Title

**Transduction of Oct6 or Oct9 gene concomitant with Myc family gene induced
osteoblast-like phenotypic conversion in normal human fibroblasts**

**N. Mizoshiri^{1,2}, T. Kishida¹, K. Yamamoto^{1,3}, T. Shirai², R. Terauchi², S. Tsuchida²,
Y. Mori^{1,2}, A. Ejima¹, Y. Sato^{1,3}, Y. Arai², H. Fujiwara², T. Yamamoto³, N.
Kanamura³, O. Mazda^{1*}, T. Kubo²**

**¹Department of Immunology, Kyoto Prefectural University of Medicine, Kyoto,
Japan**

²Department of Orthopaedics, Kyoto Prefectural University of Medicine, Kyoto, Japan.

³Department of Dental Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan

***Corresponding author: O. Mazda, MD PhD, at Kyoto Prefectural University of
Medicine, Kamikyo, Kyoto 602-8566, Japan.**

E-mail: mazda@koto.kpu-m.ac.jp

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Abstract

Introduction: Osteoblasts play essential roles in bone formation and regeneration, while they have low proliferation potential. Recently we established a procedure to directly convert human fibroblasts into osteoblasts (dOBs). Transduction of Runx2 (R), Osterix (X), Oct3/4 (O) and L-myc (L) genes followed by culturing under osteogenic conditions induced normal human fibroblasts to express osteoblast-specific genes and produce calcified bone matrix both *in vitro* and *in vivo*. Intriguingly, a combination of only two factors, Oct3/4 and L-myc, significantly induced osteoblast-like phenotype in fibroblasts, but the mechanisms underlying the direct conversion remains to be unveiled. **Materials and Methods:** We examined which Oct family genes and Myc family genes are capable of inducing osteoblast-like phenotypic conversion. **Results:** As result Oct3/4, Oct6 and Oct9, among other Oct family members, had the capability, while N-myc was the most effective Myc family gene. The Oct9 plus N-myc was the best combination to induce direct conversion of human fibroblasts into osteoblast-like cells. **Discussion:** The present findings may greatly contribute to the elucidation of the roles of the Oct and Myc proteins in osteoblast direct reprogramming. The results may also lead to establishment of novel regenerative therapy for various bone resorption diseases.

Keywords

osteoblast; direct reprogramming; Oct9; N-Myc

Abbreviations

dOBs: directly reprogrammed osteoblasts; Oct: Octamer-binding transcription factor; NHDF (Adult): Normal human dermal fibroblasts (Adult); iPS: induction of pluripotent stem

Introduction

Bone does not only support various organs in the body of vertebrates by forming a skeleton, but plays important roles such as in motion and organ protection. If an osseous defect such as a bone fracture occurs, it is repositioned and temporarily fixed, callus form, and endogenous bone union occurs. However, if a wide-ranging osseous defect occurs due to trauma or a neoplastic disease, in some cases, endogenous bone regeneration capacity may be insufficient. It has been reported that the clinically optimal option in the case of a wide-ranging osseous defect is to perform autologous bone transplant to the extent possible [1]. Meanwhile, there has also been a report on severe pain at the bone sampling sites such as the ilium, and serious complications [2]. Therefore, a bone regeneration method which is less invasive and causes fewer complications than autologous bone transplantation is required for wide-ranging osseous defects.

In the bone, reconstruction and absorption are constantly performed by osteoblasts and osteoclasts, and the amount of osteocytes is maintained at a certain level. Osteoblasts are cells which are the basis of bone formation, and play an important role in endogenous bone regeneration. In the post-natal organism, osteoblasts differentiate from skeletal stem cells, a subset of bone marrow stromal cells [3], and the molecular mechanism of this was clarified in recent years. Main signals such as Wnt [4], BMP [5, 6], Notch [7] are involved, and through the expression of transcription factors such as Runt-related transcription factor 2 (Runx2) [8], and Osterix [8, 9], mature osteoblasts differentiate from bone marrow stromal stem/progenitor cells (BMSCs) via pre-osteoblasts and immature osteoblasts. In the early stage and intermediate stage of osteoblast differentiation, the volume of expression of Alkaline Phosphatase (ALP) and Osteopontin (OPN) increases, and in the late stage of differentiation, Osteocalcin is induced [10]. Therefore, Osteocalcin is most frequently used as a marker for osteoblast differentiation.

Since osteoblasts have low proliferation potential, they are induced by using BMSCs. BMSCs have differentiation potential such as to bone, cartilage, and adipocytes [3]. However, since BMSCs may have low cell proliferation potential due to the age of the sample and the tissue, and have low differentiation potential to osteoblasts, there remain issues with using BMSCs as transplantation cells for regenerative medicine [11, 12]. Therefore, if an osteoblast creation method other than that using BMSCs is established, it would make a significant contribution to regenerative medicine.

In recent years, there have been reports on direct reprogramming (or direct conversion) which directly induces somatic cells to various differentiated cells without mediation by pluripotent stem cells. The direct programming method enables the induction of cells such as cardiomyocytes [13], neurons [14], chondroid cells [15, 16] and hepatocytes [17], from fibroblasts. According to these reports, inducing transcription factors which have an important role in target cell differentiation induction leads to phenotypic conversion. Moreover, some of these reports state that, in addition to such transcription factors related to differentiation induction, inducing transcription factors related to the initialization of somatic cells enables fate conversion of fibroblasts [15, 16].

We discovered a method of conversion to directly-reprogrammed osteoblasts (dOBs) by inducing several genes into human fibroblasts [19]. In other words, we used a retroviral vector and induced the genes of the four factors of Runx2 (R), Osterix (X), Oct3/4 (O) and L-myc (L) to fibroblasts, and successfully converted them to osteoblasts which generate calcified bone matrix both *in vitro* and *in vivo*, by strongly expressing osteoblast markers. The fact that these RXOL-induced cells have a comprehensive gene expression profile analogous to normal osteoblast, and that the CpG methylation pattern in the Osteocalcin gene promoter area was changed, suggest that epigenetic changes were induced. Since the osteoblast phenotype was maintained even when the four factors to which genes were induced were temporarily expressed and thereafter the expression disappeared, it

was suggested that these osteoblasts were programmed epigenetically.

The mechanism of direct conversion from these fibroblasts to osteoblasts has not been clarified. However, from amongst the 4 RXOL factors, only Oct3/4 was required in osteoblast induction. While the other three factors still make a contribution in that they have an action of induction in cells which are highly analogous to normal osteoblasts, they were not required in the osteoblast-like phenotype induction. In particular, it was possible to induce the osteoblast-like phenotype with only the two factors of Oct3/4 and L-myc [19]. However, Oct3/4 is originally expressed in pluripotent stem cells, is a transcription factor that is required in maintaining stem cell phenotype, and it is not expressed in osteoblasts. Moreover, L-myc is not a transcription factor related to the differentiation of osteoblasts. A deeply interesting question is why the two factors of Oct3/4 and L-myc, which do not contain osteoblast-specific transcription factors, can convert fibroblasts to osteoblast-like cells. In order to understand what kinds of roles Oct3/4 and L-myc play, it is necessary to clarify whether transcription factors other than Oct3/4, and which belong to the Oct family, are replaceable with Oct3/4, and whether Myc family members other than L-myc can replace L-myc.

Hence, in the present study, we used all Oct family members and Myc family members in investigating which combinations will induce an osteoblast-like phenotype in fibroblasts.

Materials and Methods

Cells

Normal human dermal fibroblasts (adult) (NHDFs (Adult)) (Kurabo) and the PLAT-GP packaging cell line were purchased from Toyobo Life Science (Osaka, Japan) and Cell Biolabs, Inc. (San Diego, CA, USA), respectively. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FBS, 100 mM non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin (complete medium).

Retrovirus vectors

The coding sequences for human Oct1, Oct2, Oct5/7, Oct6, Oct8, Oct9, Oct11 and N-myc genes were amplified by RT-PCR using KOD plus Neo DNA polymerase (Toyobo Life Science). The amplified fragments were cloned into EcoRI-digested pMXs vector (Cell Biolabs) using Gene Art system (Invitrogen), and their nucleotide sequences were confirmed. pMXs vectors containing Oct3/4, c-myc, L-myc and GFP genes were kindly provided by Professor S. Yamanaka (Kyoto University). PLAT-GP packaging cells were seeded onto gelatin-coated 100 mm dishes at a density of 3×10^6 cells/dish and cultured overnight. They were co-transfected with 2.5 µg each of pCMV-VSV-G and one of the pMX plasmids containing transcription factor genes as above using X-treme Gene 9 transfection reagent (Roche Applied Science, Penzberg, Germany) diluted in 500 µL

of Opti-MEM. Twenty-four hours later, the culture supernatant was replaced by anti-biotic free culture medium. After culturing for another 24 h, the supernatant was harvested and filtered through a 0.45 μ m pore-size filter.

Infection and induction into osteoblasts

Infection and induction of fibroblasts into osteoblast-like cells were performed as described [19]. Briefly, NHDFs were resuspended in complete medium and seeded onto 35 mm dishes on day -1. On the next day, supernatants containing each retroviral vector were mixed, supplemented with 4 μ g/mL poly-brene (Nacalai Tesque), and added to the fibroblast culture. Twenty-four hours later, the virus-containing medium was replaced by fresh complete medium supplemented with 50 μ g/mL ascorbic acid, 10 mM β -glycerol phosphate and 100 nM dexamethasone (osteogenic medium). The culture medium was changed every 2-3 days without trypsinization or re-seeding for 28 days.

Alizarin Red S staining

Cells were fixed with 95% ethanol and stained with Alizarin Red S solution (Sigma Aldrich). For quantitative measurement of Alizarin Red S staining, each culture was dissolved with 10% Triton-X, and optical density for each cell lysate was measured at 550 nm.

Real time RT-PCR

Total RNA was extracted from cells using ISOGEN II (Nippon Gene), and reverse transcribed

using ReverTra Ace qPCR RT Master Mix (Toyobo). Real-time RT-PCR was carried out using Real-time PCR Master Mix (Toyobo) and probes and primers specific for Runx2 and osteocalcin genes (Applied Biosystems) with the 7300 Real-Time PCR System instrument (Applied Biosystems). All values were normalized with respect to β -actin mRNA level in each sample and expressed relative to corresponding value in NHDFs.

Immunostaining

For immunostaining of osteocalcin and Runx2, the cells were fixed in 4% paraformaldehyde at 4°C for 30 min. After washing with PBS, the cells were incubated with anti-osteocalcin (AbD Serotec, Kidlington, UK) and anti-Runx2 (Abnova, Taipei, Taiwan) antibodies at 4°C for 24 hour. For visualization, secondary antibodies conjugated with Alexa fluor 488 (Invitrogen) was added for 1 hour. In some experiments, cell nuclei were also stained with DAPI. The staining was observed under a fluorescent microscope.

Data analysis

Data are expressed as means $\pm$ standard deviation (S.D.). Statistical significance was analyzed using Student's *t*-test. $p < 0.05$ was considered significant.

Results

When either Oct3/4, Oct6 or Oct9 was introduced together with L-myc to fibroblasts, the expression of mRNA of Runx2 genes was induced, and calcified bone matrix was generated.

Since we have found that the induction of the two factors of Oct3/4 and L-myc can induce human fibroblast to differentiate into osteoblast-like cells, we investigated whether Oct family members other than Oct3/4 can replace the function of Oct3/4. Each of all the Oct family members was individually or in combination with L-myc introduced to NHDFs, and the expression of mRNA of Runx2, which is a transcription factor with an important role in osteoblast differentiation, was investigated through quantitative RT-PCR after culturing. The results were that the cells transduced with either Oct3/4, Oct6 or Oct9 alone expressed mRNA for Runx2 gene at low, but significant, levels, while transduction with other Oct family members or L-myc alone failed to provoke fibroblasts to express Runx2 (Fig 1). Co-transduction experiments demonstrated more significant expression of Runx2 mRNA in the fibroblasts transduced with Oct3/4, Oct5/7, Oct6, or Oct9 in combination with L-myc. Among others, Oct9 plus L-myc induced the expression of Runx2 mRNA at the highest level in fibroblasts (Fig 1). Therefore, this suggested that even if Oct6 or Oct9 instead of Oct3/4 is introduced together with L-myc, Runx2 expression is still induced.

Next, we performed Alizarin Red S staining for cells to which Oct3/4 plus L-myc, Oct6 plus L-myc and Oct9 plus L-myc were introduced, in order to evaluate the capability of generating calcified bone matrix. The result showed that the cultures of all cells were strongly stained with Alizarin Red S (Fig 2A top). When evaluating the strength of staining with optical density, significantly high values were found in all cells to which Oct3/4 plus L-myc, Oct6 plus L-myc and Oct9 plus L-myc were introduced compared to the control (Fig 2A bottom). Therefore, it was found that introducing L-myc in a combination with either Oct3/4, Oct6 or Oct9 enables the conversion of

fibroblasts to osteoblasts which have calcified bone matrix generation capacity.

Oct3/4 and Oct9 expressed Runx2 in fibroblasts when introduced in combination with L-myc.

Next, we performed immunostaining in order to evaluate Runx2 expression in cells to which L-myc is introduced in combination with either Oct3/4, Oct6 or Oct9. Cells to which Oct3/4 plus L-myc or Oct9 plus L-myc was introduced clearly expressed Runx2 in comparison with the control. However, there was no difference between the control and cells to which Oct6 plus L-myc was introduced (Fig 2B). Therefore, it was suggested that a combination of L-myc with Oct3/4 or Oct9 can induce the cells to a phenotype closer to normal osteoblasts, than can a combination of Oct6 and L-myc.

The introduction of N-myc in combination with Oct3/4, Oct6 or Oct9 induced the mRNA of Runx2 and osteocalcin more strongly than other Myc family members did.

Following the above, we introduced combinations of either Oct3/4, Oct6 or Oct9 and various Myc family members, namely c-myc, L-myc and N-myc, to fibroblasts and investigated the expression of the mRNA of Runx2 and osteocalcin through real-time RT-PCR. Every Myc family member gene significantly augmented the Runx2 expression induced by Oct3/4 as well as by Oct6 and Oct9. The Oct6 plus N-myc was the combination that induced Runx2 mRNA expression at the highest level. The osteocalcin mRNA was also highly induced in fibroblasts by some combinations of genes such as Oct3/4 plus N-myc and Oct9 plus N-Myc. Therefore N-myc was considered the most appropriate myc family gene for induction of osteoblast-like phenotype in fibroblasts in cooperation with either Oct 3/4, Oct6 or Oct9 gene.

200

201 The combination of Oct9 and N-myc most strongly induced the expression of Runx2 and
202 Osteocalcin.

203 Lastly, we combined either Oct3/4, Oct6 or Oct9 and N-myc, introduced them together to
204 fibroblasts, and evaluated Runx2 and Osteocalcin on immunostaining. While the expression of
205 Runx2 was strongly induced by Oct3/4 plus N-myc and Oct9 plus N-myc, it was not induced almost
206 at all with Oct6 plus N-myc. The combination of Oct9 plus N-myc induced the expression of Runx2
207 more strongly than Oct3/4 plus N-myc did (Fig 4A). Moreover, the expression of Osteocalcin was
208 strongly induced through all the combinations, namely Oct3/4 plus N-myc, Oct6 plus N-myc and
209 Oct9 plus N-myc, and there were no clear differences between each combination (Fig 4B). Thus, it
210 was considered that the combination of Oct9 plus N-myc has the strongest capability of inducing
211 osteoblast-like phenotype.

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Discussion

We have shown that the introduction of the two factors, namely Oct3/4 and L-myc, can induce an osteoblast-like phenotype in human fibroblast [19]. In the present study, we found that the introduction of a combination of Oct9 and L-myc by replacing Oct3/4 with Oct9 can induce higher osteoblast-type phenotype than Oct3/4 plus L-myc can and that N-myc is more favorable than L-myc.

The Oct protein is a subclass transcription factor of the POU family. POU family transcription factors are POU-specific domains (POUs) of amino terminals and POU homeodomains (POUh) of carboxy-terminals. These subdomains are specifically bound with octamer motifs (ATGCAAAT) and are closely related to various promoters and enhancer function adjustments [20]. POU protein subclasses are defined based on the sequence structure of binding with DNA and are categorized into six types, from class I to class VI. Since POU classes I, IV and VI do not show high affinity with general octamer motifs, they are called non-octamer-binding factors. Meanwhile, since POU classes II, III and V have a high affinity with octamer motifs, they are called Octamer-binding proteins (Oct proteins). Oct1 (POU2F1), Oct2 (POU2F2) and Oct11 (POU2F3) are categorized as POU class II; Oct6 (POU3F1), Oct5/7 (POU3F2), Oct8 (POU3F3) and Oct9 (POU3F4) are categorized as class III; and Oct3/4 (POU5F1) is categorized as class V. In the present study, a combination of Oct9 and Myc family induced osteoblast-like phenotype similar to or higher than that induced by a combination of Oct3/4 and L-myc. Therefore, it was shown that Oct3/4 and Oct9 are Oct proteins which can induce the strongest osteoblast-type phenotypes in dermal fibroblasts. Moreover, Oct6 was still able to induce osteoblast-like phenotype, although it was weak. These Oct proteins belong to POU class III or V in the POU class category, and none of the Oct in POU class II could induce osteoblast-like phenotypes. The Oct3/4 protein is specifically expressed in ES cells and germ cells

which have totipotency, it plays a central role in adjusting and maintaining pluripotency, and is widely used as a pluripotency marker [21]. The expression of the Oct6 protein is limited to ES cells, the developing brain, Schwann cells, stellate cell precursors, the skin, and the testes [22-24], and it has been reported that it adjusts the formation of myelin sheaths in peripheral nerves and is involved in the adjustment of epidermal keratinocyte differentiation [25]. It is also believed that Oct9 protein is a transcription factor involved in the differentiation of nerve cells, and the expression of Oct9 is necessary in the differentiation of neural stem cells [26]. Also, it has an extremely important role in the generation of the inner ear, and a deficiency of Oct9 can give rise to hearing impairment [27]. However, there have been no reports on the relationships between Oct3/4, Oct6 and Oct9 and osteoblasts. The result of the present study showed for the first time the possibility that Oct6 and Oct9 may be involved in osteoblasts.

Myc family proteins are a type of basic helix-loop-helix zipper (bHLHZ) protein, and form myc-associated factor X (Max) and heterodimers and facilitate transcription by binding to specific sequences in the promoters of target genes [28]. Myc is related to cell proliferation, cell growth factors, differentiation and apoptosis. Proteins belonging to the Myc family include c-myc, N-myc and L-myc. C-myc is highly expressed in many types of cancer such as Burkitt's lymphoma. In the same way, the amplification of N-myc is highly frequently observed in neuroblastoma and rhabdomyosarcoma, and that of L-myc is highly frequently observed in small cell lung cancer and ovarian cancer [29]. C-myc is a classical Yamanaka factor which induces iPS cells, and it is still possible to induce iPS cells without using c-myc, although this is not efficient. Nakagawa et al. reported that the Myc family facilitates the initialization of cells and that either L-myc, c-myc or N-myc can induce iPS cells when combined with three factors, namely Oct3/4, Sox2 and Klf4 [30]. In the present study, by introducing either Oct3/4, Oct6 or Oct9 and one of the Myc family members

together in a skin fibroblast it was possible to induce osteoblast-like phenotype regardless of the combination. However, in the Myc family, c-myc had the highest osteoblast-like phenotype induction effect followed by L-myc and N-myc.

It has been reported that Oct3/4 is expressed in certain types of cancer [31, 32], and it has been suggested that it may be involved in tumor formation. However, there have been no reports stating that Oct9 is involved in tumor formation. Moreover, while it is known that N-myc is involved in neural origins such as for neuroblasts and the onset of neuroendocrine type tumors, its involvement in bone tumor is unknown [33]. Therefore, it is considered that osteoblasts induced through the introduction of Oct9 and N-myc pose the lowest risk of tumor formation, and further investigations are necessary on this aspect.

It has been reported that it is not possible for the Oct family members other than Oct3/4 to induce iPS cells with existing methods [34, 35]. Therefore, there is a possibility that Oct family members may induce direct reprogramming to osteoblasts by adjusting the transcription of downstream gene groups differently from the case of iPS cell induction. Although there have been few reports on the downstream genes of Oct6 and Oct9, once the downstream genes of Oct6 and Oct9 are found this may lead to the clarification of the mechanism of direct reprogramming from skin fibroblasts to osteoblasts. Transduction of Oct6 or Oct9 gene in combination with a Myc family member gene significantly converted normal human fibroblasts into osteoblast-like cells. Among others, Oct9 plus N-myc most effectively induced the cell fate change. These findings may lead to clarification of the mechanism of direct conversion from fibroblasts to osteoblasts. Moreover, the procedure may provide a novel regenerative therapy against osteolytic bone diseases.

Competing interests

The authors declare no conflicts of interest.

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Legends to the Figures

Fig 1. Some Oct family members in combination with L-myc induced human fibroblasts to express Runx2 mRNA.

NHDFs were transduced with the indicated genes and cultured in osteogenic medium for 4 weeks. RNA was extracted from the cells, and real-time RT-PCR was performed to evaluate Runx2 mRNA. Data represent the means $\pm$ S.E. (n=3). * p <0.05 vs. non-transduced control; ⁺ p <0.05 vs. L-myc alone.

Fig 2. Either Oct3/4 or Oct9 induced osteoblast-like phenotypic conversion in human fibroblasts in the presence of L-myc.

NHDFs were transduced with the indicated genes and cultured as in Figure 1. (A) Cells were stained with Alizarin Red S (Upper)(Scale bar= 500 μ m). Cells were lysed, and optical densities at 550 nm were measured (Lower). Data represent the means $\pm$ S.E. (n=4). * p <0.05 vs. un-transduced cells (control). (B) Cells were stained with anti-Runx2 antibody and DAPI (nuclear staining). Fluorescence images are shown. (Scale bar= 500 μ m).

Fig 3. N-myc can be substituted for L-myc in induction of osteoblast-like phenotype in cooperation with the Oct family genes.

NHDFs were transduced with the indicated genes and cultured as in Figure 1. RNA was extracted from the cells, and real-time RT-PCR was performed to evaluate Runx2 and osteocalcin mRNA levels. Data represent the means $\pm$ S.E. (n=3). * p <0.05 vs. non-transduced control; ⁺ p <0.05 vs. Oct3/4 alone; [#] p <0.05 vs. Oct6 alone; [‡] p <0.05 vs. Oct9 alone.

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394 Fig 4. Oct3/4 plus N-myc and Oct9 plus N-myc most remarkably induced fibroblasts to produce
395 Runx2 and osteocalcin.

396 NHDFs were transduced with the indicated genes and cultured as in Figure 1. Cells were
397 immunostained with either anti-Runx2 (A) or anti-osteocalcin (B) antibodies, while the cell nuclei
398 were also stained with DAPI. (Scale bar= 500 μ m).

Fig 1

Fig 2

A

B

Fig 4

A

Runx2

DAPI

Control

Oct3/4 + N-myc

Oct6 + N-myc

Oct9 + N-myc

B

Osteocalcin

DAPI

Control

Oct3/4 + N-myc

Oct6 + N-myc

Oct9 + N-myc