Interestingly, miR-206 expression did not affect *Runx2* mRNA expression, indicating that miR-206 regulates osteoblast differentiation independently of *Runx2* (Fig. 2*C*).

Because it has been shown that expression of miR-206 induces myogenic differentiation (24, 27, 28), we asked whether miR-206 expression induces myogenic transdifferentiation of osteoblasts; however, no expression of myogenic genes such as *MyoD* or *Myf5* (31) was detected, indicating that miR-206 does not induce myogenic differentiation (Fig. 2*C*).

Since overexpression of miR-206 inhibits osteoblast differentiation, we next asked whether decreasing miR-206 expression would accelerate their differentiation. Indeed, knockdown of miR-206 significantly induced osteoblast differentiation (Fig. 2*D*). Taken together, these results demonstrate that miR-206, which is expressed in the osteoblastic cell lineage, physiologically regulates osteoblast differentiation.

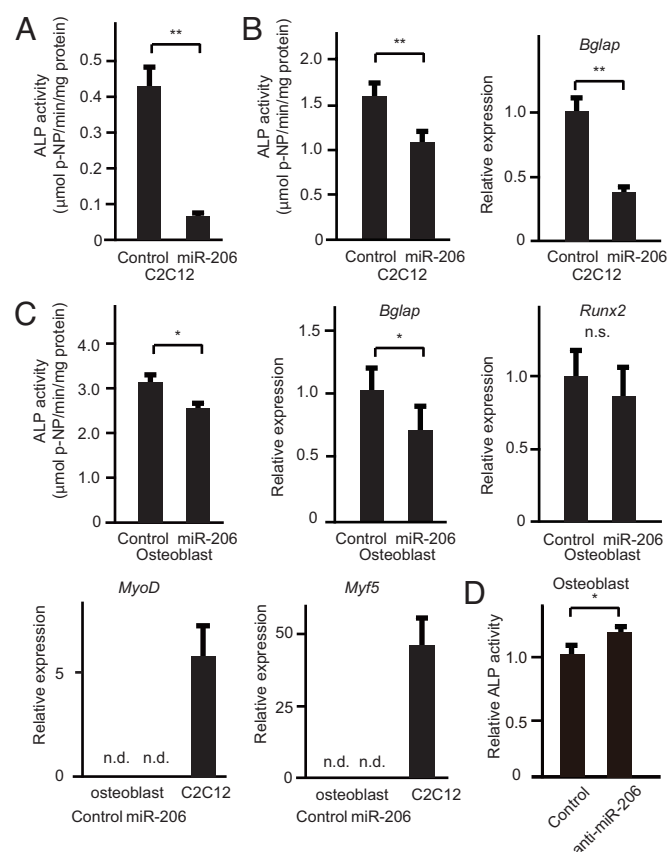


Fig. 2. Regulation of osteoblast differentiation by miR-206. (A and B) Effect of miR-206 expression on BMP-2-dependent C2C12 cell differentiation: C2C12 cells constitutively expressing miR-206 (A) or transiently transfected with miR-206 (B). Alkaline phosphatase activity (A and B, Left) and *bglap* gene expression (B, Right) were analyzed. Note the decreased osteoblastic differentiation in miR-206 expressing cells. **, $P < 0.01$, $n = 6-8$. (C) Effect of miR-206 continuous expression on primary mouse osteoblast differentiation: primary mouse osteoblasts infected with pAd-miR-206 or control adenovirus were cultured. Subsequently, alkaline phosphatase activity assay (Top Left) and quantitative RT-PCR analysis for the indicated genes were analyzed. GAPDH was used as an internal control. n.s., not significant. n.d., not detected. *, $P < 0.05$, $n = 6-8$. (D) Effect of miR-206 knockdown on osteoblast differentiation: osteoblasts were transfected with a miR-206 inhibitor or control. Subsequently, alkaline phosphatase activity was analyzed. Note the significant increase by the miR-206 inhibitor. *, $P < 0.05$, $n = 6$.

Connexin 43 Is One Molecular Target of miR-206 in Osteoblasts. We next studied the molecular mechanism by which miR-206 inhibits osteoblast differentiation. To identify target genes of miR-206, we relied on a computational approach using two different established databases (32, 33). Among the many genes that were predicted to be potential targets by both databases, we focused on *Cx43*, a gap junction protein expressed in osteoblasts that plays a major role in osteoblast differentiation and function; indeed, *Cx43*-deficient mice display low bone mass due to osteoblast dysfunction (34, 35). Two putative target sequences for miR-206 were found in the 3'UTR region of *Cx43* (Fig. 3*A*). At first, we tested if miR-206 regulated *Cx43* expression using a reporter plasmid in which the two putative binding sites of the *Cx43* 3'UTR were cloned into the 3'UTR of the luciferase gene (Fig. 3*B*). As expected from *in silico* analysis, ectopic expression of miR-206 significantly decreased luciferase activity (Fig. 3*B*); furthermore, ectopic expression of miR-206 downregulated endogenous *Cx43* protein expression without affecting *Cx43* mRNA expression (Fig. 3*C*). Taken together, these results identify *Cx43* as a bona fide target of miR-206 *in vivo*.

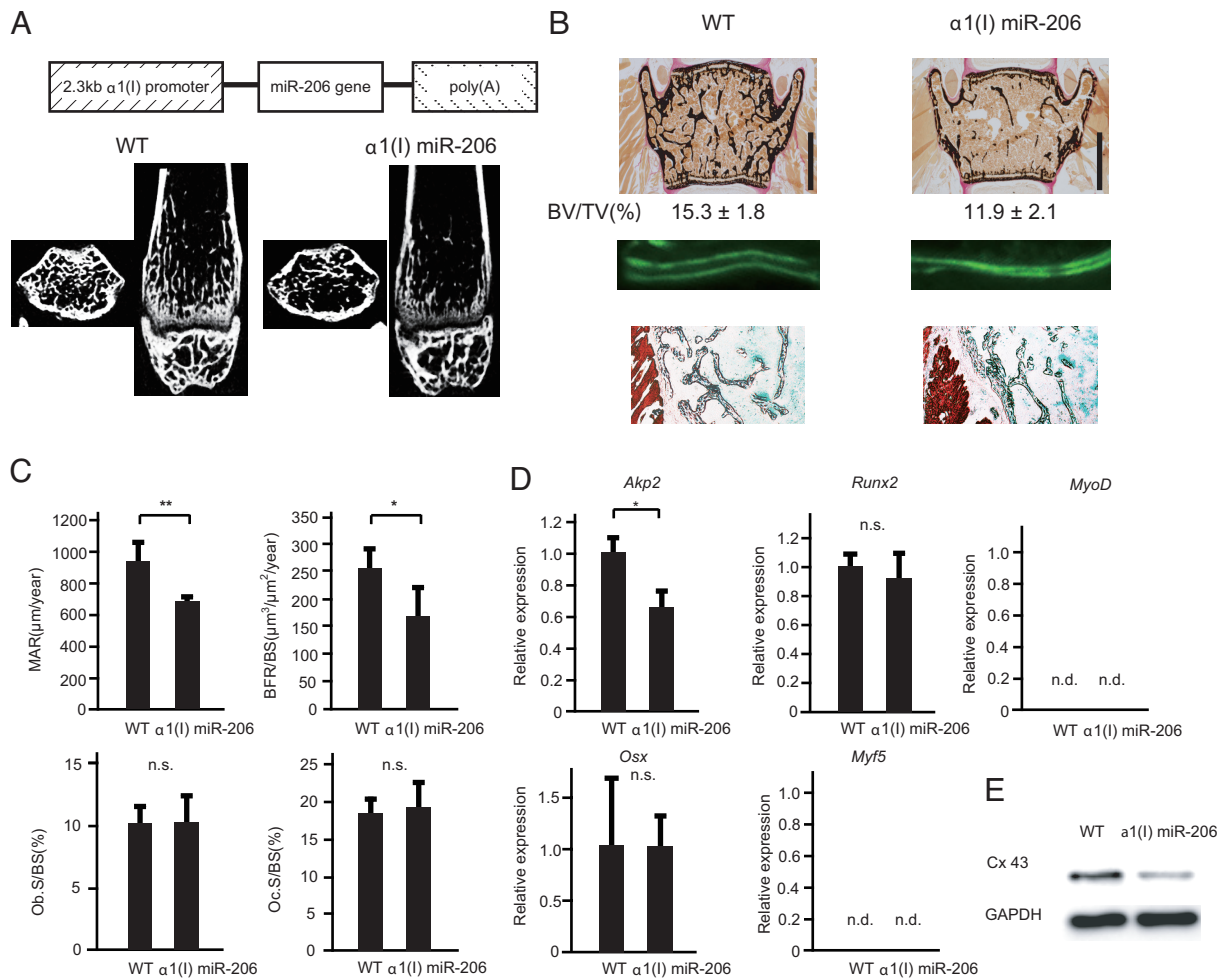


Fig. 4. Low bone mass in $\alpha 1(I)$ miR-206 tg mice due to reduced bone formation. (A) Structure of the construct for osteoblast-specific $\alpha 1(I)$ miR-206 tg mice (Top). μCT analysis of the femurs of 6-week-old female wild-type (WT) or $\alpha 1(I)$ miR-206 tg mice (Bottom). Axial section (Left) and coronal section (Right). (B) Histological analysis of the vertebrae of 6-week-old female WT or $\alpha 1(I)$ miR-206 tg mice. Von Kossa staining (Top). Bone volume per tissue volume (BV/TV). (Scale bars, 1 mm.) The distance between the two calcein labels represents the bone formation rate (middle). Note the significant decrease in bone formation in $\alpha 1(I)$ miR-206 tg mice. Immunohistochemical staining for Troponin I (Bottom). Note the absence of Troponin I immunoreactivity (brown) in the vertebrae of $\alpha 1(I)$ miR-206 tg mice in contrast to the intense staining in skeletal muscle. (C) Histomorphometric analysis of the vertebrae of 6-week-old female mice. Mineral apposition rate (MAR), bone formation rate over bone surface area (BFR/BS), osteoblast surface area over bone surface area (Ob.S/BS), osteoclast surface area over bone surface area (Oc.S/BS). n.s., not significant. *, $P < 0.05$, **, $P < 0.01$, $n = 6-7$. (D) Gene expression in $\alpha 1(I)$ miR-206 tg mice. Quantitative RT-PCR analysis of osteoblastic genes (*Akp2*, *Runx2*, and *Osx*) and myogenic genes (*Myf5*, *MyoD*). Primary calvarial osteoblasts were isolated from WT or $\alpha 1(I)$ miR-206 tg mice and used for subsequent analyses. n.s.: not significant. *, $P < 0.05$, $n = 6$. (E) Western blot analysis of calvarial bones of 2-week-old female mice. Note the decrease in Cx43 protein expression in $\alpha 1(I)$ miR-206 tg mice.

accelerated their differentiation demonstrates that miR-206 is expressed in osteoblasts and plays a role in their differentiation.

Muscle-specific miRNAs comprise a well-defined family consisting of miR-1, miR-133, and miR-206. Interestingly, while miR-1 and -133 are expressed in *Drosophila* and vertebrates, miR-206 is not expressed in *Drosophila*. Instead, it is only expressed in vertebrates. This suggests that miR-206 evolved at a different period from miR-1 and miR-133 and thus may play a role other than the regulation of myogenic differentiation exhibited by miR-1 and miR-133.

Our observation that the inhibitory effect of miR-206 on osteoblast differentiation was rescued by the restoration of Cx43 suggests that Cx43 is a bona fide target of miR-206 in osteoblast differentiation. Indeed, while miR-206-expressing osteoblasts have a defect in osteoblast differentiation, they do not show any proliferative abnormality. This result is consistent with the normal proliferation of Cx43-deficient osteoblasts (34, 35). However, given that a miRNA can regulate multiple target genes

(8), the effect of miR-206 may not depend solely on Cx43. Indeed, although osteoblast-specific Cx43-deficient mice have a normal mineral apposition rate (34), $\alpha 1(I)$ miR-206 tg mice have a 30% decrease in the same parameter, suggesting the involvement of other molecules in miR-206-mediated bone formation defects.

Interestingly, parathyroid hormone (PTH), a well-known regulator of osteoblast differentiation, has been shown to regulate Cx43 expression through a posttranscriptional modification of Cx43 mRNA (38), and the anabolic response of PTH is attenuated in Cx43-deficient mice (34). Because miR-206 regulates both osteoblast differentiation and Cx43 mRNA stability, we tested whether PTH regulates Cx43 through a modification of miR-206 expression. However, PTH did not affect miR-206 expression.

It is interesting that miR-206 is strongly expressed in perichondrium, whereas its expression in trabecular bone is less (Fig. 1F). Considering that osteoblasts are derived from immature

osteoprogenitor cells located in the perichondrium (39), strong expression of miR-206 in the perichondrium suggests that miR-206 may work to keep osteoblast immature and decrease of miR-206 expression is important for proper osteoblastic differentiation, which is in agreement with our *in vitro* observations. Currently, the molecular mechanism accounting for the down-regulation of miR-206 expression during osteoblast differentiation is unknown. Because miR-206 is expressed in myogenic (24–28), adipocytic (40), and osteoblastic cells, all of mesenchymal origin, and miR-206 regulates both myogenic and osteoblastic differentiation, it is tempting to hypothesize that transcription factors involved in the differentiation of mesenchymal stem cells into specific cell lineages also regulate miR-206 expression. In this context, myogenic factors were shown to bind the upstream sequences of miR-206 (26). Therefore, it is possible that essential transcription factors for osteoblast differentiation, such as Runx2 and Osterix, also regulate miR-206 expression. Indeed, there are many putative binding sites for these factors in the sequence upstream of miR-206.

In conclusion, we demonstrated a regulatory role of miRNA in osteoblast differentiation *in vivo*. From a clinical point of view, inhibiting miRNA expression (41) may lead to therapies for bone degenerative diseases such as osteoporosis.

Materials and Methods

Cell Culture, Microarrays, Alkaline Phosphatase Assay, and Dual-Luciferase Reporter Assay. Primary osteoblast and C2C12 cells were cultured and alkaline phosphatase activity (Wako, LabAssay ALP) was measured as previously described (42). Microarray analysis was performed as previously described (23).

- Karsenty G, Kronenberg HM, Settembre C (2009) Genetic control of bone formation. *Annu Rev Cell Dev Biol*, in press.
- Komori T (2006) Regulation of osteoblast differentiation by transcription factors. *J Cell Biochem* 99:1233–1239.
- Khosla S, Westendorf JJ, Oursler MJ (2008) Building bone to reverse osteoporosis and repair fractures. *J Clin Invest* 118:421–428.
- Rosen CJ (2005) Clinical practice. Postmenopausal osteoporosis. *N Engl J Med* 353:595–603.
- Sambrook P, Cooper C (2006) Osteoporosis. *Lancet* 367:2010–2018.
- Franceschi RT, Ge C, Xiao G, Roca H, Jiang D (2007) Transcriptional regulation of osteoblasts. *Ann N Y Acad Sci* 1116:196–207.
- Venter JC, et al. (2001) The sequence of the human genome. *Science* 291:1304–1351.
- Hobert O (2008) Gene regulation by transcription factors and microRNAs. *Science* 319:1785–1786.
- Kloosterman WP, Plasterk RH (2006) The diverse functions of microRNAs in animal development and disease. *Dev Cell* 11:441–450.
- Stefani G, Slack FJ (2008) Small non-coding RNAs in animal development. *Nat Rev Mol Cell Biol* 9:219–230.
- Mattick JS (2001) Non-coding RNAs: The architects of eukaryotic complexity. *EMBO Rep* 2:986–991.
- Mattick JS, Makunin IV (2006) Non-coding RNA. *Hum Mol Genet* 15:R17–29.
- Li Z, et al. (2008) A microRNA signature for a BMP2-induced osteoblast lineage commitment program. *Proc Natl Acad Sci USA* 105:13906–13911.
- Luzi E, et al. (2008) Osteogenic differentiation of human adipose tissue-derived stem cells is modulated by the miR-26a targeting of the SMAD1 transcription factor. *J Bone Miner Res* 23:287–295.
- Mizuno Y, et al. (2008) miR-125b inhibits osteoblastic differentiation by down-regulation of cell proliferation. *Biochem Biophys Res Commun* 368:267–272.
- Kobayashi T, et al. (2008) Dicer-dependent pathways regulate chondrocyte proliferation and differentiation. *Proc Natl Acad Sci USA* 105:1949–1954.
- Davis BN, Hilyard AC, Lagna G, Hata A (2008) SMAD proteins control DROSHA-mediated microRNA maturation. *Nature* 454:56–61.
- Tuddenham L, et al. (2006) The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells. *FEBS Lett* 580:4214–4217.
- Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ (2005) The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proc Natl Acad Sci USA* 102:10898–10903.
- Watanabe T, et al. (2008) Dnm3os, a non-coding RNA, is required for normal growth and skeletal development in mice. *Dev Dyn* 237:3738–3748.
- Sugatani T, Hruska KA (2007) MicroRNA-223 is a key factor in osteoclast differentiation. *J Cell Biochem* 101:996–999.
- Katagiri T, et al. (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *J Cell Biol* 127:1755–1766.
- Hohjoh H, Fukushima T (2007) Marked change in microRNA expression during neuronal differentiation of human teratocarcinoma Ntera2D1 and mouse embryonal carcinoma P19 cells. *Biochem Biophys Res Commun* 362:360–367.
- Kim HK, Lee YS, Sivaprasad U, Malhotra A, Dutta A (2006) Muscle-specific microRNA miR-206 promotes muscle differentiation. *J Cell Biol* 174:677–687.

The activities of luciferase were determined by the dual-luciferase reporter assay (Promega). Further details are provided in the *SI Text*.

Cloning and Gene Expression. Genomic fragments of miR-206 precursors were amplified by PCR. miRNA expression was detected by an RNase protection assay using a mirVANA miRNA kit (Ambion) or quantitative RT-PCR with Mx3000P (Stratagene). Northern blot analysis was performed as previously reported (43). 3'-UTR of Cx43 was subcloned into downstream of the luciferase gene for Cx43-3'-UTR reporter construction.

Western Blot Analysis, Immunohistochemistry, and *In Situ* Hybridization. Western blot analysis and immunohistochemistry were performed according to a standard protocol (42). *In situ* hybridization was performed using DIG labeled probe [miR-206, miR-scramble and $\alpha 1(I)$ collagen] and ^{35}S -labeled riboprobe (Runx2) as reported in ref. 44 with modifications. Further details are provided in the *SI Text*.

Transgenic Mice, Histology, and Histomorphometry. The genomic fragment of the miR-206 precursor was cloned into a plasmid containing a 2.3-kb $\alpha 1(I)$ collagen promoter and microinjected as described in ref. 37. We performed histomorphometric analysis using the Osteomeasure System (Osteometrics) as described in ref. 42. Further details are provided in the *SI Text*.

Statistics. All data are presented as means \pm SE. ($n \geq 6$). We performed statistical analysis by Student's *t* test, and $P < 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS. We thank Dr. Gerard Karsenty for discussion and Takako Usami for technical assistance. This work was supported by the Japan Society for the Promotion of Science grants (to Y.A., K.S., and S.T.) and Uehara Memorial Foundation grant (to S.T.).

- Politz JC, Zhang F, Pederson T (2006) MicroRNA-206 colocalizes with ribosome-rich regions in both the nucleolus and cytoplasm of rat myogenic cells. *Proc Natl Acad Sci USA* 103:18957–18962.
- Rao PK, Kumar RM, Farkhondeh M, Baskerville S, Lodish HF (2006) Myogenic factors that regulate expression of muscle-specific microRNAs. *Proc Natl Acad Sci USA* 103:8721–8726.
- Rosenberg MI, Georges SA, Asawachaicharn A, Analau E, Tapscott SJ (2006) MyoD inhibits Fstl1 and Utrn expression by inducing transcription of miR-206. *J Cell Biol* 175:77–85.
- Sweetman D, et al. (2008) Specific requirements of MRFs for the expression of muscle specific microRNAs, miR-1, miR-206, and miR-133. *Dev Biol* 321:491–499.
- Sambrook J, Fritsch EF, Maniatis T (1989) in *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp 7.2–7.87.
- Reue K (1998) mRNA quantitation techniques: Considerations for experimental design and application. *J Nutr* 128:2038–2044.
- McKinsey TA, Zhang CL, Olson EN (2002) Signaling chromatin to make muscle. *Curr Opin Cell Biol* 14:763–772.
- Krek A, et al. (2005) Combinatorial microRNA target predictions. *Nat Genet* 37:495–500.
- Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120:15–20.
- Chung DJ, et al. (2006) Low peak bone mass and attenuated anabolic response to parathyroid hormone in mice with an osteoblast-specific deletion of connexin43. *J Cell Sci* 119:4187–4198.
- Lecanda F, et al. (2000) Connexin43 deficiency causes delayed ossification, craniofacial abnormalities, and osteoblast dysfunction. *J Cell Biol* 151:931–944.
- Tiso N, et al. (1997) Fine mapping of five human skeletal muscle genes: alpha-tropomyosin, beta-tropomyosin, troponin-I slow-twitch, troponin-I fast-twitch, and troponin-C fast. *Biochem Biophys Res Commun* 230:347–350.
- Dacquín R, Starbuck M, Schinke T, Karsenty G (2002) Mouse alpha1(I)-collagen promoter is the best known promoter to drive efficient Cre recombinase expression in osteoblast. *Dev Dyn* 224:245–251.
- Mitchell JA, Ou C, Chen Z, Nishimura T, Lye SJ (2001) Parathyroid hormone-induced up-regulation of connexin-43 messenger ribonucleic acid (mRNA) is mediated by sequences within both the promoter and the 3' untranslated region of the mRNA. *Endocrinology* 142:907–915.
- Caplan AI, Pechak DG (1987) in *Bone and Mineral Research*, ed Peck WA (Elsevier, New York), pp 117–183.
- Walden TB, Timmons JA, Keller P, Nedergaard J, Cannon B (2009) Distinct expression of muscle-specific MicroRNAs (myomirs) in brown adipocytes. *J Cell Physiol* 218:444–449.
- Care A, et al. (2007) MicroRNA-133 controls cardiac hypertrophy. *Nat Med* 13:613–618.
- Sato S, et al. (2007) Central control of bone remodeling by neuromedin U. *Nat Med* 13:1234–1240.
- Saito K, et al. (2006) Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome. *Genes Dev* 20:2214–2222.
- Obernosterer G, Martinez J, Alenius M (2007) Locked nucleic acid-based *in situ* detection of microRNAs in mouse tissue sections. *Nat Protoc* 2:1508–1514.