In Vivo Bioluminescence Imaging of Cell Differentiation in Biomaterials: A Platform for Scaffold Development

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In vivo testing is a mandatory last step in scaffold development. Agile longitudinal noninvasive real-time monitoring of stem cell behavior in biomaterials implanted in live animals should facilitate the development of scaffolds for tissue engineering. We report on a noninvasive bioluminescence imaging (BLI) procedure for simultaneous monitoring of changes in the expression of multiple genes to evaluate scaffold performance *in vivo*. Adipose tissue-derived stromal mesenchymal cells were dually labeled with *Renilla* red fluorescent protein and firefly green fluorescent protein chimeric reporters regulated by cytomegalovirus and tissue-specific promoters, respectively. Labeled cells were induced to differentiate *in vitro* and *in vivo*, by seeding in demineralized bone matrices (DBMs) and monitored by BLI. Imaging results were validated by RT-polymerase chain reaction and histological procedures. The proposed approach improves molecular imaging and measurement of changes in gene expression of cells implanted in live animals. This procedure, applicable to the simultaneous analysis of multiple genes from cells seeded in DBMs, should facilitate engineering of scaffolds for tissue repair.

Introduction

THE OBJECTIVE of tissue engineering (TE) is the production of functional tissue replacements using biological and synthetic materials, often in combination with cells and suitable biochemical factors. Development of materials for bioengineering purposes requires the analysis of multiple of these elements interacting in complex manners. Thus, agile analytical procedures for *in vivo* evaluation of cell-material interactions would allow scaffold development strategies based on iterative cycles of material production-modification followed by *in vivo* testing.

The main aim of the current work has been to demonstrate a noninvasive procedure to image differentiation of cells in live animals that should aid in the development of scaffolds for tissue regeneration.

Due to the capacity of visible-light photons to transverse living tissues, bioluminescence imaging (BLI) allows low cost, real-time monitoring of location, proliferation, and differentiation of luciferase-expressing cells in living tissues.^{1–6}

By using tissue-specific promoters to regulate expression of luciferase reporters introduced in living cells, changes in promoter activity translate into measurable changes in photon fluxes that correlate with transcriptional activity.⁷ Thus,

noninvasive BLI may be used as a convenient analytical tool to monitor the behavior of cell-seeded biomaterials implanted in live animals.⁸

Demineralized bone matrix (DBM), consisting of acid-extracted rat cortical bone allograft,^{9,10} provides a convenient testing ground to demonstrate imaging procedures applicable to the development of new materials for bone formation. Porous DBM allows infiltration and colonization by host cells that, instructed by osteoinductive signals and growth factors remaining within the collagen structure, supports osteogenesis.^{11,12}

Correct bone healing requires not only stem cell populations capable of osteogenic differentiation, but also the formation of a vascular bed to support metabolic needs.¹³ In its absence, the ensuing hypoxia results in cell loss and no formation of bone. Different phenotypic markers can be used to monitor sequential stages during bone formation and healing. Bone gamma-carboxyglutamate protein or osteocalcin (OC), the expression of which is limited to cells of the osteoblasts lineage,¹⁴ is the major noncollagenous bone matrix protein expressed in bone. Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a frequently used marker constitutively expressed in endothelial cells, platelets, and specific immune system cells.¹⁵ Finally, genes such as lactate

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dehydrogenase A¹⁶ and phosphoglycerate kinase 1 (PGK1)¹⁶ can be used as proxy reporters responsive to hypoxia activation during bone formation and healing.

Human adipose tissue mesenchymal stromal cells (hAMSCs) have been proposed for bone repairing in TE,¹⁷ due to their capacity to differentiate into cell lineages involved in osteogenic and vascular development, ease of isolation, abundance, and long-term stability during *in vitro* expansion.¹⁸

In the current work, we labeled hAMSCs with lentiviral vectors for the expression of chimeric photoproteins with two types of activities: bioluminescence for noninvasive BLI and fluorescence for cell enrichment and histological analysis.

In addition, to take into consideration the inherently noisy environment of measurements in live animals, we adopted an improved luciferase-labeling strategy, where constitutively active and tissue-specific promoters are simultaneously used to regulate light production by two different luciferases in the same cell. Thus, imaging of constitutively expressed *Renilla reniformis* luciferase (RLuc), regulated by the cytomegalovirus (CMV) promoter, is used to evaluate the cell number, while imaging of *Photinus pyralis* luciferase (PLuc), regulated by inducible tissue-specific promoters (PECAM-1,¹⁹ OC,²⁰ or hypoxia²¹), is used to evaluate cell differentiation and hypoxia. In this manner, changes in tissue-specific reporter expression are calculated in relation to the level of constitutive reporter expression, and allow taking into account different types of system's noise, including that resulting from changes in the cell number.

We show that this is a convenient and sensitive strategy to noninvasively monitor changes in tissue-specific gene expression during osteogenesis *in vivo* that also facilitates subsequent histological validation through fluorescence imaging.

Materials and Methods

Generation of luciferase-fluorescent protein reporters regulated by a specific human OC promoter, human PECAM-1 promoter, and hypoxia-response element promoter

A pLox:PLuc:green fluorescent protein (GFP) lentiviral vector containing a fusion reporter comprising PLuc and GFP was obtained by polymerase chain reaction (PCR) amplification and standard cloning procedures using the PLuc and GFP genes from plasmid pGL4.10:PLuc (Promega Corporation) and pEGFP-N1 plasmid (Clontech Lab.). PLuc was amplified from pGL4.10:PLuc using paired primers: 5'-CTCGA GATGGAAGATGCCAAAACATTAAGAAG-3' and 5'-AGATCTCCATGGAGGCGATCTTGCCGCCCTTC-3' and cloned into the pCR2.11 vector (Invitrogen). After, the PLuc gene was then removed from the pCR2.11 plasmid using *NcoI/SpeI* enzymes and cloned into the pEGFP-N1 vector cleaved with *NcoI/XbaI*. Finally, the in-frame fusion reporter gene was excised from the pEGFP-N1 plasmid using *EcoRI* enzyme and cloned into the *EcoRI* site of the pLox lentiviral vector, to obtain the pLox:PLuc:GFP vector.

The 800-bp human OC promoter (*hOCp*) was PCR-amplified from genomic DNA using specific primers: 5'-CTGCAGGGT CAGGAGGAGAAT-3' and 5'-GGGCTGCTGCTCAGGACT-3' as described¹⁶ and cloned into the pCR2.11 vector (Invitrogen). The promoter sequence was then removed from the pCR2.11 vector using *XbaI/SpeI* enzymes and cloned into the *SpeI* site of

the pLox:PLuc:GFP lentivirus vector. Human PECAM-1 promoter (HPECAM-1p) was kindly provided by Dr. Carmelo Bernabéu (Centro de Investigaciones Biológicas CSIC, Madrid, Spain) and cloned into pLox:PLuc:GFP using *XbaI/SpeI* and *SpeI* enzymes, respectively. The hypoxia-response element promoter (HRE-12p) artificial promoter was constructed as described.¹⁷ Briefly, two self-complementary 79 oligonucleotide sequences corresponding to the HRE-binding sequence of lactate dehydrogenase A (LDHA), phosphoglycerate kinase 1 (PGK1), and enolase 1 (ENO1) were annealed to generate a dsDNA monomer fragment with *XbaI*- and *SpeI*-compatible cohesive ends. After self-ligation of this monomer and agarose gel electrophoresis, a repetition of 12 monomers was selected from the gel and cloned into the *SpeI* site of the pLox:PLuc:GFP lentivirus vector.

The CMV:RLuc:RFP:ttk lentiviral vector containing a tri-functional chimeric construct comprising the RLuc reporter gene, the monomeric red fluorescent protein (RFP), and a truncated version of the herpes simplex virus thymidine kinase gene sr39tk (*ttk*) under transcriptional control of CMV was a kind gift of Professor S.S Gambhir (Dept. Radiology, Stanford University, US).

Lentiviral particle production

Viral particles were produced in human embryonic kidney cells 293T grown in Dulbecco's modified Eagle's medium-high glucose (DMEM-hg; Sigma), 10% heat-inactivated fetal bovine serum (FBS; Sigma), 2 mM L-glutamine (Sigma), 50 units/mL penicillin/streptomycin (Sigma), and 2 mM HEPES. The day before transfection, 3×10^6 trypsinized cells were seeded on 10-cm² poly-D-lysine-treated plates (Sigma). Previously described lentiviral transfer vector DNA (pLox-hOCp:PLuc:GFP; pLox-hPECAM-1p:PLuc:GFP; pLox-HRE-12p:PLuc:GFP; and CMV:hRLuc:RFP:ttk) (15 µg) was mixed with viral envelope plasmid (pMD-G-VSV-G, 5 µg), packaging construct (pCMV DR8.2, 10 µg), 450 µL of water, and 50 µL of 2.5 M CaCl₂, and then added dropwise to 500 µL of HEPES-buffered saline 2×(pH 7) and incubated at room temperature for 20 min. This DNA solution was then added dropwise to the plate containing the 293T cells in a growth medium, swirled gently, and then incubated for 16 h at 37°C with 5% CO₂. The following day, the transfection solution was removed, and the cells were rinsed with phosphate-buffered saline (PBS) 1×, and the medium without FBS was added to the cells. After a 48-h incubation, the supernatant was collected, centrifuged at 400 g to remove cell debris, and filtered through a 0.45-µm low-protein-binding filter (Corning). The filtered supernatant was then loaded in thin-wall polyallomer tubes and ultracentrifuged using the SW41.Ti rotor at 26,000 rpm for 90 min at 4°C in a L-100XP (Beckman Coulter) ultracentrifuge. The virus pellets were resuspended in PBS and kept at -80°C for storage. Viral titers were determined using the HIV-1 p24 antigen EIA (Beckman Coulter) 96 test kit.

hAMSC transduction and differentiation

hAMSCs were isolated from adipose tissue derived from cosmetic subdermal liposuctions, with patient consent. Liposuction samples were obtained after written informed consent by anonymous donors from Hospital de la Santa Creu i Sant Pau, Barcelona, Spain. Work with human

samples was approved by written consent by the Ethics Committee of Clinical Investigation of Hospital Santa Creu i Sant Pau, Barcelona, Spain; and Bioethics Subcommittee of Superior Council of Scientific Research. Briefly, lipoaspirate was suspended in $1 \times$ collagenase type I (Invitrogen) solution and incubated at 37°C and inactivated by addition of DMEM+10% FBS. hAMSCs were isolated by plastic adherence technique. hAMSCs were grown in DMEM-hg with 20% FBS (Hyclone), 2 mM L-glutamine (Sigma), and 50 units/mL penicillin/streptomycin (Sigma), expanded for until 70% of, and frozen at passage 2. Three-donor pool at passage 4 was used for the study.

hAMSCs were transduced using CMV:hRLuc:RFP:ttk-concentrated lentiviral stock (2×10^6 transduction units/mL, multiplicity of infection [MOI]=21) during 48 h. The highest 11% RFP-expressing cells were selected by fluorescence-activated cell sorting (FACS). Sorted cells were divided in three aliquots and transduced again with either concentrated lentiviral PLox-hOCp:PLuc:GFP, PLox-HRE-12p:PLuc:GFP or PLox-hPECAM-1p:PLuc:GFP stocks (2×10^6 transduction units/mL, MOI=21) during 48 h to obtain double-transduced cells hOCp:PLuc:GFP/CMV:RLuc:RFP:ttk-hAMSCs (hOCp:hAMSCs), HRE-12p:PLuc:GFP/CMV:RLuc:RFP:ttk-hAMSCs (HRE-12p:hAMSCs), and hPECAM-1p:PLuc:GFP/CMV:RLuc:RFP:ttk-hAMSCs (hPECAM-1p:hAMSCs), respectively.

For *in vitro* osteogenic differentiation, cells were cultured in a StemPro Osteogenesis Differentiation Kit (Gibco) during a 14-day period, following the manufacturer's instructions. For *in vitro* endothelial differentiation, cells were cultured in Endothelial Cell Growth Medium-2 (Lonza) during a 14-day period. For hypoxia activation, cells were grown under hypoxic conditions in an incubator supplied with 1% O_2 during 48 h.

BLI of luciferase activity *in vitro*

BLI of cultured double-transfected cells hOCp:hAMSCs, HRE-12p:hAMSCs and hPECAM-1p:hAMSCs was performed at the end of the differentiation or hypoxia activation process. For BLI, the medium was removed from plates (24-well plates), and cells were rinsed twice with PBS $1 \times$ and immediately imaged after addition of 100 μL /well of PLuc or RLuc substrate stock reagent (Promega Corporation). PLuc and RLuc activities were imaged in consecutive days. For imaging, a plate was placed in the detection chamber of a high-efficiency ORCA-2BT Imaging System (Hamamatsu Photonics) provided with a C4742-98-LWG-MOD camera fitted with 512×512 -pixel charge-couple device (CCD) cooled at -80°C . Images were acquired during 30 s using 1×1 arrays (binning 1×1) for RLuc detection, and during 1 min using an 8×8 array (binning 8×8) for PLuc detection. To register the position of the light signal, an additional image was obtained using a white light from a lamp in the detection chamber. Image analysis was performed using Wasabi software (Hamamatsu Photonics). Recorded light fluxes were expressed as photon counts (PHCs) after discounting background using the formula, $\text{PHCs} = (\text{total number of PHCs in the area of interest}) - (\text{number of pixels in the area of interest} \times [\text{background average PHCs/pixel}])$.

Arbitrary color scales are used to represent standard light intensity levels for PLuc (blue=lowest; red=highest) and for RLuc (black=lowest; blue=highest).

Cell implantation in DBM chambers and Matrigel

Six-week-old severe combined immunodeficiency disease (SCID) mice were purchased from Charles River and maintained in a specific pathogen-free environment throughout the experiment. All animal-related procedures were performed with the approval of the Animal Care Committee of the Cardiovascular Research Center and the Government of Catalonia. Mice were anesthetized by intraperitoneal injection of a mixture containing 100 mg/kg ketamine (Merial) and 3.3 mg/kg xilacine (Henry Schein). Three DBM chambers were subcutaneously implanted in the backs of six SCID mice 1 week before seeding with cells. DBMs were prepared as described²² and had the ends sealed with Millipore filters (Millipore Ltd.) to prevent leakage. Trypsinized cells, 3×10^5 suspended in 15 μL of α -MEM, were injected through the mouse skin and the sealed end of the DBM using an insulin syringe. The distribution of DBM chambers in mice is indicated in Figure 2A diagram.

A mixture of 50 μL Matrigel with 3×10^5 cells suspended in 50 μL of PBS was subcutaneously injected in the paraspinal space of the mouse (four independent injection sites per animal; total $n=2$) using a 21–25G-size needle.

RNA extraction and real-time PCR

Total RNA was extracted from DBM scaffolds implanted in mice, at 3-week intervals and from *in vitro* samples at the end of differentiation or hypoxia activation process. RNA extraction was performed using the RNeasy mini kit (Qiagen). One μg of total RNA was reverse-transcribed using the Revertaid First Strand cDNA Synthesis Kit (Fermentas), and product cDNA was then real-time PCR-amplified, using ABI PRISM 7000[®] (Applied Biosystems). The glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) gene was used as an internal control. Due to the low amounts of cDNA obtained from DBM scaffolds, a preamplification stage was performed with TaqMan[®] PreAmp Master Mix (Applied Biosystems).

FAM-labeled primer/probes were purchased from Applied Biosystems: *PECAM-1* (Hs00169777_m1), *GAPDH* (Hs99999905_m1), *BGLAP* (Hs01587813_g1), *LDHA* (Hs00855332_g1), and *PGK1* (Hs00943178_g1). The threshold cycle (Ct) method was used to quantify relative expression for each gene using *GAPDH* as endogenous reference.

Noninvasive BLI of luciferase activity from implanted DBMs and Matrigel

For *in vivo* BLI, anesthetized mice bearing DBMs seeded with PLuc-expressing cells were intraperitoneally injected with 150 μL of luciferin (16.7 mg/mL in physiological serum; Braun). Alternatively, for *in vivo* BLI of RLuc-expressing cells, mice were intravenously (tail vein) injected with 25 μL of benzyl coelenterazine (hCTZ, 1 mg/mL in 50/50 propylene glycol/ethanol; Nanolight Technology, www.nanolight.com) diluted in 125 μL of water.

PLuc and RLuc activities were imaged in consecutive days. Images were acquired during 5 min. In all cases, an additional image of the animal was obtained using a white-light source inside the detection chamber, to register the position of the luminescence signal in the animal.

To increase detection sensitivity, readout noise of the recorded signal was reduced by simultaneous reading of light events recorded in arrays of 8×8 adjacent pixels (binning

8×8) in the camera CCD. Mice were monitored during the 8-week period for DBMs and during 6 days for Matrigel at the indicated times. The photons recorded in images were quantified and analyzed using Wasabi image analysis software (Hamamatsu Photonics).

Fluorescence angiography and confocal microscope localization of hAMSCs in DBMs

An fluorescein isothiocyanate (FITC)-conjugated high-MW (2,000,000 Da) dextran, FITC-dextran (Sigma), was used as a fluorescent tracer of microvascular structures in DBM scaffolds. FITC-dextran 200 μ L (10 mg/mL) was injected through the lateral tail vein of anesthetized mice. Ten minutes after inoculation, mice were sacrificed, DBM chambers retrieved and fixed in 10% formalin solution (Sigma) during 24 h, and sectioned for microscopy. Laser confocal microscopy was used to analyze microvascular structures connected to the host vascular system in DBMs and their relationship with implanted fluorescent-labeled hAMSCs. Three DBMs were retrieved and analyzed at 3-week intervals.

Histological analysis

DBM chambers were retrieved from sacrificed animals at days 21, 42, and 56 postimplantation and were fixed in 10% formalin solution (Sigma) for 24 h. The chambers were then decalcified and dehydrated before embedding in paraffin. Blocks were sectioned into slices of 5- μ m thickness and stained by hematoxylin and eosin (H&E). Immunofluorescence stain samples were incubated with the primary antibodies against *HIF1- α* , *hOC* (5 μ g/mL), or *hPECAM-1* (4 μ g/mL; Abcam). Secondary antibody conjugated with Cy5 (3 μ g/mL; Jackson ImmunoResearch) was applied, and sections were counterstained with 4,6-diamidino-2-phenylindole (Hoechst, Sigma). The signal was visualized with confocal laser scanning microscopy (Leica TS1 SP2). For anti-human CD31 immunostaining, samples were incubated with mouse monoclonal antibody, anti-human CD31 (DAKO; dilution 1:50). Secondary antibody conjugated with biotin anti-mouse (Vector Laboratories; dilution 1:200) was added, and after color reaction with 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution, contrast staining was conducted using hematoxylin.

Statistical analysis

Statistical analysis was performed using a two-tailed Student's *t*-test. Values are presented as mean $\pm$ s.d. One-way ANOVA with Tukey *B post hoc* analysis was applied to determine significance among more than 2 groups. Descriptive statistics were performed with SPSS Statistics (15.0.1 version; SPSS Inc.). Statistical tests were considered significant when $p < 0.05$.

Results

HRE-12p-, hPECAM-1h-, and hOCp-regulated photoproteins can be used as reporters of hypoxia state, endothelial, and osteogenic differentiation in vitro

Expression of hPECAM-1p-, hOCp-, or HRE-12p-regulated genes is an indicator of cell differentiation to the endothelial and osteoblastic lineages and hypoxic state.

To show that *PECAM-1*-, *OC*-, and *HRE*-promoter driven expression of photoprotein reporters can be used to monitor endogenous gene expression, hAMSCs were transduced as described in the Materials and Methods section using lentiviral vector constructs described in Figure 1A.

To analyze the relationship between expression of endogenous hypoxia response, *PECAM-1* and *OC* genes, and light production driven by the corresponding promoters, photoreporter-transduced cells were grown either under normoxic or hypoxic conditions, in control media and differentiation-inducing media, and analyzed by BLI to determine PLuc and RLuc activities (Fig. 1B, C left). After BLI, mRNA was extracted for real-time PCR analysis (Fig. 1C right).

HRE-12p:hAMSCs grown during 48 h under hypoxic conditions (1% O₂) in a control medium showed a ratio of PLuc to RLuc activity 5.5-times higher than that of cells grown under normoxic conditions (20% O₂), while expression of hypoxia-induced genes *PGK1* and *LDH*, measured by real-time PCR, was twofold higher under hypoxic than under normoxic conditions.

After a 14-day period of growth in an either differentiation-inducing or control medium, hPECAM-1p:hAMSCs grown in an endothelial differentiation medium showed a ratio of PLuc to RLuc activities 3.5-times higher than that of control cells, while hOCp:hAMSCs grown in an osteogenic-inducing medium showed a ratio of PLuc to RLuc activities 2.6-times higher than that of control cells (Fig. 1C). Real-time PCR quantification of mRNA from the same cells, after BLI analysis, showed a similar trend, and the level of *PECAM-1* and *OC* expression was 13-fold and 17-fold higher in cells induced to differentiate to the endothelial and osteogenic lineages, respectively, than that of undifferentiated cells. In all cases, differences were statistically significant. Thus, in the three independent cases, changes in the expression of a specific promoter regulated luciferase and reflected changes in the expression of the corresponding endogenous promoter-regulated gene.

Noninvasive BLI monitoring of hypoxia activation, endothelial, and osteogenic differentiation of hAMSCs seeded in DBM scaffolds in SCID mice

We used DBM as an established model of biomaterial scaffold that provides an osteoinductive environment to demonstrate the use of BLI for noninvasive analysis of changes in gene expression in live mice. To do this, hAMSCs double transduced with the inducible and constitutive photoprotein reporters were seeded in DBM matrices implanted in the backs of six SCID mice (three DBM chambers/mice), according to the diagram in Figure 2A, and regularly imaged during a 56-day period (Fig. 2A).

Quantification of PHCs from the hAMSCs in the scaffolds shows that the ratio PLuc/RLuc activity (Fig. 2B) changed by 125-fold (day 28) for hPECAM-1p (Fig. 2B.2) regulated luciferase, and by 15-fold (day 35) for hOCp (Fig. 2B.3) regulated luciferase, both relative to the day of implantation (day 0), indicative of the changes in gene expression associated with differentiation to the endothelial and osteogenic lineages, respectively. In the case of scaffolds seeded with hAMSCs expressing an HRE-12p regulated luciferase, the ratio PLuc/RLuc achieved no significant increase by 56 days (Fig. 2B.1).

FIG. 1. *In vitro* activity of luciferase reporters regulated by the human PECAM-1 promoter (hPECAM-1p), human OC promoter (hOCp), or hypoxia-response element promoter (HRE-12p) promoters correlates with mRNA levels for the corresponding endogenous genes. Human adipose tissue mesenchymal stromal cells (hAMSCs) transduced to express *Renilla reniformis* luciferase (RLuc) regulated by the cytomegalovirus (CMV) promoter and *Photinus pyralis* luciferase (PLuc) regulated by either hOCp, hPECAM-1p, or HRE-12p promoters were grown under inducing conditions and analyzed by bioluminescence imaging (BLI) to image luciferase activities. After BLI, expression of platelet endothelial cell adhesion molecule-1 (PECAM-1), osteocalcin (OC), phosphoglycerate kinase 1 (PGK1) and LDHA, genes was analyzed by reverse transcription-polymerase chain reaction of mRNA extracted from the same cells. **(A)** Diagram showing the luciferase reporter constructs used for cell transductions. **(B)** Representative images of luciferase activity from cells grown in control (left panel) or inducing medium (right panel). Top row, PLuc regulated by specific promoter; bottom row, RLuc regulated by CMV promoter. **(C, left panel)** Quantification of image data from **(B)**. The histograms show average values for photon counts (PHCs) recorded in images for control cells and induced cells (RLuc regulated by the CMV promoter, line gray bars for control cells and black for induced cells; PLuc regulated by specific promoters, gray bar for control cells, and dark gray for induced cells). **(C, center panel)** Histograms showing the ratios of PLuc/RLuc activity calculated from BLI images, (control cells, gray bars; induced cells, black bars). **(C, right panel)** Histograms showing mRNA levels from induced hAMSCs versus uninduced. AU, arbitrary light units. Histograms and bars represent the average of four wells and SD, respectively; **p* < 0.05. Arbitrary color bars represent light intensity levels for PLuc (blue, low; red, high) and RLuc (green, low; blue, high). Color images available online at www.liebertpub.com/tea

FIG. 2. Noninvasive BLI of hAMSC differentiation in subcutaneous implanted demineralized bone matrix (DBM) scaffolds. DBM scaffolds were subcutaneous implanted in the back of severe combined immunodeficiency (SCID) mice and seeded with hAMSCs previously transduced with the CMV:RLuc:mRFP reporter (a constitutively expressed reporter of cell number) and a cell differentiation reporter (PLuc:eGFP) regulated by hPECAM-1p, hOCp, or HRE-12p, and imaged at the indicated times. **(A)** Diagram illustrating the DBM implantation sites (numbered squares) and seeded cell types. Representative BLI and b&w composite images showing bottom row, RLuc; top row, PLuc, ($n=6$). **(B)** (Top panel 1-3) Quantification of image data from **(A)**. The graphs show average values for PHCs recorded in the images (RLuc regulated by CMV promoter, square symbol; PLuc regulated by specific promoter, round symbols). (Bottom panel 1-3) Histograms showing the ratio PLuc/RLuc calculated from photon fluxes recorded in BLI images. Arbitrary color bars represent light intensity levels for PLuc (blue, low; red, high) and RLuc (green, low; blue, high). * $p < 0.05$. HAMSC labeling scheme: DBM-1: PLox-HRE12p:PLuc:eGFP + CMV:RLuc:mRFP:ttk; DBM-2: PLox-hPECAM-1p:PLuc:eGFP + CMV:RLuc:mRFP:ttk; DBM-3: PLox-hOCp:PLuc:eGFP + CMV:RLuc:mRFP:ttk. Color images available online at www.liebertpub.com/tea

Real-time PCR was also used as independent procedure, to analyze mRNA extracted from the hAMSCs seeded in DBMs at days 21, 42, and 56, and validate BLI data. Changes in levels of *PECAM-1*, *OC*, *PGK1*, and *LDHA* gene expression are shown in Table 1. At the indicated days, mRNA levels were 8.86, 9.41, and 18.85-fold for *PECAM-1*, and 5.48-fold, 9.96-fold, and 22.34-fold for *OC*, relative to those from hAMSCs grown in a noninducing medium during the same time period. Also in agreement with BLI data, the modest, not significant, increase in HRE-12p-regulated luciferase ex-

pression correlated with a nondetectable change in the level of mRNA expression of two genes, *PGK1* and *LDHA* that are upregulated under hypoxic conditions.

Thus, *in vivo*, changes in the activity of PLuc regulated by promoters of genes that are expressed in osteogenic and endothelial cell lineages or during hypoxia correlate with changes in the levels of mRNA expression for the corresponding endogenous promoter-regulated gene, supporting the use of BLI to noninvasively report changes in gene expression.

TABLE 1. *In Vivo* PGK1/LDH, PECAM-1, AND OSTEOCALCIN GENE EXPRESSION

Fold ^a (vs. control cells)								
21 days			42 days			56 days		
PGK1/LDHA	PECAM-1	OC	PGK1/LDHA	PECAM-1	OC	PGK1/LDHA	PECAM-1	OC
0/0	8.96	5.48	0/0	9.41	9.96	0/0	18.85	22.39

^aAvarage C_t values from at least three independent experiments performed in duplicate were used to calculate fold changes in gene expression (2^{-ΔΔC_t}) using GADPDH as reference.
OC, osteocalcin; PECAM-1, platelet endothelial cell adhesion molecule-1; PGK1, phosphoglycerate kinase 1; LDHA, lactate dehydrogenase.

Angiogenesis in hAMSC seeded DBM scaffolds in live mice

To visualize development of microvasculature in the implanted DBMs, at days 19, 30, and 56 postimplantation, mice were perfused by injection trough the lateral tail vein with a

high-molecular-weight (2,000,000 Da) dextran–fluorescein conjugate (Fig. 3A). After harvesting, perfused DBMs were lightly fixed in formaldehyde, sectioned, and prepared for imaging by laser confocal microscopy (see Supplementary Video S1 online; Supplementary Data are available online at www.liebertpub.com/tea).

FIG. 3. Imaging of microvessel colonization of hAMSC-seeded DBMs in SCID mice. DBM-bearing mice were injected through the lateral tail vein with FITC-conjugated dextran (MW 2,000,000 daltons). After fluorescent dextran perfusion, DBM implants were harvested, formalin fixed, and sectioned for laser confocal microscope imaging of microvascular structures. **(A)** Diagram illustrating FITC–dextran injection procedure. **(B)** Dextran-perfused DBM scaffolds harvested at the indicated times were imaged for dextran fluorescence using an IVIS spectrum (Caliper Life Sciences) (left), and by laser confocal microscopy at low (10×) magnification to reveal fluorescent microvascular networks within the DBM section (right) (Scale bar: 50 μm). **(C)** High-magnification (63×) images from dextran-labeled capillary network regions. Each set of images shows (top right) red fluorescent cells corresponding to the implanted hAMSCs present in all implants, (top left) cells expressing GFP regulated by specific promoter, and FITC-labeled microvessel (bottom left) Hoechst-stained nuclei, and (bottom right) red, blue, and green composite image (scale bars: 20 μm). Color images available online at www.liebertpub.com/tea

As shown in Figure 3B, DBMs harvested by day 21 and thereafter were fluorescent, and fluorescence intensity was directly proportional to the elapsed time after implantation (5.15×10^8 , 6.67×10^8 , and 8.06×10^8 p/s/cm²/sr for days 21, 42, and 56*, respectively * $p < 0.005$), indicative of the existence of a vascular system, well connected to that of the host. The fluorescent capillary bed connected to the host vascular system that invaded the DBM matrix could be easily visualized by low-magnification confocal microscopy of DBM sections. To localize the hAMSCs originally seeded in DBMs, we further exploited the inclusion of fluorescent protein activities associated with the luciferases in the chimeric photoreporters. Confocal microscope observation at high magnification showed, in all cases, the presence of red fluorescent cells within the DBM matrix (Fig. 3C), corresponding to hAMSCs constitutively expressing RFP regulated by the CMV promoter. Moreover, in the case of DBMs seeded with hPECAM-1p:hAMSCs, red fluorescent cells frequently expressed green fluorescence, indicating endothelial differentiation (shown as yellow in composite images) (Fig. 3C and Supplementary Video S2 online). What is more, such cells had endothelial morphology and were found lining thin vascular structures filled with dextran. Additional support for the endothelial lineage of these cells was obtained by staining with an antihuman-PECAM-1-specific antibody, conjugated to a Cy5 fluorescent second antibody, chosen to generate a far-red fluorescent signal, easily distinguishable from that of the RFP signal (Fig. 4B).

In the case of DBMs seeded with hOCp:hAMSCs, the implanted red fluorescent cells also expressed green fluorescence (appearing yellow in composite images) (Fig. 3C). However, in this case, the cell phenotype was not endothelial, but corresponded with that of cells from the osteogenic lineage, and green cells did not appear associated with vascular structures. In addition, the green fluorescent cells stained positively with an antihuman-OC-specific monoclonal antibody, conjugated to a Cy5 fluorescent second antibody, providing further evidence of their osteogenic lineage (Fig. 4C).

Our assessment showed that most of eGFP-expressing cells also expressed RFP, ruling out CMV promoter silencing as the cause for the decrease in production RLuc. Moreover, to ascertain that the increase in the ratio PLuc/RLuc observed by BLI was not due to silencing of the CMV promoter regulating RLuc:RFP expression, we counted the number of red and green fluorescent cells and only green fluorescent cells (that would be generated if the CMV promoter had been silenced). Our results show that in the 12 sections, the counted 579 cells were red + green simultaneously, and only 14 (2.4%) were only green fluorescent (Supplementary Table S1).

Finally, in serial sections from DBMs seeded with HRE-12p:hAMSCs, contained red fluorescent cells, which showed no green fluorescence (Fig. 3C), indicating that few, if any, of the seeded hAMSCs were under hypoxic stress, in support of the BLI and real-time PCR data. This result was further supported by the absence of cells that stained with an anti-HIF1- α monoclonal antibody, conjugate to a Cy5-labeled second antibody (Fig. 4A).

Moreover, the DBMs were extracted from mice at the end of the BLI experiment, sectioned, and stained with anti human-PECAM antibody (Fig. 4D) and H&E (Fig. 4E) to show vascular structures and calcium deposits indicative of bone formation. After, parallel sections from the same location

were also imaged by laser confocal microscope to determine the presence of red and green fluorescent cells.

Our results show vascular-like structures in the DBM interior that stain with anti-hPECAM antibody and correspond with cells that expressed RFP and eGFP regulated by the PECAM promoter in parallel sections. Also, H&E-stained DBM sections showed purple-staining calcium deposits that also corresponded with cells expressed RFP and eGFP regulated by the OC promoter in parallel sections. Number of demineralized bone matrix implanted and mice in time is represented in Supplementary Table S2. To show that osteoblastic differentiation is specific of DBM and does not take place in other nonosteoinductive materials, hAMSCs expressing firefly luciferase regulated by either the OC or the PECAM promoters (as a positive control) were seeded in Matrigel, a proangiogenic material, subcutaneous implanted in the back of SCID mice and monitored during a 6 day period (Supplementary Figure S1).

Discussion

Development of TE strategies proceeds through multiple stages, including material design, *in vitro* testing, and *in vivo* demonstration of effectiveness. This complex process would benefit from agile longitudinal noninvasive real-time monitoring of biomaterial–stem cell interactions in live animals. Noninvasive imaging is particularly suited to evaluate the performance of cells *in vivo*, and could be integrated in design strategies based on iterative cycles of material engineering, *in vivo* testing, and re-engineering aimed at improving the performance of scaffolds.

In the current work, we demonstrate an improved procedure to noninvasively image and measure changes in the expression of several genes during differentiating cells seeded in a model biomaterial implanted in live mice.

The physicochemical and instrumental aspects of bioluminescence and its measurement are well established and relatively easy to control in isolated enzymatic systems and cells *in vitro*.^{23,24} However, noninvasive BLI of cells implanted in live animal models presents additional challenges resulting from local differences in the physiological environment and optical properties of intervening tissues that difficult a quantitative analysis of cell behavior.

To minimize the effect of these issues during noninvasive analysis of cell differentiation, we use a simple imaging strategy based on labeling the same therapeutic cell with a reference luciferase–fluorescent protein (RLuc-RFP) regulated by a constitutively active promoter, and with a different luciferase–fluorescent protein combination (PLuc-GFP) regulated by the promoter of the target gene we wish to investigate (specific reporter). This double luciferase approach facilitates interpretation of inherently noisy *in vivo* reporter data, on a per cell basis, for example, independently of the proliferation state of the implanted cells. In addition, the use of chimeric luciferase–fluorescent protein reporters allows both, the selection of reporter expressing cells after labeling and the validation of *in vivo* imaging data by standard histological procedures.

We established that luciferase expression regulated by tissue-specific promoters could be used as correlates of endogenous gene activity. In hAMSCs dually labeled to express tissue-specific promoter- (hOCp, hPECAM-1p, and HRE-

FIG. 4. Immunofluorescence staining, immunostaining, and staining for detection of specific proteins *hPECAM-1*, *hOC*, and *HIF- α* and bone formation in DBM-implanted hAMSCs. hAMSC-seeded DBM sections were imaged by laser confocal microscopy to reveal GFP and red fluorescent protein (RFP)-positive cells and then incubated with primary antibodies against *HIF1- α* (A), *hPECAM-1* (B), or *hOC* (C), followed by a Cy5-conjugated secondary antibody (3 mg/mL; Jackson ImmunoResearch) and 4,6-diamidino-2-phenylindole (Hoechst, Sigma) for nuclear staining. In each row, images show, from left to right, green fluorescence protein regulated by tissue-specific promoter; RFP regulated by constitutively active CMV promoter in all implanted hAMSCs; specific ab-detection of the corresponding endogenous protein marker; Hoechst nuclear stain; and overlay of four images (scale bars: 10 μ m). (D) From left to right, the panel shows *hPECAM* *in vivo* staining of a DBM section ($\times 5$); higher magnification of the image showing positive *hPECAM* cells in capillary structure ($\times 80$); cells expressing GFP regulated by *PECAM* promoter from parallel section; cells expressing RFP regulated by the CMV promoter from the same section; previous images merged with also Hoechst stained nuclei (blue). Scale bar: 10 μ m. (E) From left to right, the panel shows hematoxylin and eosin (H&E)-stained DBM section ($\times 5$); higher magnification of the same section showing cells positive for deposited calcium ($\times 20$); cells expressing GFP regulated by *hOCp*, from parallel section; cell expression RFP regulated by the CMV promoter from the same section; previous two images merged with also Hoechst-stained nuclei (blue). Scale bar: 25 μ m. (a) DBM-1. PLox-HRE12p:PLuc:eGFP + CMV:RLuc:mRFP:ttk. (b) DBM-2. PLox-hPECAMp:PLuc:eGFP + CMV:RLuc:mRFP:ttk. (c) DBM-3. PLox-hOCp:PLuc:eGFP + CMV:RLuc:mRFP:ttk. Color images available online at www.liebertpub.com/tea

12p) regulated PLuc, and CMV-regulated RLuc, and induced to differentiate *in vitro*, the ratio of PLuc/RLuc activity was statistically significantly higher (2.6, 3.5, and 5.5 times, respectively) than that of control, uninduced cells. These changes in luciferase activity correlated with changes in mRNA levels for the corresponding genes regulated by the endogenous promoters (17-fold, 13-fold, and 2-fold, respectively) in the same induced cells, as verified by an independent procedure using real-time PCR.

Differences in the trends of BLI and real-time PCR values may reflect the inevitable use abbreviated promoter constructs with structures not entirely corresponding to those of the endogenous ones, or the insertion of the reporter constructs at random chromosomal locations.

Due to the preservation of the three-dimensional, as well as molecular, information in DBM, this material is a convenient scaffold model in which to demonstrate the capacity to *in vivo* image changes in gene expression during bone development.

hAMSCs labeled to express luciferases regulated by tissue-specific and constitutively active promoters were seeded in DBMs subcutaneously implanted in the back of SCID mice and imaged weekly during a 56-day period. In these experiments, imaging data showed that there was an increase in the ratio PLuc/RLuc indicative of differentiation of the implanted hAMSCs to the osteogenic and endothelial cell lineages (dynamic range of 15-fold and 125-fold, respectively). However, there was only a slight, not statistically significant, increase in the level of *HRE* promoter-regulated PLuc, indicative of little hypoxia-related stress in the environment of the implanted cells. Analysis by real-time PCR of mRNA levels in DBMs at specific times during the experiment showed also significant increases in the expression of *OC* and *PECAM-1* genes (dynamic range of 22.3-fold and 18.8-fold, respectively), but not of hypoxia-induced genes, again in agreement with BLI data.

Interestingly, *in vivo* PLuc/RLuc ratios were higher than those found *in vitro*. This could result, in part, from animal tissues having a higher opacity to the shorter wavelength of RLuc-generated photons (480 nm), than to longer-wavelength PLuc photons (578 nm). However, probably more important is the fact that DBMs were implanted in a physiological environment, and that their natural origin provides a nearly optimum combination of the structural and molecular cues required to induce cell differentiation.

Inoculation of mice at different times of the experiment with a high-MW fluorescent dextran followed by confocal microscope imaging revealed a complex capillary network, functionally connected to the host vascular system that colonizes the DBMs. Within the invading vascular structures, hAMSCs seeded in DBMs appeared to have differentiated to endothelial and osteoblastic lineages as shown by the presence of simultaneously red and green fluorescent cells. In the case of hPECAM-1:hAMSCs, red and green fluorescent cells had spindle shapes and were found in close association lining the capillaries, while in the case of hOCp:hAMSCs, red and green fluorescent cells were found scattered through the material, but not in association with capillaries.

Further support that DBM-seeded hAMSCs had differentiated to the endothelial and osteoblastic lineages was provided by their staining with antibodies against PECAM-1 and OC proteins.

Small changes in BLI-detectable luciferase activity regulated by the *HRE* promoter *in vivo* correlated with the absence of GFP-expressing RFP cells, no increase in the expression of hypoxia-inducible gene mRNA levels, the absence of cells detectable with an anti HIF- α ab, and with the presence of a host capillary network penetrating the scaffolds, to provide necessary oxygen and nutrients.

In summary, we show that differentiation of cells seeded in biomaterials implanted in live animals can be reliably monitored using BLI by introducing in the target cells' two different luciferase reporters, one of them regulated by a tissue-specific promoter and the other regulated by a constitutively expressed promoter. By measuring the change in activity of the tissue-specific reporter relative to that of the constitutively expressed reference reporter, it is possible to obtain information on changes in gene expression relative to an internal standard indicative of cell number, free of artifacts produced by cell death or proliferation. Moreover, by including both reporters in the same cell, noise resulting

from uneven distribution of cells with different labels is largely avoided, and comparison between different samples is facilitated. An additional advantage of the reporter strategy was the use of chimeric luminescent/fluorescent reporters. This approach had a double advantage, allowing the use of FACS for purification of labeled cells, previous to seeding in scaffolds, and providing very convenient fluorescent tracers for histological detection of implanted and differentiated cells after *in vivo* BLI analysis.

Although in the current work we demonstrate the use of three different inducible promoters to regulate a luciferase, there is no limit in the number of genes that could be simultaneously monitored in different samples of biomaterial. Moreover, the availability of additional luciferases with different substrate requirements would allow increasing the number of gene activities that could be monitored in the same cell, or biomaterial sample, widening the scope of biological parameters that could be monitored during scaffold development.

Conclusions

Dual labeling of hAMSCs with constitutively active and tissue-specific promoter-regulated luciferase reporters allows quantitative noninvasive BLI monitoring of gene expression during vascular and bone differentiation, which is independently validated by molecular and histological procedures. This approach could be the basis of an optimization procedure based on iterative cycles of modification-validation for scaffold development.

Acknowledgments

The authors wish to thank Dr. Esther Peña for technical assistance in laser confocal microscopy. We also like to thank Dr. S.S. Gambhir and Dr. Carmelo Bernabeu for the kind donation of the CMV-Rluc-RFP and PECAM-1 promoter, respectively. This research was supported by collaborative project no. 214402 Acronym Angioscaff (EU-FP7) and in part (reporter vector construction) by grant SAF2009-07102 (Ministerio de Ciencia e Innovación). BIO2009-13903-C02-01, PLE2009-0163, PI10/02529 and the Andalusian Governments (P07-CVI-2781, PI-0729-2010, and PAID BIO217 (DBM scaffold production)).

Disclosure Statement

No competing financial interests exist.

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Received: February 6, 2012

Accepted: September 21, 2012

Online Publication Date: December 19, 2012