Core binding factor α-1 (Cbfa1), known as an essential transcription factor for osteogenic lineage, has two major N-terminal isoforms: Pebp2αA and Til-1. To study the roles of these isoforms in bone regeneration, we applied an adenoviral vector carrying their genes to transduce primary osteoprogenitor cells in vitro and in vivo. Overexpression of the two isoforms induced rapid and marked osteoblast differentiation, with Til-1 being more effective in vitro, by examination of the alkaline phosphatase activity, calcium content, and Alizarin red staining. Til-1 overexpressing cells/porous ceramic composites were transplanted into subcutaneous and bone defect sites in Fischer rats (cultured bone transplantation model) and markedly affected in vivo bone formation and osteoblast markers. The results demonstrated that the reconstitution of bone tissues, such as cortical bone and trabecular bone was accelerated by implantation of Til-1 overexpressing cells/porous ceramic composites. Moreover, the new bone formation by Til-1 overexpression appeared to reflect replacement of new bone within the implant boundaries. To ascertain whether implanted Cbfa1 overexpressing cells could differentiate into osteogenic cells to create bone or whether it stimulated the surrounding recipient tissue to regenerate bone, implanted male donor cells were visualized by fluorescent in situ hybridization analysis. The proportion of implanted cells in the presumptive bone forming region was over 80% and did not change throughout from 3 days to 8 weeks after implantation. These findings suggested that the newly formed bone in the porous area of the scaffold is mostly produced by the implanted donor cells or their derived cells, effectively by Til-1 overexpression.

Core binding factor α-1 (Cbfa1) is an essential transcription factor for the osteogenic/chondrogenic and odontogenic lineages. Several studies have established that Cbfa1 is essential for in vivo bone formation (1–3), maturation of hypertrophic chondrocytes (4, 5), and osteoblast differentiation (6, 7). In addition to the study of Cbfa1 function in osteoblasts by the analysis of Cbfa1 knock-out mice, the effect of Cbfa1 overexpression in mesenchymal stem cells (MSCs) and preosteoblasts has attracted much attention not only from basic biologists but also from clinicians. Because transplantation of genetically engineered non-osteogenic cells with Cbfa1 overexpression into bone defects is expected to be a powerful tool in bone tissue engineering with gene therapy for repair of large bone defects.

As a means of bone regeneration, a technique for the transplantation of cultured bone (8–10) has been developed to overcome the disadvantages of autologous bone grafts and simple implantation of biomaterial into bone defects, problems such as a limited bone supply for bone graft, risk of infection, unsuitability for large bone defects, etc. This technique will be applicable to patients who have lost large segments of bone because of bone tumor etc. The procedure essentially involves the in vivo transplantation of MSC-derived osteoprogenitor cells and porous ceramic composite, to form bone-like tissue at ectopic (subcutaneous and intramuscular) or orthotopic sites, providing tissue engineering to patients with skeletal defects. A representative model of cultured bone transplantation was reviewed by Ogushi et al. (11) and recent examples using novel ceramic materials and culture techniques have been reported by our group (12–17). Briefly, rat bone marrow-derived osteoprogenitor cells and porous ceramic composite were cultured in vitro with osteogenic supplements: dexamethasone, β-glycerophosphate, and ascorbic acid. This was followed by a subcutaneous transplantation resulting in new bone forming porous internal areas in vivo. Before this system can be applied clinically, however, several tasks need to be performed, such as elucidation of the mechanism of in vivo bone formation, improvement of ceramic scaffolds, and the advancement of culture techniques of MSC-derived cells. Above all, it is necessary to shorten the culture period for MSCs in vitro because long-term culture causes MSC-derived cells to cease their lineage progression or to become cancerous.

One approach to accelerating the process of differentiation involves the use of gene therapy in which viral or non-viral vectors are utilized to overexpress osteogenic factors for transplantation. Non-viral vectors are comparatively easy to make, however, they are usually less efficient as delivery vehicles. They also lack the ability to target specific cell types and tissues (18). Among them, adenovirus is a relatively safe vector compared with human immunodeficiency virus-based vectors including retroviral vectors. The rate of infection with adenovirus is also known to be almost 100% for adherent cells, such as osteoblasts. Osteogenic cytokines like bone morphogenetic protein (19–21) and transforming growth fac-
tor-β (22) are expected to be effective as osteogenic factors, however, as a potential intrinsic factor with a key role in osteoblast differentiation, transcription factor is expected to accelerate the differentiation and induce a more effective bone formation in vivo upon their overexpression. Cbfa1, Dlx-5 (23), Bapx1 (24), Msx2 (25), Scleraxis (26), and Sox-9 (27) have been reported to be representative transcription factors related to the osteoblast and chondrocyte lineage. Cbfa1 is a key osteogenic transcription factor and was found to be indispensable for osteoblast differentiation.

Some reports have been published regarding the effect of Cbfa1 overexpression on differentiation of an osteoblast cell line (28, 29), however, several basic research studies using primary cells in vitro and in vivo are needed before its clinical application. First of all, there exist two major N-terminal isoforms of Cbfa1, Pebp2Aα (starting with the sequence MRIPKV) (30) and Til-1 (starting with the sequence MAANNS) (31), each regulated by distinct promoters. Harada et al. (32) reported that the Cbfa1 isoforms had functional differences in osteoblast differentiation. Benerjee et al. (33) reported a clear difference between the two N-terminal isoforms, Pebp2A and Til-1, in response to bone morphogenetic protein-2 during development of the osteoblast phenotype. Previous reports regarding the effects of Cbfa1 overexpression on osteoblast differentiation focused only on Pebp2Aα, however, no examination of the difference between the two N-terminal isoforms has been performed. We hypothesized that overexpression of the Cbfa1 gene promotes the rapid differentiation of osteoblasts in vitro and in vivo and have explored the use of adenoviral vectors carrying the two Cbfa1 isoforms (Pebp2Aα and Til-1, as shown in Fig. 1A) to transduce MSC-derived osteoprogenitor cells. Results suggested that the adenoviral vector induced the transfer of the Cbfa1 gene, markedly stimulating the differentiation of osteoblasts in vitro. Til-1 overexpression, we transplanted Til-1-overexpressing osteoprogenitor cells/porous ceramic composites into ectopic (subcutaneous) and orthotopic (bone defect) sites in Fischer rats (cultured bone transplantation model). The formation of bone was markedly increased by the overexpression, with negligible risk of tumorigenesis during the experimental period. Moreover, for investigating the mechanism of bone formation, we examined the in vivo bone formation in this model by fluorescence in situ hybridization (FISH) of the Y-chromosome of the osteoblastic cells from male Fischer rats transplanted into female rats.

MATERIALS AND METHODS

Cells and Culture—Experiments were performed in accordance with the guidelines of the Japanese Government for the care and use of laboratory animals. Rat bone marrow-derived osteoprogenitor cells were obtained from the femora of male Fischer 344 (7-week-old) rats using the method of Maniatisopoulou et al. (34). The epiphyses of both bone sides were removed and the marrow cavity was flushed out with medium A (minimal essential medium (Nakarai Tesque, Kyoto, Japan) containing 15% fetal bovine serum (Sigma) and antibiotic-antimycotic (Invitrogen) using a syringe. The primary cells were cultured at 37 °C in a humidified atmosphere of 95% air with 5% CO2 until nearly confluent. Cells were trypsinized with 0.05% trypsin and 0.02% EDTA, harvested by low speed centrifugation, and resuspended in fresh medium at a density of 5 × 10^6 cells/ml. The cells were treated with 0.1% trypsin including 0.02% EDTA, and subcultured under the appropriate conditions for the following experiments.

Construction of a Recombinant Adenoviral Vector Carrying the Cbfa1 Gene—The plasmid pSG5TS (mPebp2Aα) and pSG5S (mTil-1) carrying Cbfa1 cDNA were kindly provided by Sumitomo Pharmaceutical Co. Ltd. Two kinds of cDNA encoding mouse Cbfa1/Pebp2Aα (GenBank™ accession number D14636) and Cbfa1/Til-1 (GenBank accession number AF102824) were obtained by digestion with the BamHI and BglII restriction enzymes, respectively. Recombinant adenoviral vectors that carried an expression cassette containing the cytomegalovirus IE enhancer, chicken β-actin promoter, mouse Cbfa1 (Cbfa1/Pebp2α or Cbfa1/Til-1) cDNA, and rabbit β-globin polyA signal were constructed using an Adenovirus Cre/loxP kit (TaKaRa, Shiga, Japan). The ends of these cDNAs were blunted for subcloning into the Sau3A site of the pAXCALNLw cosmid. Each cosmid and adenoviral DNA-terminal complex protein was co-transfected into the E1-transcomplemental cell line 293. The recombinant adenoviruses (Adv-pebp2αA or Adv-til-1) generated by homologous recombination were isolated, and the insertion of each cDNA was confirmed by digestion using restriction enzymes. Two adenoviral vectors, Adv-cre (Cre recombinase) and Adv-β-galactosidase, attached to an Adenovirus Cre/loxP kit, were used accordingly to the host cell and adenoviral infections. Adv-cre was co-transfected with the vector of interest to induce the gene expression.

Alkaline Phosphatase Activity—The cells were seeded at 3 × 10^3 cells/well on 6.5-cm dishes (three dishes for each group) in medium B (medium A containing an osteospecific supplement (10 mM β-glycerophosphate (Sigma), 50 μg/ml ascorbic acid phosphate (Wako, Osaka, Japan), and 10 mM demethasone (Sigma)). Twenty four hours later (day 0), the cells were exposed to Adv-pebp2αA or Adv-til-1 at a multiplicity of infection of 400 and Adv-cre at a multiplicity of infection of 400 in 100 μl of minimal essential medium containing 5% fetal bovine serum for 1 h. Then, 3 ml of medium B was added to dilute the viruses. On the days indicated after infection, cells were detached by low speed centrifugation, and Trs (pH 7.4) containing 1 Triton X-100 and 5 mM MgCl2 by sonication. After centrifugation at 6,000 × g for 10 min, supernatants were collected. Aliquots of 2.5 μl from each sample were added to 250 μl of alkaline phosphatase substrate buffer containing 0.056 mM 2-amino-2-methyl-1,3-propanediol and 16.7 U/ml alkaline phosphatase, and the enzymatic activity of alkaline phosphatase was calculated after measuring the absorption of the p-nitrophenol product at 405 nm on a microplate reader (Bio-Rad). The activity was expressed as millimol of p-nitrophenol/10,000 cells. 

Calcium Content—Ten, 14, and 21 days after infection, cells were fixed with 10% formalin-neutralized buffer for 15 min and extracted with 0.6 M HCl at 4 °C overnight. The calcium content of each sample was determined with a calcium assay kit (Sigma) according to the manufacturer’s instructions.

Alizarin Red Staining—Fourteen and 21 days after infection, cells were fixed with 10% formalin-neutralized buffer for 15 min, stained with 1% Alizarin red (pH 4.0) for 2 min, and then washed with deionized water. For control experiment, the uninfected cells and the cells infected only with Adv-cre were stained.

Northern Blotting—On the days indicated after infection, total RNA was isolated from osteoblast cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. Aliquots (30 μg) of total RNA were separated on a 1% agarose, 5.5% formaldehyde gel and blotted onto Hybond™-XL membrane (Amersham Biosciences). Probes for cDNA encoding mouse Cbfa1 (GenBank accession number AF102824), rat osteocalcin (GenBank accession number X04141), and human GAPDH (GenBank accession number NM002046) were labeled with [32P]dCTP (3000 Ci/mmol, Amersham Biosciences) using Rediprime™ (Amersham Biosciences). After hybridization at 68 °C for 3 h in PerfectHyb™ Plus Hybridization Buffer (Sigma) with the labeled CDNA probe (2 × 10^6 cpm/ml), the membrane was washed with 2 × SSC, 0.1% SDS, and then twice with 0.5 × SSC, 0.1% SDS. The washed blot was exposed to Kodak X-AR film for 1 day.

Cultured Bone Transplantation Model—β-TriCalcium phosphate (β-TCP) cubic blocks (5 × 5 × 5 mm³) and 2 × 2 × 2 mm³ were kindly provided by Olympus Optical Co. Ltd. (Tokyo, Japan). The solid and porous components of the microstructure are well interconnected. The average pore is 200 μm in diameter. After 10 days of primary culture, the cells were treated with 0.25% trypsin and 0.02% EDTA, harvested by low speed centrifugation, and resuspended in fresh medium at a concentration of 2 × 10^6 cells/ml. β-TCP blocks were soaked in the cell suspension and incubated for 2 h in a CO2 incubator. The blocks were transferred to 24-well plates (single block per well) and cultured in medium B for 24 h. The cells/β-TCP composites were incubated in minimal essential medium with 5% fetal bovine serum containing Adv-til-1 (2 × 10^6 plaque-forming units/ml) and Adv-cre (2 × 10^6 plaque-forming units/ml) for 2 h. Then the composites were transferred to fresh plates and cultured for 24 h. Uninfected cells/β-TCP composites were used as a control. As shown in Fig. 3, the composites (5 × 5 × 5 mm³) were implanted subcutaneously in the back of Fischer rats; three infected cells composites were implanted separately on the right side, and control composites were implanted separately on the left side. As an in vivo bone defect model, the lateral side of the distal end of the rat femur shaft was exposed and incised surgically. Bilateral bone defects were
Quantitative RT-PCR—Total RNA was isolated from collected composites using TRIzol reagent. For reverse transcription, the reaction mixture contained 2 μg of RNA, 2.5 μl oligo(dT) primer, and 5 units of avian myeloblastosis virus reverse transcriptase (TaKaRa) in a total volume of 20 μl. The reaction was run for 1 h at 42 °C and stopped by heating for 5 min at 99 °C. Aliquots (0.5 μl) of the reverse transcriptase products were amplified in a reaction mixture (20 μl) containing LightCycler™FastStart DNA Master SYBR Green I, 0.5 μM of each primer, and 3 μM MgCl2 using a LightCycler™ (Roche Molecular Biochemicals). After preincubation at 95 °C for 10 min, a PCR was performed with 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 5 s, and elongation at 72 °C for 10 s. A single fluorescence reading was taken in each cycle following the elongation step. The primers used were as follows: total Cbfa1/Til-1 (exogenous and endogenous Cbfa1 expression, GenBank accession number AF102804), 5′-TCGACTAAGTGCTGCTG-3′ and 5′-TTAGCTCTTGGAATACCTT-3′; c-jun (rat c-jun mRNA, GenBank accession number X17163), 5′-AGTCTCCAG-GACCCGATACAG-3′ and 5′-TTTGTGCTTCAAGGTTTT-3′; c-myc (rat c-myc mRNA, GenBank accession number Y00859), 5′-GACAGATGCCCTCTGTTGGA-3′ and 5′-CTGGCGCTTCCTCCGTAAG-3′; fos (rat c-fos mRNA, GenBank accession number X06769), 5′-GAGGC- CGTCAAGAACATTA-3′ and 5′-GAGGAAACGAGACAGCTC-3′; glyceraldehyde-3-phosphate dehydrogenase (GenBank accession number M32033) as an internal control as described previously (35).

RESULTS

In Vitro Effect of Cbfa1/Til-1 Overexpression in Primary Osteoprogenitor Cells—First, we examined the effect that the overexpression of Cbfa1 has on the differentiation of rat bone marrow-derived primary osteoprogenitor cells, cultured by the methods of Maniotopoulos et al. (34).

Adenovirus-mediated Gene Expression in Primary Osteoblasts—Rat bone marrow-derived osteoprogenitor cells were infected with a recombinant adenovirus encoding the two Cbfa1 isoforms (mouse Pebp2Aa and mouse Til-1) (Fig. 1A). To confirm that the Cbfa1 gene was expressed in these experiments, Northern blotting of Cbfa1/Til-1, c-fos, c-myc, c-jun, and regenerated bone area were calculated, expressed as the arithmetic mean ± S.E., and plotted in the graphs. Parallel t test was used to evaluate the statistical significance of the differences between the experimental and control groups were established at the p < 0.05 (*) and p < 0.01 (**) levels.

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RESULTS
greater than the control value at 10 days. Because the expression of Cbfa1 was maximal at 3 days, the peak in alkaline phosphatase activity at 10 days means that the effect of Cbfa1 on the activity was delayed.

Calcium Content—The effect of overexpression of Cbfa1 in osteoprogenitor cells was found to be remarkable in comparison of calcium content among the three groups as shown in Fig. 2B. The effect began at 10 days after infection and was notable until day 21, the calcium content of Adv-til-1-infected cells being over 10 times that of uninfected cells. Comparisons among the three groups suggested that the Adv-til-1-infected cells deposited more calcium mineral than the Adv-pebp2A-infected cells.

Alizarin Red Staining—Alizarin red staining of infected and uninfected cells also revealed a greater effect in the Adv-til-1-infected cells in vitro, showing markedly strong calcification compared with the Adv-pebp2A-infected cells and the (uninfected and Adv-cre infected) control groups as shown in Fig. 2C.

Mineral deposition is known to accompany cell death in culture. To rule out the alternative explanation that the increase of mineralization is because of an increase in programmed cell death, we performed a control experiment of Alizarin red staining of infected and uninfected cells, respectively. Therefore, Adv-pebp2A infection without cre recombinase does not work for Pebp2A overexpression but serves as a kind of control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 2. A, alkaline phosphatase activity of osteoprogenitor cells infected with two types of recombinant adenoviral vectors (Adv-pebp2A and Adv-til-1). B, calcium content of osteoprogenitor cells. Each experiment was done over 5 times independently (n = 3). C, Alizarin red-S staining of osteoprogenitor cells infected with recombinant adenoviral vectors Adv-(pebp2A + cre), Adv-(til-1 + cre), Adv-cre, or uninfected (control).

In Vivo Effect of Cbfa1 (Til-1) Overexpression in a Cultured Bone Transplantation Model—Based on the results of the in vitro experiments described above, we chose Til-1 for the in vivo experiment. Histochemical, Biochemical, and FISH Analysis of Implanted Bone Marrow-derived Osteoprogenitor Cells—To examine the effect of Cbfa1 overexpression on bone formation in vivo, the transplantation experiments of Cbfa1-overexpressing cells/porous ceramic composites into subcutaneous (ectopic) and bone defect (orthotopic) sites of rats were performed. The procedure for transplantation is outlined schematically in Fig. 3 (described under “Materials and Methods”). Fig. 4A shows the H&E staining of subcutaneously implanted porous ceramic block/Adv-til-1-infected (Fig. 4A, d–f) and uninfected (Fig. 4A, a–c) osteoprogenitor cells composites at 3 days and 3 and 8 weeks postimplantation. At 3 days postimplantation, the negligible difference between Adv-til-1-infected and uninfected cells composites was observed (Fig. 4A, a and d). Immunohistochemical analysis showed that Adv-til-1-infected cells have already expressed alkaline phosphatase, osteocalcin, and osteopontin, at 3 days postimplantation, whereas these osteoblast-
tive markers in uninfected osteoprogenitor cells were very weak (Fig. 4B, a–f). There was no significant difference in the localization and the intensity of these osteoblastic markers between Adv-til-1-infected composites and uninfected composites when compared at 8 weeks postimplantation. Alkaline phosphatase and osteopontin were localized to cells inside the pore of blocks, especially at boundaries between the mineralized region (bone) and pore. On the contrary, osteocalcin was localized to the mineralized region (bone). The results of H&E staining at 3 and 8 weeks postimplantation indicated a rich formation of bone in Adv-til-1-infected groups. Bone formed in the Adv-til-1-infected cells composites at 8 weeks was thicker and with wide distribution compared with 3 weeks postimplantation (Fig. 4A, e and f). Alkaline phosphatase activity and osteocalcin expression levels suggested that alkaline phosphatase activity was enhanced by Til-1 overexpression 2.0- and 4.7-fold at 3 and 5 weeks postimplantation, respectively, whereas osteocalcin expression was enhanced 2- and 1.5-fold at 3 and 5 weeks postimplantation, respectively, as shown in Fig. 4C. Fig. 4D shows the results of image analysis for new bone formation in the uninfected and Adv-til-1-infected cells composites at 3 and 5 weeks postimplantation. The area of the new formed bone in the porous area in the composite was enhanced by Til-1 overexpression 5.6- and 8.0-fold at 3 and 5 weeks postimplantation, respectively, as shown in Fig. 4D. These results show that the overexpression of Cbfa1 (Til-1) also affected the ability of transplanted osteoprogenitor cells to form bone in vivo (at subcutaneous sites) similar to in vitro.

To study the mechanism of in vivo bone formation on transplantation, we detected the cells derived from donor (implanted) cells in recipient rats by FISH analysis for Y-chromosomes in the modified cultured bone implantation model, with Adv-til-1-infected osteoprogenitor cells derived from bone marrow of male Fischer rats implanted into subcutaneous sites in female Fischer rats. Fig. 5A represents the FISH analysis of transplanted cells and their derived cells in the recipient rat. The red-labeled Y-chromosomes, seen as bright points in nuclei (blue), are identified and distributed homogeneously in the region of new bone formation in the porous area of the scaffold, as shown in a and c of Fig. 5A. At 3 days postimplantation, cells are distributed in the pore and strongly observed close to the pore surface. FISH images taken at higher magnification demonstrate that the Y-chromosome-positive cells are derived from donor cells or their derived cells. By counting the Y-positive and the 4,6-diamidino-2-phenylindole-positive cells, the percentage of Y-chromosome-positive cells among all the cells was calculated to be 86.6 ± 3.8%. At 2 and 4 weeks postimplantation, the percentage of Y-positive cells was 82.2 ± 2.8 and 88.8 ± 2.4%, respectively, with clear new bone formation. At 8 weeks postimplantation, cells were distributed in the new bone inside the pores. The percentage of Y-positive cells was calculated to be 84.2 ± 5.1%. In this period, the proportion of Y-positive cells was high and did not change (Table I). The results suggested that the newly created bone in the porous area of the scaffold is mostly produced by the implanted donor cells or their derived cells.

To examine whether the overexpression of Cbfa1 was originated from the virally transplanted donor cells related to its differentiation (bone formation), we performed quantitative RT-PCR to detect adenovirus-derived mouse Cbfa1 (Adv-derived Cbfa1) expressions and total Cbfa1 (exogenous Cbfa1 and endogenous Cbfa1) expressions in the donor cells from the subcutaneous implant, as shown in Fig. 5B. Adv-derived Cbfa1 was amplified by RT-PCR using primer specific to its 3’-untranslated region of the vector and primer specific to its open reading frame, omitting the amplification of endogenous Cbfa1. The control experiment showed that, in the absence of Adv-derived Cbfa1 expression, the total Cbfa1 expression once decreased to a minimum at 7 days postimplantation and gradually increased with time. In the transplanted cells, Adv-derived Cbfa1 increased to a peak at day 7 after the implantation and decreased to a low level at day 21, which was maintained until day 56. Because the adenoviral vector lacks E1A, E1B, and E3 genes, infection and amplification of virus does not occur, thus the overexpression of Cbfa1/Til-1 is transient. The total Cbfa1 expression of infected cells composites maintained a high level until day 7, decreased to a minimum on day 21, and then increased again on day 56. The value of total Cbfa1 was close to the sum of exogenous Adv-derived Cbfa1 and endogenous donor-derived Cbfa1 (control).

The increase in the time course of the Cbfa1/Til-1 expression in Adv-til-1-infected cells can be explained by the enhancement of the original expression levels in the uninfected cells, by Cbfa1/Til-1 overexpression. The results shown in Fig. 5, A and B, suggested that the new-formed bone in the porous area of the scaffold is mostly produced by the implanted donor cells or derived cells, continuing to express Cbfa1/Til-1 in vivo during the experimental period.

Next we performed orthotopically implantation (bone defect site) of porous ceramic block/Adv-til-1-infected osteoprogenitor cells composites, and analyzed them by H&E staining and immunohistochemical staining. H&E staining data after 1 week (Fig. 6A, a and d) showed a negligible difference in bone formation, however, differences between infected and uninfected cells composites were observed in Fig. 6C (g–l). Osteocalcin immunostaining was negative in uninfected cells composites, whereas alkaline phosphatase and osteopontin immunostaining were weakly positive as shown in Fig. 6C (g–i). In the infected cells composites, all three osteoblastic markers were positive and their localization was different to each other (Fig. 6C, j–l). This is reasonable, considering the differentiation stage dependence of these osteoblastic marker expressions, which osteocalcin is known to express later than alkaline phosphatase and osteopontin. In addition, cells attached to the pore surface and inside the pore showed strong immunostaining of alkaline phosphatase and osteopontin, whereas osteocalcin expression was localized to the pore surface and area surrounding the pore. H&E staining data after 4 weeks implantation demonstrated more abundant bone formation, especially at the surgically incised presumptive cortical bone region, which was partly replaced by new bone (Fig. 6A, b and e). In contrast, little bone formation was observed in the control, the hole with the implanted ceramic composite remain-
The region of strong osteocalcin staining moved along the composite by replacing the marrow tissues in the infected cells composites, whereas a strong signal was detected only around the boundary region of the composite in the control (Fig. 6B, b and c). At 8 weeks postimplantation, significant bone regeneration was observed in the infected cells composites.
ite: at this stage, the incised cortical bone was not only almost entirely replaced by new bone, but the implanted composite in the bone cavity had almost disappeared and was replaced with bone marrow tissue (Fig. 6, A–C, a–f). On the contrary, the implanted composites remained both in the cortical bone and bone marrow cavity in the control (Fig. 6, A–C, c). A strong signal of osteocalcin was detected at the area of the surgically incised cortical bone area, not in the bone cavity area in infected cells composites, while the whole area of the implanted composite showed the signal in control (Fig. 6, B and C, c and f). These results suggest that Til-1 overexpression in osteoprogenitor

**TABLE I**

| Time after implantation | 3 days | 2 weeks | 4 weeks | 8 weeks |
|-------------------------|--------|---------|---------|---------|
| Y-chromosome positive cells/all cells (%) | 86.6 ± 3.8 | 82.2 ± 2.8 | 88.8 ± 2.4 | 84.2 ± 5.1 |

FIG. 5. A, FISH analysis of donor cells derived from male rats implanted into subcutaneous sites of recipient female rats. Images of the composites at 3 days (a, b) and 8 weeks (c, d) postimplantation with lower (a, c; ×20) and higher (b, d; ×100) magnifications are shown. Cells were stained with 4,6-diamidino-2-phenylindole (blue) for nuclear chromatin and rhodamine (red) for Y-chromosome FISH. Bars: 100 μm. B, time course of the mRNA expression of adenovirus-derived exogenousCbfa1/Til-1 and total Cbfa1/Til-1 (including exogenous adenovirus-derived Cbfa1/Til-1 and endogenous Cbfa1/Til-1) in Adv-til-1 infected (Adv-infected) and uninfected (Control) cells composites at 3, 7, 21, and 56 days after implantation. Each experiment was done three times independently (n = 2). The Adv-derived Cbfa1/Til-1 was only detected in the case of the Adv-til-1-infected cells composite.

FIG. 6. H&E staining (A) and immunostaining of osteocalcin (B and C, a–f) of serial sections from orthotopically implanted porous ceramic block/Adv-til-1-infected (d–f) and uninfected (a–c) osteoprogenitor cells composites at 1 (a, d), 4 (b, e), and 8 (c, f) weeks postimplantation. Bars: 1 mm. Continuous serial sections were individually stained with H&E or immunostaining and verified for each stain. Although the strength of immunostaining (osteocalcin) was variable in contrast to H&E staining, the detected region of bone area was overlapped. C, g–l, immunostaining of alkaline phosphatase (g, j) and osteocalcin (h, k) and osteopontin (i, l) of Adv-til-1-infected (i–l) and uninfected (g–i) osteoprogenitor cells composites at 1 week postimplantation. Bar: 100 μm. All images correspond to the boxed regions (a and f).
cells and porous ceramic composite in bone defect sites markedly enhanced in vivo bone formation. Moreover, the differentiation stages of osteoblasts in the composites depend on the deposition site within the composite. The cells placed close to the bone marrow are likely to be more differentiated.

Fig. 7 shows the image analysis of the regenerated bone area in uninfected and Adv-til-1-infected cells composites orthotopically implanted into bone defects at 1, 4, and 8 weeks postimplantation. It increased with time, demonstrating the progressive bone formation in pores. The area of the new-formed bone in the porous area in the composite was enhanced by Til-1 overexpression 4.6- and 1.4-fold at 4 and 8 weeks postimplantation, respectively. The enhancement at 4 weeks postimplantation demonstrates the accelerated bone formation by Cbfa1/Til-1 overexpression.

To examine the risk of tumorigenesis of the donor (implanted) cells, a quantitative RT-PCR analysis of oncogenes was performed on the harvested cells and ceramic composites. Fig. 8 shows the time course of the ratio of mRNA expression of c-jun, c-myc, and c-fos of the Adv-til-1-infected cells composites to uninfected cells composites. The ratio of c-jun and c-fos had a similar time course with slight enhancement at 7 days postimplantation, but did not deviate much from 1. Paired t test was used for statistical evaluation. The calculated p value is larger than 0.1 for data in Fig. 8, indicating statistically no significant difference in expressions of oncogenes between Adv-til-1-infected and uninfected cells composites. Each experiment was done two times independently (n = 2).

**DISCUSSION**

Bone tissue engineering is expected to be approved for clinical application in the near future, following in the path of skin tissue engineering. Some problems remain to be overcome and basic studies are still necessary, however, before this technique can be used for patients with skeletal defects. One is that long-term culture in vitro causes MSC-derived cells to cease their lineage progression or to become cancerous. One approach to accelerating the osteoblast differentiation process involves the use of gene therapy in which viral or non-viral vectors are utilized to overexpress osteogenic factors in cells for transplantation. Intrinsice acceleration of osteoblast differentiation might be achieved by transcription factors, which are expected to promote differentiation and induce a more effective bone formation in vivo. In this paper, we described an application of an adenoviral vector carrying the Cbfa1 gene to transduce MSC-derived osteoprogenitor cells in a cultured bone transplantation model. Two isoforms of Cbfa1 were examined for their effect on osteoblast differentiation upon overexpression, with Til-1 being more effective in vitro than Pebp2aA. The difference between the two Cbfa1 isoforms stems from the NH2 terminus as shown in Fig. 1A. Pebp2aA and Til-1 were originally discovered by Ogawa et al. (30) and Stewart et al. (31), respectively. A recent paper (33) on the regulation of the isoforms in osteoblasts described distinct differences between the two; Pebp2aA is expressed in nonosseous mesenchymal tissues and in osteoblast progenitor cells, and does not change with differentiation status. In contrast, the Til-1 transcript accumulates during the differentiation of primary osteoblasts. The presence of the ubiquitous Pebp2aA isoform in nonosseous cells and before bone morphogenetic protein-2-induced expression of the Til-1 isoform suggests a regulatory role for Pebp2aA in the early stages of mesenchymal cell development, whereas Til-1 is necessary for osteogenesis and maintenance of the osteoblast phenotype. The Til-1 isoform is expected to be more effective in osteoblasts for bone formation, which is consistent with the above in vitro results. This is our new finding, that Til-1 overexpression might provide more effective gene therapy than Pebp2aA. We thus chose to overexpress Til-1 in osteoprogenitor cells and porous ceramic composites to be transplanted into ectopic (subcutaneous) and orthotopic (bone defect) sites in Fischer rats, the result being a rich formation of bone in comparison with the uninfected cells composites. The in vivo examination, especially by orthotopic transplantation, is quite important even if a strong effect of Cbfa1 overexpression is confirmed in vitro, because in vivo osteoblastic differentiation in bone is dynamically complex. A study reported by Liu et al. (36) indicated that Cbfa1 transgenic mice showed osteopenia with multiple fractures caused by the maturational blockage of osteoblasts. Their results seem contradictory to ours. However, our data shown in Fig. 5B clearly showed that Adv-til-1 (Cbfa1)-infected cells express Adv-derived Cbfa1 (adenovirus-derived mouse Cbfa1) at maximum level at 1 week after transplantation and decreased down to a low level at 3 weeks after transplantation. The increase in the time course of total Cbfa1/Til-1 expression in Adv-til-1-infected cells can be explained by the enhancement
of the original expression levels in the uninfected cells, by Adv-derived Cbfa1/Til-1 overexpression. In this point of view, our experimental system is quite different from the system by Liu et al. (36) and it is reasonable that our system did not show the similar effect of maturational blockage of osteoblasts by Cbfa1 overexpression. Moreover it can be said that this short lifetime of Adv-derived Cbfa1 in the infected donor cells in our system gives a great advantage to our application of Cbfa1 overexpression to bone tissue engineering.

The second problem is our poor understanding of the mechanism of in vivo bone formation in transplantation models. No report has been published about the behavior of donor cells and interaction between recipient cells and donor cells after implantation. We need to investigate whether the transplanted cells are undergoing full differentiation or whether they are simply overexpressing a set of development markers, inducing the surrounding cells to be osteogenic. This investigation is also important to justify our model system, compared with the previous report about Cbfa1 transgenic mice (36). Here we performed an additional experiment to elucidate the mechanism of in vivo bone formation as shown in Fig. 5, A and B. FISH analysis of Y-chromosomes in the modified subcutaneous transplantation model in Fig. 5A demonstrated that the newly formed bone tissue was mostly produced by donor cells. Combining the data of Adv-derived and total Cbfa1/Til-1 mRNA expressions in Fig. 5B it is concluded that the newly formed bone tissue was mostly produced by donor cells continuing to express Cbfa1/Til-1 in vivo.

The results from the bone defect model showed that the implanted Adv-til-1-infected osteoprogenitor cells/ceramic composites were almost completely replaced by new cortical bone and bone marrow tissue at 8 weeks. Here we emphasize that the new bone formation by Til-1 overexpression appeared to reflect existing tissue boundaries: the incised cortical bone was replaced by new bone, whereas, the implanted composites in the bone cavity almost disappeared and was entirely replaced with bone marrow tissues. This result is quite promising for clinical application.

A previous cultured bone transplantation model needed about 2 weeks for in vitro culture (5–10), however, the present implantation model with adenoviral infection to osteoprogenitor cells required only 2 days after subculture. Based on the FISH analysis, we concluded that newly formed bone was derived from donor cells. However, the donor cells might become cancerous. To confirm the safety of this therapy, we performed an additional experiment to elucidate the mechanism of in vivo bone formation in transplantation models. No report has been published about the behavior of donor cells and interaction between recipient cells and donor cells after implantation. We need to investigate whether the transplanted cells are undergoing full differentiation or whether they are simply overexpressing a set of development markers, inducing the surrounding cells to be osteogenic. This investigation is also important to justify our model system, compared with the previous report about Cbfa1 transgenic mice (36). Here we performed an additional experiment to elucidate the mechanism of in vivo bone formation as shown in Fig. 5, A and B.

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