Direct conversion of human fibroblasts into functional osteoblasts by defined factors

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Osteoblasts produce calcified bone matrix and contribute to bone formation and remodeling. In this study, we established a procedure to directly convert human fibroblasts into osteoblasts by transducing some defined factors and culturing in osteogenic medium. Osteoblast-specific transcription factors, Runt-related transcription factor 2 (Runx2), and Osterix, in combination with Octamer-binding transcription factor 3/4 (Oct4) and L-Myc (RXOL) transduction, converted ~80% of the fibroblasts into osteocalcin-producing cells. The directly converted osteoblasts (dOBs) induced by RXOL displayed a similar gene expression profile as normal human osteoblasts and contributed to bone repair after transplantation into immunodeficient mice at artificial bone defect lesions. The dOBs expressed endogenous Runx2 and Osterix, and did not require continuous expression of the exogenous genes to maintain their phenotype. Another combination, Oct4 plus L-Myc (OL), also induced fibroblasts to produce bone matrix, but the OL-transduced cells did not express Osterix and exhibited a more distant gene expression profile to osteoblasts compared with RXOL-transduced cells. These findings strongly suggest successful direct reprogramming of fibroblasts into functional osteoblasts by RXOL, a technology that may provide bone regeneration therapy against bone disorders.

Significance

Osteoblasts produce bone matrix and contribute to bone remodeling. We have established a procedure to directly convert human fibroblasts into osteoblasts by transducing some defined factors and culturing in osteogenic medium. Osteoblast-specific transcription factors, Runx2 and Osterix, in combination with Oct4 and L-Myc, drastically induced fibroblasts to produce calcified bone matrix and express osteoblast-specific markers. The directly converted osteoblasts (dOBs) showed similar gene expression profiles as normal osteoblasts and contributed to bone repair after transplantation into mice with bone defects. Furthermore, dOBs did not require continuous expression of the exogenous genes to maintain their phenotype. These findings strongly suggest successful direct reprogramming of fibroblasts into osteoblasts, which may be applicable to bone regeneration therapy.

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The authors declare no conflict of interest.

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE52817).

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differs (8, 28), along with three reprogramming factors, Oct4 (O), L-Myc (L), and c-Myc (M), because of their potential to reprogram cell fates (13, 18, 20, 21, 29). Retrovirus vectors encoding these factors were infected into human gingival fibroblasts either individually or in various combinations. The cells were cultured in osteogenic medium, followed by analyses of osteoblast-like characteristics, including mRNA expression of alkaline phosphatase (ALP) and osteocalcin and staining properties by the von Kossa method. As shown in Fig. 1, Oct4 alone moderately induced the osteoblast-like phenotypes. Similar results were also obtained by the two-factor combinations containing Oct4 (e.g., Runx2 plus Oct4, Osterix plus Oct4). Therefore, Oct4 may play a crucial role in induction of the osteoblast-like phenotype. Among the two-factor combinations, Oct4 plus L-Myc (OL) induced high levels of both ALP and osteocalcin mRNA, and evoked massive production of calcified bone matrix. The most remarkable phenotypic change was elicited by a four-factor combination: Runx2, Osterix, Oct4, and L-Myc (RXOL); therefore, we analyzed RXOL and OL in the subsequent experiments.

**RXOL- and OL-Transduced Cells Significantly Produced Bone Matrix and Expressed Osteoblast-Related Genes.** Human gingival fibroblasts were transduced with RXOL and OL retrovirus vectors. ALP staining showed that transduction with RXOL induced ALP activity in ~80% of the fibroblasts at 14 d after infection, and OL infection also induced this osteoprogenitor marker (Fig. 2A, Left). Alizarin Red S staining showed that the RXOL transduction induced apparent calcium deposition on day 14, and the calcified body occupied almost the entire surface of the culture dish on day 28 (Fig. 2A, Right).

We examined whether fibroblasts of other origins were also induced to produce bone matrix. Human dermal fibroblasts infected with RXOL or OL were positive for ALP activity at 14 d after gene transfer, and Alizarin Red S and von Kossa staining revealed calcium deposition and bone formation nodules on day 28 (Fig. S1A).

To further elucidate osteoblast-like characteristics of the RXOL- and OL-transduced cells, we infected gingival fibroblasts with the retrovirus vectors and tested for mRNA expression of osteoblast-related genes. As shown in Fig. 2B, RXOL induced expression of osteocalcin, ALP, and Runx2 mRNA at levels comparable to those in normal osteoblasts and mesenchymal stem cell (MSC)-derived osteoblasts (MSC-OBs). The RXOL-transduced cells expressed osteopontin, BSP, and Osterix mRNA at levels between those of osteoblasts and MSC-OBs. The transduction of OL significantly induced osteocalcin, osteopontin, BSP, and ALP mRNA. OL-transduced cells expressed Runx2 at a low level and did not significantly express mRNA for the Osterix gene (Fig. 2B).

Human dermal fibroblasts infected with RXOL and OL also expressed mRNA for osteocalcin, ALP, and BSP genes at significant levels (Fig. S1B).

**RXOL-Transduced Cells Shared Similar Global Gene Expression Signature with Osteoblasts.** We performed DNA microarray analysis of mRNA from RXOL-transduced, OL-transduced, and untransduced fibroblasts, osteoblasts, MSCs, and MSC-OBs. In hierarchical clustering analysis, we focused on osteoblast-related differentiation and signaling genes, and found that the expression patterns of these osteoblast-related genes in RXOL-transduced cells were the most similar to those in osteoblasts (Fig. 3A). Genome-wide gene expression profiles also indicated that RXOL-transduced cells were most similar to osteoblasts among all the cells tested (including MSC-OBs), and vice versa (Fig. 3B).

These findings strongly suggest that the RXOL-transduced cells share similar gene expression profiles with osteoblasts. Meanwhile, OL-transduced cells were less similar to osteoblasts than
RXOL-transduced cells in terms of the expression patterns of both osteoblast-related genes (Fig. 3A) and whole genes (Fig. 3B). Based on these findings, RXOL-transduced cells were used in further detailed analyses as representative directly converted osteoblasts (dOBs).

Epigenetic Status and Immunostaining Properties of dOBs. In experiments performed to assess the epigenetic status of dOBs, we found that in osteoblasts, genomic DNA was mostly unmethylated at CpG dinucleotides at the osteocalcin gene upstream region, whereas in fibroblasts, the CpG sequences were heavily methylated (Fig. 3C). These results are consistent with previous reports on epigenetic markers at the osteocalcin gene loci in rat osteoblasts (30) and mouse osteoblasts (31). dOBs showed an intermediate level of CpG methylation at this osteoblast-specific gene locus, suggesting that the epigenetic status of dOBs had drastically changed from that of fibroblasts, but had not reached that of osteoblasts.

Immunostaining of cells indicated that dOBs produced osteocalcin and osteopontin (Fig. 4A). On day 21 after the transduction, the proportion of the osteocalcin-positive cells among the RXOL-infected cells was 79.9 ± 3.5%, whereas that among gingival fibroblasts was only 0.47 ± 0.18% (Fig. 4B). dOBs strongly expressed retroviral transgenes (Fig. 5A). It may be possible that the continuous expression of transgenes is

Fig. 3. Characteristics of dOBs induced from fibroblasts. (A) RNA extracted from the indicated cells was subjected to DNA microarray analysis. Heat map and hierarchical clustering analysis of the DNA microarray data showing the genes for transcription factors and signaling molecules involved in osteoblast differentiation. RXOL and OL represent human fibroblasts infected with the corresponding retrovirus vectors. In the heat map, genes with increased expression are colored red, and genes with decreased expression are in blue (see the color range below the heat map). The expression level of each gene was normalized to median signal intensity. Clusters indicate that RXOL-transduced cells showed the greatest similarity to osteoblasts. (B) RNA obtained from the indicated cells were analyzed by the GeneChip human Gene 1.0 ST (Affymetrix), consisting of 29,096 probes. (Upper) In the heat map, red color indicates high correlation between the two cells, whereas black color shows low correlation (see color range). (Lower) Correlation coefficient values. (C) DNA extracted from human gingival fibroblasts, dOBs (RXOL-transduced fibroblasts), and osteoblasts were tested for CpG methylation at the osteocalcin gene upstream region.

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### Fibroblasts Were Converted into dOBs Without Passing Through an Intermediate Pluripotent Stage

Human fibroblasts infected with RXOL were immunostained with anti-Nanog antibody every 2 d from day 1 to day 15 after infection. More than 1,000 cells were observed at each time point under fluorescent microscopy. No significant expression of Nanog, which is an indispensable transcription factor for pluripotency, was detected in any cell over the entire experimental period. These results demonstrate that fibroblasts were directly converted into dOBs without passing through an immature pluripotent stage.

We also estimated mRNA expression for the Rex-1 and Nanog genes during the conversion from fibroblasts to dOBs. Real-time RT-PCR analysis revealed no detectable expression of these pluripotent stem cell-specific genes in fibroblasts or in RXOL-infected fibroblasts at 7, 14, and 21 d after transduction.

We considered the possibility that the osteoblast-like cells could have arisen from MSCs that were a contaminating factor in the original fibroblast population. The fibroblasts were cultured under adipogenic, osteogenic, and chondrogenic conditions. Adipocytes, osteoblasts, and chondrocytes were not induced from the fibroblasts, whereas adipose-derived MSCs cultured as a positive control were induced into these mesenchymal lineages under the corresponding conditions. The results and the aforementioned high efficiency of conversion with ~80% of fibroblasts converted to dOBs clearly indicate that the dOBs were not derived from MSCs that had contaminated the fibroblast population.

### Transient Expression of RXOL Was Sufficient to Induce dOBs

The dOBs strongly expressed retroviral transgenes. It may be possible that the continuous expression of transgenes is...
required for the dOBs to maintain their osteoblast-like phenotypes. Alternatively, once established by the transduced factors, the converted cells may remain stably osteoblast-like even in the absence of sustained expression of the exogenous genes.

To address this issue, we constructed retroviral vectors that express R, X, O, L, and GFP under control of the tetracycline-inducible (Tet-On) promoter. After infection, doxycycline was added to the culture during the first 3 or 7 d, followed by further culturing without doxycycline. Osteogenic medium was used only after the withdrawal of doxycycline. The cells showed significant production of calcified bone matrix and expression of osteocalcin mRNA (Fig. 5B and C) on day 28, when the transgene was no longer expressed (Figs. S4 and S5).

dOBs Contributed to Bone Repair After Transplantation into Mice at an Artificial Bone Defect Region. To examine whether dOBs can enhance bone regeneration, we transplanted dOBs and fibroblasts into bone defect lesions surgically created at the femurs of NOD/SCID mice. Bone repair was estimated by macroscopic, radiographic, biomechanical, and histological examinations at 21 d after transplantation. Macroscopic observation of the femur showed that the defect parts in dOB-transplanted femurs were covered with bridging callus formation, which was not seen in the fibroblast-transplanted femurs (Fig. 6A and Fig. S7A). The dOB transplantation resulted in a significantly higher degree of callus formation compared with fibroblast transplantation (Fig. 6B). Micro computed tomography (µCT) revealed that the callus was composed of regularly aligned bone trabeculae, with no signs of pseudoarthrosis. Furthermore, the radiopacification of the defective area was higher in dOB-transplanted femurs than in fibroblast-transplanted femurs, as demonstrated by the µCT transmission images (Fig. S7B).

Histological analysis also showed bridging callus formation at the defective lesion in the dOB-transplanted femurs, but only partially formed callus in the fibroblast-transplanted femurs (Fig. 6C). Alizarin Red S staining confirmed ossification at the callus of the dOB-transplanted femurs (Fig. 6C). These results indicate that local transplantation of dOBs accelerated bone healing at bone defect sites in NOD/SCID mouse femurs.

We next assessed the distribution of, and bone matrix production by, the dOBs in the regenerated bone tissue. GFP-labeled dOBs were transplanted into the bone defect lesions, and human osteocalcin was visualized by immunohistochemical staining. GFP-positive cells were located on the surface of the callus and in the thickened periosteum, whereas human osteocalcin was widely distributed in the callus and periosteum, as demonstrated by staining with an antibody that specifically recognized human, but not mouse, osteocalcin (Fig. 6D). Sox9-positive GFP-negative chondrocytes were present in bone marrow and callus (Fig. S8).

These results strongly suggest that the donor cells retained the osteoblast-like feature and produced bone matrix in vivo.

Discussion

In the present study, we accomplished direct conversion of human fibroblasts into osteoblasts that produced mineralized bone matrix at high efficiency by introducing four transcription factor genes (R, X, O, and L) and then culturing in osteogenic medium. The resultant osteoblasts facilitated bone repair in vivo after transplantation into immunodeficient mice at the bone defect lesions. The dOBs induced by RXOL may be epigenetically reprogrammed cells that significantly expressed endogenous Runx2 and Osterix.

Interestingly, we also found that RXOL was not the minimum essential combination of factors to achieve some degree of osteoblast-like conversion of fibroblasts. A smaller number...
of factors, such as OL and even Oct4 alone, was sufficient to induce bone matrix production in fibroblasts (Fig. 1). Among the four factors, only Oct4 may have an essential role, and the other factors might not be indispensable for the induction of bone matrix production. Nevertheless, the difference between RXOL- and OL-transduced cells strongly suggests important roles of Runx2 and Osterix in converting fibroblasts into such osteoblasts that express endogenous Runx2 and Osterix and have similar global gene expression profiles as bone-derived osteoblasts (Figs. 2 and 3).

Runx2, a master regulator of osteoblast development (4, 5, 32), is a member of the Runx family of transcription factors that is specifically expressed in cells of osteoblastic lineage at various differentiation stages, from immature mesenchymal osteochondroprogenitors to mature osteoblasts. Runx2 expression is both necessary and sufficient for the differentiation of mesenchymal progenitor cells toward the osteoblastic lineages (4, 5). Runx2 enhances the transcription of all major osteoblast-related genes, such as osteocalcin, by specifically binding to the genes’ regulatory regions (28, 31). Runx2-null mice lack ossification owing to the maturational arrest of osteoblasts (33). A previous study found that Runx2 transfection accelerates osteoblastic differentiation from iPSCs (34). In other studies, forced expression of Runx2 induced osteocalcin in rodent fibroblasts (35, 36), but not in human fibroblasts (Fig. 1).

Osterix, a zinc-finger transcription factor, is specifically expressed in osteoblasts at a later stage of differentiation than Runx2-expressing cells (6, 7). Expression of Osterix depends on Runx2, whereas Osterix acts downstream of Runx2. Osterix-null mice also lack bone formation (6). The present study indicates that Runx2 and Osterix have important roles in the direct conversion of osteoblasts, consistent with previous reports stratifying indispensable roles of lineage-specific transcription factors in direct reprogramming of the corresponding tissue cells (16–18, 21, 23).

Oct4 plays central roles in maintaining pluripotency and reprogramming differentiated cells into pluripotent stem cells (12, 13, 37). Shu et al. (38) recently reported that Oct4 and Sox2 present mesendodermal and ectodermal differentiation cues, respectively, and that the balance between these counteracting cues may facilitate reprogramming. In the present study, we found that Oct4 is indispensable for the direct reprogramming of osteoblast-like cells (Fig. 1), strongly suggesting that the mesendodermal skewing induced by Oct4 may be crucially involved in the mechanisms underlying the lineage conversion toward osteoblasts.

The proto-oncogene c-Myc is another reprogramming factor, although it is not a prerequisite for iPSC cell generation (39). Recent studies indicate that L-Myc may substitute for c-Myc (29). In our experiments, L-Myc was superior to c-Myc in terms of efficiency of osteoblast induction. L-Myc may have lower tumorigenicity compared with c-Myc, which would be a significant advantage in future clinical applications of dOBs. In addition, c-Myc is considered a crucial factor in the direct reprogramming of mouse fibroblasts into neural stem cells (18, 20) and chondrocytes (21).

Transduction of OL induced osteoblast-like phenotypes in fibroblasts (Figs. 1, 2, and 3). The OL-transduced cells moderately expressed endogenous Runx2 (Fig. 2B), strongly suggesting important roles of endogenous Runx2 in the cell type conversion caused by OL. The OL-transduced cells showed less similarity to osteoblasts compared with RXOL-transduced cells in terms of genome-wide gene expression profiles (Fig. 4A and B). This “incomplete conversion” may be related to the low expression of endogenous Osterix (Fig. 2B), strongly suggesting a crucial role of Osterix in “full conversion”. The detailed molecular mechanisms involved in this cell conversion remain to be elucidated, however.

Transplantation of autologous bone marrow cells and MSCs obtained from the bone marrow or adipose tissue has been performed to treat bone loss after surgical resection of bone tumors or large bone defects caused by trauma (40). This transplantation may facilitate bone repair, probably because osteoblasts may be originated from the graft and contribute to bone regeneration. The clinical application of autologous MSC transplantation to bone regeneration faces some obstacles, however, especially in elderly patients, including limited numbers of stem cells, age-related decrease in potential for differentiation into osteoblasts, and invagination during the cell isolation procedure (41, 42). Such obstacles...
may be avoided by applying the direct reprogramming technology, in which a large number of functional osteoblasts can be obtained from fibroblasts isolated from patients or allogenic donors by a less-invasive procedure (43, 44). Fibroblasts have a high proliferative capacity, which does not decline with increasing donor age (43, 45). Thus, direct reprogramming may provide another option for such patients in whom MSC transplantation might not be an appropriate therapy.

Osteoblasts induced from a patient’s own somatic cells or allogenic cells also may be applicable to cell-based therapy against bone disrupting conditions associated with osteoporosis, bone disruption caused by rheumatoid arthritis, and alveolar bone resorption due to periodontitis. This therapy may help increase the patient’s quality of life and capacity to perform activities of daily living.

Materials and Methods

For direct reprogramming, human fibroblasts were retrovirally transduced with transcriptional factors and cultured in DMEM supplemented with 50 μg/mL ascorbic acid, 10 mM β-glycerol phosphate, 100 nM dexamethasone, and 10% FBS (osteogenic medium). hMSCs (normal human osteoblasts derived from femur of a 39-year-old male) were purchased from Lonza. Detailed information on cells, retroviral vectors, infection, induction into osteoblasts, cell staining, real-time RT-PCR, immunostaining, DNA microarray analysis, bisulfite sequencing, surgical procedure, cell transplantation, radiographic and histological assessment, and data analysis is provided in SI Materials and Methods. The sequences of real-time RT-PCR primers are provided in Table S1.

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