Fibroblasts expressing Sonic hedgehog induce osteoblast differentiation and ectopic bone formation

Naoki Kinto[a,f], Masahiro Iwamoto[c], Motomi Enomoto-Iwamoto[d], Sumihare Noji[c], Hideyo Ohuchi[e], Hidefumi Yoshioka[e], Hiroko Kataoka[a], Yasuhiro Wada[a,b], Gao Yuhao[a], Hideaki E. Takahashi[f], Shusaku Yoshiki[a], Akira Yamaguchi[a,*]

[a] *Department of Oral Pathology, School of Dentistry, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan*
[b] *Department of Orthodontics, School of Dentistry, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan*
[c] *Department of Anatomy and Cell Biology, Osaka University Faculty of Dentistry, Suita 565, Japan*
[d] *Department of Biochemistry, Osaka University Faculty of Dentistry, Suita 565, Japan*
[e] *Department of Biological Science and Technology, Faculty of Engineering, University of Tokushima, Tokushima 770, Japan*
[f] *Department of Orthopedic Surgery, School of Medicine, Niigata University, Niigata 951, Japan*

Received 6 December 1996; revised version received 26 December 1996

Abstract We investigated the role of Sonic hedgehog (SHH) in osteoblast differentiation and bone formation. The numbers of ALP-positive cells in the mouse fibroblastic cell line C3H10T1/2 and the mouse osteoblastic cell line MC3T3-E1 were increased by co-culture with chicken fibroblasts transfected with chicken *Shh* cDNA encoding amino-terminal peptide (*Shh-N*). The conditioned medium of *Shh-N*-RCAS-transfected chicken fibroblast cultures also significantly increased ALP activity in both C3H10T1/2 and MC3T3-E1 cells. Intramuscular transplantation of *Shh-N*-RCAS-transfected chicken fibroblasts into athymic mice induced ectopic bone formation. These results indicate that SHH induces osteoblast differentiation and ectopic bone formation.

Key words: Sonic hedgehog; Osteoblast differentiation; Bone formation

1. Introduction

Osteoblasts are derived from the common progenitors of other mesenchymal cell lineages such as chondrocytes, adipocytes and muscle cells. We and others have demonstrated that bone morphogenetic proteins (BMPs) belonging to the TGF-β superfamily play an important role in regulation of the differentiation pathway of these cell lineages [1]. For example, BMP-2 stimulates differentiation of osteoblasts [2–5] and chondroblasts [6], but inhibits differentiation of muscle cells [3,4].

The gene *hedgehog* (hh) is a segment polarity gene regulating embryonic segmentation and patterning in *Drosophila* and is highly conserved in vertebrates [7]. In higher vertebrates, the *Hedgehog* gene family consists of at least three members, *Sonic*, *Indian*, and *Desert hedgehog* (*Shh, Ihh*, and *Dhh*) [7]. SHH has multiple functions during formation of various organs and tissues including skeletal formation in limbs and vertebrae [8,9]. Bitgood and McMahon [10] first reported that *Ihh* is expressed in cartilage during skeletogenesis in the mouse embryo. Recently, Vortkamp et al. [11] demonstrated that *Ihh* regulated chondrocyte differentiation in chicken embryos. In the present study, we investigated the role of *Shh* in osteoblast differentiation using an in vitro culture system and in vivo transplantation experiments.

2. Materials and methods

2.1. Construction and expression of amino-terminal peptides of Sonic hedgehog

A fragment of chicken *Shh* cDNA encoding the amino-terminal functional domain [12] was subcloned into RCAS(A) retrovirus [13] with insertion of a stop codon at residue 200 (designated as *Shh-N*-RCAS). Virus-free chicken embryonic fibroblasts were transfected with *Shh-N*-RCAS by the calcium phosphate precipitation method [14]. These transfected cells were used in the following experiments except for Western blot analysis. The virus-free chicken embryonic fibroblasts infected with recombinant virus in the presence of 1 mg/ml polybrene was used for Western blot analysis.

2.2. Cell culture

Chicken embryonic fibroblasts were obtained from the torsos of 11-day-old virus-free white leghorn embryos (line M) (Nisseiken, Yamanashi, Japan). The chicken fibroblasts and that transfected with *Shh-N*-RCAS or RCAS were cultured in medium 199 (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) without antibiotics (control medium). The mouse clonal cell line C3H10T1/2 clone 8 (C3H10T1/2) [15] was obtained from the RIKEN Cell Bank (Tsukuba Science City, Japan). This pluripotent fibroblastic cell line has the potential to differentiates into muscles, adipocytes, chondrocytes or osteoblasts by treatment with either 5-azacytidine or BMPs [2,6,15]. The mouse osteoblastic cell line MC3T3-E1 [16] was provided by Dr. Masayoshi Kumegawa (Meikai University, Saitama, Japan). To investigate the interaction between *Shh-N*-RCAS-transfected chicken fibroblasts and C3H10T1/2 or MC3T3-E1 cells, these cells were cultured in the same wells separated with cell culture inserts (Becton Dickinson Labware, Franklin Lakes, NJ) with membrane filters (0.45 mm). *Shh-N*-RCAS- or RCAS-transfected chicken fibroblasts were first cultured in 24-well plates, then C3H10T1/2 or MC3T3-E1 cells were inoculated onto the membrane filters of cell culture inserts. These cultures were maintained with control medium.

2.3. Western blot analysis

To enrich SHH-N from the culture supernatants, we used heparin-agarose as described previously [17]. Western blotting was performed with an affinity-purified antibody raised against the N-terminal residues (103–170) of chicken SHH (SHH-N). Detection of SHH-N was carried out by chemiluminescence (ECL, Amersham) with a horseradish peroxidase-conjugated secondary antibody against rabbit IgG. Total protein concentrations were determined with the BCA assay (Pierce) according to the manufacturer's instructions.

2.4. Transplantation of Shh-N-RCAS-transfected cells

Shh-N-RCAS- or RCAS-transfected chicken fibroblasts (1×10^6 cells) were cultured for 12 h in 100 μl of type I collagen gel (Nitta Gelatin, Osaka, Japan) supplemented with control medium. The collagen gel containing *Shh-N*-RCAS- or RCAS-transfected cells was

*Corresponding author. Fax: (81) (3) 3784-2870.
E-mail: akirayam@dent.showa-u.ac.jp

PII S0014-5793(97)00014-8

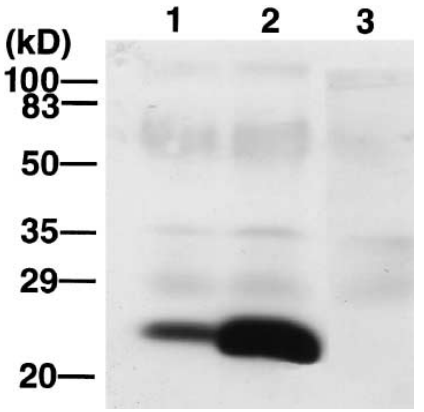

Fig. 1. Production of SHH-N in chick embryo fibroblasts. The antibody directed against an N-terminal domain of chick SHH (residues 103–170) was used for detection. The protein corresponding to the N-terminal cleavage product (SHH-N; approx. 23 kDa) was detected in the culture supernatant from chick embryo fibroblasts infected with a recombinant virus encoding a chick full-length SHH as a control (lane 1) or *SHH-N* alone (lane 2). There is no detectable band in the culture supernatant from chick embryo fibroblasts without infection (lane 3).

transplanted intramuscularly at the inguinal region in 6-week-old athymic male mice. The transplants were removed with the surrounding tissues on 3, 6, 9, 14 and 21 days after transplantation, fixed in 10% neutral buffered Formalin, and embedded in Technovit 8100 (Heraeus Kulzer GmbH, Germany). Undecalcified 4-mm sections were prepared, and stained with hematoxylin-eosin or double stained with alkaline phosphatase (ALP) and von Kossa.

2.5. ALP activity

ALP activity was determined by histochemically and biochemically as described previously [4].

2.6. Statistical analysis

The results were expressed as the means ± S.D. The data were analyzed by one-way ANOVA and differences between means was assessed using the Bonferroni/Dunn multiple comparison test.

3. Results

To obtain cultured cells producing the N-terminal half of the Sonic hedgehog protein (SHH-N) constantly, we used chick embryo fibroblasts infected with an RCAS virus containing a cDNA fragment of *Shh-N*. We confirmed production of SHH-N in the culture supernatant of the fibroblasts infected with *Shh-N*-RCAS virus by Western blot analysis as shown in Fig. 1.

Cultures of C3H10T1/2 cells contained few ALP-positive cells on day 6 when cultured with control medium, but a number of ALP-positive cells were observed in MC3T3-E1 cells under the same culture conditions. To determine the interaction between *Shh-N*-RCAS- or RCAS-transfected chicken fibroblasts and C3H10T1/2 or MC3T3-E1 cells, we cultured these cells separately using membrane inserts in the same culture wells. The numbers of ALP-positive C3H10T1/2 and MC3T3-E1 cells markedly increased, when cultured for 6 days with inserts in the same wells as *Shh-N*-RCAS-transfected chicken fibroblasts (Fig. 2). In contrast, no significant increase in the number of ALP-positive cells was induced by

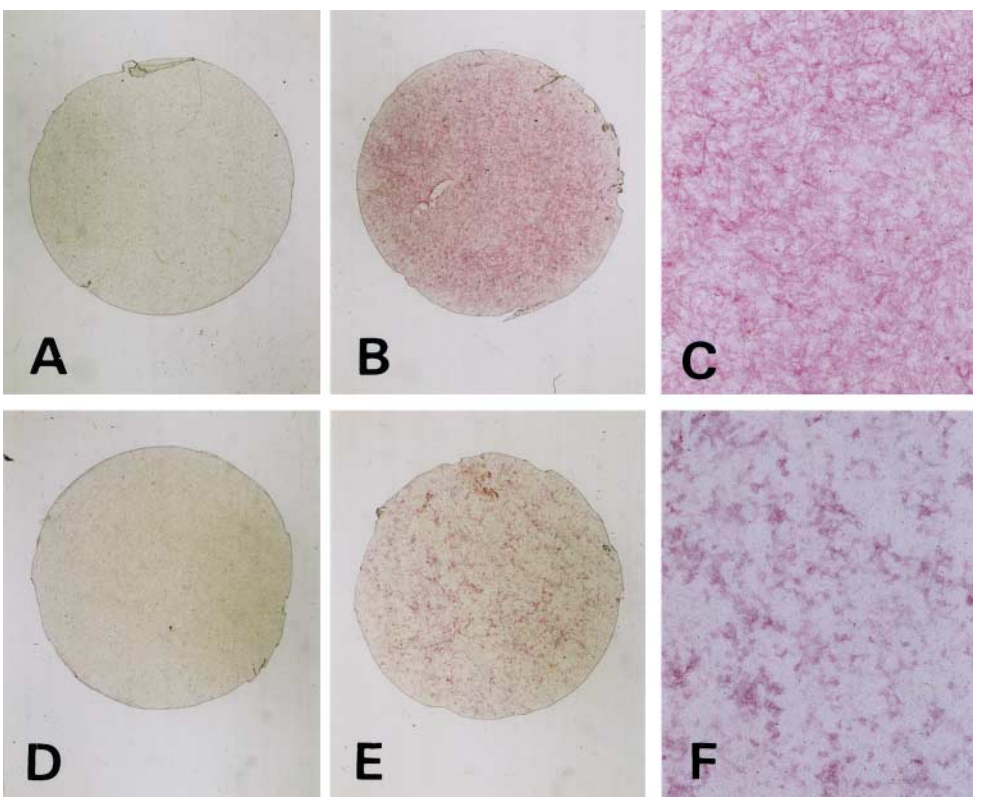

Fig. 2. Effects of co-culture with *Shh-N*-RCAS or RCAS-transfected chicken fibroblasts using cell culture inserts on the number of ALP-positive cells in C3H10T1/2 cells and MC3T3-E1 cells. Cells were cultured for 6 days, then stained with ALP. (A) C3H10T1/2 cells cultured with RCAS-transfected chicken fibroblasts; (B) C3H10T1/2 cells cultured with *Shh-N*-RCAS-transfected chicken fibroblasts; (C) higher magnification of B; (D) MC3T3-E1 cells cultured with RCAS-transfected chicken fibroblasts; (E) MC3T3-E1 cells cultured with *Shh-N*-RCAS-transfected chicken fibroblasts; (F) higher magnification of E. Cells stained in red represent ALP-positive cells. (A,B,D,E) ×5, (C,F) ×50.

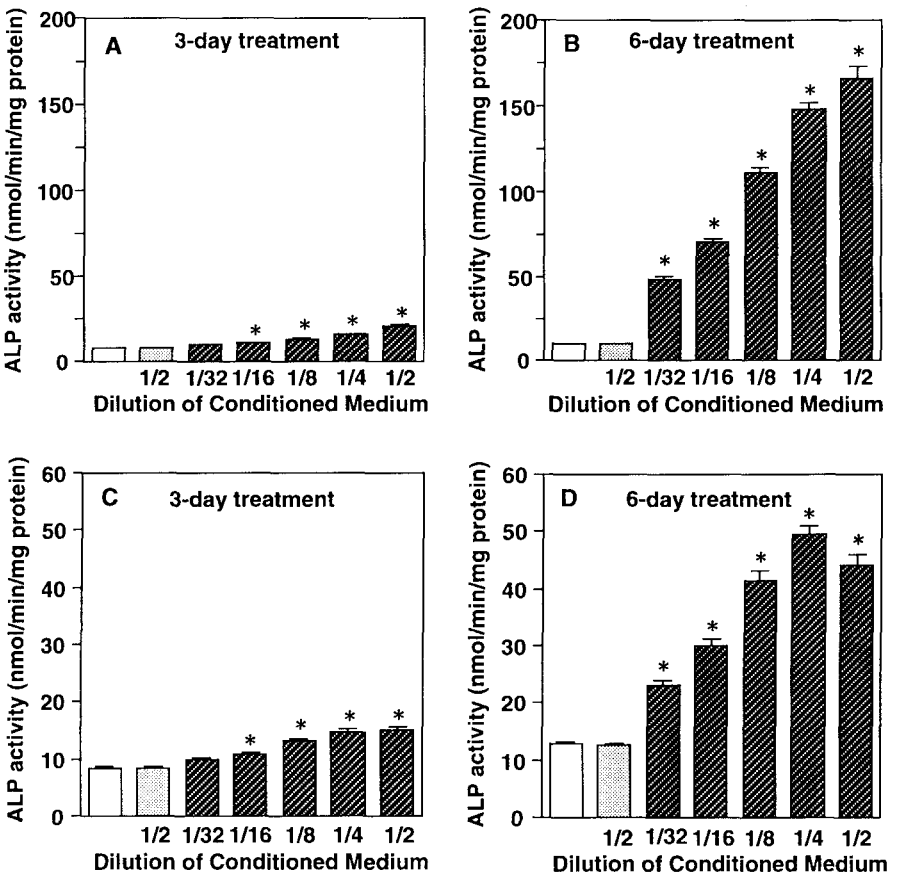

Fig. 3. Effects of conditioned media collected from *Shh-N*-RCAS- or RCAS-transfected chicken fibroblasts on ALP activity in C3H10T1/2 (A,B) and MC3T3-E1 (C,D) cells. Cells were cultured for 3 days (A,C) or 6 days (B,D) with control medium (open bars), conditioned medium collected from RCAS-transfected chicken fibroblasts (stippled bars) or various dilutions of conditioned medium collected from *Shh-N*-RCAS-transfected chicken fibroblasts (hatched bars). Data are means ± S.D. of four culture wells. Significantly different from the cells cultured with conditioned medium of RCAS-transfected chicken fibroblasts in each group at $^{*}P < 0.01$.

co-culture with RCAS-transfected chicken fibroblasts (Fig. 2). No ALP-positive cells appeared in either *Shh-N*-RCAS- or RCAS-transfected chicken fibroblasts. Accumulation of Alcian blue-positive extracellular matrix resembling cartilage matrix did not appear in either C3H10T1/2 or MC3T3-E1 cells.

ALP activity in C3H10T1/2 and MC3T3-E1 cells was significantly increased by addition of the conditioned medium of *Shh-N*-RCAS-transfected chicken fibroblast cultures (Fig. 3). The increase in the ALP activity was abolished by serial dilution of the conditioned medium with control medium (Fig. 3). In contrast, the culture supernatant collected from RCAS-transfected chicken fibroblasts had no effect on ALP activity in either C3H10T1/2 or MC3T3-E1 cells (Fig. 3).

We next performed transplantation experiments to examine whether *Shh-N*-RCAS-transfected chicken fibroblasts could induce ectopic bone formation. The collagen gel containing *Shh-N*-RCAS- or RCAS-transfected cells was retained as well-demarcated islets in muscles. No ALP-positive cells were observed at the surrounding tissue of the transplant of *Shh-N*-RCAS-transfected cells 3 days after transplantation. 6 days after transplantation, several ALP-positive cells appeared at the surrounding tissue of the transplant of *Shh-N*-RCAS-transfected cells. The islet of *Shh-N*-RCAS-transfected cells were almost completely surrounded by numerous ALP-positive cells at 9 days after transplantation (Fig. 4B). Mineralized bones associated with ALP-positive osteoblasts appeared at 14 days after transplantation. The bones became more mature by forming bone marrow at 21 days after transplantation (Fig. 4C). No apparent chondrocytes appeared during the ectopic bone formation induced by transplantation of *Shh-N*-RCAS-transfected cells. Transplantation of RCAS-transfected chicken fibroblasts induced neither ALP-positive cells nor formation of bone and cartilage at the sites transplanted (Fig. 4A). Although dystrophic calcification was observed at the transplantation sites in some cases with both *Shh-N*-RCAS- and RCAS-transfected cells, no ALP-positive cells appeared in the tissues surrounding these calcified foci.

4. Discussion

Osteoblasts express various phenotypes depending on their differentiation stage [18]. ALP appears in a wide range of differentiation stages of osteoblasts. The conditioned medium collected from *Shh-N*-RCAS-transfected chicken fibroblast cultures stimulated ALP activity in both C3H10T1/2 and MC3T3-E1 cells, but no effects were induced by the conditioned medium from RCAS-transfected chicken fibroblast cultures. These results suggested that the SHH protein produced by *Shh-N*-RCAS-transfected chicken fibroblasts induced osteoblast differentiation. This was further supported by transplantation experiments; intramuscular transplantation of *Shh-*

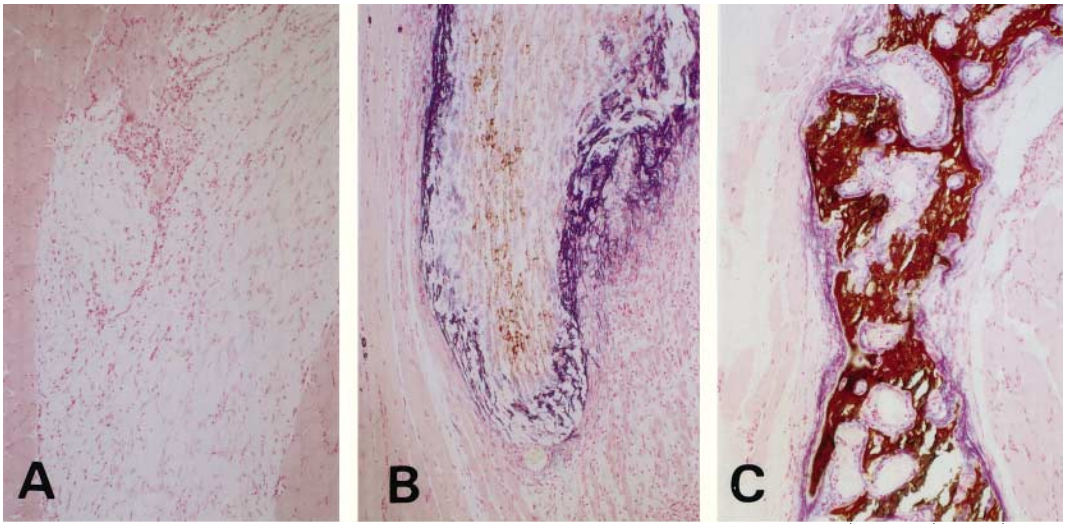

Fig. 4. Histology of transplants of *Shh-N*-RCAS- or RCAS-transfected chicken fibroblasts. Type I collagen gels containing *Shh-N*-RCAS- or RCAS-transfected chicken fibroblasts were transplanted intramuscularly in athymic mice as described in Section 2. (A) Histology of the transplant of RCAS-transfected chicken fibroblasts 9 days after transplantation. (B) Histology of the transplant of *Shh-N*-RCAS-transfected chicken fibroblasts 9 days after transplantation. (C) Histology of the transplant of *Shh-N*-RCAS-transfected chicken fibroblasts 3 weeks after transplantation. These are undecalcified sections stained with ALP and von Kossa. Cells stained in blue represent ALP-positive cells, and matrix stained in brown represents mineralized bone. (A,B) ×50, (C) ×66.

N-RCAS-transfected chicken fibroblasts into athymic mice induced ectopic bone formation.

Recent studies indicated that SHH induced *Bmp* during morphogenesis in several organs [7,19,20]. These suggest that SHH produced by *Shh-N*-RCAS-transfected chicken fibroblasts stimulates synthesis of BMPs in 10T1/2 and MC3T3-E1 cells, then the BMPs induce osteoblast differentiation in an autocrine fashion. However, the conditioned media from *Shh-N*-RCAS-transfected fibroblasts induced no apparent changes in levels of *BMP-2, BMP-4* and *BMP-7* mRNA expression in C3H10T1/2 cells as judged by RT-PCR (unpublished observation). It is unlikely that BMPs derived from chicken fibroblasts are involved in the mechanism of induction of osteoblast differentiation in these cell lines, since the levels of *BMP-2* and *BMP-4* mRNA expression were almost the same between *Shh-N*-RCAS- and RCAS-transfected chicken fibroblasts (unpublished data). However, there is the possibility that Shh regulates the responsibility to these BMPs or induces other types of BMPs in C3H10T1/2 cells and MC3T3-E1 cells. Further studies are necessary to reveal the precise interaction between Shh and BMPs in osteoblast differentiation and bone formation.

Transplantation of BMPs into subcutaneous or intramuscular sites usually induces endochondral bone formation; cartilage formation occurs first followed by bone formation [21]. In the present study, transplantation of *Shh-N*-RCAS-transfected fibroblasts induced bone formation without any histological evidence of preceding cartilage formation. Although BMP-2 and BMP-7 induced C3H10T1/2 cells to differentiate into both osteoblasts and chondrocytes [6,22], the culture supernatant collected from *Shh-N*-RCAS-transfected fibroblasts appeared to induce C3H10T1/2 cells to differentiate into only osteoblasts. Recently, Vortkamp et al. [11] reported an important role of *Ihh* in chondrocyte differentiation. They indicated that the target cells for *Ihh* were located in the perichondrium, where subsequent bone formation occurred, as judged by the expression patterns of the hedgehog receptor gene *Patched* [23,24] and the hedgehog-responding gene *Gli*. These prompt us to speculate that Ihh produced in cartilage may induce adjacent cells to differentiate into osteoblasts. Since SHH and IHH have similar functions [11], it is likely that vertebrate hedgehog proteins induce osteoblast differentiation preferentially over chondrocyte differentiation. It is of considerable interest to investigate molecules induced by SHH or IHH during osteogenesis, and such studies are currently under investigation in our laboratory.

Riddle et al. [8] demonstrated that the implantation of *Shh*-expressing cells into anterior limb buds of chick embryos induced mirror-image duplications associated with formation of extra skeletons. In contrast, Pagan et al. [25] recently reported that surgical removal of *Shh*-expressing cells in polarizing region (zone of polarizing activity: ZPA) resulted in severe pattern defects along anteroposteior axis with skeletal defects. These results suggest that Shh plays important roles in not only the anteroposterior patterning but also skeletogenesis in the developing limb.

References

[1] Yamaguchi, A. (1995) Semin. Cell Biol. 6, 165–173.
[2] Katagiri, T., Yamaguchi, A., Ikeda, T., Yoshiki, S., Wozney, J.M., Rosen, V., Wang, E.A., Tanaka, H., Omura, S. and Suda, T. (1990) Biochem. Biophys. Res. Commun. 172, 295–299.
[3] Yamaguchi, A., Katagiri, T., Ikeda, T., Wozney, J.M., Rosen, V., Wang, E.A., Kahn, A.J., Suda, T. and Yoshiki, S. (1991) J. Cell Biol. 113, 681–687.
[4] Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J.M., Fujisawa-Sehara, A. and Suda, T. (1994) J. Cell Biol. 127, 1755–1766.
[5] Yamaguchi, A., Ishizuya, T., Kintou, N., Wada, Y., Katagiri, T., Wozney, J.M., Rosen, V. and Yoshiki, S. (1996) Biochem. Biophys. Res. Commun. 220, 366–371.
[6] Asahina, I., Sampath, T.K. and Hauschka, P.V. (1996) Exp. Cell Res. 222, 38–47.
[7] Fietz, M., Concordet, J.P., Barbosa, R., Johnson, R., Krauss, S., McMahon, A.P., Tabin, C. and Ingham, P.W. (1994) Development (Suppl.), 43–51.

[8] Riddle, R.D., Johnson, R.L., Laufer, E. and Tabin, C. (1993) Cell. 75, 1401–1416.
[9] Fan, C.-M. and Lavigne, M.T. (1994) Cell. 79, 1175–1186.
[10] Bitgood, M.J. and McMahon, A.P. (1995) Dev. Biol. 172, 126–138.
[11] Vortkamp, A., Lee, K., Lanske, B., Segre, G.V., Kronenberg, H.M. and Tabin, C.J. (1996) Science 273, 613–622.
[12] Porter, J.A., von Kessler, D.P., Ekker, S.C., Young, K.E., Lee, J.J., Moses, K. and Beachy, P.A. (1995) Science 374, 363–366.
[13] Hughes, S.H., Greenhouse, J.J., Petropoulos, C.J. and Sutrave, P. (1987) J. Virol. 61, 3004–3012.
[14] Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752.
[15] Taylor, S.M. and Jones, P.A. (1979) Cell 17, 771–779.
[16] Sudo, J., Kodama, H., Amagai, Y., Yamamoto, S. and Kasai, S. (1983) J. Cell Biol. 96, 191–198.
[17] Lopez-Martinez, A., Chang, D.T., Chiang, C., Porter, J.A., Ros, M.A., Simandl, B.K., Beachy, P.A. and Fallon, J.F. (1995) Current Biology 5, 791–796
[18] Rodan, G.A. and Rodan, S.B. (1984) in: Bone and Mineral Research (Annual 2) (Peck, W.A. ed.) pp. 244–285, Elsevier, Amsterdam.
[19] Francis, P.H., Richardson, M.K., Brickell, P.M. and Tickle, C. (1994) Development 120, 209–218.
[20] Laufer, E., Nelson, C.E., Johnson, R.L., Morgan, B.A. and Tabin, C. (1994) Cell 79, 993–1003.
[21] Wozney, J.M. (1993) in: Cellular and Molecular Biology of Bone (Noda, M. ed.) pp. 131–167, Academic Press, San Diego, CA.
[22] Wang, E.A., Israel, D.I., Kelly, S. and Luxenberg, D.P. (1995) Growth Factors 9, 57–71.
[23] Stone, D.M., Hynes, M., Armanini, M., Swanson, T.A., Gu, Q., Johnson, R.L., Scott, M.P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J.E., De Sauvage, F. and Rosenthal, A. (1996) Nature 384, 129–134.
[24] Marigo, V., Davey, R.A., Zuo, Y., Cunningham, J.M. and Tabin, C. (1996) Nature 384, 176–179.
[25] Pagan, S.M., Ros, M.A., Tabin, C. and Fallon, J.F. (1996) Dev. Biol. 180, 35–40.