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Enhanced reporter gene assay for the detection of osteogenic differentiation

G. A. Feichtinger, T. J. Morton, A. Zimmermann, D. Dopler, A. Banerjee, H. Redl, M. van Griensven

Ludwig Boltzmann Institute for Experimental and Clinical Traumatology / AUVA Research Center, Vienna, Austria

The Austrian Cluster for Tissue Regeneration, Vienna, Austria

European Institute of Excellence on Tissue Engineering and Regenerative Medicine Research (Expertissues EEIG), Vienna, Austria

Correspondence:

Georg A. Feichtinger, MSc

Ludwig Boltzmann Institute for Experimental and Clinical Traumatology

Donaueschingenstrasse 13

A-1200 Vienna, Austria

Tel +43 1 33110 461

Fax +43 1 33110 460

E-mail: georg.feichtinger@trauma.lbg.ac.at

http://trauma.lbg.ac.at/

Abstract

Detection of osteogenic differentiation is crucial for bone tissue engineering. Despite established standard end point assays, there is increasing demand for methods allowing non-invasive kinetic differentiation monitoring. Reporter gene assays employing tissue-specific promoters and suitable reporter genes fulfill these requirements. Many promoters, however, exhibit only weak *cis*-activating potential, thus limiting their application to generate sensitive reporter gene assays. Therefore, the aim of this study was to design a reporter gene assay employing elements of the murine osteocalcin promoter coupled to a viral enhancer for signal amplification. Additionally, the system's practicability was enhanced by introducing a secreted luciferase as a quantifiable reporter gene. The constructs were tested in C2C12 cells stimulated with recombinant human bone morphogenetic protein 2 (rhBMP2) for osteogenic differentiation in 2D and 3D culture.

Osteogenic differentiation was confirmed by standard assays for osteogenesis. The reporter gene signal was detected through a secreted luciferase or fluorescence microscopy for enhanced yellow fluorescent protein. The constructs exhibited strong activation upon treatment with rhBMP2. Weak background expression was observable in negative controls, attributed to the *pan*-active viral enhancer. In conclusion, a novel enhancer/tissue-specific promoter combination allows specific signal-amplified, kinetic monitoring of osteogenic differentiation in a non sample-destructive manner.

Keywords

Osteocalcin promoter, reporter gene assay, osteogenic differentiation, BMP2, metridia

luciferase

Introduction

All present bone regeneration approaches intend to induce cellular differentiation as a central criterion, i.e. the bone forming osteoblast phenotype. The assessment of the successful acquirement of this phenotype is mandatory in validating the therapeutic benefit of newly devised methods or treatment options. Today, several standard assays have been established providing the viable information of successfully induced osteogenic differentiation to the researcher (e.g. *in vitro* or *in vivo* enzymatic alkaline phosphatase assay (1), *in vivo* ectopic bone formation assay (2) and bone matrix specific staining methods like *von Kossa* (3) and Alizarin red staining). Furthermore, PCR quantifiable or antibody detectable differentiation specific markers (4-8) seem to provide more than a satisfying array of diagnostic tools for the detection of osteogenesis.

None of these methods, however, are suitable for highly sensitive and quantifiable on-line monitoring without the need for sample-destructive processing. The necessity of obtaining this kind of data becomes obvious in large-scale osteoinductive substance screening, during *ex vivo* stem cell differentiation or while monitoring the progression of bioreactor constructs towards bone-like tissue. In large-scale screening assays, the option of on-line monitoring is crucial for identification of lead compounds. *Ex vivo* cellular therapies and bioreactor approaches rely on the optimization of culturing methods to force the cells towards osteogenic differentiation. Assay technologies offering this information could either reduce sample number and thus labour time for large scale screening assays and provide non-invasively obtained information on the status of preconditioned stem cells or bioreactor constructs prior to implantation.

Additionally, these systems could refine *in vivo* testing in animals, thus substantially reducing animal experiments carried out to test the osteoinductive capacity of future drugs and therapeutic approaches.

A well-known method derived from the genetic toolbox could meet these requirements, namely the reporter gene assay that has been originally used for the identification and characterization of endogenous gene-regulatory (*cis*-acting) sequences. Detailed insights into the signal transduction cascades and gene-regulatory mechanisms underlying osteogenesis provided several tissue-specific promoter elements that have already been partially exploited to design reporter-vectors (9-11) that employ fluorescent or bioluminescent reporter genes. Furthermore, not only information on differentiation but also on cellular viability can be obtained, since this type of assay relies on the transcriptional machinery of viable cells, ceasing its function upon cellular death.

Osteocalcin (12, 13), a genuine marker-gene transcribed by differentiated osteoblasts with its extensively studied and precisely described promoter sequence (9, 12, 14) offers regulatory sequences that can be used to design novel reporter gene constructs for the detection of osteogenesis. The main limitation of its proximal promoter sequence, however, is its generally low transactivation potential. Therefore, this promoter reaches only low expression levels when applied ectopically to drive the expression of a reporter gene. Strategies to overcome this limitation have been studied in previous efforts, such as the artificial multimerization of short specific regulatory sequences of this promoter like the multimerized osteoblast specific element 2 (6xOSE2) (10), enabling the design of a functional reporter gene assay for osteocalcin expression. This study, in contrast to the strategy described above, aimed at the generation of an artificial, chimeric *cis*-acting regulatory sequence for signal amplification through combination of a *pan*-active viral enhancer with a tissue specific

promoter element. Taking the relatively low transcriptional activation potential of the murine osteocalcin 2 promoter (mOG2P) into account, the primary objective of this work was to investigate whether the cytomegalovirus immediate early enhancer (CMVE) (15, 16) is capable of amplifying specific expression. The CMVE, as already reported (17, 18), can be used to amplify expression from cell-type specific, weak endogenous promoters, but with different impact on specificity depending on the employed promoter (19).

The design of constructs that employ reporter genes suitable for quantification without sample destructive processing and preferably suitable for real-time monitoring of osteoblastic differentiation was the secondary aim of this study in order to expand the range of application of our system to bioreactor monitoring. Therefore, we selected a novel secreted bioluminescent luciferase from the copepod *Metridia longa* (MetLuc) (20), which can be simply assayed and absolutely quantified by medium sampling, thus offering bioluminescence quantification without manipulation of the culture and under optimal imaging conditions.

Materials and Methods

Growth factors

Recombinant human (CHO-derived) rhBMP2 (InductOS) was purchased from Wyeth (Madison, NJ, USA). Recombinant human FGF2 (*Escherichia coli* derived) and recombinant human VEGF-A (*Escherichia coli* derived) were purchased from Peprotech (Rocky Hill, NJ, USA).

Reporter plasmids and chimeric cis-acting elements

All PCR synthesis reactions were carried out using a Hot-*Taq* polymerase enzyme (PeqLab Biotechnologie GmBH, Erlangen, Germany) according to the manufacturer's instructions using the appropriate annealing temperatures for each of the outlined primer pairs. The proximal murine osteocalcin-2 promoter fragment (mOCP) (ranging from –174 to +3 relative to the transcriptional start site) was amplified from the plasmid pDRIVE-OG2 (m) v08 purchased from InvivoGen (San Diego, CA, USA) using the primers mOCPs1 (GATGGATCCCCTGCAGGGCCCACTAGT) and mOCPas (GATAAGCTTGGTGTCTGCTAGGTGTGC) for the unenhanced control reporter plasmid pmOCP-EYFPHis and mOCPs2 (CCAATTCGGATCTGTCCTGCAGGGCCCACTAGT) and mOCPas for subsequent fusion PCR with the cytomegalovirus immediate early enhancer (CMVE). The CMVE fragment (ranging from –524 to –120) was amplified from the plasmid pCDNA3 (Invitrogen, Lofer, Germany) using the primers CMVs (GATAGATCTGCAGGCGTTACATAACTTACGG) and CMVas

(ACAGATCCGAATTGGGTGAAAACAAACTCCCATTGA).

The purified PCR fragments were employed in equimolar concentrations for a ligation PCR reaction using the primer overhangs (underlined in the primer sequences) for hybridization and the primers mOCPs and CMVas for subsequent amplification. The generated element was cloned into the BglII/HindIII digested pCDNA3 vector. The mOCP fragment from the first mOCPs1/mOCPas reaction was cloned into the BglII/HindIII digested pcDNA3 vector. The reporter genes EYFPHis and MetLuc were then ligated into the multiple cloning site of the intermediate pcDNA3-CMVE/mOCP plasmid to produce the reporter plasmids pCMVE/mOCP-EYFPHis and pCMVE/mOCP-MetLuc (Figure 1, A, B). The unenhanced control reporter plasmids pmOCP-EYFPHis (Figure 1, C) and pmOCP-MetLuc (Figure 1, D) were produced by ligation of EYFPHis or MetLuc into the multiple cloning site of the mOCP containing pCDNA3 intermediate plasmid. The EYFPHis fragment was created by HindIII/EcoRI digestion of the pEYFP (Clontech, Palo Alto, CA, USA) derivate pEYFPHis. The MetLuc fragment was generated by HindIII/XbaI or HindIII/NotI restriction digest of the pMetLuc reporter plasmid purchased from Clontech. All designed plasmids were verified by control restriction digests and sequenced (data not shown).

Cell culture and transient transfection

The mouse C3H muscle myoblast precursor cell line C2C12 (#ACC565), purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) were cultured in Dulbecco's modified eagle's medium (DMEM) (Sigma Aldrich, Vienna, Austria) containing 4.5 g/L D-Glucose, supplemented with 2 mM L-glutamine (Sigma Aldrich, Vienna, Austria) and 5% fetal calf serum (FCS)

(Lonza Ltd, Basel, Switzerland) (21).

For the testing, cells were seeded into 24-well plates at a cell density of $0.5x10^5$/well 24-hours prior to transfection ($2.8x10^4/cm^2$). The next day, these cells were either transfected with 2 µg of the pCMVE/mOCP-EYFPHis reporter plasmid or with 2 µg of the unenhanced control reporter plasmid pmOCP-EYFPHis for fluorescence readout. Co-transfection with 1 µg of the plasmids pmOCP-MetLuc or pCMVE/mOCP-MetLuc with 1 µg of the internal control plasmid pCBR (Promega, Madison, WI, USA) was carried out for subsequent bioluminescence readout. Another co-transfection using 1 µg pMetLuc-Control (Clontech) and 1 µg of pCBR was carried out in order to generate the positive control samples required to calculate expression capacity relative to the control plasmids SV40 promoter. All transfections were carried out using Lipofectamine 2000 (Invitrogen, Lofer, Germany). The medium was changed 4 hours after transfection to remove the remaining DNA/liposome complexes.

Secreted osteocalcin reporter gene assay in 2D culture

A medium change to serum reduced DMEM (1% FCS) was carried out in all wells 24 hours after transfection. The fluorescent reporters pmOCP-EYFPHis and pCMVE/mOCP-EYFPHis were studied using 0 ng/ml and 500 ng/ml of rhBMP2. Further characterization was then carried out using the systems pmOCP-MetLuc and pCMVE/mOCP-MetLuc for bioluminescent quantification and comparison of produced signal intensities with or without CMV-enhancer. Direct comparison of mOCP and CMVE/mOCP activity was carried out using 0 ng/ml, 100 ng/ml and 300 ng/ml rhBMP2, followed by a detailed characterization of pCMVE/mOCP-MetLuc. For these experiments, the medium was supplemented with 50, 100 and 300 ng/ml of rhBMP2

or with the described growth factor controls FGF2 and VEGF-A or left unsupplemented as negative control. The cells were induced for 5 days with the described supplemented culture media before readout without medium change. A medium change was carried out in the pmOCP-MetLuc and pCMVE/mOCP-MetLuc transfected wells 24 hours before supernatant sampling (50 µl samples) in order to reduce background activity potentially present due to initial expression before specific induction.

Secreted osteocalcin reporter gene assay in 3D culture

C2C12 cells were transfected in 2D culture prior to incorporation into fibrin clots with the pCMVE/mOCP-MetLuc reporter plasmid (according to 2D culture transfection protocol). 24h after transfection, the cells were mixed into fibrin clots (Tisseel, Baxter) at cell density of 10^6 cells/clot (200 µl fibrin, ø 7.4mm, height 4mm) through resuspension of the cells into the thrombin (4IU/ml) component before mixing with the fibrinogen component (final concentration: 12,5 mg/ml fibrinogen). The fibrinogen component was supplemented with 1 µg InductOS rhBMP2 (final concentration in clot 5 µg/ml rhBMP2) in the osteoinduction group. No growth factor was added in the control group. The clots were then cultured in serum-reduced medium (1% FCS) for 6 days. A medium change was carried out 24h before sampling. 50 µl of supernatant surrounding the clot was then sampled 24h later for readout according to the 2D reporter gene assay protocol.

Fluorescence microscopy

The pmOCP-EYFPHis and pCMVE/mOCP-EYFPHis transfected wells were subjected to live fluorescence microscopy using a confocal laser scanning microscope (CLSM; Zeiss, Oberkochen, Germany) with a 488 nm laser for excitation and a 520 nm long pass filter for emission detection of the fluorescent reporter gene EYFP. A second channel using 488 nm excitation without emission filter was employed to generate phase contrast microscopy like light microscopy overlays to document the transfection efficacy and reporter plasmid activity relative to the total cell numbers that were photographed. The depicted images represent overlays of these channels.

Secreted Metridia Luciferase assay

50 µl of supernatant from the pmOCP-MetLuc, pCMVE/mOCP-MetLuc and pMetLuc-Control transfected wells covered with 50 µl 1x PBS were stored at -80°C until processing. *Metridia Luciferase* activity was assessed in the 50 µl supernatant samples after thawing using the Secreted Luciferase Assay Kit (Clontech) according to manufacturer's instruction in conjunction with the CCD imaging system (IVIS100 imaging station; Caliper Life Sciences GmbH, Russelsheim, Germany) in 96-well plates. The transfection efficiency internal control was carried out by quantifying pCBR mediated intracellular click beetle luciferase activity (CBRLuc) after thawing the frozen cell samples. CBRLuc activity was assessed using the Luciferase Assay Kit purchased from Promega (Madison, WI, USA) according to manufacturer's instruction.The obtained values were then used to normalize the observed MetLuc activity for the

individual transfection efficiency of each well occurring during serial transfections. After normalization, the relative light units of pmOCP and pCMVE/mOCP-MetLuc transfected samples were calculated relative to the averages obtained for the pMetLuc-Control positive control samples, representing normalized values promoter activation per 24 hours of the reporter construct relative to the constitutive SV40 promoter of the pMetLuc-Control vector according to the following equation (Figure 2).

Alkaline phosphatase (ALP) activity

Enzymatic alkaline phosphatase assays were employed to additionally assess osteogenic differentiation with an established standard method and to test the reporter systems interfere with differentiation. The cells were frozen at -80°C prior to measurement and then lysed with 100 µl per well of an alkaline ALP-assay buffer (pH 10.5) containing 0.25% Triton X-100 for 1 hour at room temperature. Enzymatic activity was quantified in cleared supernatants after centrifugation by providing 20 mM p-nitrophenylphosphate in 50 µl ALP-assay buffer as substrate. p-nitrophenylphosphate is converted to p-nitrophenol by ALP, which was quantified by its absorbance at 405 nm. The reaction was stopped 20 minutes after addition of the substrate with 50 µl of 0.2 M NaOH. The enzymatic activity was then expressed as nmoles of p-nitrophenol liberated per minute.

Von Kossa staining for mineralization

Von Kossa staining for mineralization was carried out after 14 days of differentiation (with 300 ng/ml rhBMP2) in order to investigate if the reporter signals overlap with centers of mineralization. The cells were washed three times with 1x PBS without calcium and magnesium (Cambrex East Rutherford, NJ, USA). The cells were fixed with a 4% aqueous solution of formaldehyde and stained with a 5% (w/w) silvernitrate solution for 30 min at room temperature followed by three times washing with ddH_2O. Development of the staining was carried out with 5% (w/w) Na_2CO_3 in a 25% aqueous solution of formaldehyde followed by three times washing with ddH_2O. Final fixation was carried out in a 5% (w/w) $Na_2S_2O_3$ solution in ddH_2O for 2 min. After washing the cells three times with ddH_2O, the staining was observed by phase contrast microscopy.

Osteocalcin mRNA expression

Total RNA was isolated from pCMVE/mOCP-MetLuc transfected cells after treatment with 0 ng/ml, 100 ng/ml and 300 ng/ml of rhBMP2 using peqGOLD TriFast™ (PeqLab Biotechnologie GmBH, Erlangen, Germany) according to manufacturer's instruction (23). 2 µg of total RNA was transcribed to cDNA using an Oligo dT18 primer with an AMV-reverse transcriptase (Finnzymes, Espoo, Finland) according to the manufacturer's instructions.

Quantitative real time PCR for the osteocalcin transcript was carried out with 40 ng of total cDNA per reaction using the primers qOC2s (GAACAGACAAGTCCCACACAG) and qOC2as (CTGCTTGGACATGAAGGCTTTG) for osteocalcin and qHPRTs (AGTCCCAGCGTCGTGATTAG) and qHPRTas (TGGCCTCCCATCTCCTTCAT) for the hypoxanthine guanine phosphoribosyl transferase (HPRT) as standard gene using the KAPA SYBR Fast qPCR kit (PeqLab Biotechnologie GmBH, Erlangen, Germany)

and a Biorad CFX96 real-time PCR cycler. Expression was calculated with relative quantification by the comparative CT-method (24).

Statistical Analysis

The average ± SEM were calculated for all variables tested. Statistical analysis of 2D cell culture data was performed by one-way ANOVA and statistical significance was accepted at $p<0.05$. Statistical analysis of 3D cell culture bioluminescence data was performed by Mann-Whitney test. Statistical analysis of ALP-assays, qPCR and the bioluminescent data of the direct comparison of pmOCP-MetLuc and pCMVE/mOCP-MetLuc was carried out using Dunn's multiple comparison test.

Results

Fluorescent osteocalcin reporter gene assay

The pmOCP-EYFPHis reporter plasmid, as expected, exhibited only weak signal intensity upon osteogenic differentiation (Figure 3, A) and no detectable background signal in the negative control (Figure 3, B). In contrast, strong EYFP fluorescence was detectable in C2C12 cells upon osteogenic differentiation 6 days after transient transfection with the enhanced reporter pCMVE/mOCP-EYFPHis (Figure 3, C). Only weak background signals were observable in the negative myogenic differentiation control incubated with DMEM + 1% FCS after 6 days (Figure 3, D).

Secreted osteocalcin reporter gene assay in 2D culture

The pCMVE/mOCP-MetLuc reporter system exhibited a strong and concentration dependent bioluminescent response to induction with increasing amounts of rhBMP2, whereas no activation above negative control background levels was observable upon treatment with non-osteoinductive growth factors such as FGF2 and VEGF-A (Figure 4, A).

Additionally, a strong correlation was observed between the applied amount of osteoinductive growth factor rhBMP2 and the induced expression of *Metridia* Luciferase as quantified by bioluminescence assays (Figure 4, B).

Furthermore, the mOCP-MetLuc transfected cells also showed a rhBMP2 dose dependent induction of bioluminescence signal in the direct comparison with the enhanced system (Figure 5), although at approx. 250-fold lower levels than the CMVE-

enhanced version. Significant signal induction was only detectable in the 300 ng/ml treated group for pmOCP-MetLuc.

Secreted osteocalcin reporter gene assay in 3D culture

The CMVE/mOCP-MetLuc system exhibited a significant signal increase in the rhBMP2 treated group in 3D after 6 days of differentiation (Figure 6) compared to negative control clots without rhBMP2 induced osteogenic differentiation.

Alkaline phosphatase (ALP) activity

As shown in Figure 7A, the levels of ALP increased with the addition of different amounts (50, 100, 300 ng) of rhBMP2. It showed a significant elevation of the p-nitrophenol signal in the group of 300 ng rhBMP2. There was no induction of ALP activity observable in the FGF2 and VEGF-A groups. Furthermore, no difference in ALP induction was observable between untransfected, pmOCP-MetLuc transfected and pCMVE/mOCP-MetLuc transfected cells as shown in Figure 7B.

Von Kossa staining for mineralization

Positive induction of mineralization at cellular condensations was observable in the rhBMP2 treated samples (Figure 8, B, C), whereas no mineralization was found in the 0 ng/ml rhBMP2 control group (Figure 8, D). The observed *von Kossa* positive centers

of mineralization (Figure 8, B) in cellular condensations overlap with the EYFP-positive cell clusters (Figure 8, A) in the rhBMP2-induced pCMVE/mOCP-EYFPHis reporter system..

Osteocalcin mRNA expression

Quantitative real-time PCR for endogenous osteocalcin mRNA expression in pCMVE/mOCP-MetLuc transfected cells (Figure 9) showed a dose dependent increase of the osteocalcin transcript and significant induction of osteocalcin expression in the 300 ng/ml rhBMP2 treated samples.

Discussion

The data obtained in this study strongly suggest that a novel artificial *cis*-acting sequence for osteoblast specific expression has been created by adding a viral enhancer element to a specific minimal murine osteocalcin promoter element. Signal amplification (approx. up to 250-fold compared to the unenhanced control) of the osteocalcin coupled osteoblast specific reporter gene expression was achieved through the addition of the CMVE to the proximal promoter without substantially impairing the specificity of the mOCP element and with no detectable impact on osteogenic differentiation of transfected cells. Furthermore, we were able to demonstrate the practicability of a secreted bioluminescent reporter gene *Metridia* luciferase, which allowed detection of osteogenic differentiation through supernatant sampling in 2D and 3D cultures. This allows true real-time monitoring of osteogenic differentiation, significantly reducing sample numbers.

Fluorescent reporter gene assay

The strong fluorescent signals observed after 6 and 14 days of culturing of pCMVE/mOCP-EYFPHis reporter transfected C2C12 cells in osteogenic medium (Figure 3C, Figure 8A) were specifically induced in cellular condensations. These clusters of transdifferentiated C2C12 cells, were comparable to mesenchymal condensations that form during ossification in embryonic development (25), and have been confirmed to actually mineralize by von Kossa staining (Figure 8B). Furthermore, alkaline phosphatase assays proved that osteogenic differentiation was induced with recombinant rhBMP2 (Figure 7). Regarding the overlapping activity of the reporter

gene assay with these observations we conclude that the system is specifically activated upon osteogenic differentiation. The background activity in myoblastic differentiated C2C12 cells, which is independent of osteoblastic differentiation could be attributed to the employed CMV-enhancer element, which is capable of binding multiple general transcriptional activators present in CMV permissive cells (16). Since the observed results suggest that the background expression in the developed assay reached only very low levels and expression was specifically enriched in cellular clusters undergoing differentiation dependent on the applied amount of osteoinductive growth factor, we conclude that the addition of the CMV-enhancer does not impair specificity of the system in this specific combination and cellular context.

Interestingly, sequence analysis (data not shown) showed that the CMVE contains also consensus sequences potentially bound by transcription factors associated with osteogenic differentiation, such as C/EBP and AP1 (26). Therefore, it can be assumed for the main responsible fact for signal amplification of the mOCP, that the enhancement is not exclusively mediated by general expression activation through the CMVE without contribution of osteogenic differentiation pathway dependent signalling.

Secreted osteocalcin reporter gene assays

Metridia luciferase, a secreted luciferase, was employed to modify the initially designed fluorescent reporter plasmid for bioluminescence readout to enable supernatant-based quantification of the induced reporter gene signals after osteogenic differentiation and therefore, to provide a system which will be suitable for application in large-scale screening assays as well as for bioreactor construct monitoring.

This system was tested not only with different concentrations of the osteoinductive growth factor rhBMP2 to calculate the correlation of its activity to the amount of rhBMP2 (Figure 4B) but also with two unrelated growth factors (not osteoinductive in C2C12 cells) in 2D culture (Figure 4A). The significant increase in signal intensity with increasing concentrations of the osteoinductive growth factor rhBMP2 confirmed that the designed system is specifically activated upon the induction of endogenous osteocalcin expression since a raise in endogenous osteocalcin levels was detected in C2C12 cells by qPCR (Figure 9) in accordance with the literature (21). This increase in endogenous osteocalcin expression was in parallel to the observed increase in reporter system activity.

The concentration dependent increase of reporter gene expression or bioluminescence respectively demonstrates the feasibility of this system to be used in bioactivity screening of produced recombinant growth factors and small compounds mediating osteoinduction. Using the CMV-enhanced system, it was possible to detect osteocalcin expression activating amounts of rhBMP2 down to concentrations of 2 nM with a strong linear correlation observed within a range of 2-10 nM (Figure 4B). The systems practical implementation for growth factor bioactivity assays for release kinetics from novel biomaterials has already been demonstrated (29).

Although other reporter gene approaches such as the BRE-Luc system have been developed (27) that are far more sensitive for BMP growth factor testing (from pM up to nM concentration) than the devised method, it is the first approach that employs

highly sensitive non-invasive osteocalcin expression monitoring. Therefore, in contrast to the above described methods that are very suitable for growth factor bioassays, this system allows to monitor bioactivity and osteoinductivity of many different osteoinductive substances not limited to a growth factor family since osteocalcin is the downstream target of Runx2, the osteogenic master regulatory transcription factor, where many osteoinductive signalling pathways converge (28). Through this interconnection our assay is not limited to a certain growth factor family. Its activation is differentiation specific and not signalling cascade specific. .

The MetLuc modified system was also tested in 3D culture with C2C12 cells embedded into a hydrogel matrix in order to assess the suitability of MetLuc for real-time bioreactor monitoring. MetLuc was discharged into the surrounding medium from the tissue-like constructs and osteoinduction could be detected. Specific activation of the system in 3D culture was demonstrated by the significant increase in MetLuc activity (Figure 6) in the supernatant of rhBMP2 treated clots compared to the negative controls. Therefore, this novel reporter gene is ideal for the proposed purpose, because it is not limited by the employed scaffolding system of the tissue-like constructs and easily diffusing into the surrounding medium, where it can be assessed without manipulating or destroying the construct.

Impact on osteogenic differentiation

Since the developed systems exploit the activation of the osteocalcin promoter for signal generation, a negative impact of the transfected heterologous osteocalcin promoter on endogenous osteocalcin expression could occur that in turn can impair differentiation of reporter-transfected cells. ALP-assays showed that there was no significant alteration of ALP-activity associated with reporter transfection (Figure 7, B). Osteocalcin qPCR in pCMVE/mOCP-MetLuc transfected cells has shown potent (100 fold) induction of osteocalcin expression through application of 300 ng/ml rhBMP2 (Figure 9), suggesting no direct impact of the system on endogenous osteocalcin expression in C2C12 cells. Von Kossa staining furthermore showed effective mineralization after 14 days of differentiation of pCMVE/mOCP-EYFPHis transfected cells (Figure 8B), proving their ability to retain the mineralizing phenotype after reporter transfection. Therefore, we conclude that the pCMVE/mOCP reporter systems do not impair osteogenic differentiation as defined by the observed parameters,

Signal amplification and specificity

The cytomegaloviral enhancer has been used to amplify expression from several tissue specific promoters with fair results concerning the maintenance of tissue specificity. So far this has been successfully demonstrated with neuronal (17), cardiac and lung tissue (19) specific constructs but not for bone tissue specific signal amplification. This work represents another CMV-enhanced version of a tissue specific promoter suitable for restricted expression to be added to this collection of chimeric promoter elements that retain their specificity.

Nevertheless, there is general consensus that there is always the risk of impairing or even abolishing the tissue specificity of the employed tissue specific promoter by using

this strategy for signal amplification (19) depending on the employed promoter and the cellular context (CMV permissive or non-permissive cell type, active signal transduction cascades, etc). Therefore, the tissue specificity of novel hybrid constructs that contain a CMVE has to be assessed carefully in order to reveal and estimate potential background activity limiting the future application of the designed system. Concerning the bone specific hybrid CMVE and osteocalcin promoter system it is very unlikely that there might be unspecific activation by other growth factors, since there was no activation of the system above background levels observable in the unrelated growth factor controls FGF2 and VEGF-A. Therefore, it can be assumed that there is no substantial reduction of tissue specificity occurring within our system regarding potential activation of the CMVE by other growth factor signal transduction cascades.

Future work will include the generation of a CMVE/mOCP-MetLuc and CMVE/mOCP-EYFPHis stable cell line to further enhance the practicability of the system (elimination of the transfection step; all cells in the assay are responsive cells) and potentially its sensitivity (through multi-copy insertion of the reporter cassette) as well as the application of the MetLuc version in mesenchymal stem cells in a bioreactor for bone tissue engineering.

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Author Disclosure Statement

No competing financial interests exist.

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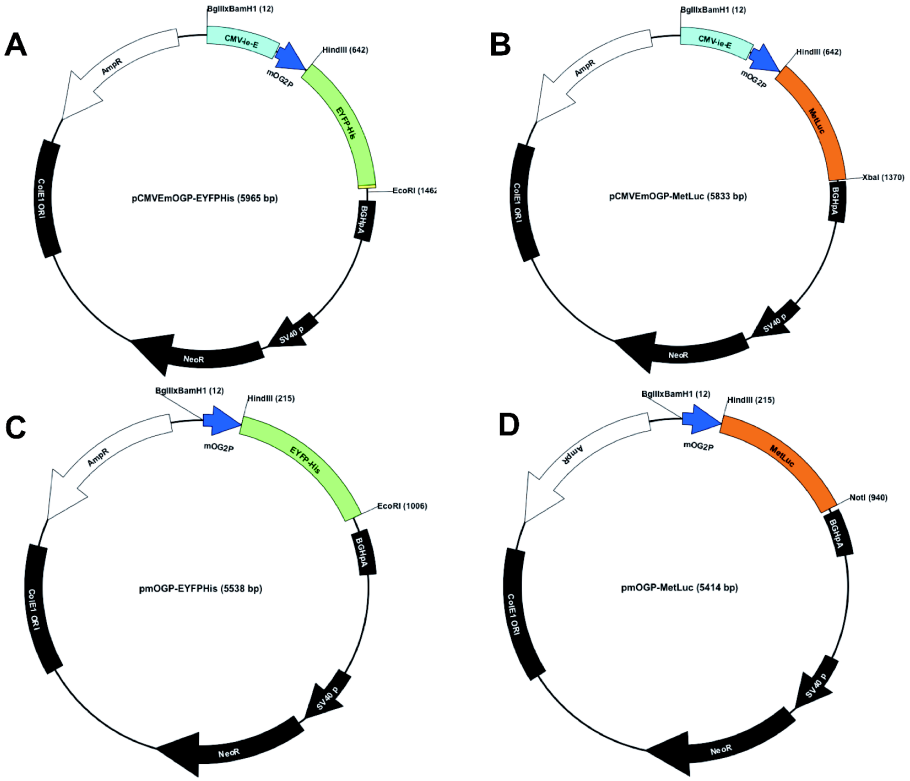

Figure 1: pCMVE/mOCP-EYFPHis fluorescent reporter plasmid (A) and pCMVE/mOCP-MetLuc bioluminescent reporter plasmid (B). pmOCP-EYFPHis unamplified fluorescent reporter plasmid (C) and pmOCP-MetLuc unamplified bioluminescent reporter plasmid (D). The Cytomegalovirus immediate early enhancer (CMVE) is depicted in light blue, the minimal osteocalcin2 promoter in dark blue. The enhanced yellow fluorescent reporter gene (EYFPHis) is shown in green, the *Metridia* luciferase (MetLuc) reporter gene in orange.

$$\text{Relative reporter activation to constitutive SV40-promoter (pMetLuc-Control)} = \frac{\dfrac{\text{photons/sec/cm}^2\text{ (24hrs sample reporter)}}{\text{photons/sec/cm}^2\text{ (pCBR-Control)}}}{\dfrac{\text{photons/sec/cm}^2\text{ (24hrs sample pMetLuc-Control)}}{\text{photons/sec/cm}^2\text{ (pCBR-Control)}}}$$

Figure 2: Equation used to calculate the relative activation of the reporter systems using the bioluminescence values of 2 different luciferase enzymes detected through the CCD-imaging system. Individual *Metridia* luciferase activity of each well is set relative to the internal transfection control Click beetle luciferase (CBRLuc) to normalize for varying transfection efficacies in serial transfection. The

relative values were then calculated relative to a constitutive *Metridia* positive control, representing normalized values of relative activation of the differentiation specific sequence to the strong constitutive SV40 promoter of the pMetLuc-Control vector transfected cells.

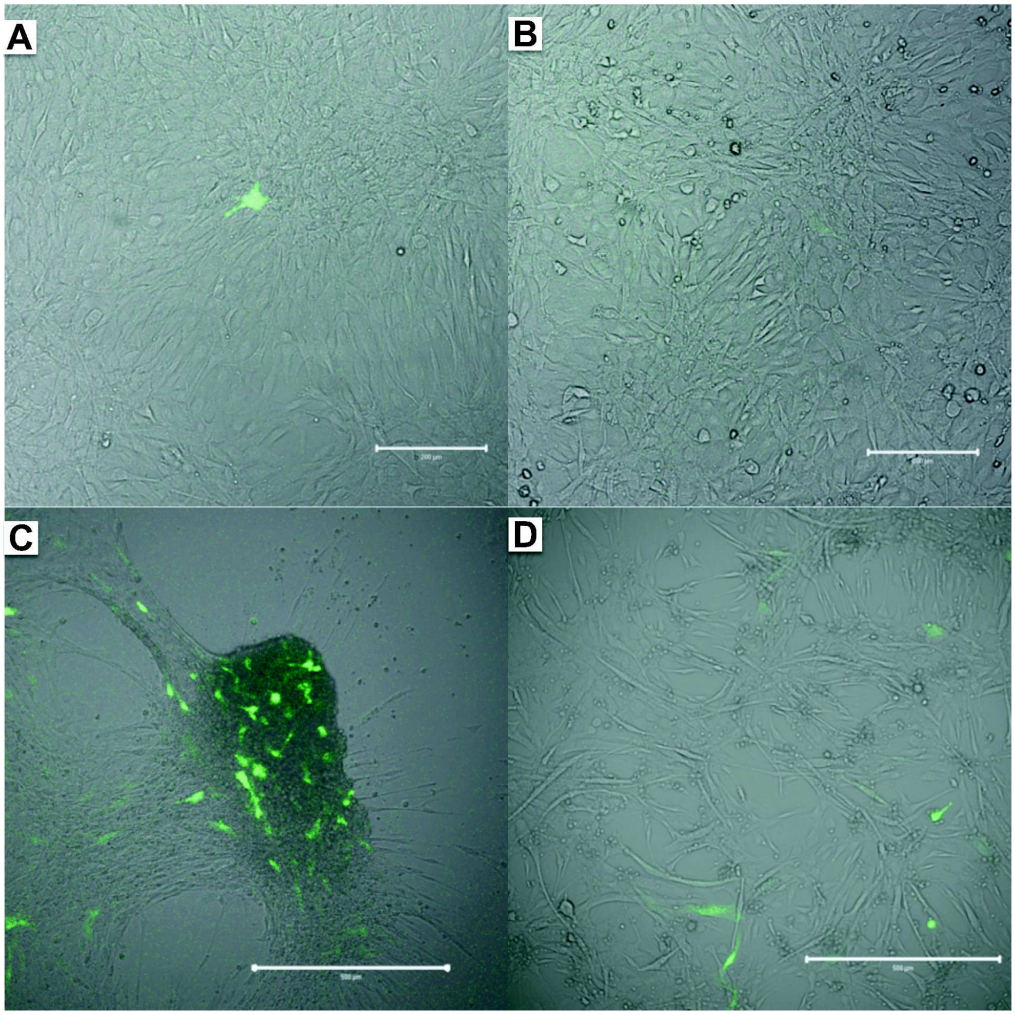

Figure 3: (A) pmOCP-EYFPHis activation in C2C12 cells cultured in DMEM + 1% FCS, 200 mM L-glutamine and 500 ng/ml recombinant human bone morphogenetic protein 2. Scale bars represent 200µm. (B) pmOCP-EYFPHis activation in C2C12 cells cultured in Dulbecco's Modified Eagles Medium (DMEM) + 1% fetal calf serum (FCS) and 200 mM L-glutamine as negative control for 6 days.
(C) pCMVE/mOCP-EYFPHis activation in C2C12 cells undergoing osteogenic differentiation. The cells were cultured in Dulbecco's Modified Eagles Medium + 1% fetal calf serum and 200 mM L-glutamine for 6 days. Osteogenic transdifferentiation of C2C12 cells was induced with 500 ng/ml of recombinant human bone morphogenetic protein 2. The systems specific and strong activation upon osteogenic differentiation and osteocalcin promoter activation is observable by the green fluorescent signal emitted by the reporter gene enhanced yellow fluorescent protein (EYFPHis) at 520 nm. (D) pCMVE/mOCP-

EYFPHis activation in C2C12 cells cultured in Dulbecco's Modified Eagles Medium + 1% fetal calf serum and 200 mM L-glutamine as negative control for 6 days. Successful myogenic differentiation is observable by the multinuclear myotube phenotype. The systems unspecific background activity levels are observable by the green fluorescence of the enhanced yellow fluorescent protein (EYFPHis) emission at 520 nm. Scale bars represent 500 µm.

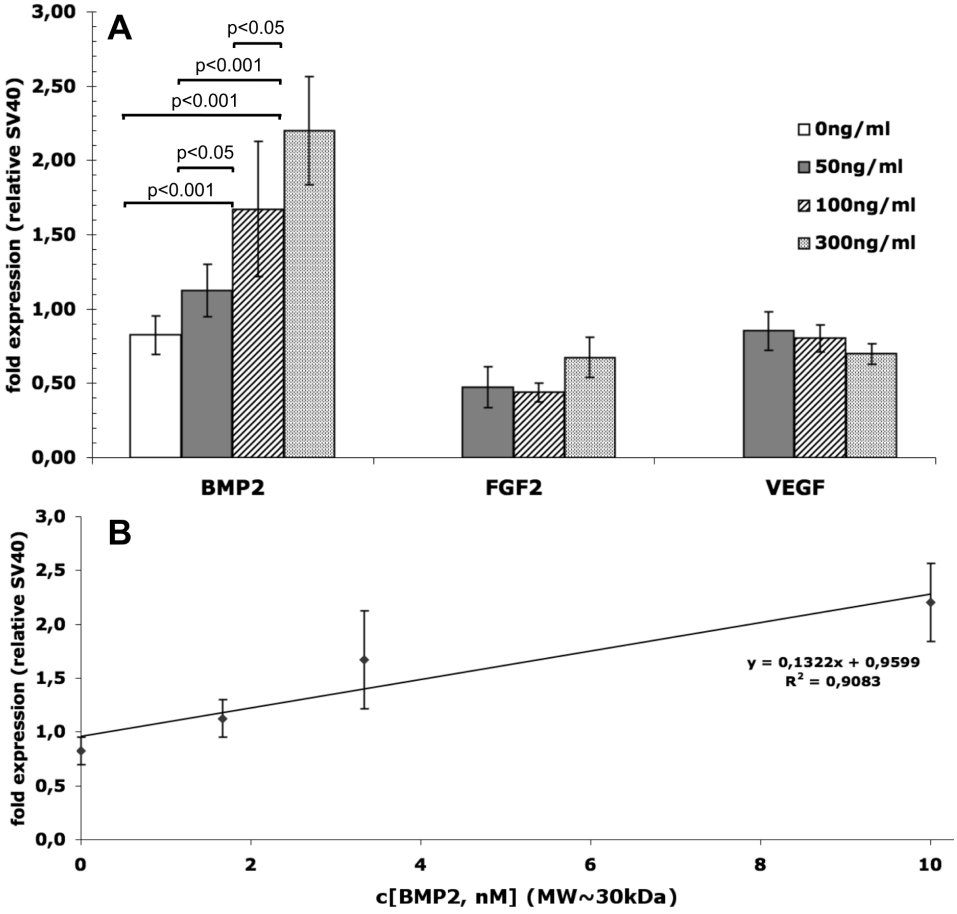

Figure 4: (A) Activation of the pCMVE/mOCP-MetLuc enhanced bioluminescent osteocalcin reporter system upon induction with different growth factors in 2D culture. A significant induction of *Metridia* Luciferase expression was observable in cells treated with osteoinductive recombinant human bone morphogenetic protein 2 (BMP-2). No activation was observable in samples treated with the growth factor controls human basic fibroblast growth factor 2 (FGF-2) or human vascular endothelial growth factor A (VEGF-A). n = 6, values represent average ± standard deviation.

(B) Correlation between the applied nanomolar (nM) concentration of recombinant human bone morphogenetic protein 2 (BMP-2) and the activation of the pCMVE/mOCP-MetLuc enhanced bioluminescent reporter system assuming an approximate molecular weight of 30 kDA for BMP-2. n=6, values represent average ± standard deviation.

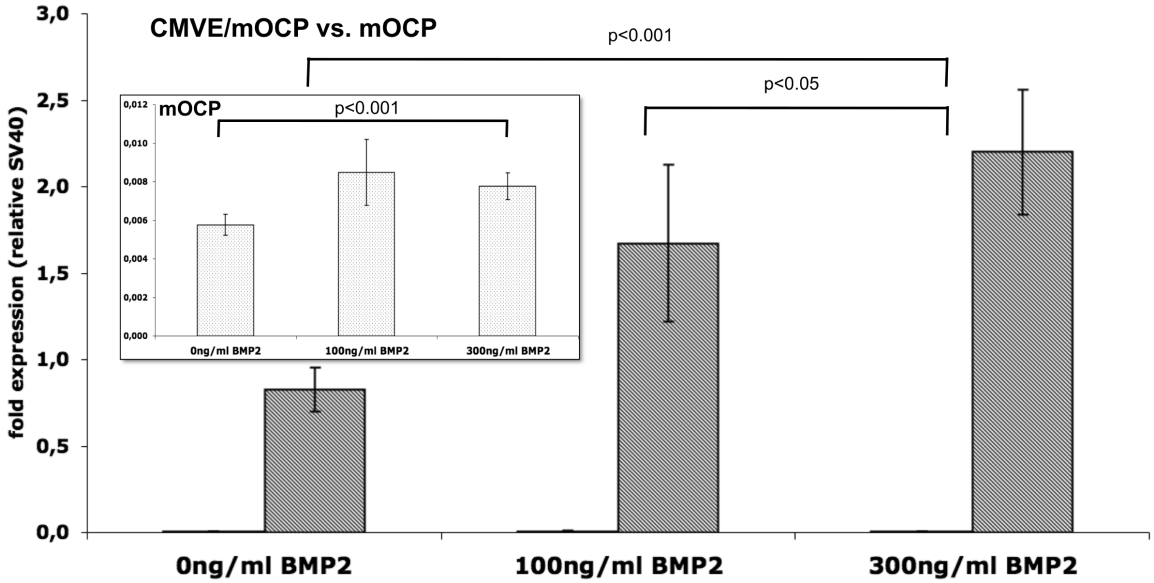

Figure 5: Direct comparison of bioluminescence readouts for the enhanced (CMVE/mOCP) and the unenhanced (mOCP) reporter systems in response to 0ng/ml, 100ng/ml and 300ng/ml rhBMP2. Readouts are depicted as relative expression levels (fold expression) to the constitutive SV40 promoter. mOCP expression levels additionally provided in separate box due to scaling. CMVE/mOCP expression levels are approximately 250-fold higher than mOCP expression levels. n=6, values represent average ± standard deviation.

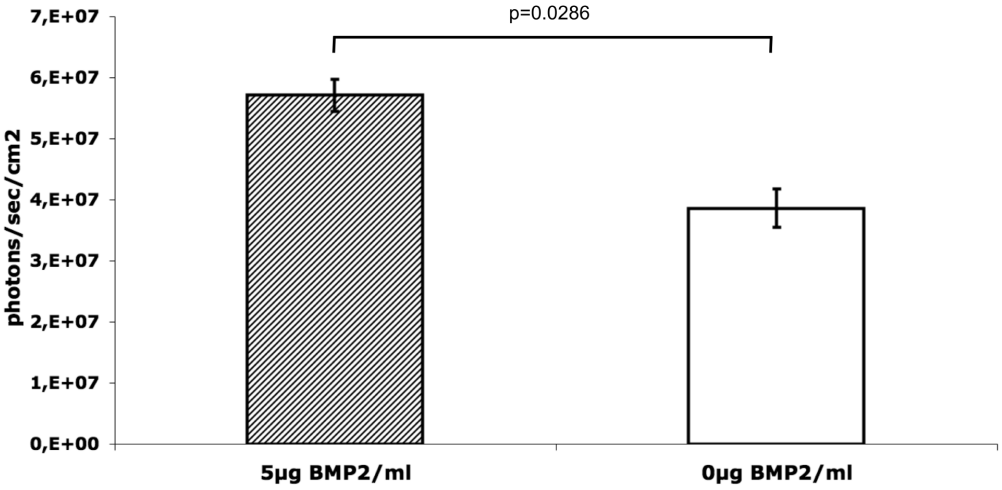

Figure 6: Activity of the CMVE/mOCP-MetLuc reporter system in fibrin clots represented as photons/sec/cm^2 luciferase activity detected in the supernatants. Osteogenic differentiation (left), negative control (right). n = 4, values represent average ± standard deviation.

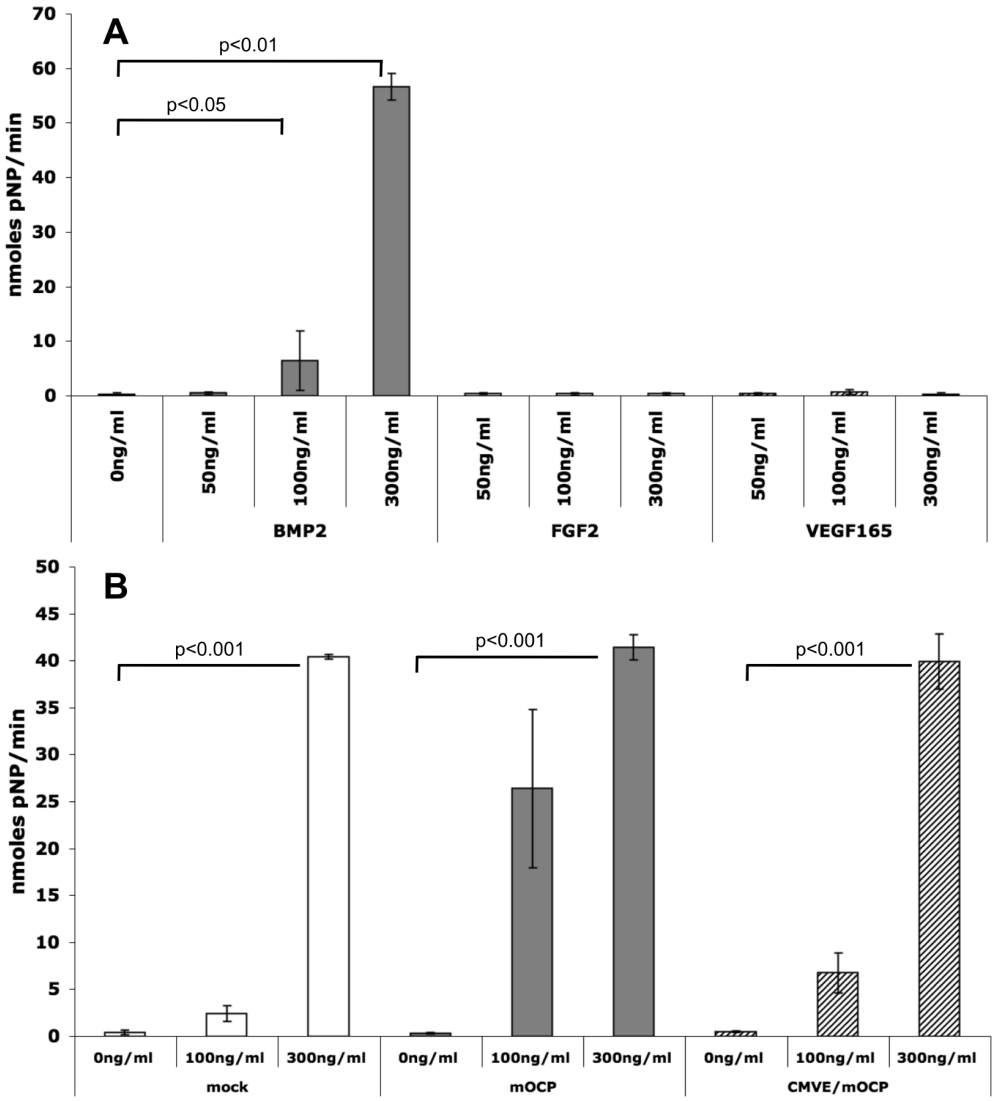

Figure 7: Enzymatic alkaline phosphatase (ALP) assay results in 2D culture.

(A) Liberation of p-Nitrophenol per minute in samples treated with different amounts of recombinant human bone morphogenetic protein 2 (BMP-2) and control growth factors. n = 6, values represent average ± standard deviation. (B) Comparison of ALP-activities of untransfected (mock), pmOCP-MetLuc transfected (mOCP) and pCMVE/mOCP-MetLuc transfected cells (CMVE/mOCP). n = 4, values represent average ± standard deviation.

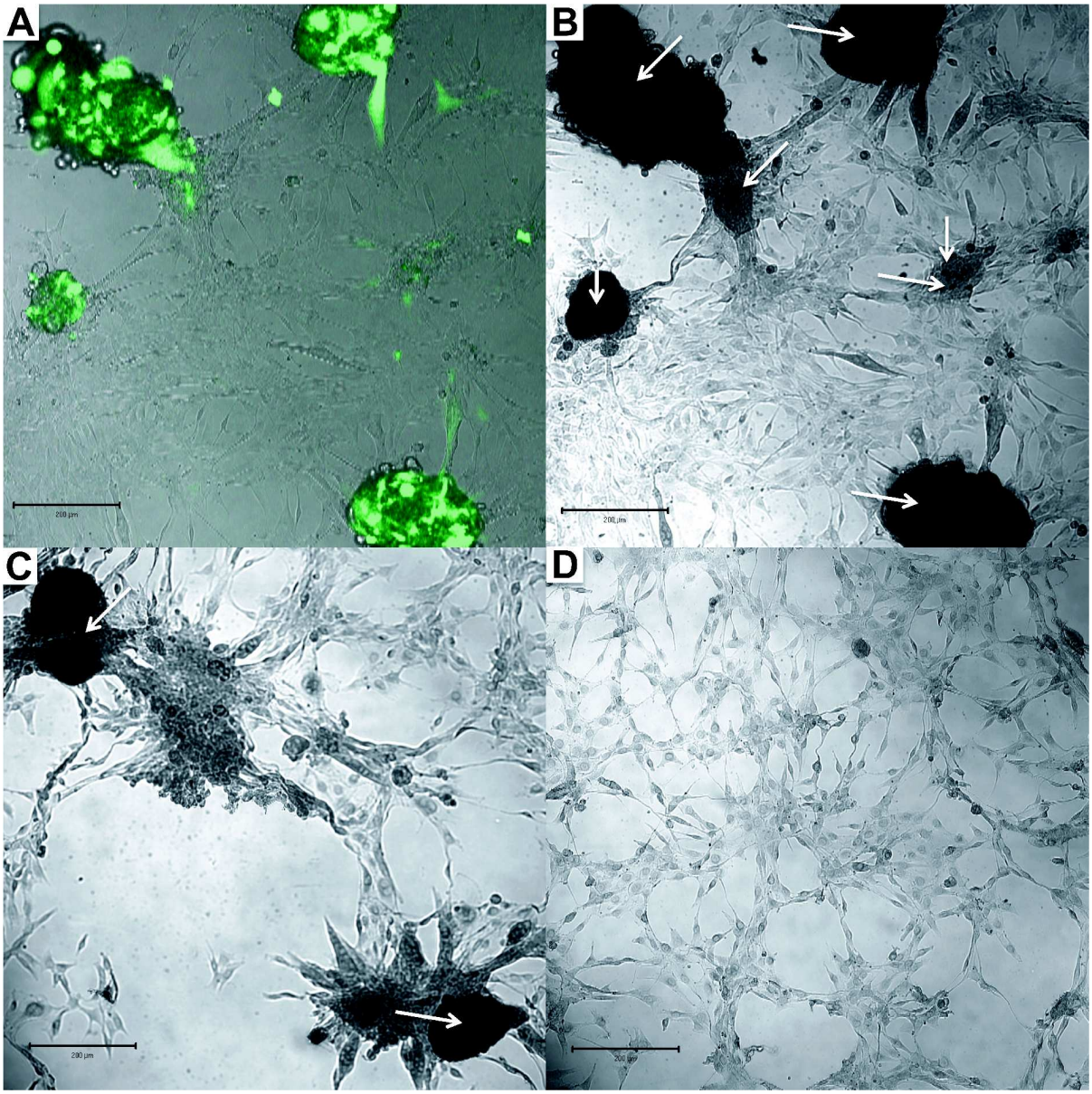

Figure 8: (A) pCMVE/mOCP-EYFPHis fluorescence signal after 14 days of differentiation with 300ng/ml rhBMP2. (B) Same field of view after *von Kossa* staining for mineralization, centers of mineralization (arrows) overlap with fluorescent signals in A. (C) Positive control after 14 days of differentiation with 300ng/ml rhBMP2 stained with *von Kossa*. (D) Negative control *von Kossa* staining after 14 days. Scale bars represent 200µm.

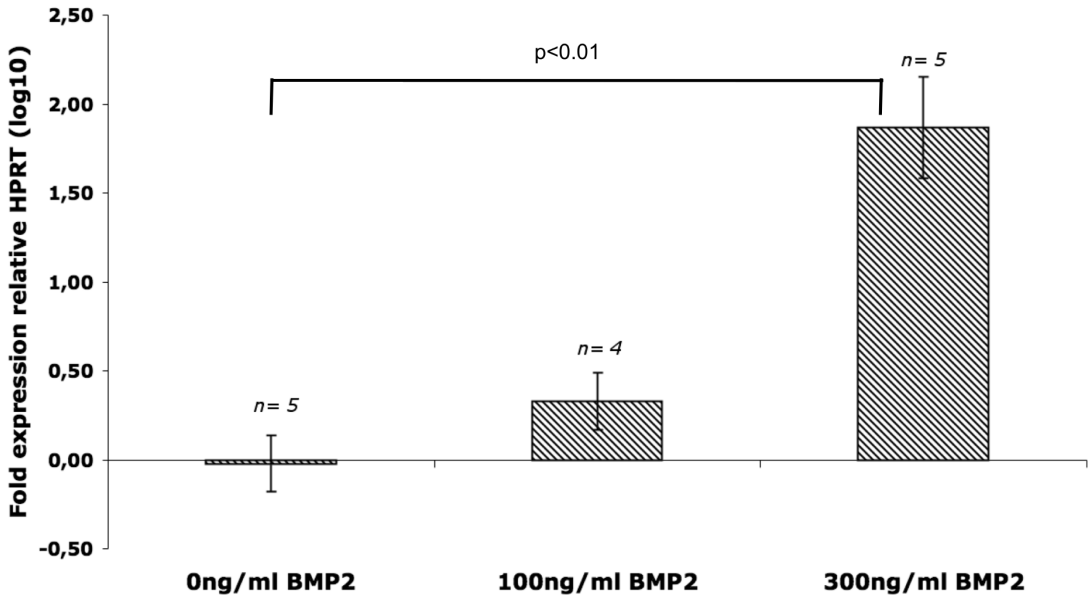

Figure 9: Osteocalcin expression levels in pCMVE/mOCP transfected cells (6 days of differentiation) as determined by quantitative real-time PCR (relative quantification with comparative Ct-method) in response to 0ng/ml, 100ng/ml and 300ng/ml rhBMP2. Readouts are depicted as fold changes of osteocalcin normalized to HPRT gene expression in logarithmic scaling. Values represent average ± standard deviation. n = 5 for 0ng/ml, n = 4 for 100ng/ml and n = 5 for 300ng/ml of applied rhBMP2.