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Watching Osteogenesis: Life Monitoring of Osteogenic Differentiation Using an Osteocalcin Reporter

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ABSTRACT

Human bone marrow-derived mesenchymal stem cells have the potential to differentiate into several cell types such as osteoblasts, chondrocytes, and adipocytes. When cultured under appropriate medium conditions stem cells can be directed toward the osteoblast lineage in vitro. Progression of osteogenic differentiation is accompanied by changes in the expression pattern of several marker proteins including bone-specific alkaline phosphatase (bALP), collagen I (Col I), and osteocalcin (OC) and can be analyzed by well-established methods like immunohistochemical staining and quantitative RT-PCR. Furthermore, expression of fluorescent protein driven by an osteogenesis promoter facilitates online monitoring of proceeding osteogenic differentiation in transiently transfected human bone marrow-derived cells. In the present study we established a new double reporter gene construct comprising OC promoter-driven expression of green fluorescent protein and constitutive expression of red fluorescent protein-tagged histone H2B for transient transfection of primary human bone cells (HBCs). Osteogenic differentiation of transiently transfected cells was visualized by fluorescence microscopy. Immunohistochemical analysis and RT-PCR confirmed the progression into the osteo-specific lineage of transfected cells. Transfection efficiency was determined by fluorescence-activated cell sorting (FACS). J. Cell. Biochem. 113: 313–321, 2012.

KEY WORDS: OSTEOCALCIN; MESENCHYMAL STEM CELLS; ONLINE MONITORING; OSTEOGENESIS

Processes like normal bone remodeling and fracture healing in adults indicate the existence and contribution of progenitor cells that are able to proliferate and differentiate into the bone lineage. Throughout life human bone marrow as well as other tissues contain a small population of mesenchymal stem cells (MSC) that possess the potential to differentiate into osteoblasts, chondrocytes, adipocytes, myoblasts, fibroblasts, and other cell types [Aubin, 1998; Pittenger et al., 1999; Nöth et al., 2002] depending on the cues that these cells sense in their microenvironment. In the last years, human MSCs attracted a great deal of attention as they represent an attractive therapeutic cell source for tissue engineering approaches [Le Blanc and Pittinger, 2005; Zhang et al., 2009]. Bone marrow-derived cells have a substantial expansion capacity and when transplanted in vivo they are capable to form new bone [Friedenstein, 1961]. For in vitro studies, MSCs can be directed into the osteoblastic lineage by cultivation in the presence of β-glycerophosphate, ascorbic acid, vitamin D_3, and a low concentration of dexamethasone [Maniatopoulos et al., 1988]. The progression of progenitor cells into the osteo-specific lineage is regulated by the differential expression of osteoblast-associated genes including those for specific transcription factors, adhesion molecules and proteins of the extracellular matrix (ECM) [Huang et al., 2007]. Initiation of maturation of osteo-progenitors is characterized by loss of cellular expansion capacity together with sequential increase of osteogenic marker expression. Primarily, cells initiate the synthesis of ECM mainly consisting of collagen I (Col I). Simultaneously, the expression of bone-specific alkaline phosphatase (bALP) increases. With ongoing differentiation cells start to produce a variety of non-collagenous proteins such as bone sialo protein (BSP), osteopontin (OP), osteonectin (ON), and osteocalcin (OC). Finally, mineralization of the ECM indicates mature osteoblasts [Jaiswal et al., 1997]. Besides well-coordinated changes of marker protein expression there are also changes in cell morphology during osteogenic differentiation [Rodríguez et al., 2004; Yourek et al., 2007; Born et al., 2009]. For discriminating stages of differentiation within the osteogenic lineage methods like immunohistochemistry or RT-PCR are well established. However, these methods are very constricted in their temporal resolution. Tools that allow online monitoring of

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defined stages of differentiation can overcome this limitation and bring a dramatic advantage for the fields of biomaterials research, tissue engineering, and high throughput drug screening. For this purpose lineage specific promoter-driven expression of fluorescent proteins offer a promising strategy [Hojo et al., 2008]. The potential for identifying pre-osteoblasts as well as mature osteoblasts by linking collagen promoter fragments to green fluorescent protein (GFP) have previously been shown by others [Dacic et al., 2001; Kalajzic et al., 2002]. In contrast to collagen the non-collagenous protein OC is thought to be specifically expressed in mature osteoblasts [Fu et al., 2010]. Hence, OC promoter-GFP constructs appear ideal for identification of a more defined population of osteoblastic cells. In previous studies, the OC promoter has been effectively used to attain the osteoblast-specific over-expression of various peptides such as human growth hormones (hGH) [Baker et al., 1992] or transforming growth factor β (TGF-β) [Erlebacher and Derynck, 1996] in transgenic mice. Furthermore, osteoblast lineage progression in transgenic mice was analyzed by Bilic-Curcic et al. [2005] using a 3.8 kb OC promoter fragment driving the expression of a topaz variant of GFP (hOC-GFPtpz). In tissues of 4 weeks old transgenic mice strong GFP expression associated with high OC expression was shown in calvaria and long bone, whereas OC promoter-driven expression of GFP was undetectable in other tissues. Recently, we have used the 3.8 kb OC promoter fragment of hOC-GFPtpz to create pOC3.8-EGFP, a construct that allows online monitoring of osteogenic differentiation of transiently transfected human bone cells (HBCs) [Born et al., 2009]. When pOC3.8-EGFP transfected HBCs were cultivated under osteo-inductive conditions the number of green fluorescent cells increased with progression of time. Due to low OC promoter activity only a small number of green fluorescent cells appeared when HBCs were cultivated under expanding conditions for 14 days. However, due to the absence of a second marker or reporter that expresses an easily detectable and measurable product it remained impossible to evaluate transfection efficiency. The cell nucleus represents a universal and easy to identify cellular compartment. As histones are the principal structural proteins of eukaryotic chromosomes they represent attractive targets for fluorescent nuclear labeling. GFP-tagged histone H2B has previously been shown to incorporate into nucleosomes without any adverse effect on the viability of HeLa cells in culture [Kanda et al., 1998]. Furthermore, Yamamoto et al. [2004] developed dual-color fluorescent HT-1080 human fibrosarcoma cells where RFP was expressed in the cytoplasm and GFP tagged to H2B was expressed in the nucleus without affecting cell cycle progression. Nucleofection is a highly efficient non-viral transfection method that has been successfully used to genetically modify a series of primary cell types including human and murine stem cells [Haleem-Smith et al., 2005; Xiao et al., 2007; Dhara et al., 2009; Balyasnikova et al., 2010]. Moreover, stable or transient introduction of transgenes by nucleofection has been shown to be a useful tool for investigating protein function in living cells without affecting differentiation patterns of human MSCs (hMSCs) [Wiehe et al., 2007]. In the present study, a double gene reporter construct containing OC promoter-EGFP and histone H2B-RFP was developed. While EGFP expression was controlled by OC promoter activity the expression of histone H2B-RFP was exclusively regulated by the constitutive active cytomegalovirus (CMV) promoter. Following transient transfection of patient-derived HBCs positively transfected cells were identified by visible expression of H2B-RFP. Subsequently, progression of osteogenic differentiation of these cells was tracked by monitoring the OC-promoter dependent expression of EGFP. At the same time, immunohistochemical staining for bALP and Col I as well as quantitative RT-PCR of osteoblastic marker genes (bALP, Col I, and OC) was performed to demonstrate the differentiation of nucleofected osteo-progenitor cells toward the osteoblast lineage. Furthermore, constitutive expression of H2B-RFP enabled fluorescence-activated cell sorting (FACS) of nucleofected human bone marrow cells (HBMCs).

MATERIALS AND METHODS

ISOLATION OF HUMAN BONE CELLS

Bone marrow samples were obtained from patients (three male and four female patients of age between 58 and 83) undergoing surgical hip replacement after informed consent. HBC and HBMC cultures were prepared from trabecular bone pieces as described previously [Beresford et al., 1984; Auf'mkolk et al., 1985; Born et al., 2009]. In brief, femur-derived bone marrow samples containing pieces of trabecular bone were incubated in isolation medium (25 mM HEPES, 128.5 mM NaCl, 5.4 mM KCl, 5.5 mM D(+)-glucose, 51.8 mM D(+)-saccharose, 0.1% BSA) overnight at 4°C. After centrifugation at 110*g* for 15 min at 4°C the supernatant was discarded. The residual pellet that included pieces of trabecular bone was repeatedly washed with isolation medium under microscopic observance to rinse out cells from the bone pieces. For isolation of HBMCs the cell suspension was filtered three-times through a 200 μm filter mesh before a 15 min centrifugation at 110*g* and 4°C. The cell pellet was resuspended in expansion medium (a-MEM supplemented with 10% FBS, 1% PSN, 1 ng/ml basic fibroblast growth factor (FGF-2)). Thereafter, 10^7 cells were seeded per T75 culture flask (CELLSTAR® T75, Greiner Bio-One GmbH, Frickenhausen, Germany) and cultured in a humidified incubator at 37°C with 5% CO_2/95% air. After 24 h non-attached cells were washed off and fresh medium was added. Medium was replaced by fresh medium twice a week and cells were passaged at pre-confluent stage. For HBC cultures several pieces of bone with a total weight of approximately 25 mg were transferred into a T75 culture flask (CELLSTAR® T75, Greiner Bio-One GmbH) containing expansion medium. After 1 week of cultivation, bone cells started to grow out onto the tissue culture flask. When cells reached a confluence between 80% and 90% (approximately after 3 weeks) 2×10^5 cells were seeded into new flasks and cultured in expansion medium until they were used for transfection. To induce osteogenic differentiation of HBCs and HBMCs the expansion medium was replaced by osteogenic medium (α-MEM supplemented with 10% FBS, 1% PSN, 10 nM dexamethasone, 50 mM ascorbic acid phosphate, 2 mM β-glycerophosphate, and 10 nM 1,25-dihydroxyvitamin D_3). Medium was replaced every 2–3 days.

CREATION OF DOUBLE GENE CONSTRUCT

For creating the double gene construct pOC3.8-EGFP/H2B-RFP, the OC3.8-EGFP fragment as well as the vector backbone of pH2B-RFP was amplified by PCR using the following

primers: CMVspeI: 5′AGTGTACTAGTCCTGCGTTATCCCCTGATT3′; SV40pAmluI: 5′ATGTTACGCGTTTAAGATACATTGATGA3′; pUC-speI: 5′TTCCGACTAGTGGCCAGGAACCGTTAAAA3′; CMVmluI: 5′TGTACACGCGTTGCGTTATCCCCTGATT3′. In the resulting construct the expression of histone H2B-RFP was controlled by the constitutively active CMV promoter and the expression of EGFP was controlled exclusively by the OC promoter.

NUCLEOFECTION OF HUMAN BONE MARROW-DERIVED CELLS

HBCs and HBMCs were collected at a confluence of 80–90% and transfected by nucleofection using the Human Mesenchymal Stem Cell Nucleofector® kit (VPE-1001, Amaxa, Lonza Cologne GmbH, Germany) following the manufacturer's protocol. For transfection cells were trypsinized and pelleted. For each transfection 5×10^5 cells were resuspended in 100 µl Nucleofector® solution. An amount of 4 µg purified plasmid DNA (EndoFree Plasmid Maxi, Qiagen, Hilden, Germany) was added to 100 µl cell solution. Cells were transfected using the Nucleofector® device (Nucleofector® II, Amaxa, Lonza Cologne GmbH). Immediately after nucleofection cells were mixed with 500 µl of pre-warmed expansion medium and transferred into culture dishes containing pre-warmed medium. Transfected cells were cultured at 37°C while medium was replaced after 24 h to remove cell debris. From that point on, half of the samples were cultured in expansion medium while the other half was cultured in osteogenic medium. Fluorescence and bright field images of cells cultured under expansion or osteo-inductive conditions were taken on days 1, 4, 7, 10, and 14 using an upright fluorescence microscope (Axio Imager.M1, Carl Zeiss, Oberkochen, Germany). Corresponding filter sets for acquisition of red and green fluorescence were used and microscope settings were kept identical within individual experiments. A minimum of 30 images was taken at random and the number of red nuclei was determined. Due to patient variations in transfection efficiency the number of analyzed cells expressing RFP-histone ranged between 38 and over 300 cells. These cells were then analyzed for appearance of green fluorescence within the cytoplasm.

IMMUNOHISTOCHEMISTRY

Transfected HBCs were plated at a density of 2×10^4 cells per 35 mm culture dish (dish bottom area 9.6 cm^2, BD Falcon™) and cultured in expansion or osteogenic medium. After indicated periods of cultivation cells were fixed using 4% paraformaldehyde for 8 min. For immunohistochemical characterization fixed cells were treated with 4% paraformaldehyde containing 0.2% Triton X-100 to permeabilize the cell membranes. To inhibit non-specific binding of antibodies, fixed cells were incubated with 5% goat serum and 1% fetal calf serum in PBS for 30 min. All antibody solutions were diluted in 1.5% skim milk/PBS. Staining against osteogenic marker proteins bALP or Col I was performed using anti-human bone alkaline phosphatase (1:1,000, Developmental Studies Hybridoma Bank, Iowa City, IA, B4-78) or anti-Col I (1:1,000, Sigma, St. Louis, MO, C 2456), respectively. Subsequently cells were incubated with either goat α-mouse IgG Alexa Fluor 488 (1:400, Molecular Probes, A11029) or goat α-mouse IgG Alexa Fluor 546 (1:400, Molecular Probes, A11030). Incubation with antibody or staining solution was performed for 1 h at room temperature. Finally, cell nuclei were stained for 1 h at room temperature using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 10 µg/ml, Sigma, D9542). Afterwards cells were washed three times with PBS and in a final step covered with 2 ml of PBS. Immunohistochemically stained samples were analyzed using an upright fluorescence microscope (Axio Imager.M1, Carl Zeiss) and filter sets corresponding to the fluorescence of interest.

QUANTITATIVE RT-PCR

HBCs were evaluated for expression of bALP, Col I, and OC mRNA at days 1, 4, 7, 10, and 14 during cultivation in expansion and osteogenic media, respectively. Cells were seeded at a density of 1.2×10^5 cells per 60 mm culture dish (dish bottom area 21.3 cm^2) for day 1 or 6×10^4 cells for all following days. Levels of mRNA on selected days were related to those on day 1 (after seeding but before change of medium type). Total cellular RNA was extracted using 800 µl of TRIzol® reagent (Invitrogen) according to the manufacturer's protocol. RNA concentrations were determined using a spectrophotometer (Nanodrop, Thermo Scientific, Waltham, MA). The reverse transcription system iScript™ (BioRad) was used for cDNA synthesis from 1 µg of total RNA. Synthesis was performed using a thermal cycler (iCycler iQTM Real Time Detection System, BioRad, Hemel Hamstead, UK) and contained the following steps: 5 min at 25°C, 30 min at 42°C and finally 5 min at 85°C. For the amplification of osteogenic marker proteins bALP, Col I, and OC (GeneBank Accession numbers NM_000478, NM_000088, and NM_199173, respectively) oligonucleotides were placed outside the junctions of two exons (alp-2-for: 5′GGACATGCAGTA-CGAGCTGA3′; alp-2-rev: 5′CCAGCAAGAAGAAGCTTTG3′; col-I-3-for: 5′CAGCCGCTTCACCTACAGC3′; col-I-3-rev: 5′TTTTGTATTC-AATCACTGTCTTGCC3′; OsteocS3: 5′GAAGCCCAGCGGTGCA3′; OsteocAS2: 5′CACTACCTCGCTGCCCTCC3′) as described previously [Born et al., 2009]. Amplification resulted in products with lengths of 111, 82, and 69 bp for bALP, Col I, and OC, respectively. 18S ribosomal RNA was used as normalization standard (18S rRNA, GeneBank Accession number NR_003286; 18S-s2: 5′GGACAGG-ATTGACAGATTGATAG3′; 18S-as2: 5′AGTCTCGTTCGTTATCGG-AAT3′). All oligonucleotides were purchased from Microsynth AG (Balgach, Switzerland). After synthesis, obtained cDNA was diluted 1:5 for amplification of osteo-specific mRNA or 1:50 for amplification of 18S rRNA. The iQ™ SYBR® Green System (BioRad) was used for PCR amplification in a total volume of 50 µl containing 5 µl of diluted cDNA. PCR reaction for each reaction was composed of 25 µl iQ™ SYBR® Supermix, 100 mM sense, 100 mM antisense primer (in 0.1 µl, respectively) and DNase free water (added up to total volume of 45 µl). All reactions were performed in triplicate. PCR amplification contained the following steps: 4 min at 95°C followed by 40 cycles of 30 s denaturation at 95°C, 50 s annealing and elongation at 57°C and final cooling to 4°C. Fluorescence was monitored and data were analyzed using the iCycler software (V2.3, BioRad) of the BioRad system. Relative changes in osteogenic marker gene expression were quantified by the delta-delta CT method by normalizing the quantified mRNA amount to the 18S rRNA. Data are presented as mean values from triplicate measurements and variances are calculated according to the laws of uncertainty propagation.

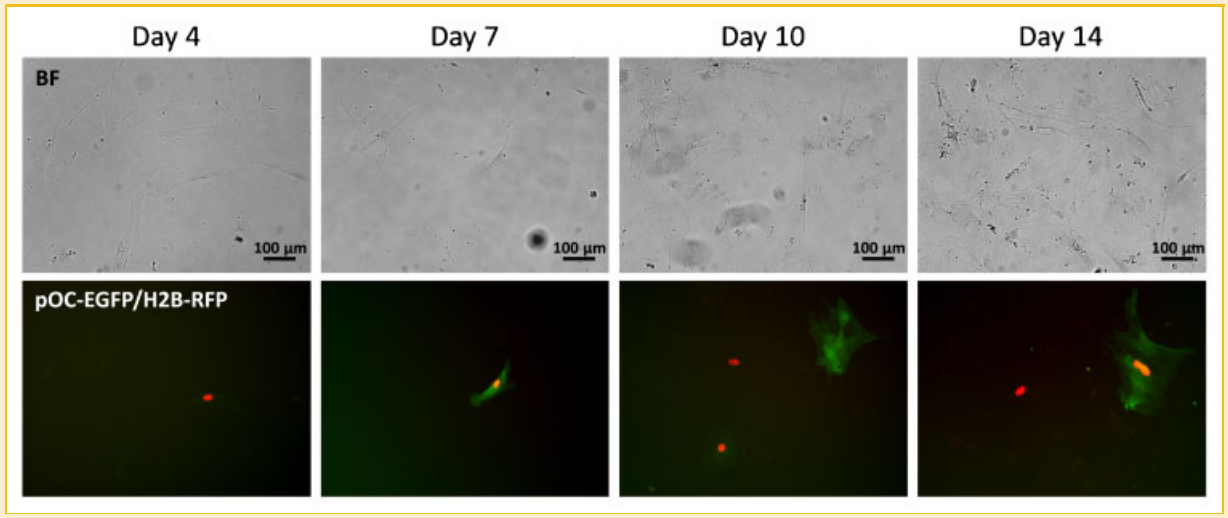

Fig. 1. Fluorescence images and corresponding bright field images of differentiating HBCs expressing the OC promoter double gene construct. Expression of H2B-RFP together with expression of EGFP driven by the activity of the human OC promoter is visualized by fluorescence microscopy at days 4, 7, 10, and 14. Post-transfection HBCs were cultured in osteo-inductive medium.

FLUORESCENCE-ACTIVATED CELL SORTING

HBMCs were nucleofected with OC3.8-EGFP/H2B-RFP as described above and culture in proliferation medium for 4 days post-transfection. Cell sorting according to RFP expression in the nuclei was performed using a FACSAria II flow cytometer (Becton Dickinson) at the Flow Cytometry Core Facility, EPFL. Untransfected HBMCs from the same patient were used to set the background level of fluorescence.

RESULTS

TRANSFECTION OF HBCs WITH pOC3.8-EGFP/H2B-RFP

HBCs derived from several donors were nucleofected with the pOC3.8-EGFP/H2B-RFP construct and subsequently cultured under expansion as well as osteo-inductive medium conditions for up to 14 days. Representative bright field and fluorescence images of cells cultured in osteogenic medium for 14 days are shown in Figure 1 (images of corresponding proliferating cells are shown in Supplementary Fig. 1). On day 1 a small fraction of cells displayed red fluorescent cell nuclei indicating the expression of H2B-RFP. The fraction of red fluorescent cells was equal in cells of both medium conditions and remained approximately similar over the observation period. EGFP expression was detectable in a very low number of cells on day 1. On day 4 the fraction of H2B-RFP expressing cells that additionally display green fluorescence was low in cells under both medium conditions indicating that OC promoter activity remained very low. While isolated EGFP expressing cells occurred on day 7 of osteogenic cells EGFP expression continued to be very low in proliferative cells. With ongoing osteogenic cultivation the number of H2B-RFP cells that display EGFP expression increased on days 10 and 14. At the same time, expanding HBCs displayed no appreciable increase in the amount of EGFP expression indicating negligible OC promoter activity. For better visualization, a representative number of H2B-RFP expressing cells was counted and analyzed for the presence of green fluorescence. The percentage of EGFP expressing cells within the population of H2B-RFP expressing cells was calculated and results are displayed in Figure 2. While on days 1, 4, and 7 the proportion of EGFP expressing cells remained low the fraction of green fluorescent cells increased remarkably on days 10 and 14 only under osteo-inductive medium conditions. OC promoter-driven expression of EGFP was increased between 3- and 10-fold on day 14 in osteogenic cells compared to expanding cells. Although the fraction of EGFP expressing cells was very different form patient to patient they all follow the same trend. Data from two additional patients are shown in Supplementary Figure 2.

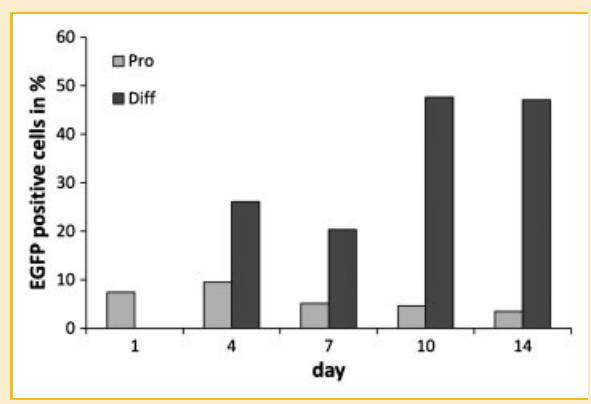

Fig. 2. Fraction of EGFP positive cells in a population of H2B-RFP expressing HBCs cultured in expansion (Pro) and osteo-inductive (Diff) medium for 4, 7, 10, and 14 days.

IMMUNOHISTOCHEMICAL STAINING FOR OSTEOGENIC MARKERS

HBCs transfected with the double gene construct pOC3.8-EGFP/H2B-RFP were cultured in expansion or osteogenic medium and fixed at days 7, 10, and 14. Subsequently, cells were stained for bALP or Col I as well as nuclei (Fig. 3). The qualification of the used bone-specific ALP antibody as a suitable marker for osteogenic differentiation has been shown previously [Born et al., 2009]. When cells in proliferation medium were stained for bALP the fluorescence intensity remained rather low. In contrast, osteogenic cells displayed distinct green fluorescence and intensity further increased with progression of osteogenesis from days 7 to 14. Col I staining of cells cultured under osteogenic conditions for 14 days displayed high fluorescence intensities compared to cells that were grown in expansion medium (Fig. 3).

GENE EXPRESSION ANALYSIS

To confirm that nucleofection with our double gene construct did not modify the potential of HBCs to undergo osteogenic

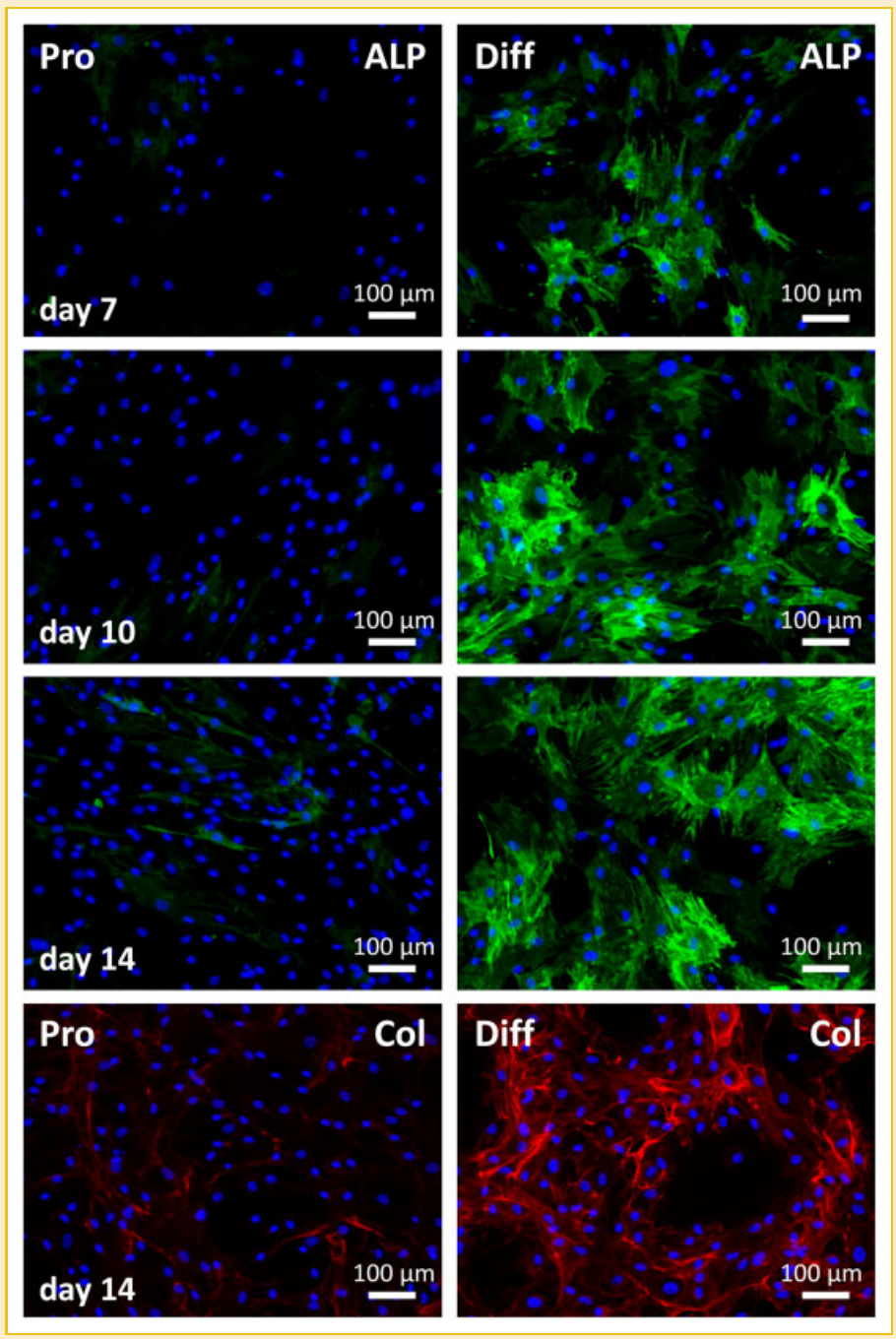

Fig. 3. Fluorescence images of HBCs cultured in expansion (Pro) or osteo-inductive (Diff) medium and immunohistochemically stained for bALP (green, days 7, 10, and 14) or Col I (red, day 14) and nuclei (blue).

differentiation the relative expression levels of osteoblastic marker genes bALP, Col I, and OC were examined in transfected cells from three different donors. Representative mRNA levels from one donor are shown in Figure 4 (see data from two additional patients in Supplementary Fig. 3). When cells from three patients were cultured in osteo-inductive medium the relative expression of bALP increased between 4- and 20-fold on day 4 relative to day 1. Bone-specific ALP expression further increased on days 7 and 10 in osteogenic cells. After 14 days of cultivation in osteo-inductive medium the relative expression of bALP was between 10- and 40-fold higher compared to expanding cells. During cultivation in proliferative conditions the relative expression of bALP remained on a low level and no increase in expression was observed. The relative expression of Col I was increased after 4 days cultivation in osteo-inductive medium when compared to expansion cells on the same day. With ongoing cultivation, the relative expression of Col I increased further. The level of Col I expression in expanding cells also increased over the time of observation but to a much lesser extent compared to osteogenic cells. The relative expression of OC was almost silent in expanding cells while in osteogenic cells from three different donors the relative expression of OC was already raised between 4- and 40-fold on day 4. With progression of osteogenesis the level of OC expression remained high compared to expanding cells. Even though data from three different patients showed high variability they follow the same trend.

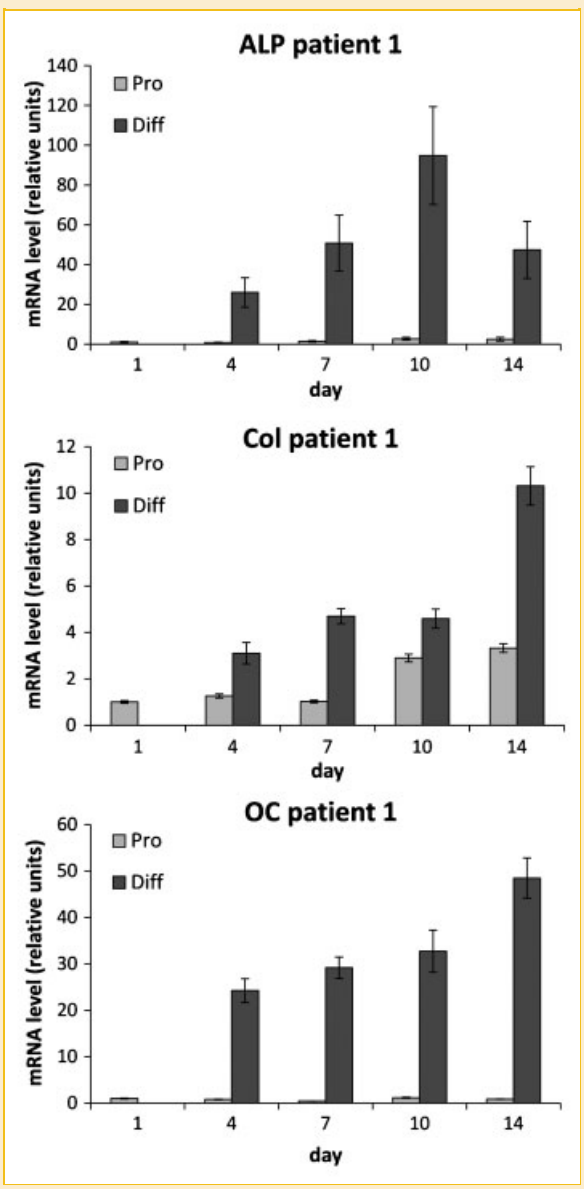

Fig. 4. Representative data of relative mRNA expression levels of bALP, Col I, and OC of HBCs cultivated under expanding (Pro) and osteo-inductive (Diff) medium conditions. Relative mRNA levels at days 1, 4, 7, 10, and 14 from one patient are shown (see data from two additional patients in Supplementary Fig. 3).

FACS ANALYSIS OF NUCLEOFECTED HBMCs

Fluorescence-activated cell sorting was performed to enrich transfected HBMCs that carry the OC double gene construct (Fig. 5). Cells from two different patients were sorted 4 days post-transfection. Expression of H2B-RFP was verified by fluorescence microscopy before sorting (Fig. 6). In both cell samples only a small fraction of cells displayed red fluorescent nuclei indicating low efficiency of nucleofection. This finding was confirmed by cell sorting as 5.6% and 5.3% of the entire cell population were sorted based on their red fluorescence, respectively. Subsequently, enriched cell populations were cultured in osteogenic medium and visualized by fluorescence microscopy. One day after sorting, a large proportion of cells expressed H2B-RFP, which indicates successful enrichment of transfected cells. However, not all cells in the sorted fraction expressed H2B-RFP and with ongoing cultivation the amount of non-fluorescent cells increased. After 5 days of cultivation in osteogenic medium, isolated cells expressed EGFP, which indicates the activation of the OC promoter. Thus, the ability to undergo osteogenic differentiation was not affected by the FACS.

DISCUSSION

In the present study, a double gene construct that mediates constitutive expression of RFP-tagged histone H2B together with OC promoter-driven expression of EGFP was established to monitor osteogenic differentiation. This double gene construct was used for transient transfection of bone marrow-derived cells from various donors. With progression of in vitro osteogenesis a remarkable increase in the number of EGFP expressing cells was observed indicating elevated activity of the OC promoter. Human bone marrow-derived MSCs possess multilineage-differentiation potential and due to their ease in isolation and cultivation, MSCs are particularly suited for research on medical implant applications and tissue engineering. The progress of in vitro osteogenic differentiation is often studied by evaluating the expression of osteogenic marker proteins and immunohistochemistry. Both methods represent end-point determinations with temporal limitations; hence OC promoter-driven expression of EGFP appears to be a suitable strategy for online monitoring of osteogenic differentiation.

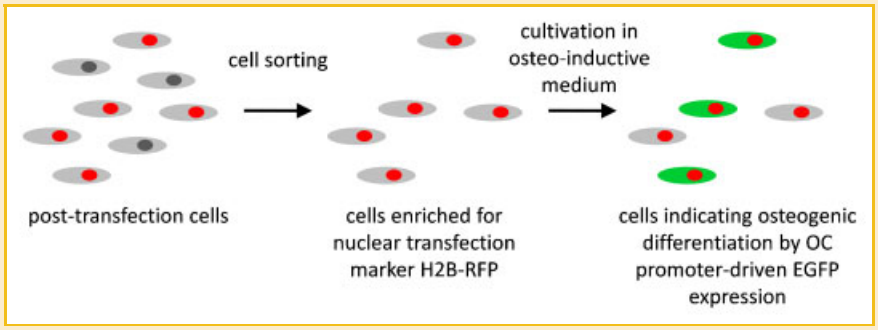

Fig. 5. Schematic principle of fluorescence-activated cell sorting of transfected cells for pre-selection of cells carrying the double gene construct. For enrichment of transfected cells that carry the double gene construct, cells are sorted based on the expression of red fluorescence protein tagged histone H2B. Sorted cells are cultivated in osteo-inductive medium for online monitoring of osteogenic differentiation.

A similar OC promoter reporter construct has already been used to generate transgenic mice whereupon high expression of GFP was found in long bones and calvaria indicating elevated OC promoter activities in these tissues [Bilic-Curcic et al., 2005]. In our previous study the qualification of the corresponding OC promoter fragment for online monitoring of osteogenesis in transiently transfected HBCs was shown [Born et al., 2009]. Development of stably transfected cells is challenging and time-consuming but when successful one obtains a cell population with identical transgene expression in individual cells. However, transgene integration into the target cell has to be monitored carefully to make sure that cell physiology is unaffected [Recillas-Targa, 2006]. Stable transgenes are most often generated from cell lines that possess consistent cellular properties over a large number of population doublings. However, it has been shown that populations of expanded primary bone marrow stromal cells lose their ability to proliferate and to differentiate into mesenchymal lineages after in vitro culture [Banfi et al., 2000], indicating that analysis of cells with low passage number is of particular importance. Consequently, transient transfection of patient-derived bone marrow stromal cells provides a practical alternative for studying cellular function during osteogenic differentiation. Bone marrow-derived cells represent a heterogeneous cell population and nucleofection was found to be challenging and resulted in only a small number of cells that carried the osteogenic double gene construct. As reported previously, transfection of HBCs with a 3.5 kb control vector for CMV promoter-driven expression of

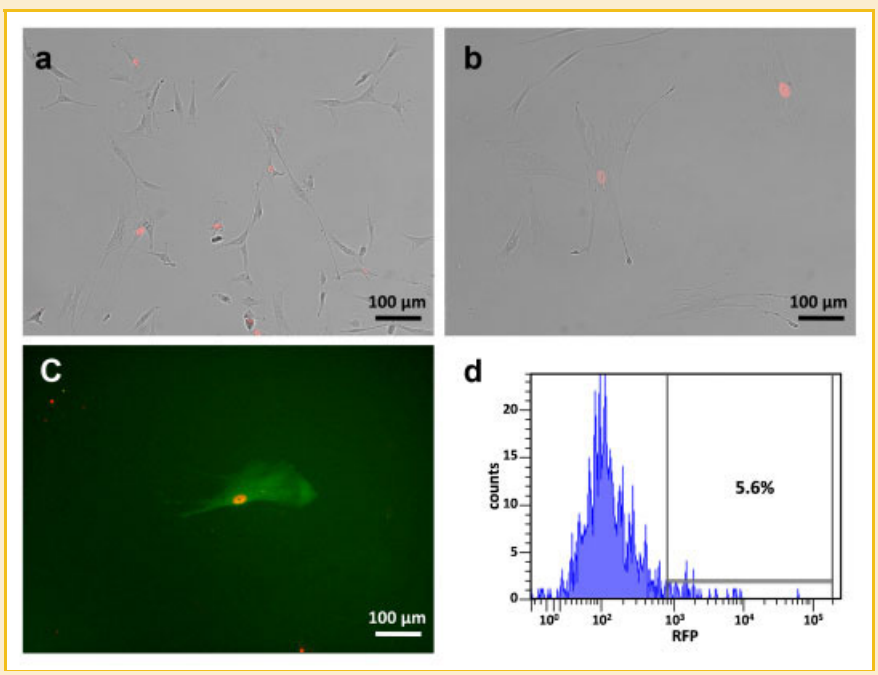

Fig. 6. Images of HBMCs nucleofected with pOC3.8-EGFP/H2B-RFP before and after cell sorting. a: Pre-sorted and (b) post-sorted HBMCs expressing H2B-RFP are displayed (overlay of bright field and RFP channel). c: Sorted cells were cultivated in osteo-inductive medium after sorting for 5 days (overlay of EGFP and RFP channel). d: FACS results of H2B-RFP expressing HBMCs.

GFP (pmaxGFP, Amaxa, Lonza Cologne GmbH) resulted in up to 25% green fluorescent cells 2 days post-transfection [Born et al., 2009]. By contrast, transfection efficiency of HBCs and HBMCs with pOC3.8-EGFP/H2B-RFP (9.8 kb) was considerably lower. As an inverse correlation between transfection efficiency and construct size has been reported recently [Rizzo et al., 2004], the low transfection efficiency of HBCs and HBMCs presented here might be due to the size of our double gene construct, to some extent. Nevertheless, besides high variability in the transfection efficiency between various donors the expression of H2B-RFP remained unchanged over the total period of observation and was restricted to nuclei in all analyzed cell populations. Cell sorting for H2B-RFP expressing cells enables us to enrich the number of cells that consequently carry the OC promoter construct. Further, the osteogenic potential of transfected HBCs was confirmed by the results of immunohistochemical staining for bALP and Col I. When performing RT-PCR assays of transfected cells we could demonstrate that the relative expression of bALP, Col I as well as OC is upregulated in the presence of osteo-inductive medium which goes in line with the increasing appearance of OC promoter EGFP positive cells. By contrast, the levels of all three marker proteins remained at a low level in expanding cells. In accordance with results from our previous study [Born et al., 2009] the expression of OC is already raised on day 4 and remains markedly higher in cells ongoing osteogenesis compared to expanding cells. Although the expression levels of osteo-specific genes show high variability between different donors the RT-PCR results obtained within our study are following the same tendency. Material-induced influence on the cells ability to undergo osteogenic differentiation is of great interest in the area of tissue engineering as well as for the evaluation of new bone implant concepts. Instead of using genetically modified stable cell lines that might be biological less relevant we propose the use of transient transfected patient-derived bone marrow stromal cells to study material effects on cellular function. Surface characteristics like topography, surface chemistry and surface energy are critical parameters for the adhesion of osteoblasts to biomaterials and have to be taken into account when new materials for medical implants are being developed [Anselme, 2000]. The elasticity of the underlying substratum was shown to influence the differentiation of MSCs with directing cells into a certain lineage [Engler et al., 2006]. Moreover, stem cell fate can also be controlled by integrin-dependent cellular interactions with the ECM as it has been shown recently by Martino et al. [2009]. The use of primary MSCs transfected and enriched cells appear appropriate to study the influence of material properties on osteogenesis. The ability of a given substrate to either facilitate or deplete osteogenic differentiation could be monitored online. Besides that, the impact of soluble factors, like hormones or growth factors present in the medium, on osteogenesis could be investigated. The use of our osteogenic reporter construct might further facilitate the development of cell-based sensors for drug screening approaches. Transfection of our reporter gene construct is not limited to primary cells presented here but can be easily transferred to other cell types as well. Consequently, our approach represents a qualified tool for online monitoring osteogenesis in studies dealing with tissue engineering.

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