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<th>Promoting effect of 1,25(OH)2 vitamin D3 on osteogenic differentiation of iPS-derived osteoprogenitors into osteocyte like cells</th>
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Promoting effect of 1,25(OH)$_2$ vitamin D$_3$ on osteogenic differentiation of iPS-derived osteoprogenitors into osteocyte like cells

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**Key Words:** Induced pluripotent stem cells, Vitamin D, Osteoblasts, Osteocytes and Drug assessment

**ABSTRACT**

We recently reported a new method to purify induced pluripotent stem (iPS)-derived osteoprogenitors (iPSop). In the present paper, we optimized the procedure and characterized cells at each process step. We observed that 4 days of treatment with FGF-2, IGF-1 and TGF-β (FIT) resulted in early phase osteoblasts and 14 days of treatment resulted in late phase osteoblasts. Vitamin D has been used as a medicine for osteoporosis for many years, but the effect of vitamin D on bone cells has not yet been entirely evaluated. We treated iPSop cells with 1,25(OH)$_2$ vitamin D$_3$, and found that the expression of osteocalcin was increased and expressions of tissue-nonspecific alkaline phosphatase and runt-related transcription factor 2 (RUNX2) were decreased in iPSop-day14 cells (cells treated with FIT for 14 days) by the treatment with 1,25(OH)$_2$ vitamin D$_3$. Therefore, iPSop-day14 cells were promoted to mature osteoblasts by 1,25(OH)$_2$ vitamin D$_3$ treatment. In addition, we found that 1,25(OH)$_2$ vitamin D$_3$ treatment for 14 days enhanced not only mineralization but also expression of osteocyte markers, including dentin matrix protein-1, fibroblast growth factor-23 and matrix extracellular phosphoglycoprotein, in iPSop cells. The results of this study suggest that 1,25(OH)$_2$ vitamin D$_3$ is a potent promoter of osteoblast–osteocyte transition.
INTRODUCTION

Induced pluripotent stem cells (iPSCs) can generate a variety of patient-specific cells and are used to explore disease mechanisms and novel therapeutic molecular targets in drug development (1-4). Since mesenchymal stem cells (MSCs) have pluripotency like iPSCs, they have been used as a cell source in regeneration medicine. However, it is known that the number of cells and proliferation potency will decrease with aging. It is expected that iPSCs could overcome these problems. Recent studies have reported on a number of different types of cells generated from iPSCs (5-7). We have previously developed an effective procedure to generate not only osteoblastic cells but also osteocyte-like cells (8, 9). Osteocytes, once considered as silent cells trapped in mineralized bone, are now identified as key regulators of bone remodeling (10, 11). Osteocytes are postmitotic, terminally differentiated osteoblasts and many are located in mineralized matrices. The most abundant cell in bone and central to bone remodeling, osteocytes secrete many soluble factors that target cells on the bone surface and target other organs. Osteocytes most abundantly express receptor activator of nuclear factor kappa-B (RANK) ligand (RANKL), which functions as a key factor in osteoclast differentiation and activation. Therefore, any drugs targeting osteocyte function and signaling pathways will have a major impact on the bone remodeling process. Currently, many researchers focus on targeting osteocytes for drug development, particularly for osteoporosis. For this reason, the generation of osteocytes is important.
Osteoporosis is a very common health problem among elderly people and is thought to affect more than 75 million people worldwide (12). With aging populations in many countries, the incidence of osteoporosis will increase further. Therefore, it is important to develop novel drugs for the treatment of osteoporosis (13-16). The bisphosphonates (BPs) are antiresorptive drugs and are most commonly prescribed for the treatment of osteoporosis (15-18). However, BPs may cause bisphosphonate-related osteonecrosis of the jaw (BRONJ), which is a rare but well recognized serious side effect of long-term bisphosphonate use (19-23). Therefore, there is a need for anabolic drugs that could effectively modulate osteoblast or osteocyte activity to regenerate bone. All therapeutic management strategies for the prevention and treatment of osteoporosis include recommendations for calcium and vitamin D supplementation (24). The active form of vitamin D, 1,25(OH)₂ vitamin D₃, contributes to a wide range of biological activities by binding to the nuclear vitamin D receptor. Many studies have shown that vitamin D has both anabolic and catabolic roles in bone homeostasis (25-28). However, the effects of vitamin D on bone tissue and bone cells have not yet been entirely evaluated. It is important to assess the precise effects of vitamin D on the osteolineage cells, osteoblasts and osteocytes.

In this study, we treated tissue-nonspecific alkaline phosphatase (TNAP) positive osteolineage cells derived from hiPSCs, referred to as iPS-derived osteoprogenitors (iPSop), with 1,25(OH)₂ vitamin D₃. We compared iPSop cells with TNAP positive osteolineage cells derived from hMSCs, referred to as MSC-derived osteoprogenitors (MSCop). We found that
osteocalcin (OCN), a late phase marker for osteoblasts, was increased and several osteocyte markers were detectable in iPSop cells but not in MSCop cells, after 1,25(OH)₂ vitamin D₃ treatment. Therefore, we suggest that iPSop cells can respond to osteogenic agents and proceed to an early phase of osteocyte differentiation.
MATERIALS AND METHODS

Cell Culture and Reagents

Human iPSCs (line 201B7; Riken Cell Bank, Tsukuba, Japan) were maintained with SNL76/7 feeder cells, clonally derived from a mouse fibroblast STO cell line transformed with neomycin resistance genes and murine leukemia inhibition factor (LIF) genes, in human ES medium [Dulbecco’s modified Eagle’s medium, nutrient mixture F-12 (DMEM/F-12, Invitrogen, Carlsbad, CA) with 20% knockout serum replacement (Invitrogen) supplemented with 1× nonessential amino acids solution (Chemicon, Temecula, CA), 2 mM L-glutamine (Chemicon), 1 mM 2-mercaptoethanol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 1% penicillin/streptomycin (Invitrogen) and 5 ng/ml human FGF-2 (ReproCELL Incorporated, Yokohama, Japan)]. Human mesenchymal stem cells (hMSCs) were purchased from Lonza (Basel, Switzerland), cultured in BulletKit® mesenchymal stem cell growth medium (Lonza) and used at passage from 3 to 5. The 1,25(OH)2 vitamin D3 was purchased from Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan). Insulin-like growth factor (IGF)-1 and transforming growth factor (TGF)-β were purchased from Wako Pure Chemical Industries Ltd.

Embryoid Body Formation and In Vitro Osteogenic Differentiation

The hiPSC colonies were dissociated with a cell scraper and transferred to low attachment petri dishes to generate embryoid bodies (EBs). EBs were maintained in suspension in human
ES medium without fibroblast growth factor-2 (FGF-2) for 6 days. On day 6, the EBs were treated with 2 μM thiazovivin (Wako Pure Chemical Industries Ltd.) in human ES medium without FGF-2 for 1 h at 37°C and collected and dissociated in 0.5 mg/ml collagenase type IV (Wako Pure Chemical Industries Ltd.) for 20 min at 37°C, followed by treatment with 0.05% trypsin-EDTA (Invitrogen) for 5 min at 37°C. Cell suspensions were rinsed with α-MEM (Invitrogen, Carlsbad, CA) with 10% FBS and cells were seeded onto cell culture dishes and cultured in osteoblast medium [OBM; α-MEM with 10% FBS, 50 μg/ml L-ascorbic acid (Wako Pure Chemical Industries Ltd.) 10 mM β-glycerophosphate (Wako Pure Chemical Industries Ltd.) and 10 nM dexamethasone (Wako Pure Chemical Industries Ltd.)]. FGF-2, IGF-1 and TGF-β (FIT) were added on the following day (day 0) at a final concentration of 25 ng/ml FGF-2, 100 ng/ml IGF-1 and 1 ng/ml TGF-β. After culture in OBM with FIT for 0 days (iPSop-day0), 4 days (iPSop-day4), 10 days (iPSop-day10) or 14 days (iPSop-day14), the cells were analyzed and isolated by flow cytometry. In addition, the hMSCs were cultured in OBM with FIT and then isolated by flow cytometry on day 4 (MSCop-day4) or day 14 (MSCop-day14).

**Flow Cytometrical Analysis and Cell Sorting**

After washing with phosphate-buffered saline (PBS), the cells were treated with 0.05% trypsin-EDTA for 10 min at 37°C. The trypsinized cells were stained with
phycoerythrin-conjugated anti-human ALP antibody (R&D Systems, Minneapolis, MN) at a concentration of 10 μl/2 × 10^5 cells for 45 min on ice. After staining, cells were washed three times with PBS, suspended in PBS containing 0.5% FBS, passed through a 40-μm mesh filter and maintained at 4°C until FACS sorting. We defined TNAP positive cells from hiPSCs and hMSCs as iPSop and MSCop, respectively. Dead cells were excluded by propidium iodide staining (2 μg/ml) and forward scatter. Flow cytometrical analysis and cell sorting were performed on a FACS Aria (Becton-Dickinson, San Jose, CA).

**Treatment with 1,25(OH)₂ Vitamin D₃**

After culture in OBM with FIT for 14 days (iPSop-day14), the cells were isolated by flow cytometry, and 1,25(OH)₂ vitamin D₃ at a concentration of 10 or 50 nM was added on the following day (day 0). The medium was refreshed every three days and the cells were analyzed on day 6 and day 12. In addition, after culture in OBM with FIT for 4 days (iPSop-day4, MSCop-day4) or 14 days (iPSop-day14, MSCop-day14), iPSop and MSCop cells were isolated by flow cytometry and were treated with 50 nM 1,25(OH)₂ vitamin D₃. The medium was refreshed every three days and the cells were analyzed on day 6 and day 12.

**RNA Isolation and Reverse Transcription Gene Expression**

Total RNA was extracted using QIAzol reagent (Qiagen Inc., Valencia, CA) according to the
manufacturer’s instructions. cDNA was synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time RT-PCR (qRT-PCR) was performed using Premix Ex Taq reagent (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. Target genes included type I collagen (COL1A1), tissue-nonspecific alkaline phosphatase (TNAP), runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), osterix (OSX), and sclerostin (SOST). 18S rRNA was used as an internal control. All primers and probes are presented in Table 1. Relative expression of genes of interest was estimated using the $\Delta \Delta Ct$ method.

RT-PCR was performed to examine some of the osteocyte specific markers using ExTaq DNA polymerase (Takara Biotechnology, Shiga, Japan). Target genes included dentin matrix protein-1 (DMP-1), fibroblast growth factor-23 (FGF-23), matrix extracellular phosphoglycoprotein (MEPE) and podoplanin (PDPN). β-actin was used as an internal control. Amplified PCR products were electrophoresed on 2% agarose gels. PCR primers used are listed in Table 2.

**Histochemistry for Osteogenesis**

The iPSoP-day 14 cells were treated with 1,25(OH)$_2$ vitamin D$_3$ for 2 weeks. The media were removed, and the cells were rinsed in PBS and fixed in 4% paraformaldehyde for 5 min at room temperature. For mineralization, plates were treated with alizarin red solution for 5 min
at room temperature. After 5 min, plates were rinsed in PBS and visually examined.

Statistical Analysis

All data are expressed as the mean ± standard deviation (S.D). When analysis of variance (ANOVA) indicated differences among the groups, multiple comparisons between each experimental group were performed using the Bonferroni test. Statistical significance was defined as $P < 0.05$. 
RESULTS

Induction of iPSop Cells

As expected, the hiPSCs expressed a large amount of TNAP. Cells separated from EBs rapidly lost TNAP expression but FIT treatment greatly increased TNAP expression. (Figure 1a, b). TNAP positive cells were isolated by FACS sorting and defined them as iPSop cells. Furthermore, we estimated osteoblast specific markers in iPSop cells at each differentiation stage during culture in OBM. COL1A1 and TNAP expression increased over time until day10. In iPSop-day10 cells, the expression of TNAP and RUNX2 was two times that in iPSop-day0 cells but OSX expression was not detected. In iPSop-day14 cells, TNAP expression was further increased, RUNX2 expression was decreased and OSX expression sharply increased (Figure 1c). Therefore, culture in OBM led to the differentiation of iPSop cells into osteoblasts over time. These results demonstrated that we could obtain osteoblasts at various stages of differentiation from iPSCs.

1,25(OH)₂ Vitamin D₃ Promotes Osteogenic Differentiation of iPSop Cells

After the treatment of iPSop-day14 cells with 1,25(OH)₂ vitamin D₃ for 6 days, the expression of COL1A1 and OCN were increased and the expression of TNAP and RUNX2 were decreased (Figure 2a). Compared to 6 days 1,25(OH)₂ vitamin D₃ treatment, these expression were more affected by 12 days 1,25(OH)₂ vitamin D₃ treatment (Figure 2b). The increases or
decreases in osteoblast markers occurred in a dose-dependent manner and the longer the treatment, the change in osteoblast marker expression was more significant.

**iPSop Cells are More Reactive to 1,25(OH)\textsubscript{2} Vitamin D\textsubscript{3} than MSCop Cells**

Furthermore, we investigated the difference in response to 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} between iPSop cells and MSCop cells. We treated iPSop-day4 and MSCop-day4 cells, which were isolated by flow cytometry after culture in OBM with FIT for 4 days, with 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3}. Every osteoblast marker in MSCop-day4 cells increased but there was little difference between 6 and 12 days of 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} treatment (Figure 3a). In iPSop-day4 cells, again every osteoblast marker increased with 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} treatment and marker expression continued to increase over time (Figure 3a). In iPSop-day14 cells, expression of COL1A1 and OCN increased and expression of TNAP, RUNX2 and OSX decreased over time with 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} treatment (Figure 3b). However, TNAP and RUNX2 expression in MSCop-day14 cells did not decrease with 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} treatment (Figure 3b). These results suggest that iPSop cells were more reactive to 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} treatment than MSCop cells, and iPSop-day14 cells were advancing to a late phase of osteoblast differentiation.

**iPSop Cells Expressed Various Osteocyte Marker Genes with 1,25(OH)\textsubscript{2} Vitamin D\textsubscript{3}**
Treatment

Osteocyte specific markers, such as DMP-1, FGF-23 and MEPE, were detected except SOST in iPSop-day14 cells treated with 1,25(OH)2 vitamin D3 for 14 days (Figure 4a). In addition, 1,25(OH)2 vitamin D3 promoted mineralization in iPSop-day14 cells, when compared with vehicle alone (Figure 4b). Therefore, these cells had proceeded to an early phase of osteocyte differentiation by the 1,25(OH)2 vitamin D3 treatment.
DISCUSSION

We recently reported a new method to purify osteoprogenitors from hiPSCs. One of the unique advantages of the method is the ability to generate osteocyte-like cells. In the present study, we used our system to evaluate the effects of 1,25(OH)₂ vitamin D₃ on osteoblast differentiation. First, we identified the optimal timing to obtain iPSoP cells by sorting TNAP positive cells at different time points. Osteoblast marker gene expression differed over time. Cells liberated from hiPSC-derived EBs after 10 days of culture in OBM with FIT were sorted as TNAP positive cells and these cells showed increased expression of RUNX2. Four days later, after 14 days of culture in OBM with FIT, OSX expression was significantly increased (Figure 1c). Therefore, it appears that cells liberated from hiPSC-derived EBs after 14 days of culture in OBM with FIT are differentiated into late-phase osteoblasts (29). Next, we investigated the effects of 1,25(OH)₂ vitamin D₃ on these iPSoP cells. We found that iPSoP cells responded to 1,25(OH)₂ vitamin D₃ and the expression of osteoblast markers in these cells was affected in a dose-dependent manner. The effects of 1,25(OH)₂ vitamin D₃ on osteoblast marker gene expression suggested that iPSoP-day4 cells were early-phase osteoblasts and iPSoP-day14 cells were late phase osteoblasts. Treatment with 1,25(OH)₂ vitamin D₃ increased all osteoblast markers in iPSoP-day4 cells and MSCoP-day4 cells. However, 1,25(OH)₂ vitamin D₃ treatment decreased the expression of some osteoblast markers (TNAP, RUNX2 and OSX) and increased OCN expression in iPSoP-day14 cells but
not in MSCop-day14 cells. Moreover, we observed an increase of mineralization in iPSop-day14 cells after 14 days of 1,25(OH)₂ vitamin D₃ treatment. Ochiai-Shino previously showed that it took 40 days for iPSop cells to express osteocyte markers (9). The current observations clearly showed that 1,25(OH)₂ vitamin D₃ could be a potent accelerator of osteodifferentiation, particularly from late to early phase osteocytes. However, we did not detect an increase in sclerostin (an osteocyte-derived inhibitor of cultured osteoblasts) expression in this study (data not shown). It is suggested that high dose 1,25(OH)₂ vitamin D₃ treatment could induce sclerostin expression in newly formed osteocytes or existing osteocytes, which in turn, could cause inhibition of Wnt signaling and accelerate the resorption of bone tissue.

Another important point is that this method could be used for research into new drugs for osteoporosis. Human cell-based in vitro assay systems are a basic requisite for drug discovery and iPSCs reprogrammed from somatic cells can provide an opportunity to establish these systems (3, 4). Recent research has revealed that osteocytes are part of the command centre for osteoremodeling, and one reason is that sclerostin is exclusively secreted from osteocytes (30). A neutralizing antibody to sclerostin has been shown to be effective in initial preclinical and early clinical trials. Postmenopausal women treated with a neutralizing antibody to sclerostin showed an increase in markers of bone formation (31). Therefore, agents regulating osteocyte functions such as sclerostin secretion could be effective in the treatment of
osteoporosis. However, screening methods using animal models may not reflect the human situation and some compounds found to be successful in cellular or animal models have failed to show effectiveness in clinical trials (32, 33). It has been postulated that drug screening using iPSCs could identify compounds that increase differentiation and promote maturation in target cells (33). To date, there are few examples of the use of high-throughput screening methods to assess differentiation. We believe that the iPSop cells generated using our method could be a valuable cell source for high-throughput screening to research new drugs for a range of diseases including osteoporosis.

CONCLUSION

We could obtain iPSoP cells as various differentiated-stage osteoblasts. Treatment with 1,25(OH)2 vitamin D3 could promote osteogenic differentiation in iPSoP cells and could accelerate the expression of osteocyte marker genes. The effect of 1,25(OH)2 vitamin D3 was more significant on iPSCs than MSCs.

ACKNOWLEDGMENTS

The human iPS cell line 201B7 (HPS0063) was provided by Riken, BRC through the National Bio-Resource Project of the MEXT, Japan.
REFERENCES


Figure legends

Figure 1. The expression of TNAP during hiPSC-to-osteogenic cell differentiation and the expression of osteoblast markers. (a): The frequency of TNAP positive cells in hiPSCs, EBs, and single cells derived from EBs cultured in OBM with FGF-2/IGF-1/TGF-β (FIT). FIT treatment greatly increased TNAP expression. (b): Flow cytometrical analysis was performed on hiPSCs, EBs, and single cells cultured in OBM with FIT for 0, 4, 10, and 14 days. (c): qRT-PCR analysis of COL1A1, TNAP, OSX, and RUNX2 was performed on iPSop-day0, 4, 10, and 14 cells. iPSop cells expressed several osteoblast marker genes. mRNA levels were normalized to those of 18S rRNA. Experiments were performed in triplicate. Values represent the mean ± S.D, n = 3. Bonferroni correction for multiple comparisons was applied. *P < 0.05, **P < 0.01.

Figure 2. Expression of osteoblast markers after 1,25(OH)2 vitamin D3 treatment. (a): After isolation by flow cytometry, iPSop-day14 cells were treated with vehicle (control), 10 nM, or 50 nM 1,25(OH)2 vitamin D3 for 6 days or (b) 12 days. The expressions of osteoblast markers affected in a dose- and time-dependent manner. Expression of COL1A1, TNAP, OCN, and RUNX2 was analyzed with qRT-PCR, and mRNA levels were normalized to those of 18S rRNA. Experiments were performed in triplicate. Values represent the mean ± S.D, n = 4. Bonferroni correction for multiple comparisons was applied. *P < 0.05, **P < 0.01.
Figure 3. Expression of osteoblast markers in iPSoP and MSCop cells at different
differentiation stages after 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} treatment. iPSoP and MSCop cells express
various osteoblast markers. Comparison of expression of osteoblast marker genes between
cells treated with vehicle and 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3}. (a): In iPSoP- and MSCop-day4 cells.
All the other osteoblast marker expressions in these cells were increased. (b): In iPSoP- and
MSCop-day14 cells. In iPSoP-day14 cells, expressions of COL1A1 and OCN were increased
and expressions of TNAP, RUNX2 and OSX were decreased. On the other hand, all the other
osteoblast marker expressions were increased in MSCop-day14 cells. These cells were treated
with vehicle (white bar) or 50 nM 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} (black bar) for 6 or 12 days.
Expressions of \textit{COL1A1}, \textit{TNAP}, \textit{OCN}, \textit{RUNX2}, and \textit{OSX} were analyzed with qRT-PCR, and
mRNA levels were normalized to those of 18S rRNA. Experiments were performed in
triplicate. Values represent the mean ± S.D, n = 3. Bonferroni correction for multiple
comparisons was applied. *P < 0.05, **P < 0.01.

Figure 4. Expression of osteocyte specific marker genes and the mineralization by alizarin red
staining. (a): Expression of osteocyte specific markers in iPSoP-day14 cells treated in OBM
without 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} (vehicle) and with 50nM 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} (vitamin D\textsuperscript{+})
for 14 days. Osteocyte specific markers, such as DMP-1, FGF-23 and MEPE, were detected
after the 50nM 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} treatment for 14 days. The expression of dentin
matrix protein-1 (DMP-1), fibroblast growth factor-23 (FGF-23), matrix extracellular phosphoglycoprotein (MEPE), and podoplanin (PDPN) were analyzed by RT-PCR. β-actin was used as an internal control. RT-PCR was performed using ExTaq DNA polymerase. Amplified PCR products were electrophoresed on 2% agarose gels. PCR primers are presented in Table 2. (b): Mineralization in iPSop-day14 cells treated with vehicle (vitamin D−) and 50nM 1,25(OH)2 vitamin D3 (vitamin D+) for 14 days with alizarin red staining. 1,25(OH)2 vitamin D3 enhanced mineralization in iPSop-day14 cells.
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Fig. 1

(a) TNAP positive cells (%) for different stages of OBM culture:

(b) Flow cytometry analysis showing TNAP positive cells at different days:

(c) Relative expression levels of COL1A1, TNAP, OSX, and RUNX2 over time.
Fig. 2

a

Day 6

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b

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Legend:
- Vehicle
- 10nM Vitamin D
- 50nM Vitamin D
Fig. 3

![Graph showing relative expression of COL1A1, TNAP, OCN, and RUNX2 in iPSoP-day4 and MSCop-day4 cells.](image)
Fig. 4

(a) 

(b)