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Bioluminescence imaging of bone formation using hairless osteocalcin-luciferase transgenic mice

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ABSTRACT

Osteocalcin is a major noncollagenous protein component of bone extracellular matrix, synthesized and secreted exclusively by osteoblastic cells during the late stage of maturation. We introduced a 10 kb human osteocalcin enhancer/promoter (OC)-luciferase (Luc) construct into a hairless mouse line. Examination of tissue RNAs from these transgenic mice showed a predominant restriction of Luc mRNA expression to bone-associated tissues. Immunohistochemical staining of calvaria tissue sections revealed the localization of Luc protein to osteoblasts. Utilizing *in vivo* bioluminescence imaging, supplementation of 1α,25-dihydroxyvitamin D_3 increased Luc activity throughout the skeleton, consistent with *in vitro* transient transfection studies in osteoblast-like cells. Moreover, we observed an abrupt decrease in bioluminescence activity as the mice reached puberty, and a further decrease gradually thereafter. Using a radius skeletal repair model, we observed enhanced bioluminescence at the fracture site in both young (14–22 weeks old) and aged (50–66 weeks old) mice. However, peak bioluminescence was delayed in aged mice compared with young mice, suggesting retarded osteocalcin expression with aging. Our *in vivo* imaging system may contribute to the therapy and prevention of various bone metabolic disorders through its effective monitoring of the bone formation process.

Introduction

Bone is a dynamic tissue that undergoes modeling during childhood and continuous remodeling throughout adulthood [1,2]. Maintenance of bone mass and density is dependent on the balance between two processes: resorption of mineralized bone by osteoclasts and *de novo* bone formation by osteoblasts. An alteration in either or both of these processes causes a change in the bone density. Bone loss and the resultant skeletal structural weakening are associated with aging as a consequence of changes in hormone levels, bone cell differentiation and function [3]. This loss of bone may be especially debilitating in post-menopausal women, giving rise to osteoporosis and the consequential morbidity and mortality associated with increased incidence of fractures [2,4]. While there is an analogous age-related skeletal deterioration in mice [5–7], additional data are needed to better describe age-dependent changes in bone structure and strength.

Osteocalcin is the most abundant noncollagenous protein expressed in bone, with its expression limited to cells of the osteoblast lineage, including mature osteoblasts, osteocytes, and hypertrophic chondrocytes [8,9]. The location of osteocalcin at bone-forming surfaces [10] and the increased bone mineralization observed in osteocalcin gene knockout mice [11] support a role for osteocalcin in the suppression of bone mineralization. Alternatively, osteocalcin has been suggested to increase bone resorption through osteoclast recruitment [10,12]. Osteocalcin also has features of a hormone, as it is synthesized as a pre-promolecule and secreted in the general circulation [9]. Further, osteocalcin acts as a regulator of insulin in the pancreas, adiponectin in adipocytes [13], and testosterone synthesis in male germ line cells [14]. Thus, osteocalcin is an important molecule that functions not only in bone, but also in energy metabolism and reproduction.

In recent years, several highly sensitive imaging technologies have been developed to detect and quantitate *in vivo* fluorescence and luminescence, without sacrificing animals [15,16]. These technologies are important in developmental studies, in gene therapy systems, and for the purpose of revealing the spatiotemporal patterns and expression intensity of target genes. Previous studies have employed a segment of the human osteocalcin (OC) promoter to analyze OC promoter regulation in mice [17–19]. Kesterson et al. [18] reported the use of transgenic mice harboring this 3.9 kb promoter sequence and 10 bp of 5′-untranslated sequence fused to a chloramphenicol acetyltransferase reporter gene, and noted its expression in bone-associated tissues. Utilizing the same

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promoter, Iris et al. and Bilic-Curcic et al. also demonstrated that luciferase and GFP, respectively, were expressed in a wide spectrum of skeletal organs, increasing several days after bone fracture [20–22]. Using *in vivo* bioluminescent imaging, the human OC promoter was also shown to exhibit a periodicity of approximately 24 h in multiple skeletal sites [23].

In this study, we produced a transgenic mouse line expressing luciferase under the control of 10 kb osteocalcin enhancer/promoter sequence. This line was backcrossed with a hairless mouse line to enable us to monitor bone formation during growth, aging, and fracture repair using *in vivo* imaging, without sacrificing the mouse. The bioluminescence monitored during fracture healing showed delayed osteocalcin expression in aged mice as compared with young mice, suggesting an age-related impairment during fracture repair. Our bioluminescence imaging system may offer a powerful tool for the non-invasive tracking of bone formation activity *in vivo* during bone development and regeneration.

Materials and methods

Plasmid construction

A pBSI-I plasmid was a kind gift from Dr. Mitsuo Oshimura (Tottori University, Tottori, Japan). This plasmid contained two copies of the 1.2 kb 5′ β-globin element derived from the chicken constitutive hypersensitive site (5′HS4) responsible for an insulating effect. pGL3-Basic and pKO Scrambler V907 were purchased from Promega (Madison, WI, USA) and Lexicon Genetics (Woodlands, TX, USA), respectively. The pGL3-Basic-AB retrieval vector was generated by inserting a 405 bp *Nhe*I/*Eco*RI-homologous fragment (termed ArmA) and a 128 bp *Eco*RI/*Nco*I-digested homologous fragment (termed ArmB) into *Nhe*I and *Nco*I sites of pGL3-Basic. ArmA and ArmB were PCR amplified from RP11-54H19 BAC clone DNA (BACPAC Resources Center at Children's Hospital Oakland Research Institute, Oakland), including human osteocalcin gene, using oligonucleotide primer sets OCP1/OCP2 and OCP3/OCP4, respectively: 5′-TTGCTAGCCGCGGACAGAGCTCAGAGTCCT-3′ for OCP1, 5′-CGAATTCAAAGGCTGCATCTAAGATAC-3′ for OCP2, 5′-CGAATTCGATATCATCATGGTCAGGCATGCC-3′ for OCP3, and 5′-TTCCATGGTGTCTCGGTGGCTGC-3′ for OCP4. To insert the 10.0 kb enhancer/promoter region of human osteocalcin gene into pGL3-Basic-AB upstream of luciferase, DY380 *Escherichia coli* [24] were transformed with RP11-54H19 BAC and the linearized retrieval vector, according to the protocol described previously [25]. To construct pOC-Luc, three DNA fragments, including 10.0 kb osteocalcin enhancer/promoter upstream of the luciferase gene and two sets of 2.4 kb 5′HS4 insulator elements, were cloned into *Sal*I and *Eco*RI sites of the pKO Scrambler V907 (Fig. 1A). For pΔOC-Luc, the 405 bp ArmB fragment was used instead of the 10.0 kb enhancer/promoter sequences.

Cell culture and reporter assays

MG-63 human osteosarcoma cells were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 U/ml penicillin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), and 0.1 mg/ml streptomycin (Meiji Seika Pharma) at 37 °C under 5% CO_2 in air. For the luciferase reporter assay, 2 μg of pOC-Luc, p-Luc, or pSV2-Luc was cotransfected into MG-63 cells (1×10^5) using TransIT-LT1 (Mirus Bio LLC, Madison, USA) with 0.1 μg of pRL-TK (TOYO B-Net, Tokyo, Japan), a plasmid carrying the *Renilla* luciferase gene driven by the thymidine kinase minimal promoter, as an internal control. DMSO or 1 nM of 1α,25-dihydroxyvitamin D_3 in DMSO (0.1% final DMSO concentration) was added following the transfection. Twenty-four hours after transfection, cells were solubilized and luciferase activities were measured using the Pikkagene Dual Luciferase Assay System (TOYO B-Net, Tokyo, Japan). The firefly luciferase activity was normalized to the *Renilla* luciferase activity of the same sample. Data was expressed as mean ± SD.

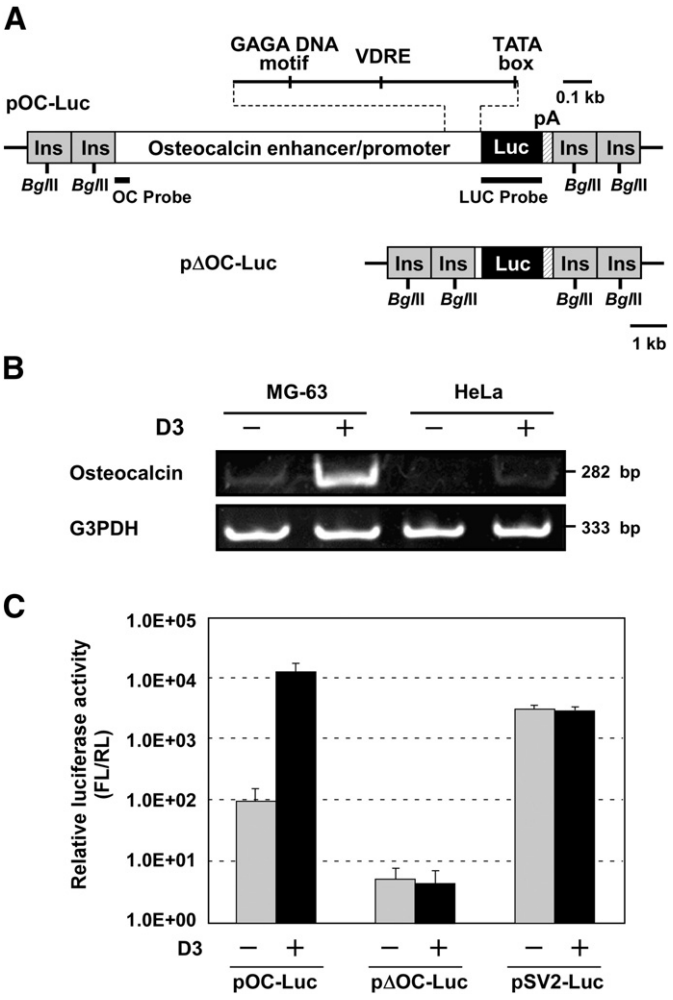

Fig. 1. Constructs for the generation of the transgenic mice. (A) A schematic map of the transgenes used in the experiment. pOC-Luc is composed of 10 kb of the human osteocalcin enhancer/promoter sequences, with 60 bp of the 5′-untranslated sequences (white box), a luciferase gene (black box), the SV40 late polyadenylation signal (striped box), and insulator sequences (gray box). OC and LUC probes used for Southern blot analysis are indicated. (B) Induction of osteocalcin expression in MG-63 cells by 1α,25-dihydroxyvitamin D_3 (D3). MG-63 and HeLa cells were treated with or without 1 nM D3 and then subjected to RT-PCR analysis. (C) Enhanced expression of pOC-Luc by D3 treatment. MG-63 cells transfected with pOC-Luc, pΔOC-Luc, and pSV2-Luc was treated with (black bars) or without (gray bars) 1 nM D3, respectively, and then subjected to luciferase reporter assay. The y axes show relative luciferase activities: firefly luciferase (FL) from the reporter plasmid was normalized by *Renilla* (RL) luciferase activity from the control vector.

Production of transgenic mice

All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals from Tottori University. To generate transgenic mice, the *Xmn*I/*Sal*I fragment from pOC-Luc was excised from an Seaplaque GTG agarose (Lonza Co., Basel, Switzerland), melted at 65 °C, purified by phenol/chloroform extraction and ethanol precipitation, and microinjected into the pronuclei of B6D2F1 × B6D2F1 fertilized eggs [26]. The incorporation of the transgene was examined by PCR analysis using genomic DNA extracted from the tail. The following oligonucleotides were used as primers: OCP3 (5′-CGAATTCGATATCATCATGGTCAGGCATGCC-3′), and LUC2 (5′-ATAAATAACGCGCCCAACAC-3′). For *in vivo* imaging, transgene-positive mice were backcrossed with Hos:HR-1 hairless mice (SLC, Inc., Shizuoka, Japan) for four generations.