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Research article

Establishment of human immortalized mesenchymal stem cells lines for the monitoring and analysis of osteogenic differentiation in living cells

Takashi Narai [a,1], Ryohei Watase [b,1], Yuji Nakayama [c,*,2], Isamu Kodani [a], Toshiaki Inoue [b], Kenji Kokura [b,d,**,2]

[a] Division of Oral and Maxillofacial Surgery, Department of Sensory and Motor Organs, School of Medicine, Tottori University Faculty of Medicine, Yonago, 683-8504, Japan
[b] Division of Human Genome Science, Department of Molecular and Cellular Biology, School of Life Sciences, Tottori University Faculty of Medicine, Yonago, 683-8503, Japan
[c] Division of Radioisotope Science, Research Initiative Center, Organization for Research Initiative and Promotion, Tottori University, Yonago, 683-8503, Japan
[d] Department of Biomolecular Science, Faculty of Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba, 274-8510, Japan

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ABSTRACT

Mesenchymal stem cells (MSCs) are expected to be useful in bone regeneration treatment for various diseases and conditions, including cleft lip and palate, fracture, and bone absorption. However, to date, MSCs have failed to produce satisfactory results in clinical settings. This is primarily due to the low rate of induced osteogenic differentiation. To realize MSC potential, it is necessary to establish methods for the isolation of MSC-derived living osteoblasts. However, no osteoblast markers have been reported to date. In an attempt to develop a method for the assessment of osteoblast differentiation, we established reporter human immortalized MSC (hiMSC) lines for *in vitro* monitoring of bone gamma-carboxyglutamate protein (BGLAP, osteocalcin) expression. To this end, we successfully knocked-in an enhanced green fluorescent protein (EGFP) gene cassette immediately downstream of the first ATG of BGLAP via CRISPR-Cas9, and established hiMSC lines expressing EGFP to monitor osteogenic differentiation. On differentiation day 7, EGFP-positive cells were collected by flow cytometric cell sorting, and the expression of EGFP and endogenous BGLAP was analyzed. During osteogenic differentiation, EGFP upregulation was found to correlate with expression of endogenous BGLAP. Moreover, mineralization was confirmed using Alizarin red-S staining after two weeks of osteogenic differentiation of the modified hiMSC lines. The modified hiMSC lines, as well as the derived differentiated osteoblasts obtained herein, are valuable tools for the monitoring osteoblast gene and protein expression, and can be used to develop novel methods for isolating living osteoblasts.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that are characterized by self-renewal and can differentiate into a variety of cell types such as osteoblasts, chondrocytes, myocytes, and adipocytes [1, 2, 3]. MSCs are considered promising seed cells to repair or regenerate various tissues including cartilage, bone, and adipose tissues. In current bone regenerative therapy, autologous bone graft is the "gold standard" method for healing bone defects [4]. However, limited bone availability and severe surgical invasion during autologous grafting necessitate the development of other strategies to augment or replace autologous bone. Therefore, clinical trials of treatments for conditions such as cleft lip and palate, fracture, and bone absorption using MSCs collected from bone marrow, fat, and dental pulp are actively being carried out across the world [5]. However, to date, MSCs have failed to produce satisfactory results, primarily due to the low rate of induced osteogenic differentiation. Using osteoblasts that originate from MSCs and committed to osteogenic differentiation might be one option to improve bone regenerative therapy.

* Corresponding author.
** Corresponding author.
E-mail addresses: yujin@tottori-u.ac.jp (Y. Nakayama), kenji.kokura@sci.toho-u.ac.jp (K. Kokura).
[1] These authors contributed equally to this work.
[2] These authors are co-corresponding authors.

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Thus far, undifferentiated MSCs have been purified using cell surface markers or RNA probes [6, 7]. However, surface markers that are capable of efficiently purifying osteoblasts are yet to be identified, since most known markers of bone differentiation are cytoplasmic, nuclear, or secreted proteins. In addition, the current data and methods obtained by in vitro assays for bone regenerative medicine have not been translated to in vivo studies [8]. To overcome this issue, it is necessary to establish efficient differentiation conditions and methods for the purification and further characterization of osteoblasts.

In this study, a human immortalized MSC line (hiMSC) [9, 10, 11] was genetically modified to allow for the *in vitro* monitoring of osteogenic differentiation. Bone gamma-carboxyglutamate protein (BGLAP, osteocalcin) is protein that is secreted by mature osteoblasts; thus, expression of BGLAP is an indicator for bone function. Previously, we established an *in vitro* [10, 12] and *in vivo* [10, 12] BGLAP luciferase assay system; however, we have not achieved this in a cell assay system. Therefore, in the present study, the EGFP gene was knocked into hiMSCs under control of the BGLAP enhancer/promoter sequence in order to monitor BGLAP expression during osteogenic differentiation in living cell. Modified hiMSC lines retained the ability to differentiate into osteogenic cells. Moreover, EGFP expression served as an indicator of osteogenic differentiation and permitted the selective sorting of differentiated osteogenic cells. Currently, no surface markers are available for the isolation of living differentiated osteoblasts. Osteogenic cells induced from the modified hiMSC lines described herein may, therefore, be used for identifying new markers capable of distinguishing differentiated osteoblasts from other cells. This work will serve to accelerate the development of methods for the establishment and purification of living differentiated osteoblasts for use in bone regenerative medicine.

2. Materials and methods

2.1. Cell culture and induction of osteogenic differentiation

Human immortalized mesenchymal stem cells (hiMSC) were gifted from Prof. Junya Toguchida (Kyoto University) [9]. Cells were maintained at 37 °C with 5 % CO_2 in high-glucose (4.5 g/L glucose) Dulbecco's modified Eagle's medium (DMEM; Nacalai tesque, Kyoto, Japan) supplemented with 10 % fetal bovine serum (FBS; Corning, Cellgro, New York, USA), and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Nacalai tesque). To induce osteogenic differentiation, hiMSC cells were seeded in 6-well plates (3×10^4 cells per well) with growth medium, and on the next day the growth medium was replaced by Mesenchymal Stem Cell Osteogenic Differentiation Medium (PromoCell, Heidelberg, Germany) with or without 100 nM vitamin D3 (VD3; Cayman Chemical, Michigan, USA). Osteogenic differentiation was continued for 1, 2, and 3 weeks.

2.2. Construction of a vector set for CRISPR/Cas9 mediated knock-in and transfection into hiMSC cells

We used a double-nicking strategy using the D10A mutant Cas9 and a pair of guide RNA expression vectors [13] for EGFP knock-in into the BGLAP locus. The pX335-U6-Chimeric_BB-CBh-hSpCas9n (D10A) was a gift from Feng Zhang (Addgene plasmid # 42335; http://n2t.net/addgene:42335; RRID: Addgene_42335) [14]. Two pX335 vectors containing different sgRNAs were constructed as follows: target sequences for sgRNAs were selected using the CRISPR design tool (http://crispr.mit.edu) [15, 16], and were as follows (PAM sequences are underlined): 5′-GGTGTCTCGGTGGCTGCGCTGGG-3′ and 5′-CCTCACACTCCTCGCCCTATTGG-3′. The targeting vector (repair template for homology-directed repair) was generated as follows: a drug-resistant gene [Blasticidin -S deaminase (BSD)] driven by the SV40 promoter (from pGL3-control vector, Promega, Madison, Wisconsin, USA) along with a bGH poly A signal sequence (from pcDNA3, Thermo Fisher Scientific, Massachusetts, USA) were generated by overlapping PCR [17] and inserted into a double-digested pBluescript II KS (+) vector (HindIII and PstI site). The left homologous region, a BamHI fragment of BGLAP promoter, and a 5′ untranslated region (~1.3 kb fragment) fused to EGFP were inserted into the BamHI site of the vector. Next, the right homologous region (a 843 bp fragment downstream to the first ATG of endogenous BGLAP gene) was amplified by PCR using hiMSC genomic DNA as a template, and was inserted into the vector via XhoI and KpnI sites. The complete insert sequence (left arm – EGFP – BSD – right arm) of the resulting vector is shown in supplementary information.

A mixture of px335 (D10A Cas9 expression plasmid) with one paired sgRNA and the targeting vector were co-transfected into hiMSC using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Massachusetts, USA), according to manufacturer's instructions. Four days after transfection, cells were selected in medium containing BSD (3 μg/mL), and BSD-resistant cell clones were isolated.

2.3. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was prepared from cells using a NucleoSpin RNA kit (MACHEREY-NAGEL, Düren, Germany) and 500 ng of total RNA were used to synthesize cDNA using a ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan), according to manufacturer's instructions. Gene expression was checked using qRT-PCR performed in triplicate on a StepOne Plus Real-Time PCR System (Applied Biosystems, California, USA), using Thunderbird SYBR qPCR Mix (Toyobo) and the following primers: human BGLAP primers forward, 5′-CACTCCTCGCCCTATTGGC-3′ and reverse, 5′-CCCTCCTGCTTGGACACAAAG-3′; human GAPDH primers forward, 5′-ACAACTTTGGTATCGTGGAAGG-3′ and reverse, 5′-GCCATCACGCCACAGTTTC-3′; human alkaline phosphatase (ALP) primers forward, 5′-CACCAACGTGGCTAAGAATG-3′ and reverse, 5′-ATCTCCAGCCTGGTCTCCTC-3′. We evaluated the quality of the qRT-PCR analysis by verifying the PCR efficiency using serially diluted cDNA as template, and performed melting curve analysis for primer check.

2.4. Genomic PCR

Genomic DNA was isolated from cell monolayers using a NucleoSpin Tissue TX kit (MACHEREY-NAGEL), as per manufacturer's protocol. PCR was performed using KOD FX Neo kit (Toyobo) under the following conditions: 2 min at 94 °C for 1 cycle, and 10 s at 98 °C, 30 s at 60 °C, and 3 min at 68 °C for 40 cycles. Primers to detect knock-in event (Figure 2C upper) were specific to the human BGLAP promoter fused to EGFP: forward, 5′- GGCAAGGGGCTTACTAGACC -3′ and reverse, 5′-TTCTCGTTGGGGTCTTTGCTC-3′. Primers to detect an endogenous unmodified BGLAP allele (from human BGLAP promoter to human BGLAP exon 1. Figure 2C, lower) were: forward, 5′-GGCAAGGGGCTTACTAGACC-3′ and reverse, 5′-CACAGGCCCACAGATTCCTC-3’.

2.5. Flow cytometry and cell sorting

The hiMSCs were trypsinized and resuspended in the fresh culture medium. The percentage of EGFP-positive cells was determined using a Gallios flow cytometer (Beckman Coulter, California, USA) at 488 nm excitation. Cell sorting was performed using a MoFlo XDP cell sorter (Beckman Coulter) at 488 nm excitation. EGFP purity and positivity in the sorted fractions were visually verified by using a fluorescence microscopy Nikon ECLIPSE T*i*-U (Nikon CORPORATION, Tokyo, Japan) and evaluated by flow cytometric analysis (Gallios). For parental hiMSCs, forward versus side scatter gating was used for cell sorting as the control. The sorted cells were cultured in osteogenic differentiation medium containing VD3 for 3–4 days prior to purification of total RNA.

2.6. Mineralization analysis

Mineralization assays for osteoblasts were performed in a 6-well plate using Alizarin Red S staining kits (Cosmo bio, Tokyo, Japan).

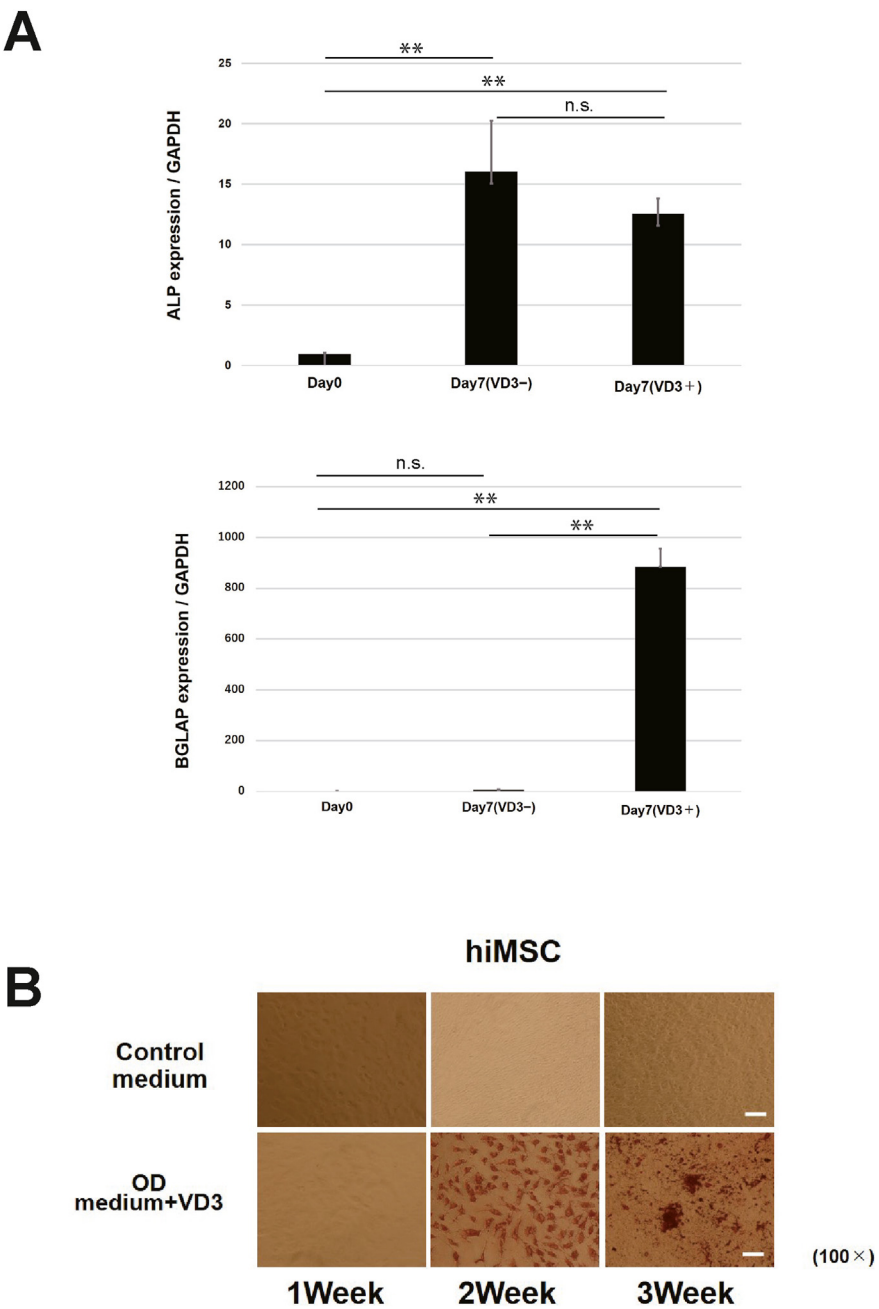


Figure 1. Analysis of osteogenic differentiation of parental hiMSC lines. (A) qRT-PCR analysis of endogenous ALP and BGLAP expression during osteogenic differentiation with or without VD3 in the parental hiMSC cell line (day 0 and day 7). Bars represent mean ± S.D. from three independent experiment. Statistical analysis was performed using student's *t*-test. ** represents statistical significance of $p < 0.01$, n.s. represents no statistical significance. (B) Mineralization assay every week during 3 weeks of osteogenic differentiation with VD3 in the parental hiMSC cell line. OD, osteogenic differentiation. Scale bar: 200 μm.

After washing three times with PBS, osteoblasts were fixed with methanol at 4 °C for 20 min. Next, methanol was removed, and osteoblasts were washed three times with purified water followed by staining with Alizarin Red S at room temperature (23–25 °C) for 5 min. Finally, cells were washed with buffer solution included in the kit and images were captured using a microscope Nikon ECLIPSE T*i*-U (Nikon).

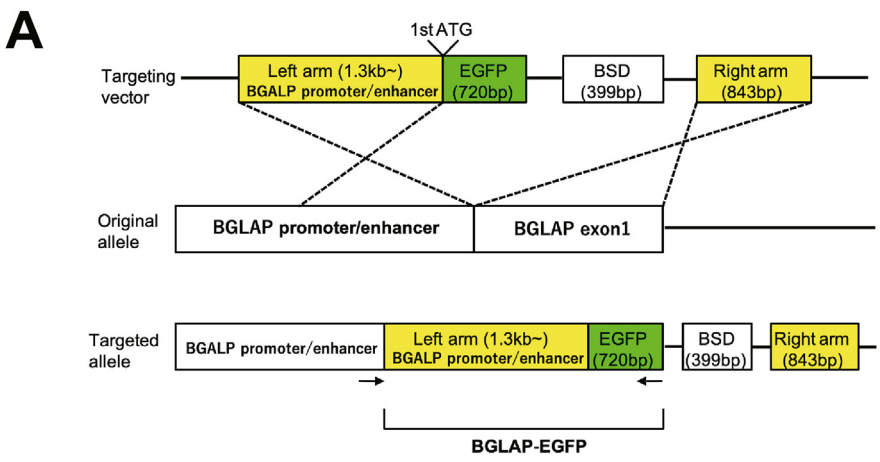
2.7. Statistical analysis

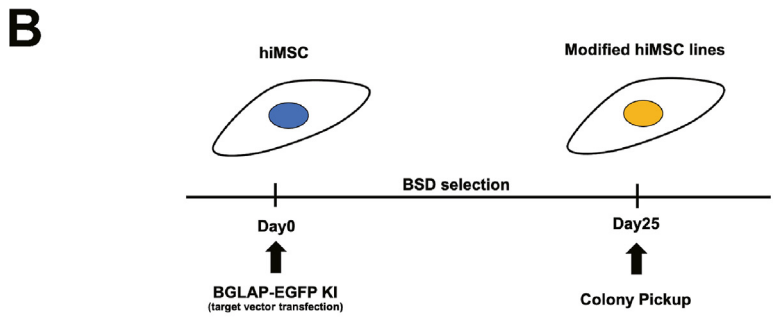
Statistical analysis was performed using BellCurve for Excel software. Using student's *t*-test, P-values of <0.01 and <0.05 were considered to be statistically significant.

3. Results

3.1. Evaluation endogenous ALP and BGLAP expression in hiMSC lines after osteogenic differentiation

ALP and BGLAP (osteocalcin) are early and late markers of osteogenic differentiation, respectively [18]. We first verified whether the endogenous expression of these markers changed after osteogenic differentiation of the parental hiMSCs. To this end, we used osteogenic differentiation medium with or without vitamin D3 (VD3). VD3 has been reported to enhance the differentiation of human MSCs to osteoblasts by affecting the expression of various genes [19, 20, 21], thereby suggesting an autocrine/paracrine effect of VD3 in osteogenic differentiation [22].





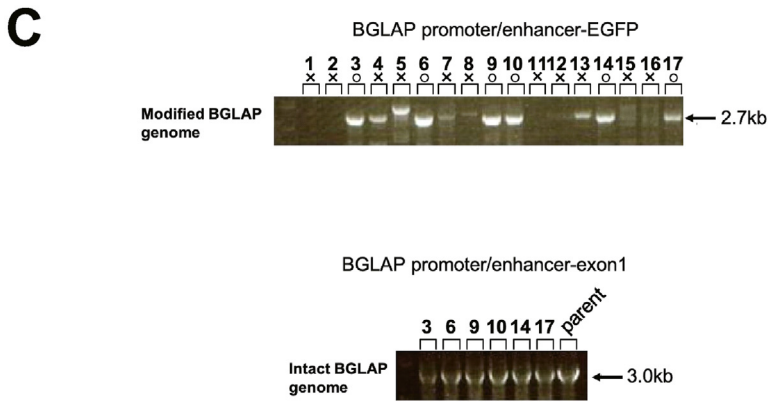


Figure 2. Genotypic analysis of the established EGFP knocked-in hiMSC lines. (A) Knock-in strategy and design of the targeting vector for CRISPR/Cas9-mediated EGFP knock-in at the BGLAP locus. The arrows indicate the PCR primers used to confirm the EGFP knock-in event at the BGLAP locus in hiMSCs after BSD-based selection. (B) Experimental schematic workflow of selecting KI clones. (C) The upper panel shows the genomic PCR analysis to detect CRISPR/Cas9-mediated EGFP knock-in, whereas the lower panel demonstrates that the other allele remained unmodified in the BGLAP locus of BSD-resistant hiMSC lines.

Our results of qRT-PCR analysis on day 7 of osteogenic differentiation showed that ALP expression increased independently of VD3, whereas BGLAP expression increased only when VD3 was added to the differentiation medium (Figure 1A). This result was consistent with our previous report as well as other studies [9, 10]. Thus, VD3 was used for osteoblast differentiation in the subsequent experiments. Additionally, mineralization was confirmed in these cells by Alizarin Red S staining after 2 weeks of osteogenic differentiation with VD3 (Figure 1B).

3.2. Cloning and evaluation of hiMSC EGFP reporter lines established by CRISPR/Cas9-mediated knock-in

It has been previously reported that the knock-in of reporters, such as genes encoding fluorescent proteins, into the genome enables the identification and purification of living cells in a tissue- and/or lineage-specific manner [23]. Thus, to monitor the expression of BGLAP during the induction of osteogenic differentiation, we used CRISPR/Cas9 system to establish a hiMSC cell line with *EGFP* knocked-in the BGLAP gene promoter. The knock-in strategy is shown in Figure 2A. The D10A mutant Cas9 (nickase mutant) was selected due to its reduced off-target effects and high knock-in efficiency [13]. We constructed two D10A Cas9 expression vectors (pX335 derivative) containing one paired sgRNA targeting the first ATG of the BGLAP coding sequence, and a knock-in targeting vector with left and right arms (~1.3 kb and 843 bp in length, respectively) for homology-dependent recombination (see Materials and Methods for details).Next, parental hiMSCs were co-transfected with two D10A Cas9 expression vectors and a knock-in targeting vector (Figure 2B). Transfected cells were cultured in BSD-selective medium, and 17 BSD-resistant clones were obtained. To verify that the gene knock-in was in the correct orientation in the selected clones, genomic PCR analysis was performed. Our results confirmed proper knock-in events in 6/17 candidate clones (Figure 2C, upper). Additional genomic PCR performed with other primer sets confirmed that the intact BGLAP allele remained in the six clones after the knock-in (Figure 2C, bottom) [9].

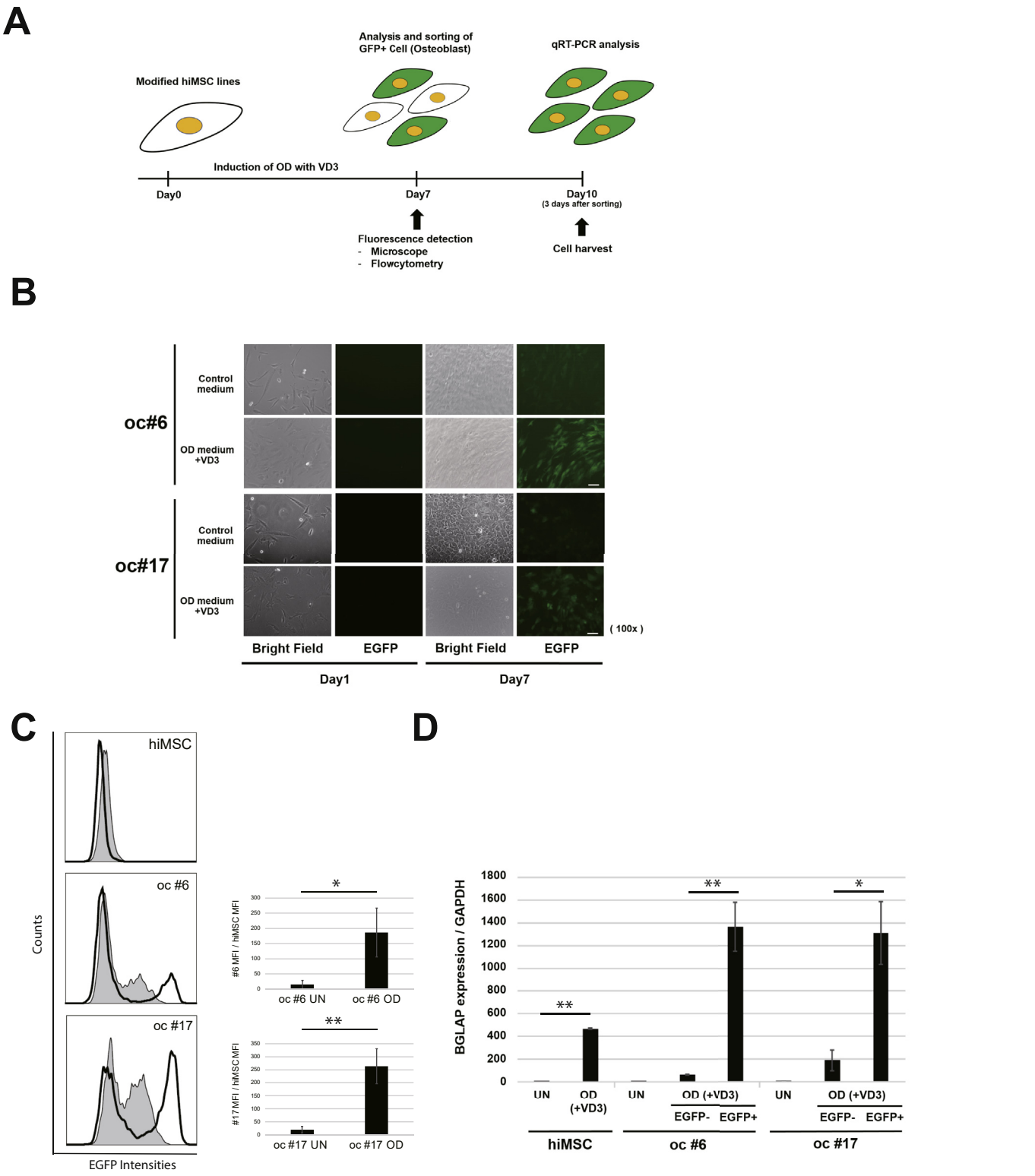


Figure 3. EGFP knocked-in hiMSC lines show increased expression of endogenous BGLAP after induction of osteogenic differentiation. (A) Experimental workflow for cell sorting and qRT-PCR. (B) Bright-field and fluorescent microscopic images of established EGFP-knocked-in hiMSCs lines (oc#6 and oc#17) after osteogenic differentiation in the presence or absence of VD3 (day 1 and day 7). Scale bar: 200 μm (C) Flow cytometric evaluation of mean fluorescence intensities (MFI) in the selected hiMSC lines after osteogenic differentiation (day 7) is shown on the right. In the histograms on the left, bold-solid lines and gray-filled spectra indicate osteogenic differentiated cells and undifferentiated cells, respectively. (D) EGFP-positive and -negative cells were sorted and subjected to qRT-PCR for analyzing the expression of EGFP and endogenous BGLAP. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method with *GAPDH* as the internal control. Cell sorting was performed twice independently. Each sample was analyzed in triplicate and the S.D. is shown. Statistical analysis in (C) and (D) were performed using student's *t*-test: ** and * represent statistical significance of $p < 0.01$ and $p < 0.05$, respectively. UN, undifferentiation; OD, osteogenic differentiation.

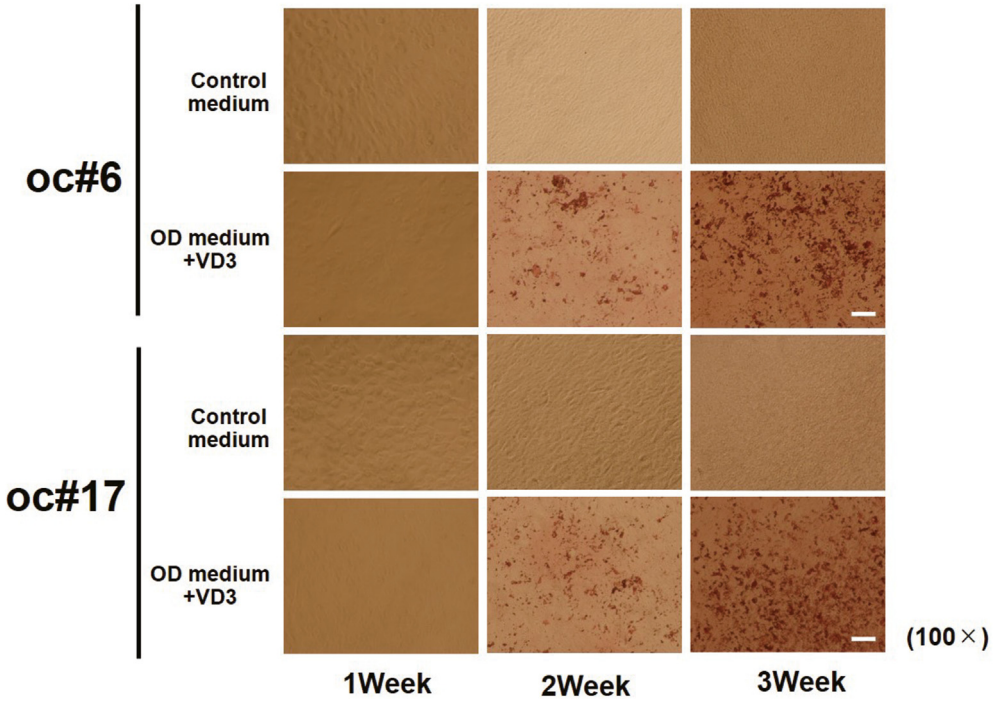


Figure 4. Mineralization assay of EGFP knocked-in hiMSC lines after induction of osteogenic differentiation. Alizarin Red S staining of EGFP knocked-in hiMSC lines after induction of osteogenic differentiation every week during 3 weeks. OD, osteogenic differentiation. Scale bar: 200 μm.

Next, we used these six clones to induce osteogenic differentiation for 7 days and confirmed EGFP expression (Figure 3A). From these six clones, we chose two clones (#6 and #17: henceforth referred to as oc#6 and oc#17) in which high EGFP expression was observed by fluorescence microscopy after induction of osteogenic differentiation (Figure 3B). Thus, to further assess changes in EGFP expression due to osteogenic differentiation we used flow cytometry to compare the mean fluorescence intensity of EGFP before and after induction of osteogenic differentiation. Although weak EGFP expression was detected in both clones before osteogenic induction (Figure 3B), this was most likely due to cytokine secretion from undifferentiated MSCs, resulting in a paracrine effect [22]; mean fluorescence intensities differed significantly for both clones after differentiation (Figure 3C). Taken together, these results suggest that the induction of EGFP expression correlated with osteogenic differentiation in our system (Figure 3B, C).

3.3. Correlation between endogenous BGLAP and EGFP expression in the modified hiMSC lines

To evaluate whether the expression of endogenous BGLAP was induced during osteogenic differentiation in established hiMSC lines, and explore its correlation with EGFP expression, we next conducted cell sorting experiments. Following induction of osteogenic differentiation for 7 days in the presence of VD3, EGFP-positive and -negative populations from the two selected clones (oc#6 and oc#17) were sorted. Total RNA was isolated from each sorted fraction and subjected to real-time qRT-PCR analysis. Our results revealed that in EGFP-positive cells, the level of endogenous BGLAP expression was more than 1000-fold higher than in undifferentiated parental hiMSCs (Figure 3D). This result suggested that in the selected clones, EGFP expression was accompanied by the induction of endogenous BGLAP expression and, therefore, indicated osteogenic differentiation. As a control, parental hiMSCs were simultaneously subjected to osteogenic differentiation and cell sorting, and endogenous BGLAP expression was analyzed in differentiated and undifferentiated cells. In parental hiMSCs, BGLAP expression showed an approximately 500-fold increase after differentiation (Figure 3D). Furthermore, to evaluate functional properties of differentiated osteoblasts from EGFP knocked-in hiMSC lines, Alizarin Red S staining was performed. As a result, calcium deposits began to appear after the second week of osteogenic differentiation (Figure 4), similar to that observed with parental hiMSC (Figure 1B).

4. Discussion

The identification of cell surface markers is critical for development of cell-based bone regenerative medicine, as they may allow the isolation and enrichment of living osteoblasts collected from individual patients. However, to date, no cell surface markers have been reported to facilitate the isolation of living osteoblasts and purification methods for isolating living osteoblasts, as well as optimized osteogenic differentiation method, are required. To this end, in the present study, we established hiMSC cell lines using a CRISPR/Cas9-mediated EGFP knock-in approach to monitor osteogenic differentiation *in vitro*. The osteoblasts differentiated from these modified hiMSC lines were able to be sorted for further analysis. Following osteogenic differentiation, EGFP-positive cells expressed high levels of endogenous BGLAP (Figure 3D), and exhibited mineralization (calcium deposit) as assessed by Alizarin Red S staining after 2 weeks of osteogenic differentiation in the presence of VD3 (Figure 4).

As such high expression of BGLAP and following mineralization after induction of osteogenic differentiation can be a good standard for the assessment of function and strength of matured osteoblast that is required in bone regeneration, the modified hiMSC lines established in this study may prove beneficial in many biological and clinical applications. For example, our cells would be useful in the screening of drugs capable of activating or accelerating osteogenic differentiation of MSCs *in vitro*, and be a good source for searching for more efficient osteogenic differentiation conditions (time, differentiation efficiency, functionality, etc.), by monitoring EGFP expression in living culture. Moreover, with respect to molecular characterization of osteoblasts, EGFP-positive cells shown in this study (Figure 3) may help to identify accurate and specific surface markers in combination with omics analysis for the development of purification methods for living osteoblasts, and/or for the discovery of additional endogenous marker genes. These novel markers would prove

useful not only for the assessment of osteogenic differentiation of MSCs [24, 25], but also for advancing the current understanding of osteogenic differentiation, Previously, we sought to assess osteoblast differentiation by using a mammalian artificial chromosomal vector (MAC) carrying a luciferase gene under the control of the BGLAP promoter (BGLAP-Luc) [10, 11, 26]. In this system, endogenous BGLAP expression was evaluated by measuring luciferase activity, and BGLAP-Luc activity was found to increase by approximately 8–10 fold after induction of osteogenic differentiation in presence of VD3 [10]. Although BGLAP-Luc is a useful tool for monitoring osteogenic differentiation, it cannot be used for monitoring live cells, as cell lysis is necessary for luciferase detection. In the present study, we overcame this limitation by using *EGFP* as a reporter gene to evaluate the expression of endogenous BGLAP, thereby demonstrating the feasibility of live-cell monitoring. Surprisingly, upon osteogenic differentiation in presence of VD3, we observed approximately 500-fold and 1000-fold increases in the expression of endogenous BGLAP in parental hiMSC and EGFP established knocked-in reporter lines, respectively (Figure 3D). Moreover, we demonstrated that functional and matured osteoblasts (Figure 4) could be enriched by means of combining cell sorting and reporter gene expression, which is consistent with the previous reports [21, 27].

A key finding of the current study is the ability to sort living differentiated osteoblast from MSCs, which presents an ideal strategy for the purification and enrichment of living osteoblasts for bone regenerative therapy. Thus, our study outcomes are a step forward towards the discovery of cell surface markers, which is pivotal to facilitate the purification of osteoblasts directly from patient MSCs without the need for genetic modification. We expect that our cell lines will be useful for the direct molecular characterization of osteoblasts as well as for advancing the development, or improvement, of osteogenic differentiation protocols.

5. Conclusion

We established modified hiMSC lines that can be used to monitor osteogenic differentiation in living cells, and to purify osteoblasts from these lines by cell sorting. The modified cell lines described herein would be useful to identify or characterize novel markers to distinguish osteoblasts from other cells. Thus, our findings provide a useful tool for future studies in bone regeneration therapy.

Declarations

Author contribution statement

T. Narai, Y. Nakayama and K. Kokura: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

R. Watase: Performed the experiments; Analyzed and interpreted the data.

I. Kodani: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

T. Inoue: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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