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„Non-viral Transient Gene Therapy for Bone Regeneration“

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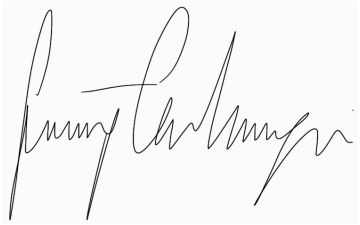
Erklärung

Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

Des Weiteren möchte ich darauf hinweisen, dass von mir in meiner Diplomarbeit (http://othes.univie.ac.at/744/, AC# AC07147280) wichtige Vorarbeiten zu dem in dieser Arbeit vorgestellten Thema geleistet wurden und aus diesem Grund die Daten zur Kinetik des TetON Systems (Kapitel II, Figure 4 (Seite 118), Figure 5 (Seite 119)) sowie das Transfektions-Screening für therapeutische Gene mittels ALP-assay (Kapitel II, Figure 2 (Seite 116)) aus meiner Diplomarbeit auch in dieser Arbeit verwendet wurden.



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Contents

Abstract

Critical size bone defects, caused by severe tissue trauma or resection surgery represent a challenging clinical problem in trauma surgery/orthopaedics. These defects can lead to non-union fractures, which fail to regenerate and are a substantial burden on patients and health-care systems worldwide. Alternative treatment strategies to conventional fracture management are bone grafting from autologous sources or the application of high doses of recombinant bone morphogenetic protein (BMP)growth factors. While grafting is associated with donor site morbidity, BMPs can lead to adverse effects by supraphysiologic dosing. Therefore, novel treatment modalities are required for the minimally invasive, cost-effective and safe treatment of bone defects, preferably omitting the use of stem cells, biomaterials and recombinant growth factors. Non-viral transient somatic gene therapy offers methods for the *in situ* manipulation of endogenous cells at the defect site for growth factor expression at relatively low cost and potential high performance, due to endogenous production and release of an active morphogen. It was the aim of this study to design and evaluate a non-viral *in vivo* gene therapy protocol for osteoinduction.

A highly osteoinductive combination of bone morphogenetic protein 2 and 7 therapeutic genes was identified in *in vitro* experiments, supported by the use of a novel enhanced reporter gene assay for osteogenic differentiation. BMP2/7 was subsequently employed in the design of novel constitutive and inducible therapeutic co-expression plasmids. Multi-cassette based BMP2/7 co-expression from such constitutive or inducible plasmids was characterized and compared to recombinant growth factor induced osteogenic differentiation *in vitro.* After successful *in vitro* testing, the novel co-expression plasmids were tested for *in vivo* osteoinduction using

passive gene delivery or active sonoporative gene transfer in ectopic and orthotopic *in vivo* rodent models.

In vitro evaluation of constitutive multi-cassette BMP2/7 co-expression plasmids revealed impaired induction of osteogenic differentiation compared to combined delivery of individual BMP2 and 7 plasmids due to transcriptional interference occurring in the single-plasmid co-expression system. This transcriptional interference related impairment of co-expression associated bioactivity was related to relative transcription cassette orientation, causing stronger interference in tandem expression cassette orientation. Inducible co-expression of BMP2/7 from a single-vector TetON inducible expression plasmid, showed potent osteoinduction and tight control over transgene expression *in vitro. In vivo* gene delivery studies in ectopic and orthotopic models showed that sonoporation could enhance non-viral gene delivery compared to passive gene transfer, increasing the overall rate of successful gene delivery and osteoinduction up to 100%. Localized ectopic and orthotopic gene expression was confirmed by bioluminescence imaging and immunohistochemistry. BMP2/7 *in vivo* gene delivery by constitutive or inducible BMP2/7 co-expression systems demonstrated potent osteoinduction *in vivo* after repeated delivery with either passive or sonoporative gene transfer. *In vivo* gene delivery using the inducible system again demonstrated tight regulation of osteogenesis via the inductor doxycycline. Orthotopic testing (0% union rate) indicated an enhancing effect of non-viral BMP2/7 gene co-delivery in critical sized femur fracture models, leading to a union rate of 33% in the treatment group.

We conclude from our *in vitro* and *in vivo* findings that BMP2/7 is a potent osteoinductive therapeutic gene combination, which can be delivered in constitutive or inducible multi-cassette co-expression plasmids for osteoinduction using passive or sonoporative gene transfer *in vivo*. Sonoporation has been shown to enhance

overall gene transfer probability. Tight control of *in vivo* transgene expression has been demonstrated using inducible systems. Therefore, the novel treatment approach of sonoporative BMP2/7 co-expression *in vivo* can be considered a promising alternative to recombinant growth factor application in tissue regeneration and could provide a potential novel therapy for clinical translation after further improvement. This includes establishing higher gene transfer efficacy & reducing the number of repetitive treatments and a reduction of DNA dosing.

Zusammenfassung

Knochendefekte kritischer Größe werden durch massives Trauma oder chirurgische Resektion verursacht und stellen ein signifikantes klinisches Problem dar. Diese Defekte können sich zu Pseudoarthrosen entwickeln und sind weltweit eine Belastung für Patienten und Gesundheitsysteme. Transplantation von autologem Knochen oder Verabreichung von hohen supraphysiologischen Mengen rekombinanter Wachstumsfaktoren sind derzeit die einzigen Behandlungsalternativen mit erheblichen Nebenwirkungen für den Patienten. Aus diesem Grund werden neue, minimal-invasive, kosteneffektive und sichere Behandlungsmethoden erforderlich, welche nicht auf der Verwendung von autologem Gewebe, autologen Stammzellen, Biomaterialien oder rekombinanten Wachstumsfaktoren aufbauen. In dieser Studie wurde nicht-virale transiente somatische Gentherapie, untersucht. die *in situ* Manipulation von endogenen Zellen zu Überexpression von Wachstumsfaktoren im Defekt ermöglicht. Dies kann zu relativ geringen Kosten und mit potentieller hoher therapeutischer Effizienz erfolgen, da im Defektareal eine kontinuierliche Freisetzung eines endogen produzierten hochaktiven Wachstumsfaktor erreicht wird .

Eine hoch-bioaktive Genkombination von bone morphogenetic protein 2 und 7 (BMP2/7) wurde in *in vitro* Experimenten mit Unterstützung eines neu entwickelten Reportergen Systems identifiziert und anschließend für die Entwicklung von neuen konstitutiven und induzierbaren Co-Expressionsplasmiden eingesetzt. Die Co-Expression von BMP2/7 von diesen konstitutiven und induzierbaren Multi-Kassetten wurde *in vitro* auf ihr osteogenes Potenzial hin untersucht und mit rekombinanten Wachstumsfaktoren verglichen. Nach erfolgreichen *in vitro* Experimenten wurden diese Plasmide in ektopen und orthotopen *in vivo* Modellen mit passivem oder Ultraschall-basiertem Gentransfer (Sonoporation) in Nagetieren getestet.

In vitro Experimente zeigten, dass die konstitutiven Multi-Kassetten BMP2/7 Co-Expressionsysteme ein reduziertes osteogenes Potenzial im Vergleich zur Co-Transfektion von BMP2 und BMP7 Plasmiden aufwiesen, welches auf transkriptionale Interferenz zurückgeführt werden kann. Diese transkriptionale Interferenz war von der Orientierung der Expressionskassetten abhängig und trat bei Tandem Orientierung der Expressionskassetten vermehrt auf. Induzierbare Co-Expression von BMP2/7 und osteogene Differenzierung *in vitro* mittels eines modifizierten TetON-induzierbaren Expressionsplasmides konnte gezielt über einen Induktor (Doxyzyklin) gesteuert werden. *In vivo* nicht-viraler Gentransfer in ektopen und orthotopen Modellen zeigte, dass Sonoporation die Wahrscheinlichkeit eines erfolgreichen Gentransfers im Vergleich zu passivem Gentransfer bis auf 100% steigern kann. Lokalisierte ektope und orthotope *in vivo* Genexpression konnte mit Biolumineszenz-Bildgebung und Immunfärbungen nachgewiesen werden. BMP2/7 *in vivo* Gentransfer mittels konstitutiver oder induzierbarer BMP2/7 Co-Expressionssystemen führte zu potenter Osteoinduktion nach wiederholtem Gentransfer. *In vivo* Verabreichung des induzierbaren Systems, ermöglichte spezifische Kontrolle der therapeutischen Genexpression über den Induktor Doxyzyklin. Orthotope Evaluierung der BMP2/7 Gentransfer-Strategie deutet auf eine verbesserte Knochenheilung (33% Heilung in der Therapiegruppe) in Femurdefekten kritischer Größe hin.

Aus den *in vitro* und *in vivo* Ergebnissen schlussfolgernd, wird festgestellt, dass BMP2/7 Co-Expression eine potente osteoinduktive therpeutische Strategie ist, welche über konstitutive oder induzierbare Co-Expressionsplasmide mit passivem oder aktivem Gentransfer verabreicht werden kann. Sonoporation führt zu einer Zunahme der Frequenz erfolgreichen Gentransfers und genaue Kontrolle der Transgen Co-Expression kann durch induzierbare Systeme *in vivo* gewährleistet

werden. Aufrgrund der Ergebnisse kann die Behandlungsstrategie der BMP2/7 *in vivo* Sonoporation als vielversprechende Alternative zu rekombinanten Wachstumsfaktoren in der Geweberegeneration angesehen werden. Die entwickelte Therapie könnte die Behandlung von Knochendefekten revolutionieren, wenn die Gentransfereffizienz gesteigert und somit die Anzahl repetetiver Behandlungen und DNA Dosis reduziert werden kann.

General Introduction

I. Bone defects & bone tissue engineering

Bone tissue, like other exceptions such as the liver, has the inherent capacity to regenerate extensive damage to an extent, which completely restores tissue function and architecture [1]. This is in contrast to tissue repair [1], which involves the replacement of damaged functional tissue with scar tissue, as in wound healing of the skin for example, which only partially restores mechanical integrity of the injured tissue but does not provide functional regeneration in the adult organism. Clinical fracture management [2] aims at supporting the endogenous regenerative capacity of bone tissue through stabilization of the fractures by repositioning/alignment of fractured bones, immobilization with casts and surgical intervention including wires, external and internal fixators, which provide mechanical stability required for regeneration. Modern clinical fracture management can thus provide sufficient means for the treatment of trauma to achieve functional regeneration. Acute tissue trauma after injury or bone resection due to infection or cancer, , however, can lead to situations, where conservative fracture management is not sufficient for regeneration. This situation leads to large segments of bone tissue missing at the defect site, causing failure of the supportive function of bone tissue. This causes significant morbidity, disfigurement, shortening of limbs and immobility in patients depending on the site of trauma. Pseudoarthroses [3], also termed non-union fractures [4], are fractures that fail to heal within 8-9 months. In these situations the fracture gap is not functionally bridged resulting in either hypertrophic, atrophic or synovial non-union

[4]. Their prevalence is depending on the general clinical situation of the patient and the fracture site [5]. These non-union fractures fail to heal with conventional treatment and represent a huge burden on patients and health care systems.

Clinicians nowadays resort to more invasive methods, such as grafting of autologous tissues to the defect in an attempt to heal critical-sized bone defects. Autologous bone grafting [5, 6] which is still the gold standard in the management of critical sized bone defects, has to be harvested from other sites in the body, such as the iliac crest. Therefore it causes donor site morbidity and is therefore not considered an ideal solution [5, 7]. Allogenous bone grafts [8], are free from issues pertaining to donor site morbidity, however have less regenerative potential and other implications such as limited availability, the risk of transmitting diseases from the donor to the new host and potential tissue necrosis [9].

The field of regenerative medicine and tissue engineering aims at providing alternatives to the currently available, limited treatment options for critical-sized segmental bone defects. The general strategy in tissue engineering is to provide an exogenous source of tissue replacement, which is compatible with endogenous regeneration through the combined or exclusive use of biocompatible biomaterials, exogenous recombinant growth factors and autologous (stem) cells [10]. This approach is currently being tailored at providing therapies for the regeneration of multiple adult tissues [11] such as bone, cartilage, skin, liver, neuronal, vascular and cardiac tissue with varying stages of clinical translation and success rates. Specific approaches for bone regeneration, which use recombinant bone morphogenetic protein (BMP) growth factors. In conjunction with a biocompatible collagen matrix carrier [12, 13] BMPs have already been clinically approved [14-16] as third line therapeutic for certain indications such as tibial, ulnar, radial, humoral, femoral and

clavicular non-unions, when autografting is considered unsuitable[1] [15, 17]. These BMPs) are recombinant versions of endogenous pleiotrophic dimeric morphogens of the TGFβ growth factor family, which among other important functions such as the development of the eye and kidney [18], dorsal-ventral axis determination [19] and neural patterning [20], have been shown to induce the formation of bone tissue *in vivo*. Urist *et al.* [21] was the first study in 1965 to show that these morphogenic growth factors can be isolated from bone tissue and induce ectopic bone formation in animals and since this discovery substantial advances have been made in cloning & recombinant production [22] as well as the delivery [23] of these proteins. A major drawback to this approach is that these proteins have to be applied in supraphysiological doses [24, 25] in humans to achieve sufficient osteoinduction, resulting in mg doses per patient. These doses exceed the entire endogenous amount of BMPs present in the human body by far [26] and can cause adverse effects such as heterotopic ossification, humoral immune response, inflammation, tissue swelling and haematoma [27-29]. logically that inflates the cost of the therapy due to the high cost of recombinant protein production, unsustainable by health care systems worldwide. The reasons for supraphysiological dosing of BMPs in human patients is still a matter of scientific debate, although it has been associated with the activity of endogenous inhibitors, stability, species specific effects and suboptimal sustained delivery *in vivo* [26, 30, 31]. Thus, if delivery and associated dosing problems cannot be solved in the near future, this form of advanced therapy needs to be reassessed in terms of cost-benefit ratio and patient safety. Such it will most likely render this approach suboptimal for modern bone defect management.

[1] http://www.eoescg.nhs.uk/Libraries/Policies_Docs/Bone_Morphogenic_Protein_Policy_Feb_2010.sflb.ashx

Stem cell therapies, another approach harnessed for bone regeneration and other tissue regenerative therapies [11, 32], rely on the isolation, optional modification, expansion and reimplantation at the defect site in order to provide a multipotent cellular source for regeneration. Due to ethical restrictions and potential hazardous adverse effects of embryonic stem cells (e.g. teratoma formation, [33]), most approaches today rely on the application of adult stem cells, mainly of mesenchymal origin such as bone marrow mesenchymal stem cells [34] and adipose derived mesenchymal stem cells [35]. Isolation of these cells, however, is still linked to invasive harvesting of donor tissue and therefore can again lead to donor site morbidity. Furthermore, *in vitro* cell expansion and cultivation prior to application requires GMP-grade facilities [36], extensive testing for pathogen contamination and the use of (autologous) human plasma instead of bovine foetal serum [37], which renders such approaches rather cost-ineffective, complicated and makes their broad-range clinical translation unlikely.

II. Ideal systems for clinical translation

An ideal novel treatment strategy for bone tissue regeneration, in contrast to the above mentioned currently exploited approaches should effectively support the endogenous regenerative capacity of the bone tissue without extensive use of exogenous cells, recombinant growth factors or biomaterials at maximum patient safety. Furthermore, such a therapy would need to be cost effective, ideally a one step procedure with a relatively quick rehabilitation time period via enhanced endogenous functional regeneration. Therefore, the therapeutic would have to be produced at lower manufacturing cost than for example recombinant growth factors

and be minimally invasive, preferably in the form of an injectable therapeutic and without the need for harvesting of autologous material as well as implantation surgery.

III. Gene therapy for tissue regeneration

A novel experimental approach in regenerative medicine, which in accordance with the above-mentioned requirements for an ideal therapy is *in vivo* gene therapy using genes encoding for morphogenic factors that are transferred to endogenous target cells at the defect site *in situ* [12, 38, 39]. The general strategy for this approach is to modify somatic cells at the defect site by *in vivo* gene transfer in order to produce a morphogenic or intracellular transcription factor locally in a sustained manner. Such GF could act at much lower doses than exogenous recombinant factors, exerting a paracrine and autocrine differentiation stimulus on target cells *in situ* (Figure 1).

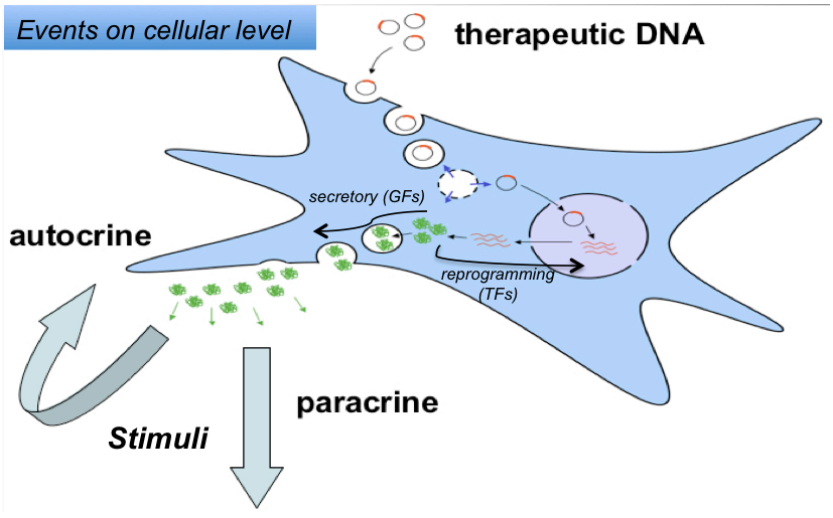

Figure 1: Schematic depiction of the principle of non-viral gene delivery for induction of differentiation in tissue regenerative gene therapy. Modes of action for Growth factor (GF) delivery vs transcription

The advantages of this forced host-cell derived *in situ* production of a growth factor for tissue regeneration [12] are that the produced factor is theoretically far more bioactive compared to recombinant production in non-human producer cell lines such

as chinese hamster ovary cells (CHO). This is potentially due to differences in protein conformation and furthermore species-specific post-translational modifications such as glycosylation patterns [40-43]. Transfected cells produce only limited amounts of the factor for any given time period, thus these modified endogenous cells can be described as sustained delivery vehicles, providing a more physiological release pattern compared to the burst release kinetics of most strategies for the delivery of supraphysiolgical amounts of recombinant factors [44].

In contrast to gene therapies for monogenetic diseases, where a lifelong, stable replacement of dysfunctional genofunction has to be established for therapeutic success, such stable modification of the genome of the target cells is not required for tissue regenerative medicine given that therapeutic gene expression is only required for the limited amount of time necessary for tissue regeneration. Therefore, in the case of tissue regenerative gene therapy one can resort to less invasive gene delivery strategies. Those rely on the application of episomal genetic entities for transient somatic *in vivo* gene therapy, resulting in the genetic alteration of a small population of cells in the patient for a limited period of time.

A crucial factor for therapeutic efficacy is *in vivo* gene transfer efficacy and therefore the selection of the gene delivery vector or strategy. Modified viruses have a long tradition of use as viral vectors for both stable and transient (episomal) gene therapy [45]. Due to their evolutionary adaptation to efficient cell penetration & nucleic acid delivery during infection, these vectors are still the most effective systems for the delivery of genes to cells *in vitro, ex vivo* and *in vivo.* Although, viral gene therapies are the most effective in terms of gene transfer efficacy and transgene production *in vivo* and the first to be clinically approved in Europe for treatment of lipoprotein lipase deficiency (Glybera®, [46]). The translatability of viral vectors is still hampered by severe adverse effects such as genotoxicity, reactivation of the modified replication-

deficient viral vector and immunogenicity [12, 47, 48]. Viral vectors were responsible for the death of patients in clinical trials [49, 50], causing a major set-back for translation of gene therapy to the clinic and a general impact on acceptance of gene therapies in the last decade due to general safety concerns.

Therefore, viral vectors are not considered ideal delivery vehicles for straightforward clinical translation of gene therapies for regenerative medicine albeit their exceptionally high gene-transfer efficacies. Due to safety restrictions and regulations extensive preclinical studies are required and approval of clinical trials by the FDA or EMA for such Advanced Therapy Medicinal Products (ATMPs) is limited due to the mentioned safety issues.

On the other hand non-viral transient somatic gene therapies (although also an ATMP) seem to be ideal for clinical translation with regards to safety. This approach, mainly based on the delivery of eukaryotic expression plasmids, is not associated with chromosomal-integration as some viral systems and is far less immunogenic than its viral counterparts [12]. Furthermore, methods for the reduction of the immunogenicity of plasmids due to unmethylated CpG islands in bacteria derived plasmid DNA [48, 51, 52], such as sequence optimization though whole-plasmid synthesis or modification to backbone-devoid minicircle plasmids are available [53, 54].

The main limitation of non-viral gene delivery, however, is its low gene transfer and thus limited therapeutic efficacy due to several barriers to non-viral gene delivery *in vivo* [55]. Several strategies for the improvement of plasmid DNA stability, plasmid DNA protection and delivery are currently being investigated. Methods such as the incorporation of additional plasmid DNA complexation and transfection reagents such as cationic polymers and liposomes are being investigated for *in vivo* performance [12, 56]. Furthermore, several studies have recently implemented auxiliary

pseudopeptides [57] and proteins for efficient cell penetration, endosomal escape and active nuclear translocation of the therapeutic plasmids [58-60] in order to increase gene expression efficacy and subsequently therapeutic efficacy of non-viral strategies. All of the above mentioned strategies for the enhancement of non-viral gene transfer efficacy rely on additional chemical adjuvants, which represent an additional component with potential adverse effects, hampering straightforward clinical translation. Therefore, with regards to simplicity and furthermore safety concerning off-target gene delivery, an ideal non-viral strategy would rely on application of unprotected naked plasmid DNA, which can only exert its functions upon triggered active transfer to the target cells and is otherwise rapidly degraded by extracellular nucleases upon systemic spread from the target site [61, 62]. More recent developments in *in vivo* non-viral gene delivery provided physical methods [63-65] for the spatially controlled delivery of naked DNA *in situ.* Methods, such as electroporation [66], femto-second infrared lasers [67] and sonoporation [68] (see IV), which rely on physical perturbation of the cytoplasmatic membrane barrier to efficient DNA uptake, could revolutionize non-viral gene delivery. Potentially such systems provide cost-effective safe application of plasmid DNA therapeutics *in vivo*, solely relying on injection of a DNA solution. In conjunction with a robust, relatively cost-effective hardware this should be compatible with one-step procedures directly in the operating theatre.

Another strategy for the enhancement of the efficacy of non-viral gene therapeutics, apart from solving the delivery problem, is the optimization of the chosen therapeutic expression strategy, which encompasses the selection of suitable therapeutic genes with maximal bioactivity for osteoinduction and the design of optimal expression plasmids [52, 69, 70].

As with the screening of pharmacologically substances in general the selection of the optimal therapeutic gene for osteoinduction can be a cumbersome process and generally relies on ectopic testing *in vivo* in animals. This is not suitable for screening several therapeutic genes or compounds as this requires large numbers of animals which is not compliant with the 3Rs (replacement, refinement and reduction) and can lead to inhomogeneous, inconclusive results as *in vivo* assay conditions are not easily controllable and sometimes not reproducible, especially when aiming at obtaining quantitative data for comparative studies. A plethora of potentially osteoinductive therapeutic genes is available for testing [71] and furthermore, recently it has been shown that gene-combinations can be even more bioactive *in vivo* than single gene delivery [72-75]. Therefore, the combinatorial possibilities further increase the number of potential therapeutic genes to be tested, rendering an *in vivo* approach impracticable for target gene identification. Ideally, a robust *in vitro* system for the screening of therapeutic genes could be applied for identification of therapeutic genes prior to *in vivo* proof of concept studies. Cell lines, such as C2C12, which robustly differentiate to the osteogenic lineage *in vitro,* can provide such a test system [76]. The detection of osteogenic differentiation, however, still relies on sample-destructive processing by using assays such as the enzymatic alkaline phosphatase assay (ALP assay, [77]) or quantitative real-time PCR detection of osteoblast-specific marker genes, such as osteocalcin. A strategy to provide online non sample-destructive assessment of the differentiation state of target cells could be provided by employing reporter gene assays using osteoblast-specific promoters in conjunction with suitable reporter genes for online monitoring and quantification, such as fluorescent proteins and bioluminescent luciferase proteins. Development of such a strategy is described in Chapter I (pages 44-70) and implemented for target gene and gene combination identification along with standard sample-destructive *in vitro*

assays in Chapter II (pages 44-70) and led to application of identified gene combinations in Chapter II (pages 78-114) and III (pages 125-192).

Careful plasmid design [52, 53] as mentioned before, can furthermore increase therapeutic efficacy and control over non-viral gene therapies. As BMP gene combinations have been shown to be more effective than single BMP gene delivery in this work and in other studies [78-80], the main focus was set on providing suitable co-expression system for the simultaneous co-delivery of 2 individual BMP-genes, which in turn leads to the formation of highly active BMP-heterodimers [81, 82] after dimerization of their monomeric precursurs prior to secretion from the cell (Figure 2).

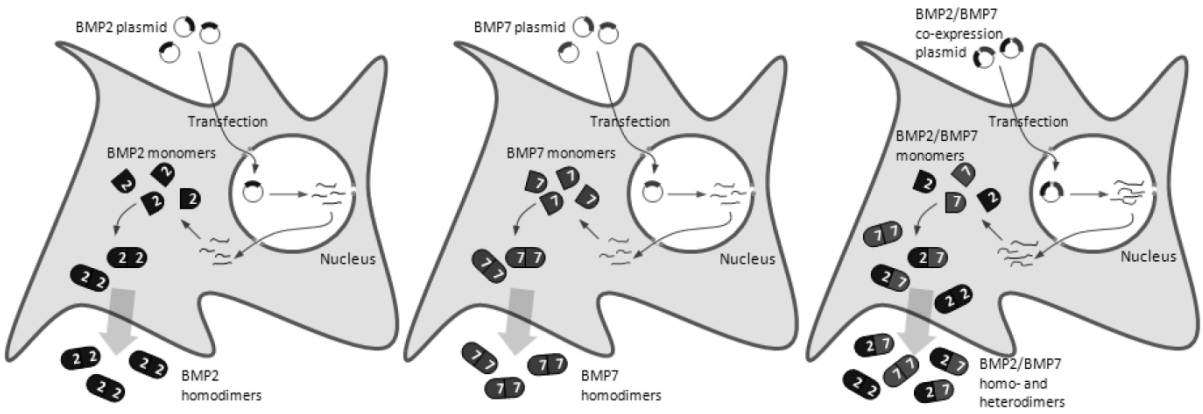

Figure 2: Schematic representation of BMP2 single gene delivery leading to the formation of BMP2 homodimers, BMP7 single gene delivery leading to the formation of BMP7 homodimers and BMP2/7 co-expression leading to the formation of BMP2/7 heterodimeric growth factors.

Furthermore, the possibility of regulating transgene expression *in vivo* through the use of inducible systems, such as the doxycycline inducible TetON system [83, 84] enables tight control over therapeutic transgene expression, expression time

windows and gene dose and furthermore enables increased safety of the therapy by providing means to shut down transgene expression in the case of adverse effects *in vivo*. The development of such inducible or alternative constitutive single vector co-expression systems is covered in Chapter II (pages 78-114) and their *in vivo* application for osteoinduction in Chapter III (pages 125-192).

IV. Ultrasound mediated non-viral gene transfer *in vivo* (Sonoporation)

The application of ultrasound for the delivery of therapeutics to cells *in vivo* has been harnessed for over a decade *in vitro* and *in vivo* [68, 85, 86] and provides a physical method for the delivery of naked plasmid DNA for minimally invasive non-viral gene therapy. The advantage of sonoporative gene delivery is its minimal invasiveness. The ultrasound trigger responsible for gene delivery can be delivered transcutaneously and focussed deep within target tissue in contrast to the necessity of inserting electrodes invasively as for example in electroporative gene delivery. Sonoporation [68, 87] utilizes ultrasound, an acoustic mechanical wave with a frequency of >20kHz. Usually, the ultrasound is generated by a piezoelectric ceramic and has been delivered as a columnar beam with frequencies of 0.8 to >1 MHz with power intensities ranging from 2-4W/cm2 spatial average, temporal peak (SATP) [88, 89] for sonoporation. The employed ultrasound generators usually deliver ultrasound not continuously during treatment time but in pulses by a duty cycle regime [89], typically adjusted between 5-100% of the total treatment time. Furthermore, it has been shown that employing a 40kHz gated over-riding pulse frequency during

ultrasound emission within the duty cycle can furthermore enhance cell poration *in vitro* [90].

High-intensity ultrasound per se has been shown to disrupt cells effectively and irreversibly, however, reversible cell membrane poration by ultrasound requires ultrasound frequencies of a specific range and power as well as adjuvant reagents called microbubbles [91, 92], which mediate a process called acoustic cavitation [93-95]. Cavitation is defined as the activity of air or gas bubbles and pockets within a liquid under the excitation of acoustic waves [95]. Such bubbles can either form through cavitation inception spontaneously or by the general presence of microscopic gas bubbles in a liquid as unstable free bubbles due to the influence of high-intensity acoustic ultrasound waves on the medium. Apart from unstable free bubbles, synthetic microbubble reagents, also termed encapsulated microbubbles (EMBs) [95] can further provide stable bubbles for cavitation as additives to the medium. These synthetic microbubbles were originally introduced as contrast agents for clinical sonography and consist of a shell of albumin, lipids or polymers, which encapsulates a gas-filled core and are usually between 2-5μm in size (Figure 3) [95].

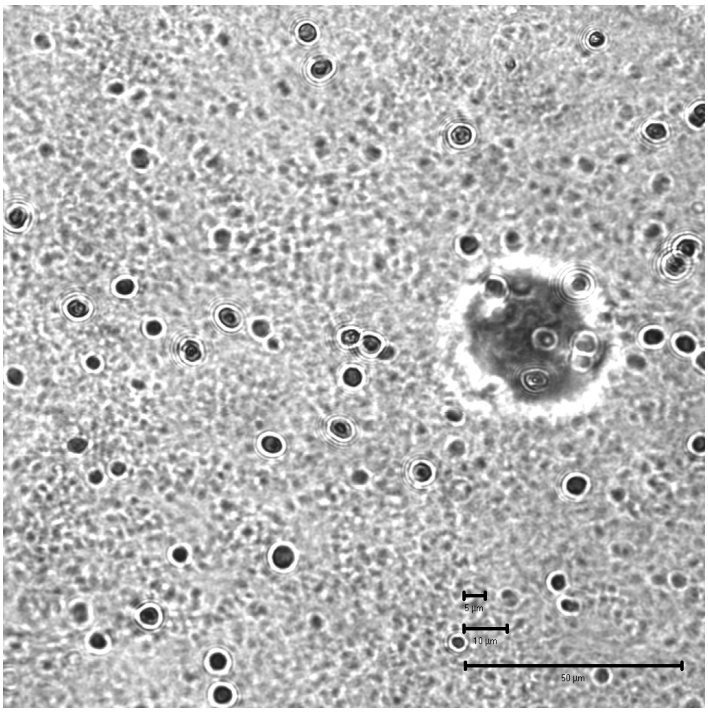

Figure 3: Microscopy image of a cell & microbubbles embedded within a matrix. Scale bars represent 5, 10 and 50 µm.

Recent aims to improve sonoporative gene transfer have focussed on producing nanobubbles and cationic bubbles for enhanced gene delivery [96, 97]. The advantage of this necessary additive for sonoporation is their long tradition of use as imaging agents, which already provides data on safety in human patients and should therefore enable straightforward translation.

Within the ultrasound field, all types of bubbles are prone to acoustic cavitation effects termed inertial (transient) and non-inertial (stable) cavitation [95] (Figure 4). Inertial cavitation is a process where over a certain threshold of the acoustic pressure amplitude, bubbles start to grow in size and then rapidly implode in a violent process, which releases high amounts of energy, where temperatures within the cavitation bubble can rise to exceptionally high temperatures of >15.000°C, produce free radicals and shockwaves. Due to the high energies depending on the gas inside of the bubble the process can even emit light due to plasma formation called sonoluminescence [98].

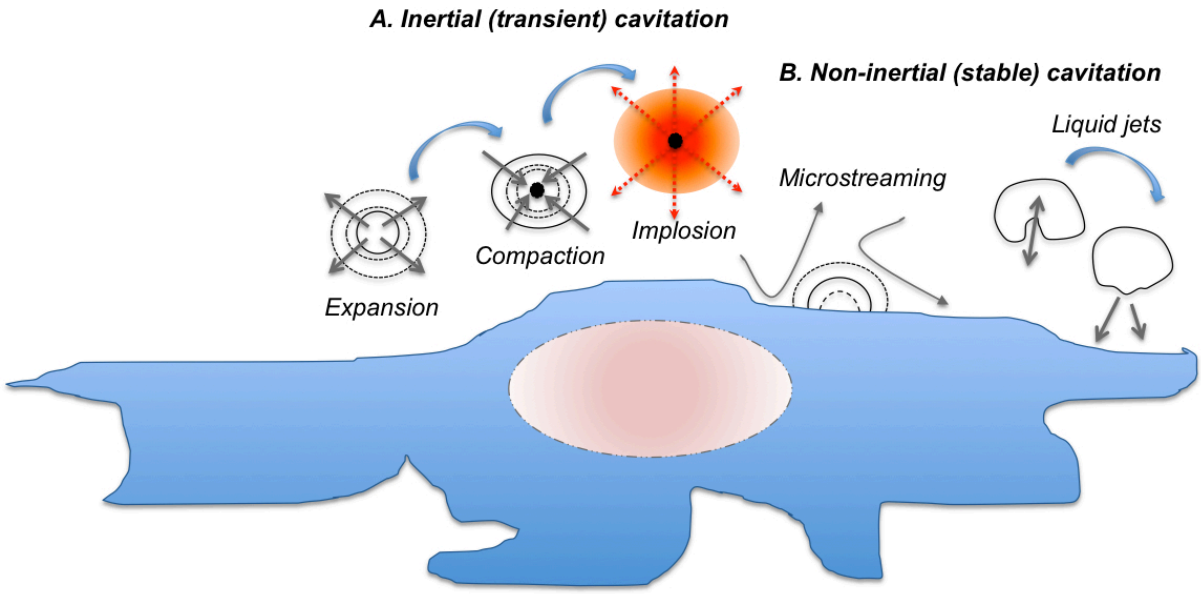

Figure 4: Schematic representation of microbubbles undergoing inertial (transient) cavitation (A) in an ultrasonic field or non-inertial (stable) cavitation leading to the exposure of a cell to different forces (arrows), which are involved in cell poration during sonoporative drug delivery.

Non-inertial (stable) cavitation [95] on the other hand is defined as the oscillation of stable bubbles within an acoustic field within defined frequency and amplitude levels. The occurrence of this resonant oscillation is dependent on the microbubble size, thus defining the appropriate frequency and amplitude of the ultrasonic field leading to successful stable cavitation. The intensity of the acoustic pressure amplitude for non-inertial cavitation is usually below the required amplitude for inertial cavitation to occur. This kind of cavitation causes microstreaming, shear stresses and liquid jets [95, 99, 100] (Figure 4) caused by bubble oscillations near a surface or in the case of sonoporation if sufficient proximity is provided, near the cell membrane. Although the exact mechanism of sonoporative cell membrane permeabilization is not clear yet, there is growing consensus that these non-inertial effects are mainly responsible for

reparable sonoporative cell membrane permabilization rather than the violent process of inertial cavitation [101].

Sonoporative non-viral DNA delivery (Figure 5) [87, 88] in a nutshell harnesses the above described cavitation mechanisms in conjunction with a solution of synthetic microbubbles and plasmid DNA. This mixture is injected to the target site and then treated with transcutaneously applied ultrasound. If sufficient proximity of all the components, microbubbles, plasmid DNA and target cells is provided, the reparable permeabilization [95] of the cell membrane enables the exogenous DNA to enter the cell and subsequently enables transgene expression.

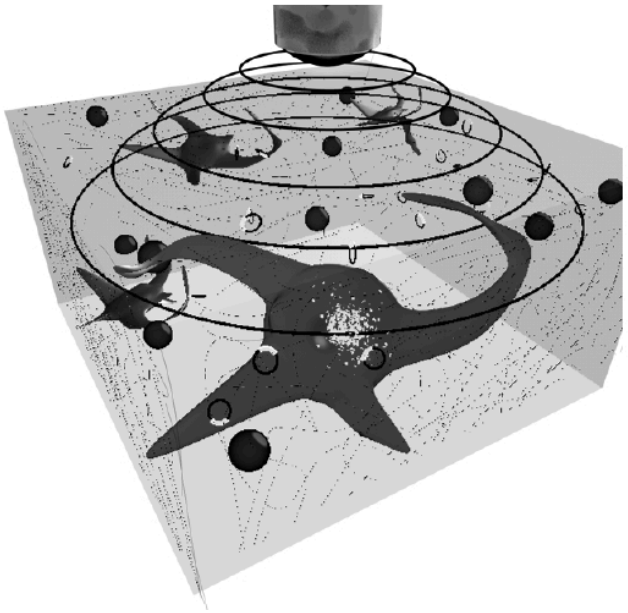

Figure 5: Schematic depiction of sonoporative gene delivery. Cells, microbubbles and plasmid DNA in close proximity within the tissue are exposed to an ultrasound field. (This figure has been kindly provided by Mag. Ara Hacobian)

An important factor to consider when applying sonoporation for drug delivery is that there is a delicate balance between ultrasound power, microbubble concentration, gene transfer rate and cell viability [89, 90]. This is due to the potential destructive influence of non-reparable sonoporation [95] caused by nonlinear oscillation of microbubbles leading to inertial cavitation, which in turn can lead to cell damage and subsequent lysis of the target cells. Furthermore, there is the potential of tissue overheating and subsequent tissue damage due to uncontrollable reflection of ultrasonic waves within the body due to different densities of different tissues present

in the target area or the presence of air at the exit site, which can lead to standing waves and constructive interference [102]. This interference can generate much higher amplitudes than the settings on the device and damage tissues at the target area or exit sites. Whilst potential damage at the ultrasound exit site can be mitigated to a certain degree by employment of a neoprene absorber pad in conjunction with ultrasound contact gel or ideally a water bath, potential standing waves within the body cannot be avoided and can their effects can only be mitigated by empirically determining the lowest possible ultrasound power at optimal gene transfer rates.

Sonoporation has been successfully applied already to transfer exogenous DNA to several tissue types *in vivo* for different gene therapeutic applications [103-105]. Its application has been shown feasible in tissue regenerative gene therapies, particularly in bone tissue engineering. Although the performance of sonoporation is still lower as compared to electroporative gene transfer [103], it can be considered a promising gene transfer modality for future minimally invasive non-viral gene delivery approaches in regenerative medicine. Minimal invasiveness, relatively low hardware costs and progress in microbubble and ultrasound targeting [96, 106, 107] leading to higher gene transfer rates and improved spatial targeting, will enable this technology to compete with and potentially outperform other methods within the field of non-viral gene therapy for tissue regeneration.

V. Aims

Taking the described requirements for an ideal system for clinical translation and the described options for optimization of non-viral gene delivery into account, the aims of this work were to:

• *Establish a non-sample destructive in vitro method for the online detection of osteogenic differentiation for the identification of therapeutic candidate genes (Chapter I, pages 44-70).*

• *Identify potent osteogenic therapeutic genes or gene combinations in vitro. Design and evaluate constitutive and inducible co-expression systems for their osteoinductive potential in vitro (Chapter II, pages 78-114).*

• *Evaluate the therapeutic potential for osteoinduction of the designed co-expression systems in conjunction with passive and with sonoporative gene transfer in vivo in ectopic and orthotopic models (Chapter III, pages 125-192).*

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PUBLICATIONS

Publication I:

Title: **„Enhanced reporter gene assay for the detection of osteogenic differentiation"**

Authors: Georg A. Feichtinger, Tatjana J. Morton, Alice Zimmermann, Daniela Dopler, Asmita Banerjee, Heinz Redl, Martijn van Griensven

Published: Tissue Eng Part C Methods. 2011 Apr;17(4):401-10. doi: 10.1089/ten.TEC.2010.0095. Epub 2010 Dec 14

Publication II:

Title: **"Constitutive and inducible co-expression systems for non-viral osteoinductive gene therapy"**

Authors: Georg. A. Feichtinger, Anna T. Hofmann, Klemens Wassermann, Alice Zimmermann, Martin Mayer, Martijn van Griensven, Heinz Redl

Currently in preparation for submission to European Cells & Materials (eCM)

Publication III:

Title: **"Evaluation of non-viral BMP2/7 *in vivo* gene transfer for ectopic and orthotopic osteoinduction"**

Authors: Georg. A. Feichtinger, Anna T. Hofmann, Paul Slezak, Sebastian Schützenberger, Martin Kaipel, Thomas Nau, Ernst Schwartz, Nathan Luedtke, Martijn van Griensven, Heinz Redl

Currently in preparation for submission to Molecular Therapy

Contributions

Contributions to Publication I

Co-Worker	Comment
G. A. Feichtinger	performed all experimental steps (unless otherwise indicated), produced all figures and prepared the manuscript
T. J. Morton	assisted with experiment design, revised the manuscript
A. Zimmermann	performed RNA isolation, cDNA synthesis & qPCR for osteocalcin transcript detection
D. Dopler	assisted with cell culture experiments
A. Banerjee	assisted with cell culture experiments
H. Redl	supervisor, assisted with study design, revised the manuscript
M. van Griensven	supervisor, assisted with study design, revised the manuscript

Contributions to Publication II

Co-Worker	Comment

G. A. Feichtinger	performed all experimental steps (unless otherwise indicated), produced all figures and prepared the manuscript
A. T. Hofmann	assisted with non-viral in vivo gene transfer, performed surgeries for animal models
K. Wassermann	successful preparation of pTetON-BMP2/7 plasmid DNA using SURE 2 *E.coli*, preparation of pVAX1-BMP2/7 co-expression plasmids
A. Zimmermann	performed RNA isolation, cDNA synthesis & qPCR for osteocalcin transcript detection
M. Mayer	performed *in vivo* µCT evaluation
M. van Griensven	supervisor, assisted with study design, revised the manuscript
H. Redl	supervisor, assisted with study design, revised the manuscript

Contributions to Publication III

Co-Worker	**Comment**
G. A. Feichtinger	performed all experimental steps (unless otherwise indicated), produced all figures and prepared the

	manuscript
A. T. Hofmann	assisted with non-viral in vivo gene transfer, performed surgeries for animal models
P. Slezak	assisted with non-viral in vivo gene transfer, performed surgeries for animal models, revised the manuscript
S. Schützenberger	assisted with non-viral in vivo gene transfer, performed surgeries for animal models, revised the manuscript
M. Kaipel	assisted with non-viral in vivo gene transfer, performed surgeries for animal models
T. Nau	assisted with non-viral in vivo gene transfer, performed surgeries for animal models, revised the manuscript
E. Schwartz	performed *in vivo* µCT evaluation, generated registered µCT images
N. Luedtke	provided F-ARA-EdU labelling agent, assisted with metabolic plasmid labelling experiment
M. van Griensven	supervisor, assisted with study design, revised the manuscript
H. Redl	supervisor, assisted with study design, revised the manuscript

Chapter I - Enhanced reporter gene assay for the detection of osteogenic differentiation

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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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Figures

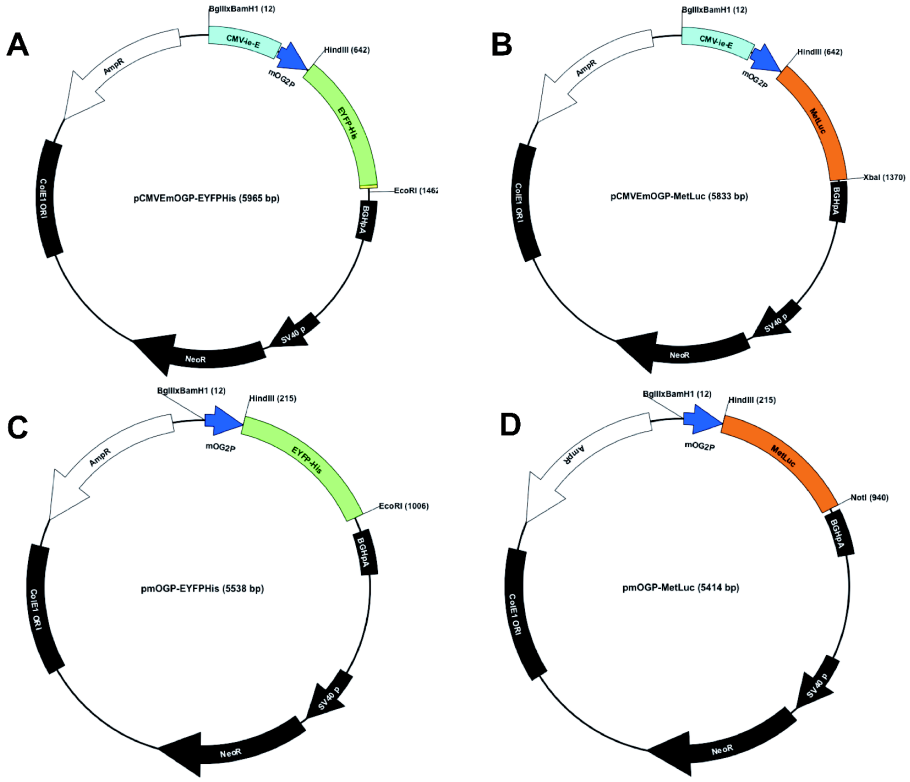

Figure 1: pCMVE/mOCP-EYFPHis fluorescent reporter plasmid (A) and pCMVE/mOCP-MetLuc bioluminescent reporter plasmid (B). pmOCP-EYFPHis unamplified fluorescent reporter plasmid (C) and pmOCP-MetLuc unamplified bioluminescent reporter plasmid (D). The Cytomegalovirus immediate early enhancer (CMVE) is 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.

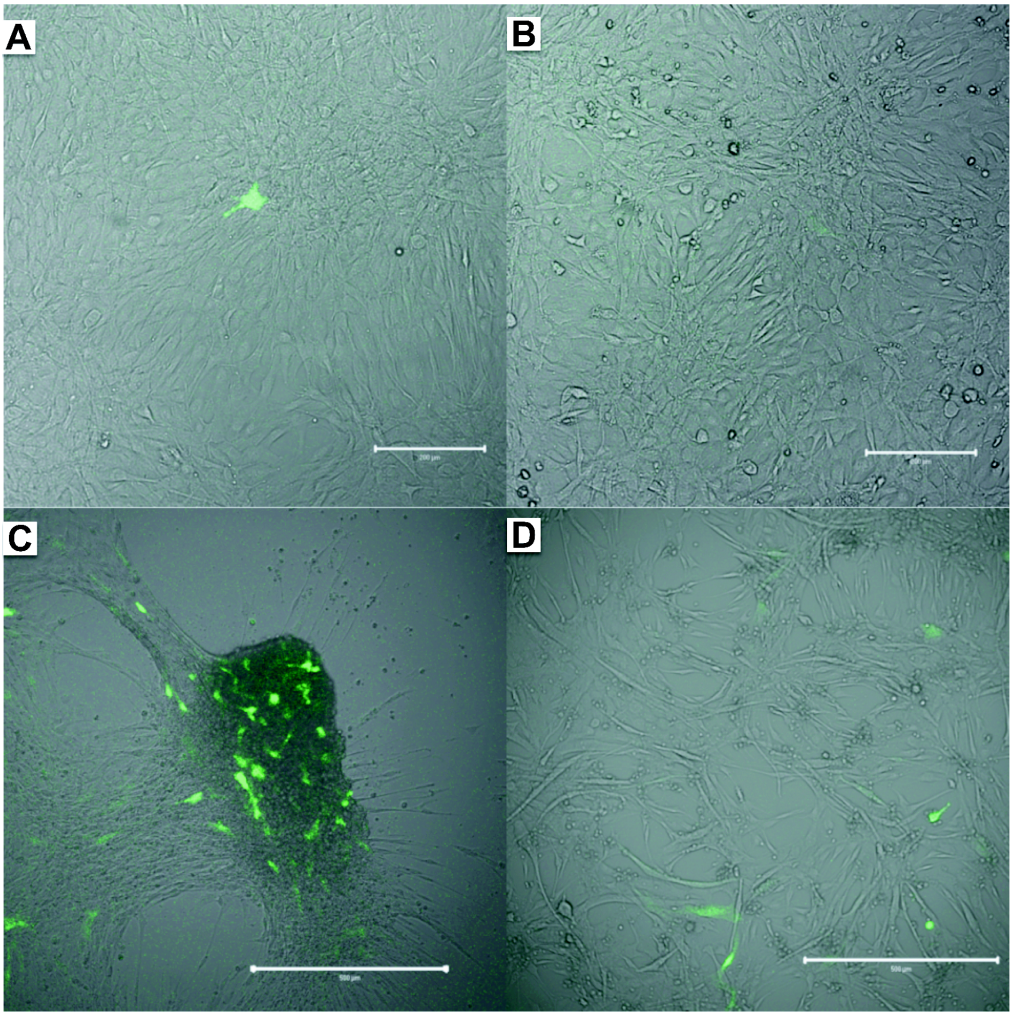

Figure 3: (A) pmOCP-EYFPHis activation in C2C12 cells cultured in DMEM + 1% FCS, 200 mM L-glutamine and 500 ng/ml recombinant human bone morphogenetic protein 2. Scale bars represent 200µm. (B) pmOCP-EYFPHis activation in C2C12 cells cultured in Dulbecco's Modified Eagles Medium (DMEM) + 1% fetal calf 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.

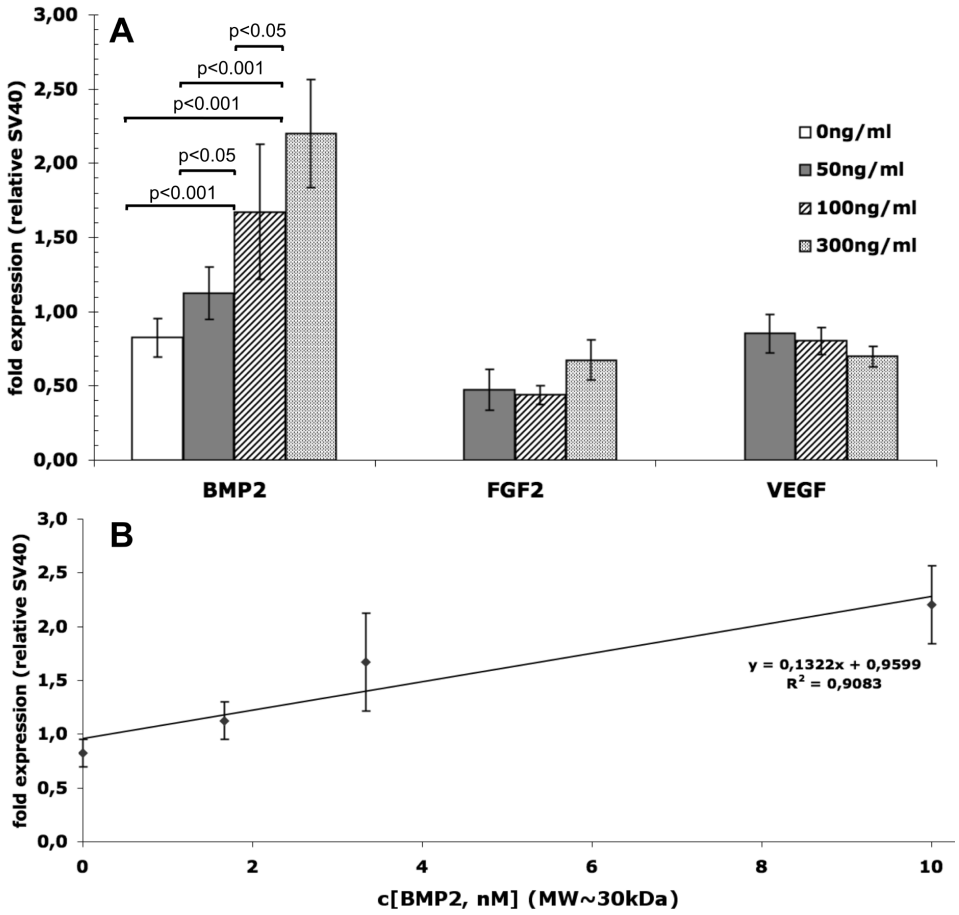

Figure 4: (A) Activation of the pCMVE/mOCP-MetLuc enhanced bioluminescent osteocalcin reporter system upon induction with different growth factors in 2D culture. A significant induction of *Metridia* Luciferase expression was observable in cells treated with osteoinductive recombinant human bone

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.

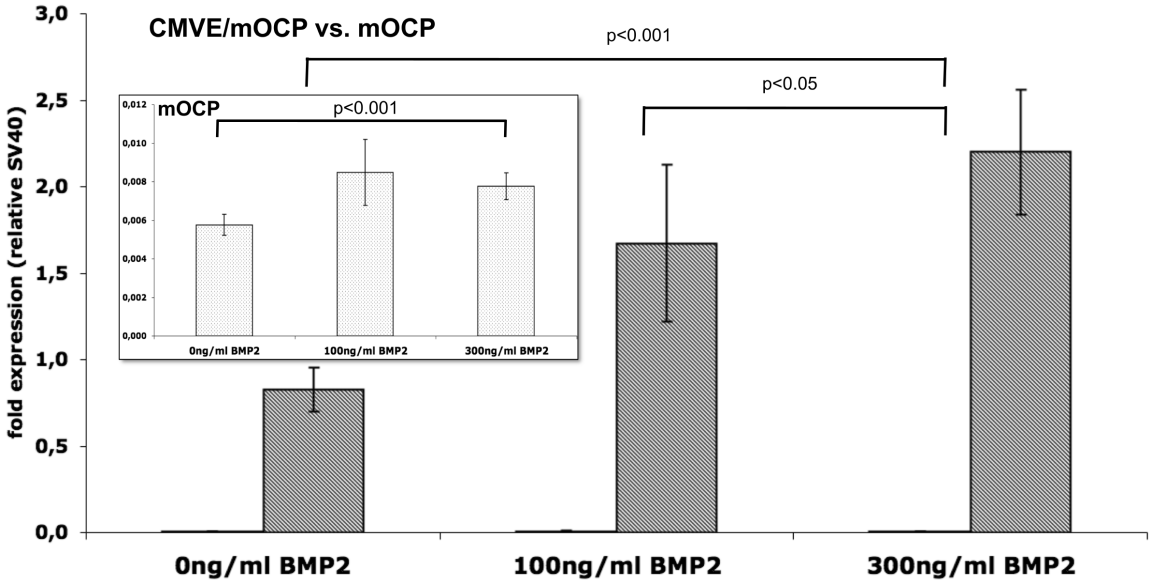

Figure 5: Direct comparison of bioluminescence readouts for the enhanced (CMVE/mOCP) and the unenhanced (mOCP) reporter systems in response to 0ng/ml, 100ng/ml and 300ng/ml rhBMP2. Readouts are depicted as relative expression levels (fold expression) to the constitutive SV40 promoter. mOCP expression 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.

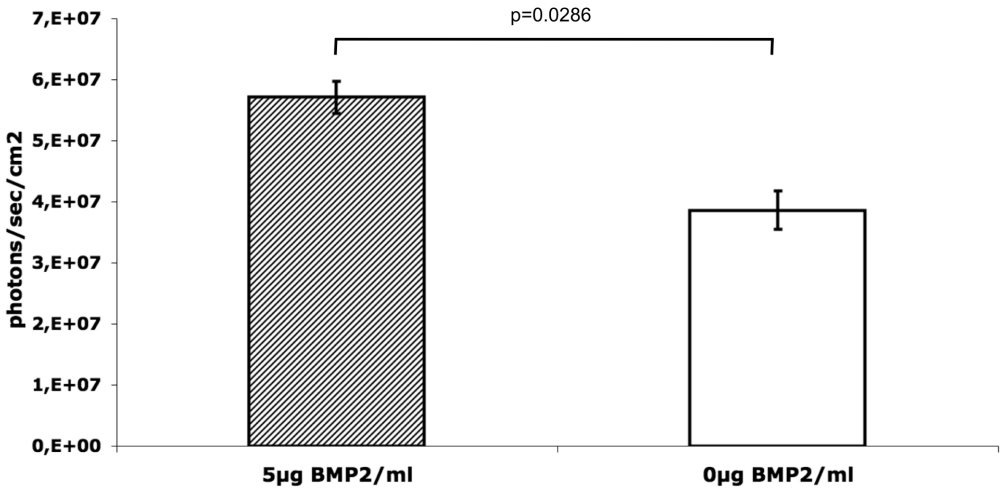

Figure 6: Activity of the CMVE/mOCP-MetLuc reporter system in fibrin clots represented as photons/sec/cm^2 luciferase activity detected in the supernatants. Osteogenic differentiation (left), negative control (right). n = 4, values represent average ± standard deviation.

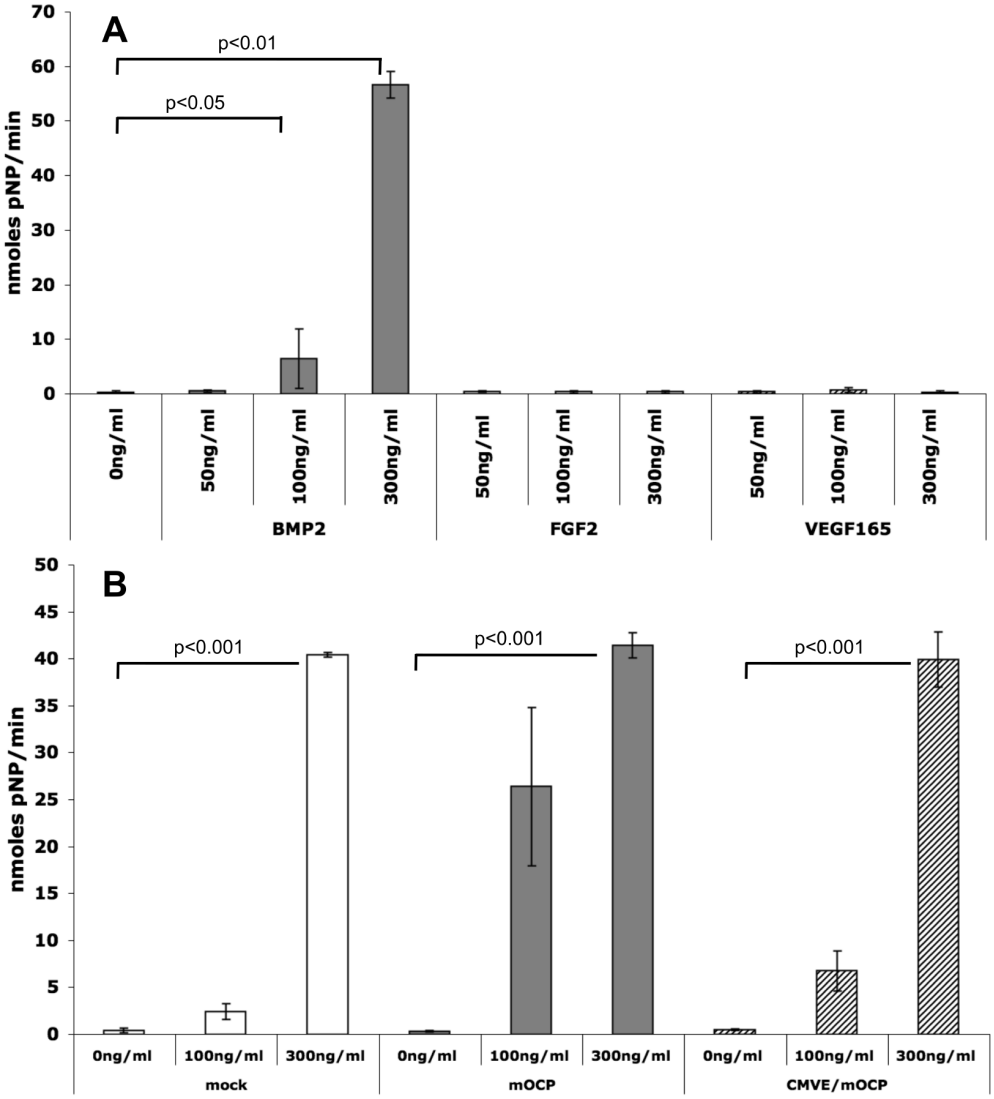

Figure 7: Enzymatic alkaline phosphatase (ALP) assay results in 2D culture.

(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.

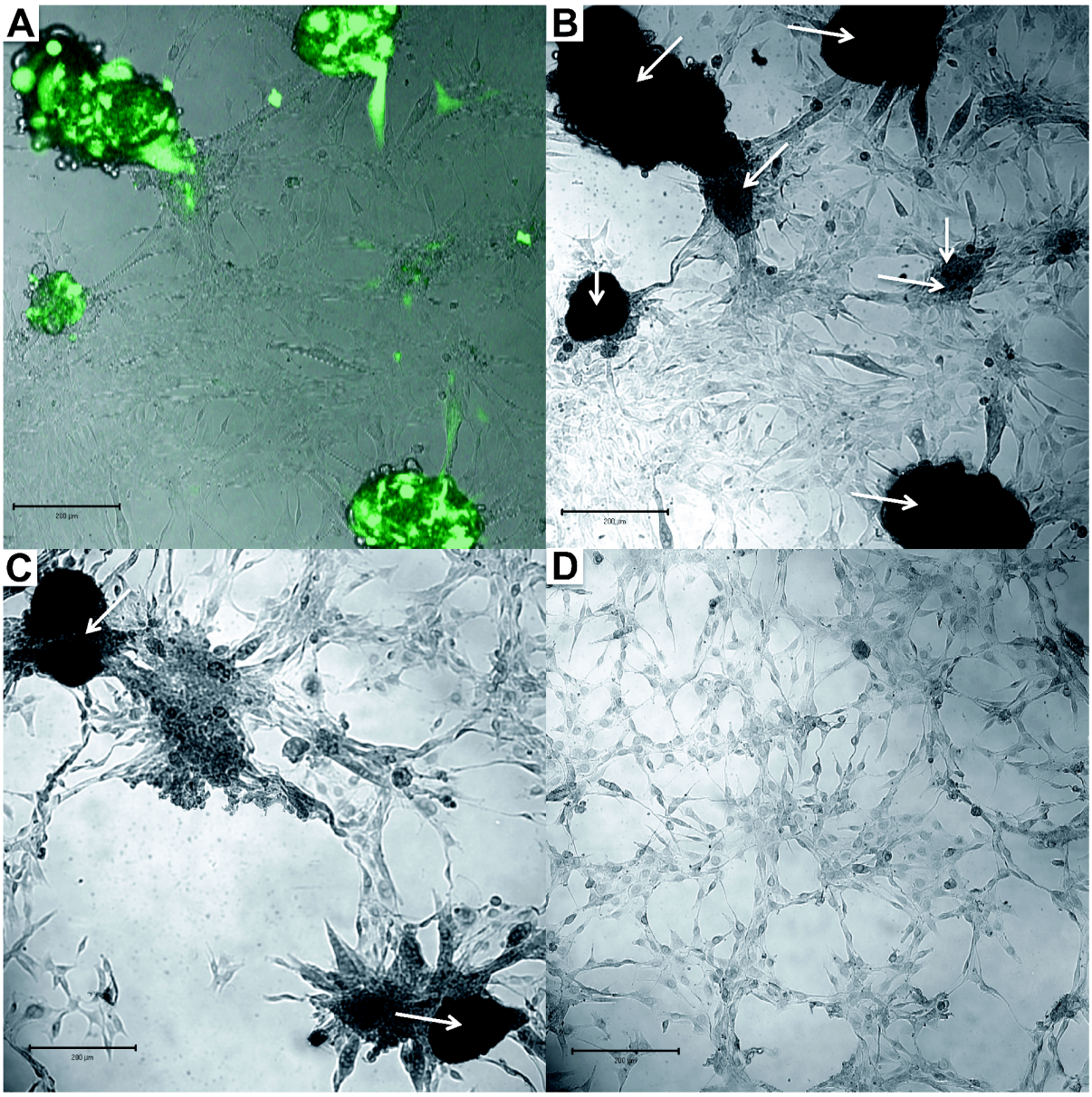

Figure 8: (A) pCMVE/mOCP-EYFPHis fluorescence signal after 14 days of differentiation with 300ng/ml rhBMP2. (B) Same field of view after *von Kossa* staining for mineralization, centers of mineralization (arrows) overlap with fluorescent signals in A. (C) Positive control after 14 days of differentiation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

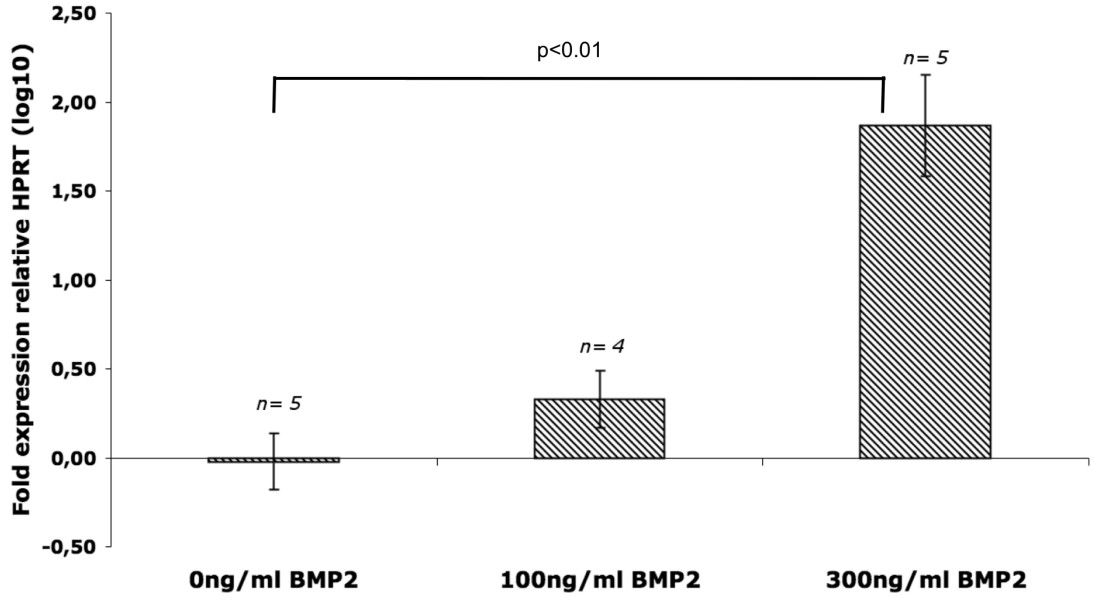

Figure 9: Osteocalcin expression levels in pCMVE/mOCP transfected cells (6 days of differentiation) as determined by quantitative real-time PCR (relative quantification with comparative Ct-method) in response to 0ng/ml, 100ng/ml and 300ng/ml rhBMP2. Readouts are depicted as fold changes of osteocalcin 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.

Chapter II - Constitutive and inducible co-expression systems for non-viral osteoinductive gene therapy

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Abstract

Tissue regenerative gene therapy requires expression strategies that deliver therapeutic effective amounts of transgenes. As physiological expression patterns are more complex than high-level expression of a singular therapeutic gene, we aimed at the design of plasmids that are capable of constitutive or inducible co-expression of 2 transgenes simultaneously.

Co-expression of human bone morphogenetic protein 2 (BMP2) and 7 (BMP7) from constitutively expressing and doxycycline inducible plasmids was evaluated *in vitro* in C2C12 cells with osteocalcin reporter gene assays and standard assays for osteogenic differentiation. The constitutive system was additionally tested in an *in vivo* pilot for ectopic bone formation after repeated naked DNA injection to murine muscle tissue.

Inductor controlled differentiation was demonstrated *in vitro* for inducible co-expression. Both co-expression systems, inducible and constitutive, achieved significantly better osteogenic differentiation than single factor expression of BMP2 or BMP7. The potency of the constitutive co-expression systems was dependent on relative expression cassette topology. *In vivo*, ectopic bone formation was demonstrated in 6/13 animals (46% bone formation efficacy) at days 14 and 28 in hind limb muscles as proven by *in vivo* µCT and histological evaluation.

We conclude from the *in vitro* findings, that the devised single vector BMP2/7 co-expression strategy mediates superior osteoinduction, can be applied in an inductor controlled fashion and that its efficiency is dependent on expression cassette topology. *In vivo* results indicate, that constitutive co-expression of BMP2/7 applied by non-viral naked DNA gene transfer effectively mediates bone formation without the application of biomaterials, cells or recombinant growth factors.

Keywords: bone morphogenetic protein, co-expression, non-viral gene therapy, inducible, BMP2/7, heterodimer, intramuscular

Introduction

Transient somatic gene transfer, enabling temporally and spatially restricted, safe expression of transgenes *in vivo,* could revolutionize the clinical treatment of organ and tissue specific diseases. Current gene transfer methods are divided in viral (high efficiency, high immunogenicity) and non-viral (low efficiency, low immunogenicity) gene transfer for transient expression of therapeutic transgenes (Bleiziffer *et al.*, 2007; Gelehrter *et al.*, 1998). Transient gene therapeutics can augment local levels of cytokines and morphogenes to compensate for pathologically down-regulated gene expression or locally augment growth factors to therapeutic levels. In contrast to the therapy of monogenetic diseases with stable, integrating viral vectors that have to provide life-long stable expression of therapeutic genes, non-viral non-integrating vectors such as plasmid DNA, are active as non-replicating episomal entities *in vivo.* The information is lost within a specific time frame with a very rare frequency of random chromosomal integration (Coelho-Castelo *et al.*, 2006; Martin *et al.*, 1999) and is therefore not associated with vector-associated genotoxicity (Baum *et al.*, 2006).

For tissue regenerative gene therapy approaches the inherent drawback of loss of information in transient gene therapy is actually a desired safety feature. Temporally regulated or restricted expression of the therapeutic is mandatory to prevent systemic effects, aberrant cellular growth and to allow complex tissue growth (Bleiziffer *et al.*, 2007; Franceschi, 2005). Plasmid DNA is cleared relatively quickly from the system and does not show substantial systemic spread or ectopic expression at off-target sites (Coelho-Castelo *et al.*, 2006; Gehl, 2003; Hengge *et al.*, 2001; Hohlweg and Doerfler, 2001). Low production costs of the non-viral plasmid therapeutic is another potential advantage (Bleiziffer *et al.*, 2007). Plasmid DNA is produced with sufficient

purity and in sufficient amounts for clinical application at relatively low cost compared to recombinant growth factors (Bonadio *et al.*, 1999; Einhorn, 2003; Johnson and Urist, 2000; Tepper and Mehrara, 2002) as advanced therapeutics. Gene therapeutics lead to an inherent sustained release of expressed therapeutic genes by host cells *in situ*, which is more effective due to higher bioactivity of the host-produced growth factor (Bleiziffer *et al.*, 2007; Bonadio *et al.*, 1999). Plasmid DNA has been successfully applied in direct *in vivo* gene transfer for wound healing and angiogenesis (Michlits *et al.*, 2007; Mittermayr *et al.*, 2008), cardiac regeneration (Sundararaman *et al.*, 2011) and musculoskeletal regeneration (Grossin *et al.*, 2003; Kawai *et al.*, 2006b; Osawa *et al.*, 2009; Osawa *et al.*, 2010).

Taking these advantages into account, non-viral vectors, such as plasmid DNA, are considered the most likely candidates for clinical translation of tissue regenerative gene therapies.

The major drawback of non-viral vectors, however, is their low transfection efficacy if used without adjuvant measures (naked) (Herweijer and Wolff, 2003; Schertzer *et al.*, 2006) and a wide range of agents, materials and physical methods to enhance the *in vivo* transfer of plasmid DNA have been developed with varying success (Bleiziffer *et al.*, 2007). An alternative strategy to increase the therapeutic effectiveness is plasmid modification (Tolmachov, 2009; Tolmachov, 2011) and the selection of an optimal therapeutic gene to compensate for the low transfection efficacy of plasmid vectors *in vivo*. Furthermore, it has been shown that gene combinations are beneficial in experimental models (Kawai *et al.*, 2006b; Steinert *et al.*, 2009; Wan *et al.*, 2012; Zhu *et al.*, 2004). Several multi-gene approaches have already been applied for tissue regenerative therapies. In bone regeneration it has been shown that combinatorial gene therapy using multiple bone morphogenetic protein (BMP)

genes, especially the combination of BMP2 and BMP7 leads to higher osteogenic bioactivity compared to single factor expression (Kawai *et al.*, 2006b; Zhu *et al.*, 2004). This BMP2/7 co-expression strategy has been shown to lead to the expression of a heterodimeric growth factor with higher bioactivity (Israel *et al.*, 1996) compared to the respective single gene derived homodimeric variants (Kawai *et al.*, 2009).

Gene combinations can be co-delivered as a mixture of different single expression cassette plasmids, as co-expression plasmids with multiple independent transcriptional entities (Kawai *et al.*, 2009) and as plasmids encoding poly-cistronic mRNA with intra-ribosomal entry sites (IRES) (Guo-ping *et al.*, 2010; Gurtu *et al.*, 1996) . Multi-cistronic plasmids allow the co-expression of multiple factors from the same plasmid molecule via bidirectional or separated, unidirectional gene expression cassettes and enable the simultaneous expression of multiple therapeutic genes at the target site.

The achievable functional expression levels, however, could be strongly related to expression cassette topology due to potential transcriptional interference phenomena (Callen *et al.*, 2004; Shearwin *et al.*, 2005) between the promoters. Furthermore, multiple expression cassettes using the same heterologous promoters could be prone to competitive effects reducing total transgene expression (Ngo *et al.*, 1993). Nevertheless, at least within the scope of the co-delivery of 2 therapeutic genes, single molecule transcriptional entities can still be advantageous *in vivo*. If both transgenes are encoded on the same molecule, then the correlated expression of both factors obtained from co-expression plasmid transfer should theoretically be higher compared to co-transfection of 2 individual plasmids. This correlated co-expression from one single molecule can be advantageous for the production of

heterodimeric growth factor variants (Kawai *et al.*, 2009) or for balanced co-expression of 2 individual synergetic factors (Banfi *et al.*, 2012).

Besides constitutive expression strategies, there are plasmid systems available with response elements that enable regulated, inductor controlled levels of therapeutic gene expression. Such, particular regulated expression kinetics could provide enhanced safety (on/off inductor dependence) and allow mimicking of physiological gene expression patterns for increased therapeutic efficacy. Systems such as the dual-component (activator and response plasmid) tetracycline (Tet) inducible or repressed (TetON, TetOFF) regulated gene expression systems (Gossen and Bujard, 1992; Gossen *et al.*, 1995; Goverdhana *et al.*, 2005) can be applied for this purpose, even for co-expression of 2 therapeutic genes (Baron U, 1995).

For therapeutic *in vivo* over-expression studies, the tetracycline-inducible system (TetON) appears to be more suitable than the tetracycline repressible TetOFF system, since the TetON system is induced by application rather than withdrawal of a small molecule an in a default off-state in its absence.

These inducible systems could help to determine the minimally required gene dose, fine-tune therapeutic expression levels in experimental models. However, its translatability to the clinic remains questionable given the complexity of such a system and the need to additionally express a heterologous transactivator transgene with potential immunogenicity (Le Guiner *et al.*, 2007). Simple constitutive co-expression systems, ideally backbone-devoid minicircle (Chen *et al.*, 2003) variants, on the other hand are more likely to be clinically translated

With regards to the above mentioned opportunities to enhance the therapeutic efficacy of non-viral plasmid based gene delivery through modification of the vector, it

was the aim of this study to design and evaluate constitutive and inductor controlled inducible BMP2/7 co-expression plasmids for osteoinductive non-viral *in vivo* gene therapy. The TetON-inducible bidirectional co-expression system was modified to contain all elements (response and activator sequences) on one single plasmid. Subsequently it was evaluated for its inductor dose dependent expression kinetics. Its osteoinductive capacity was compared to single factor expression, constitutive co-expression or application of recombinant growth factors *in vitro*. The constitutive systems were designed based on the plasmid backbone pVAX1 for DNA vaccination studies, which is compliant with FDA safety guidelines for plasmid DNA vaccines (FDA Docket No. 96N-0400) and the employed therapeutic BMP 2 and 7 cDNAs were of human origin, in order to generate constitutive systems with potential for clinical translation. These “preclinical” constitutive systems were evaluated *in vitro* for their osteoinductive capacity and tested *in vivo* in an ectopic bone formation assay using repeated non-viral intramuscular *in vivo* gene transfer.

Materials and Methods

Primers

All primers (Table 1) were ordered at Microsynth AG (Balgach, Switzerland).

Growth factors

Recombinant CHO-derived human BMP-2 was purchased from Medtronic Biopharma Sárl (Neuchatél, Switzerland, PZN#20238). Recombinant CHO-derived human BMP-7 and *E. coli*-derived BMP-2/7 heterodimer were purchased from R&D systems (Minneapolis, US, #354-BP, #3229-BM). The lyophilized growth factors were dissolved according to manufacturers instructions and then diluted to working stocks 100ng/µl and stored at -80°C prior to use.

Plasmids

The constitutive expression plasmid pVAX1 was purchased from LifeTechnologies Ltd (Paisley, UK, #V260-20). The two-component tetracycline (Tet) inducible co-expression system, consisting of the pTREtight-BI response and the pTetON-Advanced activator plasmid, was purchased from Takara Bio Europe/Clontech (Saint-Germain-en-Laye, France, #631068, #631069). The click beetle luciferase (CBR) plasmid pCBR-Control was purchased from Promega (Madison, US, #E1421). The CMV-enhanced osteocalcin reporter plasmid pCMVE/mOCP-EYFPHis was described previously (Feichtinger *et al.*, 2010). All plasmids were maintained in *E.coli* TOP10 (Life Technologies Ltd., Paisley, UK, #C404003) unless indicated otherwise and prepared as endotoxin-free maxipreps using the EndoFree Plasmid Maxi or Giga kits from Qiagen GmbH (Hilden, Germany, #12362) for *in vitro* and *in vivo* use.

Cloning of human BMP2 and BMP7 cDNAs

Full-length human BMP2 and BMP7 cDNA were cloned from human total lung RNA or kidney RNA (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France, #636524, #636529) using AMV based reverse transcription of poly-a RNA to cDNA (Promega, Madison, US, #M5108) according to manufacturers instructions and primers in Table1. Both cDNAs were subcloned into pVAX1 via & verified by sequencing (Microsynth AG, Balgach, Switzerland, data not shown).

Preparation of constitutive co-expression plasmids

The co-expression plasmids pVAX1-BMP2/7+ (tandem organization of expression cassettes, Figure 1C) and pVAX1-BMP2/7– (divergent organization of expression cassettes, Figure 1D) were constructed by transferring the complete expression cassette for BMP7 expression from pVAX1-BMP7 to pVAX1-BMP2 (Figure 1A, restriction sites for insertion marked with red lines) to the BspH1 backbone site in pVAX1-BMP2 using standard PCR (primers see Table 1) and cloning procedures.

Preparation of inducible single-vector co-expression plasmids

An inducible expression kinetics reporter system was designed by PCR-amplification and cloning of dsRed and EYFP in pTREtight-BI using standard cloning procedures. This response plasmid pTRE-EYFP/dsRed was then modified to a single-vector inducible system by cloning the entire reverse tet-transactivator (rtTA) expression cassette, obtained trough high-fidelity Phusion polymerase (New England Biolabs, Frankfurt, Germany, #M0530S) PCR amplification (primers see Table 1), from the pTetON Advanced activator plasmid (Figure 1E) into the BspLU11I site of pTRE-EYFP/dsRed (Figure 1E, insertion site marked red) to form pTetON-EYFP/dsRed

(Figure 1F). Both plasmids were used for expression kinetics studies and for a comparison of single vector TetON systems with standard double vector systems. The therapeutic inducible co-expression plasmid pTetON-BMP7/2 (Figure 1G) was created by transferring the cDNAs of human BMP2 and human BMP7 into pTREtight-BI using standard cloning procedures in the *E. coli* cloning strain SURE2 (Stratagene/Agilent Technologies, Santa Clara, US, #200152). The system was then modified to a single-vector inducible system as described for the kinetics reporter system.

Cell Culture & Transfection

The mouse cell line C2C12, capable of osteogenic differentiation (Katagiri T, 1997; Katagiri T, 1994) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany, #ACC565) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, Vienna Austria, #D6546) containing 4.5 g/L D-glucose, supplemented with 2mM L-glutamine (Sigma Aldrich, Vienna, Austria, #G6392) and 5% foetal bovine serum (FBS; Lonza Ltd., Basel, Switzerland, #14-502E).

The cells were kept subconfluent during expansion and seeded at $0{,}5x10^5$ cells/ml/well in 24-well plates to enable transfection at approx. 80% confluence unless otherwise stated. Differentiation experiments were carried out in serum-reduced DMEM+1%FCS for 6 days.

Constitutive systems

Initial transfection studies were carried out in C2C12 cells using Lipofectamine 2000 (LifeTechnologies Ltd, Paisley, UK, #11668019) according to the manufacturer's instructions. 2µg total pDNA : 2µl Lipofectamine 2000 per well in a 24-well plate was used in transfections with vectors expressing only one factor. 1µg : 1µg (2µg total pDNA) of these plasmids were used in co-transfection incubated with 2µl Lipofectamine 2000. Co-expression plasmids were transfected at an amount of 2µg:2µl of Lipofectamine 2000.

The constitutive systems were tested with the osteocalcin reporter plasmid pCMVE/mOCP-EYFPHis in a super-transfection experiment. C2C12 cells were transfected with the reporter plasmid 24h prior to seeding in a T175 flask at 80% confluence. 24h post transfection with the reporter, the cells were seeded into 24-well plates at a density of $0.5x10^5$ cells/ml. 24h post seeding the cells were super-transfected with 1µg/well of the constitutive pVAX1 over-expression plasmids, and following transfection, the medium was changed to DMEM with a reduced serum amount of 1%FCS. Fluorescence microscopy for osteocalcin-linked EYFP expression was carried out 6 days post expression vector transfection.

Inducible systems

The expression levels of the inducible reporter systems pTRE-EYFP/dsRed, pTetON-Advanced (double vector system) and pTetON-EYFP/dsRed were compared via fluorescence microscopy after transfection and induction in C2C12 cells. The double vector system was co-transfected as 1µg pTRE-EYFP/dsRed response plasmid combined with 1µg of pTetON-Advanced activator plasmid using 1µl of Lipofectamine 2000 per well in a 24-well plate. In the other group, 2µg of the single vector system

pTetON-EYFP/dsRed was transfected using 1µl Lipofectamine 2000 per well in a 24-well plate.

Both systems were induced with a single dose of doxycycline (0ng, 250ng and 1000ng/ml/well) at the start of the experiment and expression of the fluorescent reporter genes was compared 48h post induction. The expression kinetics of the single vector system were investigated after transfection into C2C12 cells and induction with a single dose of 0ng, 250ng and 1000ng/ml/well of doxycycline for up to 14 days with fluorescence microscopy.

The therapeutic single vector co-expression system pTetON-BMP2/7was transfected into C2C12 cells to test its osteoinductive capacity. Cells were incubated with a single dose 0ng, 250ng and 1000ng/ml/well of doxycycline and the cells were cultivated for 6 days in DMEM+1FCS prior to assessment of osteogenic differentiation by microscopy, ALP assays and qPCR.

The inducible system was tested with the osteocalcin reporter plasmid pCMVE/mOCP-EYFPHis in a co-culture experiment. $0{,}25x10^5$ pCMVE/mOCP-EYFPHis transfected “reporter cells” were mixed with $0{,}25x10^5$ pTetON-BMP2/7 “osteogenic cells” per well and cultured for 6 days in DMEM+1%FCS. Co-Expression of BMP2 and BMP7 was induced with a single dose of 0ng, 250ng and 1000ng/ml/well of doxycycline. Osteocalcin-coupled reporter gene expression by pCMVE/mOCP-EYFPHis was observed by fluorescence microscopy on day 6.

Alkaline phosphatase (ALP) assay

Enzymatic ALP assays were carried out as previously described in (Feichtinger *et al.*, 2010). Enzyme activity was calculated as fold induction relative to negative control samples, 6 days after induction.

Osteocalcin gene expression analysis

Osteocalcin gene expression was determined as fold induction (comparative C_T method, (Schmittgen and Livak, 2008)) relative to negative control samples by quantitative real-time PCR using SYBR green as previously described in (Feichtinger *et al.*, 2010) using the primers qHPRT_s and qHPRTas (Table 1).

***In vivo* non-viral naked DNA gene transfer**

The animal protocol review board of the City Government of Vienna, Austria approved all experimental procedures in accordance with the Guide for the Care and Use of Laboratory Animals as defined by the National Institute of Health. Female nude mice (Hsd:Athymic Nude-Foxn1^{nu}, Harlan Laboratories, Bresso, Italy, #069(nu)/070(nu/+)) of approx. 10-128 days age weighing approx. 30g were used in this study.

Plasmid Injections

20µg of pVAX1-BMP2/7 and 20µg of pCBR plasmid as internal luciferase transfection control were injected as a mixture (50µl total) into *gastrocnemius* hindlimb muscles under short inhalation anaesthesia (2 vol.% isoflurane (Forane, Abbott GmbH, Vienna, Austria) and 3 L/min air) at each day of the treatment protocol. The treatment was repeated to a total of 5 injections, resulting in a final total dose of 200µg plasmid DNA per hindlimb muscle (100µg therapeutic plasmid: approx. 3.3mg/kg therapeutic DNA per animal).

Micro Computer Tomography (µCT) analysis

In vivo µCT images of hindlimbs were obtained using a vivaCT 75 (Scanco Medical AG, Brütisellen, Switzerland) 14 days and 28 days post last gene transfer under short inhalation anaesthesia. Bone volume (mm^3) and bone mineral density (mg hydroxyapatite per cm^3) for 2 and 28 days were calculated using Scanco software and a standard density calibration phantom.

Bioluminescence Imaging

Bioluminescence imaging of CBR luciferase activity after *in vivo* gene transfer was carried out under short inhalation anaesthesia 24h post last plasmid DNA injection using a Xenogen IVIS100 Imaging system (Caliper Life Sciences GmbH, Mainz, Germany). Mice received 0,5mg D-luciferin potassium salt (Caliper Life Sciences GmbH, Mainz, Germany, # 122796) in 300µl ringer solution (Mayerhofer

Pharmazeutika GmbH, Linz, Austria, license #16.609) intraperitoneally before imaging. 20 minutes post D-luciferin administration, the mice were imaged for 2 minutes. Imaging was carried out at 24h, 48h, 72h, 9 days, 14 days and 28 days post last gene transfer. Luminescence intensity was depicted in overlay images in false colour.

Histological examination

Gastrocnemius muscles were excised and fixed with 4% paraformaldehyde in PBS for 24h, dehydrated in 50% ethanol and stored in 70% ethanol at 4°C. Samples were embedded in paraffin without decalcification, and several sections of the same sample were stained with haematoxylin and eosin (H&E) and von Kossa staining for mineralization according to standard histology protocols.

Statistical Analysis

Results are represented as average±standard deviation (AVG±SD) unless otherwise stated. Statistical testing was carried out using the non-parametric Kruskal-Wallis test in conjunction with Dunn's multiple comparison test as post-test for ALP data.
Real-time PCR data passed normality tests and was tested with parametric ANOVA analysis followed by Turkey's multiple comparison test. Bone mineral density values for 14 days and 28 days passed normality tests and were evaluated with a two-tailed t-test for significance. $p<0,05$ was considered statistically significant.

Results

Constitutive co-expression systems *in vitro*

Osteogenic differentiation using single factor expression vs. Co-Expression

ALP assays (Figure 2) showed that the co-transfection of 2 individual pVAX1-BMP2 and pVAX1-BMP7 plasmids had the strongest osteoinductive capacity of the tested constitutive systems. The co-expression plasmid pVAX1-BMP2/7- (divergent organisation of expression cassettes, Figure 1D) showed moderate ALP expression (comparable to 100ng/ml recombinant BMP2), significantly elevated if compared to the negative control. pVAXBMP2/7+ (tandem organisation of expression cassettes, Figure 1C) displayed weaker ALP-induction and failed to induce ALP levels significantly higher than the negative control. Single factor expression via pVAX1-BMP2 or pVAX1-BMP7 did not induce significant up-regulation of ALP compared to the negative control, although low-level induction was observable in pVAX1-BMP2 transfected cells. The strongest induction of ALP was achieved in the recombinant growth factor controls in which 300ng/ml recombinant BMP2 homodimer and 100ng recombinant BMP2/7 heterodimer were applied. No induction of ALP was achieved in samples treated with 100ng/ml recombinant human BMP7.

Osteocalcin reporter transfected C2C12 cells showed highest expression of the fluorescent reporter in cultures that were super transfected with the constitutive BMP2/7 co-expression plasmid pVAX1-BMP2/7- (Figure 3D). The cultures furthermore displayed altered morphology compared to the negative control. Single factor delivery of pVAX1-BMP2 or pVAX1-BMP7 induced detectable reporter gene expression at lower EYFP positive cell numbers (Figure 3B, C). The frequency of reporter positive cells was slightly higher in BMP2 transfected cells compared to

BMP7 transfected cells. pVAX1-BMP2 transfected cells (Figure 3B) displayed an altered morphology compared to the negative control.

Inducible co-expression systems *in vitro*

Single vs. double vector TetON co-expression systems

Both Tet-inducible co-expression systems, the two-component (response+activator plasmid, Figure 1E) and the one component (response/activator plasmid, Figure 1F) system, displayed comparable, dose dependent activation of the EYFP and dsRed co-expression reporter genes 48h post treatment with a single dose of doxycycline (Figure 4, B&E, C&F). Basal expression was absent in inactive controls not treated with doxycycline (Figure 4, A&D).

The majority of reporter expressing cells emitted both, EYFP and dsRed fluorescence. Some exclusively EYFP or dsRed positive cells were observed in both groups, in particular in samples treated 250ng/ml doxycycline.

TetON co-expression kinetics

Fluorescence imaging of the inducible single vector co-expression plasmid pTetON-EYFP/dsRed for 10 days revealed doxycycline dose dependent expression kinetics (Figure 5). Expression was observable as early as 12h post administration of 250ng/ml or 1000ng/ml of doxycycline. A peak of fluorescence positive cells was observed at 48h post administration of a single dose of 1000ng/ml doxycycline. After 120h post administration lower numbers of cells were positive and exhibited exclusively dsRed fluorescence. This fluorescence decreased further on day 10.

No basal expression of EYFP or dsRed fluorescence was detected in control samples not induced with doxycycline.

Inducible osteogenic differentiation

Induction of osteogenic differentiation (ALP assays, Figure 2) by BMP2/7 co-expression showed a doxycycline dose dependency. Induction with 250ng/ml doxycycline stimulated expression of ALP at levels comparable to cells treated with 100ng/ml recombinant BMP2. 1000ng/ml of doxycycline triggered ALP expression to the similar levels as treatment with 100ng/ml recombinant BMP2/7 heterodimer or 300ng/ml recombinant BMP2 homodimer and was significantly higher than the negative control.

No induction of ALP expression was detectable in samples not treated with doxycycline. These findings were paralleled by morphology observations (Figure 6A-C) that revealed changes only in samples treated with 250ng/ml or 1000ng/ml of doxycycline.

TetON-BMP2/7 cells co-cultured with osteocalcin reporter transfected cells (Figure 6D-F) induced osteocalcin-specific fluorescence reporter gene expression upon treatment with doxycycline. Fluorescence accumulated in cellular clusters of differentiation.

Endogenous expression of osteocalcin, quantified by qPCR, increased significantly in TetON-BMP2/7 transfected cells upon treatment with increasing doses doxycycline (Figure 6G). A statistically significant, dose dependent difference in osteocalcin expression was observed between 250ng and 1000ng/ml doxycycline. Both induced groups exhibited significantly higher osteocalcin expression compared to the control without doxycycline.

In vivo gene transfer

Bioluminescence monitoring of gene transfer efficacy

Detection of luciferase expression (Figure 7) 24h post last plasmid injection confirmed gene transfer and expression for 8 out of 13 animals (gene transfer efficacy of 61%) treated. Luciferase expression levels were highly variable among animals, no specific peak was detected within the observed period of 28 days and some animals showed luciferase activity for more than 21 days (data not shown).

In vivo µCT Imaging

µCT images of hindlimbs at day 14 and day 28 (Figure 7) confirmed ectopic bone formation in *gastrocnemius* muscles after treatment with pVAX1-BMP2/7- for both time points in 6 out of 13 animals (46% bone formation efficacy). Furthermore, resorption and remodelling of ectopic bones was observable in 28-day images in comparison with the 14-day images. The ectopic bones displayed varying morphology, ranging from singular structures of notable size in some animals to several smaller centres of ossification in others. Total bone volumes were variable for both time points (14 days: 0,5903±0,2787mm3; 28 days: 0,2905±0,1658mm3). Bone mineral density significantly increased from 181,5±7,914mg/cm^3 hydroxyapatite (14 days) to 229,8±17,64mg/cm^3 (28 days) during the observed time period (Figure 8).

Histological examination

The formed ectopic bone structures displayed a compact layer of bone, including osteocytes and bone synthesizing lining osteoblasts, haematopoiesis and a bone marrow like lumen with progenitor cells and adipose cells observed in HE stained

samples (Figure 9 A, B). Von Kossa staining for mineralization confirmed ectopic structures as mineralized bone tissue (Figure 9C, D).

Discussion

The study presented herein aimed at designing and evaluating co-expression systems for the constitutive and inducible co-delivery of 2 different BMP genes for induction of osteogenic differentiation via non-viral gene therapy. Co-expression of BMP2 and BMP7 has been demonstrated to be more effective than single factor expression *in vitro*. This particular co-expression strategy displayed potent osteoinductive capacity, with a potency comparable to the application of recombinant growth factors *in vitro.* The potency of the differentiation induced by expression from constitutive single molecule co-expression plasmids was dependent on expression cassette topology and associated potential transcriptional interference.

Furthermore, it was possible to demonstrate the feasibility of applying this strategy in an inducible fashion, triggering the expression of 2 transgenes simultaneously in a doxycycline dependent manner. This system enabled tight control of BMP expression and induction of differentiation *in vitro*, being silent in the non-induced state. *In vivo* ectopic evaluation of the constitutive co-expression system with convergent orientation of expression cassettes clearly demonstrated an osteoinductive potential. Repeated injection into mouse hindlimb muscles was leading to formation of ectopic bone in 46% of treated animals after 5 injections of only 20µg therapeutic DNA per day, displaying similar efficacy at lower daily and total doses when compared to approaches using BMP2 gene delivery only (Osawa *et al.*, 2010).

In vitro evaluation

Constitutive co-expression systems

Multi-gene approaches, in which more than one therapeutic gene is expressed simultaneously, can enhance efficacy of gene therapies *in vivo.* Therefore, a plasmid based constitutive co-expression system was investigated for the correlated co-delivery of BMP2 and BMP7 genes.

BMP2/BMP7 co-expression was chosen as a proof of concept in this study given its high osteoinductive potential *in vitro* and *in vivo* (Kawai *et al.*, 2006a; Zheng *et al.*, 2010; Zhu *et al.*, 2004), which has been shown to trigger expression of heterodimeric BMP2/7 (Kawai *et al.*, 2009), a growth factor with high bioactivity (Israel *et al.*, 1996; Zheng *et al.*, 2010). Higher osteoinductive potential of BMP2/7 co-delivery in comparison with single gene delivery has been confirmed by ALP assays and osteocalcin reporter gene assays *in vitro* in this work. It has been observed that the co-transfection of individual BMP2 and BMP7 encoding plasmids leads to a higher osteoinductive stimulus *in vitro* when compared to single co-expression plasmid delivery. Lower activity of the single plasmid co-expression at an even higher dose (1 BMP2=1 BMP7 pmoles expression cassettes; 2µg/well) compared to higher activity at a lower dose (0,77 BMP2+ 0,77 BMP7 pmoles expression cassettes; 1µg+1µg/well) of individual expression plasmids strongly suggests interference phenomena impairing expression in the constitutive CMV-based co-expression systems.

Therefore, the influence of transcriptional interference phenomena (Shearwin *et al.*, 2005) needs to be taken into account when working with multiple expression cassettes. CMV-enhancer elements were shown to compete for transcription initiation complex formation in plasmids, through interference of their respective enhancer

elements (Andersen *et al.*, 2011), which might partially account for the lower expression efficacy and osteoinductive potential of both constitutive co-expression systems *in vitro.* However we observed better osteoinduction with the divergent promoter system compared to tandem organization indicating the presence of stronger downstream interference phenomena in the tandem configuration. Given the limited predictability of transcriptional interference and the potential compound effect of multiple forms of transcriptional interference on one specific construct, such effects have to be elucidated empirically (Curtin JA, 2008) with different cassette arrangements as demonstrated in the current study.

Given that BMP-heterodimer formation relies on BMP dimerization within the endoplasmatic reticulum of the cell (Degnin *et al.*, 2004), co-transfection of a single molecule is still preferable, when aiming at heterodimer production for maximum bioactivity. Co-transfection efficacy and balanced expression of 2 transgenes *in vitro* has been demonstrated to be highly variable (Schwake *et al.*, 2010) with only a low number of cells positive for both transgenes when using individual plasmids for co-transfection, thus indicating a potential advantage of delivering 2 transgenes via single vector strategies (Kerrigan *et al.*, 2011).

Inducible co-expression systems

Regulation of therapeutic gene expression levels and kinetics allows controlling of optimal dosing and can help to mimic physiological gene expression patterns. Furthermore, bi-directional inducible systems (Baron U, 1995) can deliver 2 transgenes simultaneously under the control of an inducible bidirectional promoter. These systems, however, require the co-transfection of an activator plasmid, expressing the doxycycline responsive transactivator protein required for expression

from the response plasmid. This situation represents another setup, where co-delivery of all transcriptional on the same molecule might offer and advantage over simple co-transfection of 2 individual plasmids. Therefore, we investigated whether modification of the original 2-component system to a single plasmid system would substantially alter the systems performance. The comparison of fluorescence microscopy images of doxycycline controlled co-expression of EYFP and dsRed from the original 2-component system and the novel single molecule system, showed no substantial differences in expression levels as well as well as in background expression without induction. This suggests that the novel single molecule TetON co-expression system performs in a similar way as the original system.

Using fluorescence-microscopy, it was possible to demonstrate that EYFP and dsRed fluorescence peaks at 48h post administration of doxycycline and fluorescence persists for up to 10days in the case of dsRed. The devised single vector TetON system responded to doxycycline with expression of functional fluorescent proteins as early as 12h post induction, a substantial drop of positive cells already after 120h and no basal expression without addition of doxycycline. These findings are in line with the kinetic data obtained in similar studies ((Puttini *et al.*, 2001) where maximal expression was obtained with 1000 ng/ml doxycycline at 48h and returned to the off-state within 36h. Individual turnover rates of the employed reporter genes need to be taken into account for kinetic data and indeed it has been shown Tet-regulated gene expression studied with enhanced high turnover reporter constructs (Voon *et al.*, 2005) responds even faster (within 4h) than detectable with common reporters. We therefore conclude according to the mentioned studies and our findings that transcriptional activation of Tet-regulated co-expression can be very fast within few hours and that gene regulation turns effective with a specific delay accountable to mRNA half life, protein maturation time and turnover rate (Voon *et al.*,

2005). In our experiments using EYFP (maturation time: 8-12h; half life: ~24hrs, (Clontech protocol PT2040-1, Clontech application note 2003)) and dsRed (maturation time: 8-12h; half life: ~4,5 days, (Mirabella *et al.*, 2004; Verkhusha *et al.*, 2003)), kinetics were in the range of 12h-48h for EYFP and 12-240h for dsRed, indicating an approximate effective therapeutic time window of 5 days with a single dose of doxycycline *in vitro* for protein expression.

Co-delivery of BMP2 and BMP7 with this expression strategy enabled tightly inductor controlled expression, which lead to subsequent differentiation. The extent of osteogenic differentiation, as determined by microscopy, ALP assays, osteocalcin reporter gene assay and quantitative real-time PCR clearly indicated at direct link between doxycycline dose and differentiation outcome. 1000ng/ml induced cultures showed the most potent osteoinduction of all tested systems *in vitro* and no induction of osteogenic differentiation was observed without induction with doxycycline. Tightly regulated gene co-expression was observed after transfection of 1 singular plasmid unit *in vitro*, narrowing the effective time window of BMP2/7 co-expression down to approximately 4 days for C2C12 cells. This system will provide a useful tool in future *in vivo* studies to demonstrate control over *in vivo* bone formation via doxycycline triggered gene expression and to determine minimally required therapeutic BMP-expression time windows for bone induction.

***In vivo* gene transfer**

Intramuscular gene transfer by naked plasmid injection is a method already used for a long time *in vivo* (Herweijer and Wolff, 2003) even clinically (Rauh *et al.*, 2001) and muscle tissue is an excellent ectopic environment for preliminary bone formation assays (Scott *et al.*, 2012) as well as for the generation of transplantable bone in clinical applications (Warnke *et al.*, 2004). It has previously shown that repeated non-viral BMP2 gene transfer can induce bone formation at ectopic sites *in vivo* (Osawa *et al.*, 2010) with fair efficacy depending on the amount of repetitive treatments. BMP2/7 co-delivery has already been shown to outperform BMP2 delivery after electroporative gene transfer *in vivo* and is perceived as an ideal gene combination for osteoinductive gene therapies (Kawai *et al.*, 2006b; Zhao *et al.*, 2005; Zhu *et al.*, 2004).

In line with these studies we tested how the constitutive pVAX1 BMP2/7 co-expression plasmid would perform in an *in vivo* test for ectopic bone formation using passive DNA delivery. Ideally by using this BMP2/7 co-expression strategy, we aimed at generating results comparable to the work of Osawa *et al.* at a reduced number of repetitive treatments (5 vs. 8) and lower total (500 vs. 100µg) and daily DNA dose (20µg vs. 62,5µg). Furthermore, to enable non-invasive gene expression monitoring, we incorporated an internal luciferase control plasmid. The bone formation efficacy of 46% observed in this study is within the range of efficacy reported by Osawa *et al.* for repeated BMP2 gene delivery, where 4 injections of 125µg BMP2 DNA led to 57,1% and 8 injections of 62,5µg BMP2 DNA led to 62,5% bone formation. Therefore, we conclude that BMP2/7 co-expression *in vivo* induces bone formation at lower DNA doses to a similar extent as BMP2 passive delivery. Direct comparison of BMP2 with BMP2/7 co-expression *in vivo* by Kawai *et al.* using intramuscular

electroporation already clearly demonstrated the higher osteoinductive potential of BMP2/7 co-delivery and thus supports this interpretation (Kawai *et al.*, 2006b).

Similar results for gene transfer efficacy estimation based on luciferase expression (61% positive, 8/13 animals) and bone formation (46%, 6/13 animals) demonstrated fair gene transfer efficacies and the feasibility of employing an internal luciferase control in gene transfer studies. However, if compared to previous unpublished single-injection data of only luciferase plasmids from our group, the luciferase levels observed herein are lower than expected after gene transfer *in vivo* if co-delivered with the therapeutic plasmid. This could indicate that competitive effects (Ngo *et al.*, 1993) between two independent expression systems (CBR luciferase system & BMP2/7 co-expression system) can occur *in vivo* thus potentially hampering luciferase expression monitoring or therapeutic efficacy and are currently under investigation *in vitro* and *in vivo*. The generally high variability of luciferase expression and bone volumes among animals within this study, however, should be mainly attributed to the plethora of parameters that affect passive naked DNA intramuscular gene delivery and its variability (Wolff and Budker, 2005 Wolff, 1991 #21), which are difficult to control, especially in a treatment protocol that relies on multiple injections per site. Injection volumes and injection speed have been shown to influence transgene expression *in vivo* (Andre *et al.*, 2006) potentially through additional hydrodynamic poration, which can substantially increase DNA uptake in addition to the postulated default receptor-mediated endocytosis of DNA in muscle (Satkauskas *et al.*, 2001) mainly responsible for intramuscular naked DNA transfer.

Another caveat of passive BMP gene transfer is that the employed DNA doses are still relatively high (~3mg/kg for BMP2/7 up to ~15mg/kg for BMP2 delivery) and that a single injection of BMP-plasmids is not sufficient in order to obtain efficient expression for ectopic bone formation (Osawa *et al* . and own unpublished data).

Therefore, given that passive intramuscular gene transfer is difficult to control and still requires multiple treatments, it might be of higher practical use to introduce an additional, preferably non-invasive physical gene transfer method in future studies. This could increase and unify global transfection efficacy while reducing the total DNA amount applied. Sonoporation (Osawa *et al.*, 2009; Zhang X, 2006) and electroporation (Kusumanto *et al.*, 2007; McMahon and Wells, 2004) are such minimally invasive methods employing naked DNA without chemical agents that can provide increased transfection efficacy *in vivo* and indeed electroporation has already been used for the delivery of BMP2/7 co-expression plasmids *in vivo* (Kawai *et al.*, 2006a; Kawai *et al.*, 2009).

The developed, inducible and constitutive BMP2/7 co-expression plasmids are useful tools for investigating the potential of non-viral gene therapeutics and the results obtained with these systems herein provide further evidence for the feasibility of developing co-expression based gene therapeutics, paving the way for further preclinical research on the performance of BMP2/7 co-expression in orthotopic models for functional bone regeneration. Gene transfer efficacy, consistency and expression vector design are variables critically influencing success of such strategies and still require further improvement in future studies.

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Figures

Primer Name	Sequence (5' to 3')	Purpose
hBMP2fls	GCCACCATGGTGGCCGGGACCCGCTGT	BMP2 cloning
hBMP2flas	CTAGCGACACCCACAACCCTC	
hBMP7fls	GGGAAGCTTGCCACCGCGATGCACGTGGCG	BMP7 cloning
hBMP7flas	TTTGCGGCCGCTGCTGCTCATGTTTCCTAATA	
TetONBsp-F	TACACATGTGGCACATGTCCAACATTACCGCCATGTTGAC	rtTA cloning
TetONBsp-R	GCAACATGTTTGGTCGAGCTGATACTTCCCGTCC	
TREtighBspOUT	CTGGCCTTTTGCTGGCCTTTTGC	single system
TREtightBspIN	CAGAATCAGGGGATAACGCAGG	PCR mapping
hBMP7NdeIs	TACCATATG GCCACCATGCACGTGCGCTCACTGCGAGC	BMP7 subcloning
hBMP7BglIIas	AGATCTCTAGTGGCAGCCACAGGCCCGGACCACC	to pTREtight-BI-MCSII
NheIdsRed sense	TAC GCTAGC GCCACCATGGCCTCCTCCGAGG	BMP7 subcloning
HindIIISTOP dsR as	AAGCTTCTATTACAGGAACAGGTGGTGGCGGCC	to pTREtight-BI-MCSI
pVAXBIas	ACATGTCGATGTACGGGCCAGATATACGC	BMP7 casette subcloning
pVAXBIs	ACATGTGTTCGCTTGCTGTCCATAAAACC	in pVAX1-BMP2
pVAXBspIN	GAAATGTGCGCGGAACCCCTATTTG	pVAX-BMP2/7 PCR mapping
pVAXBspOUT	CTGACGCTCAGTGGAACGAAAACTC	
qHPRT_as	TGGCCTCCCATCTCCTTCAT	qPCR for HPRT transcript
qHPRT_s	AGTCCCAGCGTCGTGATTAG	
qOC2_as	CTGCTTGGACATGAAGGCTTTG	qPCR for osteocalcin transcript
qOC2_s	GAACAGACAAGTCCCACACAG	

Table1: Primer designations and sequences used in this study.

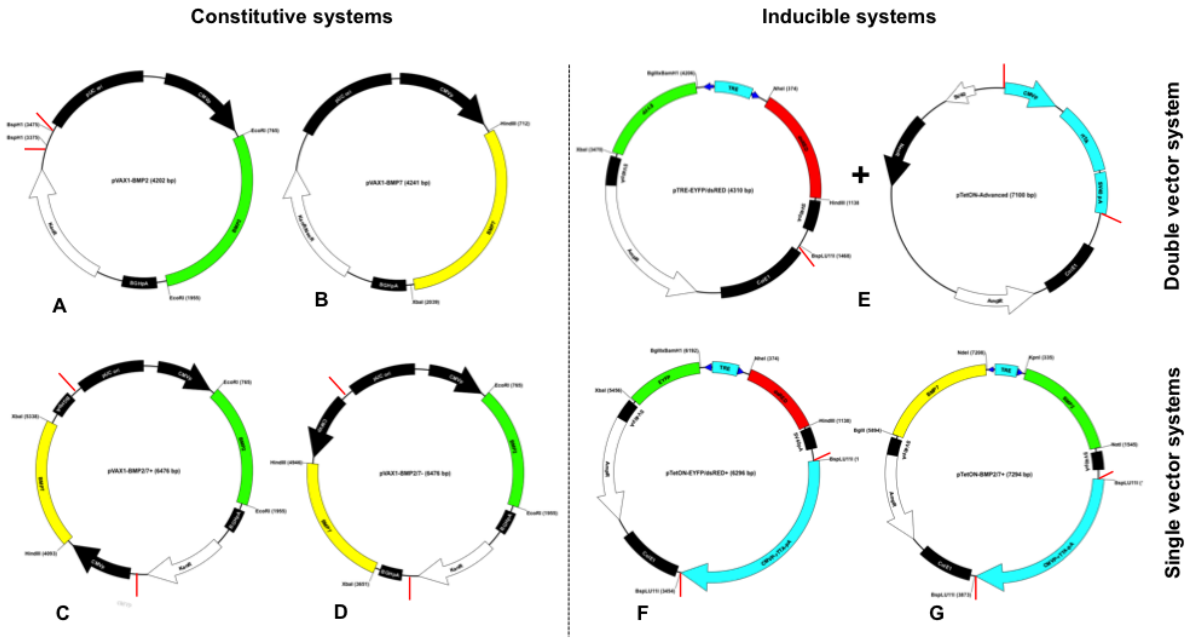


Figure 1 – Constitutive and Inducible Expression Plasmids used in this study.

Constitutive systems (A-D): BMP2 expression plasmid pVAX1-BMP2 (A), BMP7 expression plasmid pVAX1-BMP7 (B), BMP2/7 co-expression plasmid with BMP7 cassette in tandem orientation, pVAX1-BMP2/7+ (C), BMP2/7 co-expression plasmid with BMP7 cassette in inverted orientation, pVAX1-BMP2/7-. BMP2 cDNA sequence (green), BMP7 cDNA (sequence (yellow), CMV-promoter (CMVp, black arrow), bovine growth hormone poly-adenylation signal (BGHpA, black box), pUC origin of replication (pUC ori, black box), Kanamycine resistance gene (KanR, white arrow). Target BspH1 target sites for transfer of BMP7 cassette into pVAX1-BMP2 and borders of integrated cassette marked in red.

Inducible systems (E-G): Dual component vector system (E): Response co-expression plasmid pTRE-EYFP/dsRed and activator plasmid pTetON-Advanced. Single component vector systems: Response/activator EYFP/dsRed co-expression plasmid pTetON-EYFP/dsRed+ (F), Response/activator BMP2/7 co-expression plasmid pTetON-BMP2/7+. EYFP cDNA (green), dsRed cDNA (red), Tet response element (TRE, light blue box), minimal bidirectional CMV promoters (blue arrows), reverse tet-transactivator (rtTA) expression cassette (light blue elements): CMV promoter

(CMVp), rtTA cDNA (rtTA), bovine growth hormone poly adenylation signal (BGHpA), ColE1 *E.coli* origin of replication (ColE1, black box), Simian virus 40 (SV40) promoter (SV40p, white arrow), SV40 poly-adenylation signal (SV40pA, black box), ampicillin resistance gene (AmpR, white arrow), neomycin resistance cassette (NeoR, black arrow). BspLU11I target site for rtTA expression cassette transfer into pTRE-EYFP/dsR (analogue site exists in pTRE-BMP2/7 precursor) and borders of integrated cassette depicted in red.

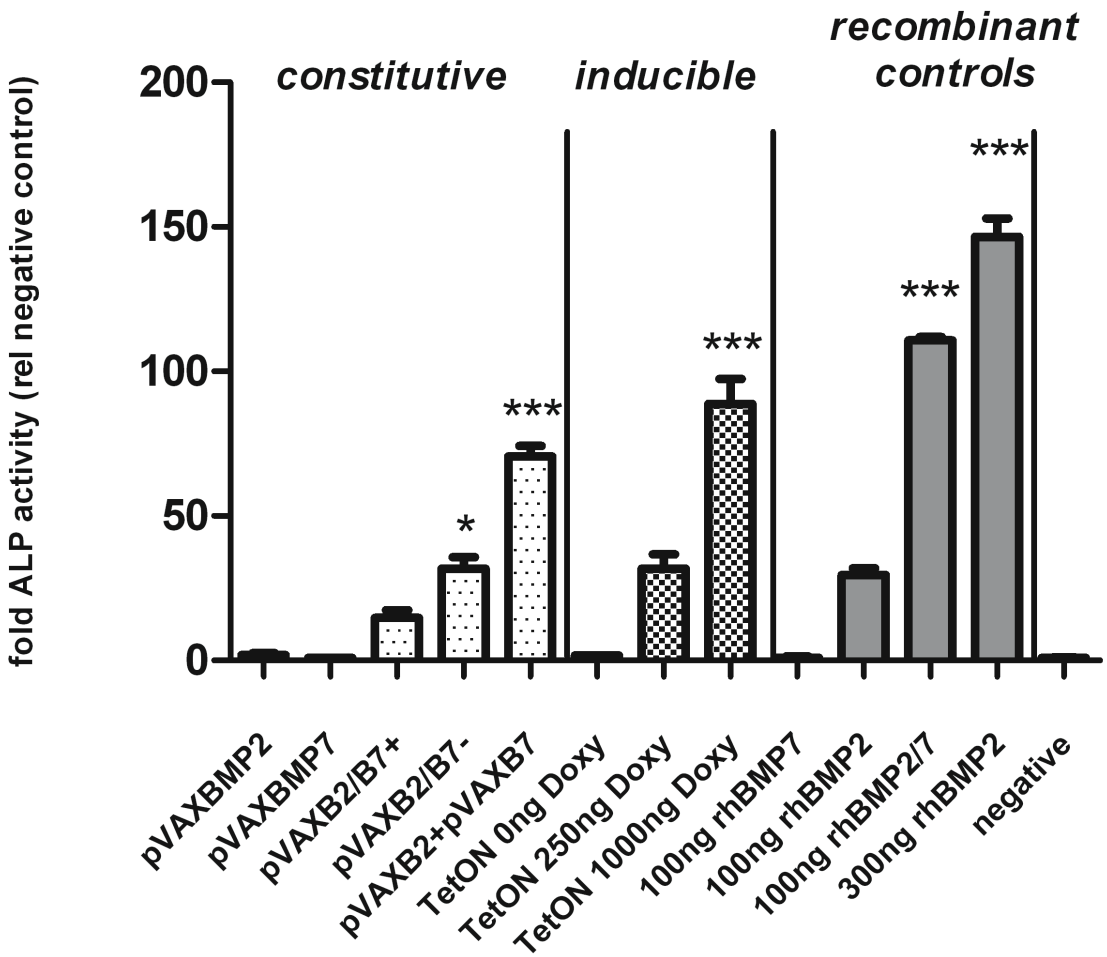


Figure 2: Enzymatic alkaline phosphatase (ALP) assays of transfected C2C12 cells, 6 days post transfection. ALP activation in fold change relative to negative (non-transfected control, n=10).

Constitutive systems: BMP2 transfected cells (pVAX1-BMP2, n=4), BMP7 transfected cells (pVAX1-BMP7, n=4), cells transfected with BMP2/7 co-expression plasmid with inverted topology of expression cassettes (pVAX1-BMP2/7+, n=10), cells transfected with BMP2/7 co-expression plasmid with tandem

topology of expression cassettes (pVAX1-BMP2/7-, n=10), Co-transfection of 2 individual BMP2 and BMP7 expression plasmids (pVAX1-BMP2+pVAX1-BMP7, n=10).

Inducible systems: ALP activity of C2C12 transfected with the inducible BMP2/7 co-expression system without doxycycline (pTetON-BMP2/7 0ng Doxy, n=4), transfected cells induced with 250ng/ml doxycycline (TetON-BMP2/7 250ng Doxy, n=4), transfected cells induced with 1000ng/ml doxycycline (TetON-BMP2/7 1000ng Doxy, n=15).

Recombinant Controls: ALP activity of positive controls stimulated with recombinant human BMPs (rhBMP), 100ng/ml recombinant BMP7 homodimer (100ng rhBMP7, n=4), 100ng/ml recombinant BMP2 homodimer (100ng rhBMP2, n=4), 100ng/ml recombinant BMP2/7 heterodimer (100ng rhBMP2/7, n=4), 300ng/ml recombinant human BMP2 homodimer (300ng rhBMP2, n=10). Average ± standard error of the mean (AVG±SEM).

Parts of the data in this graph is have already been established in G. A. Feichtinger's Master Thesis in 2008 (http://othes.univie.ac.at/744/, AC# AC07147280).

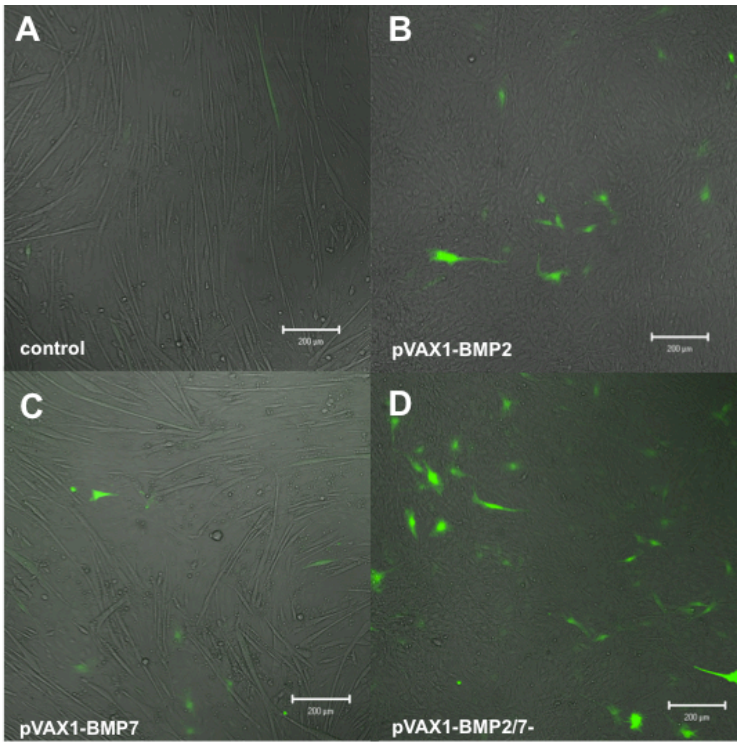


Figure 3: Fluorescence microscopy based pCMVE/mOCP-EYFPHis osteocalcin reporter gene assay with constitutive co-expression systems. Transiently transfected osteocalcin reporter cells without supertransfection (A), pVAX1-BMP2 supertransfected, BMP2 expressing reporter cells (B), pVAX1-

BMP7 supertransfected, BMP7 expressing reporter cells (C), pVAX1-BMP2/7- supertransfected, BMP2/7 co-expressing reporter cells (D). Scale bars represent 200µm.

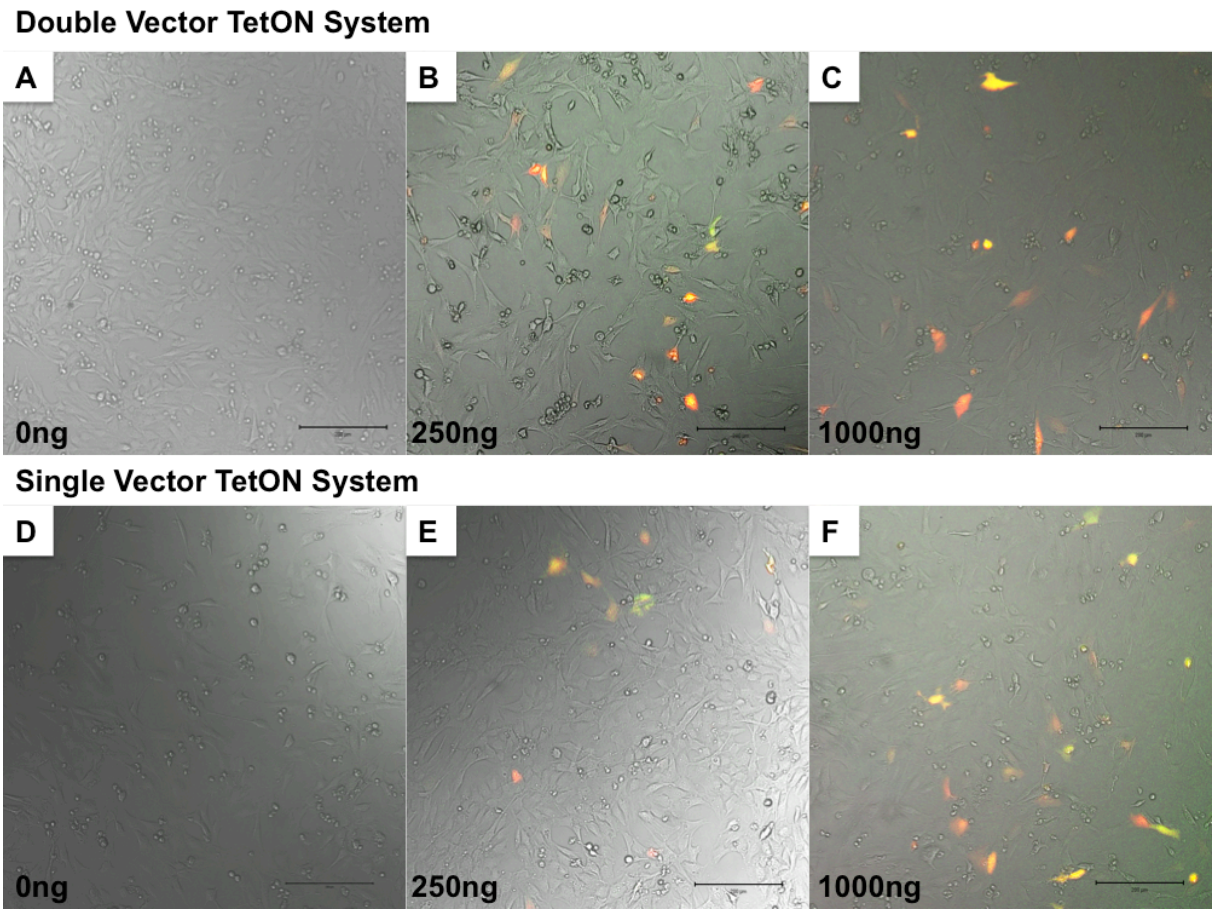


Figure 4: Inducible EYFP/dsRed co-expression systems.

Fluorescence microscopy of EYFP/dsRed expression of the dual component TetON system pTRE-EYFP/dsRed+pTetON-Advanced (4A-C) and the single component response/activator EYFP/dsRed co-expression system pTetON-EYFP/dsRed (4D-F). Scale bars represent 200µm.

Parts of the data in this graph is have already been established in G. A. Feichtinger's Master Thesis in 2008 (http://othes.univie.ac.at/744/, AC# AC07147280).

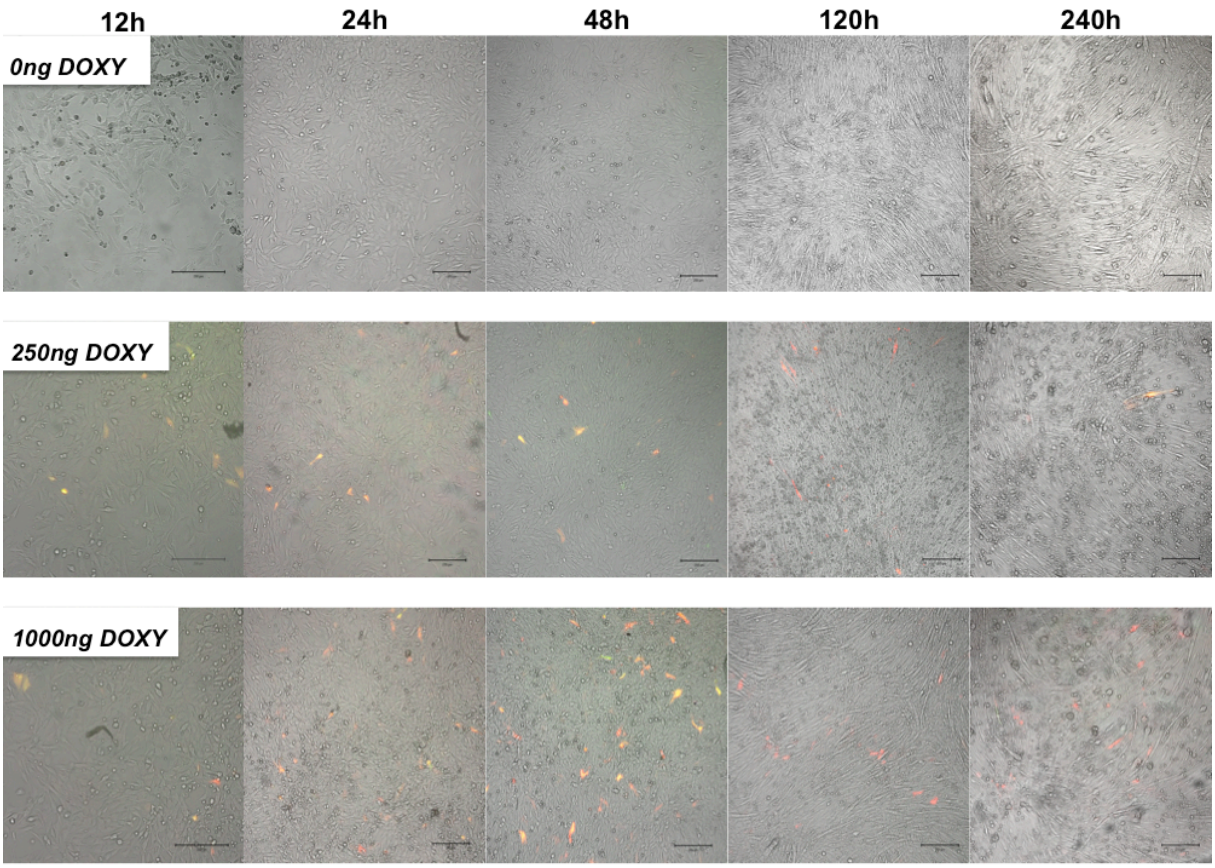
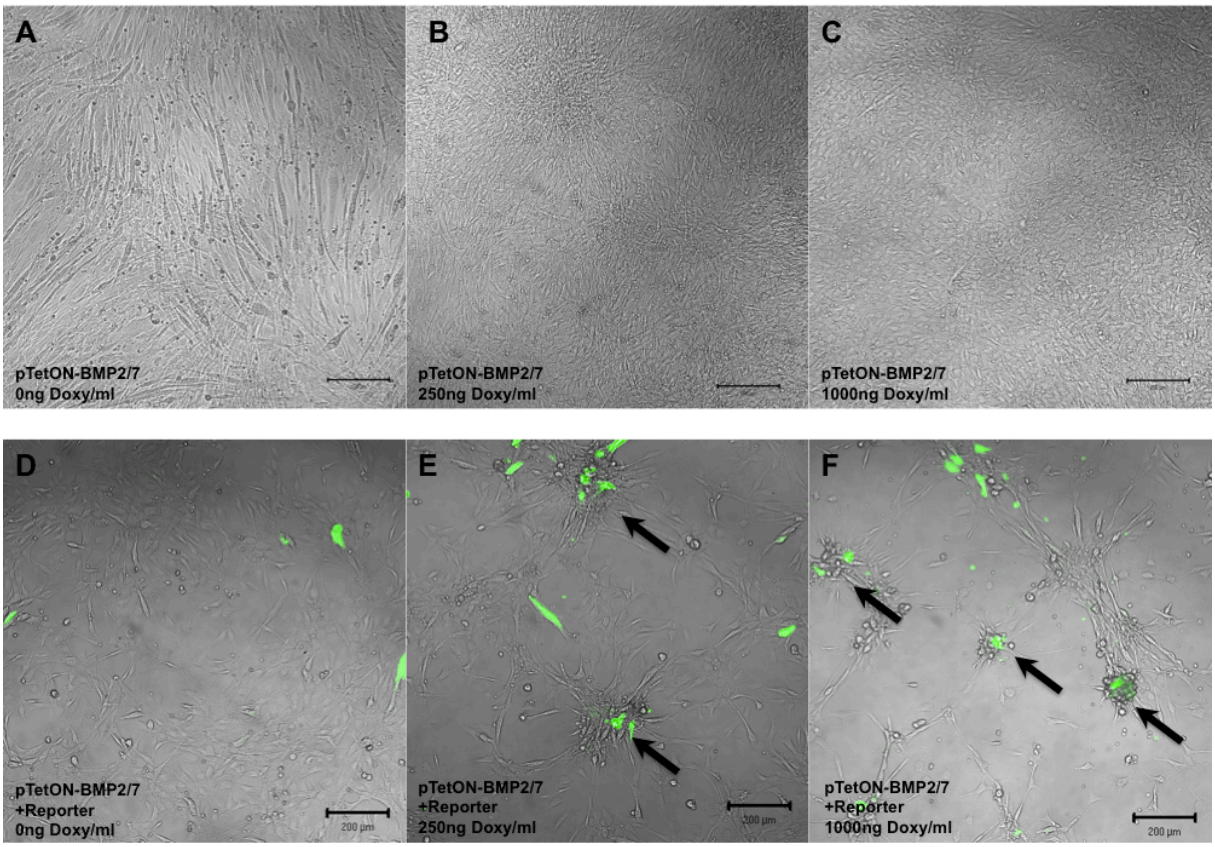


Figure 5: Fluorescence microscopy of EYFP/dsRed co-expression kinetics of the response/activator EYFP/dsRed co-expression system pTetON-EYFPdsRed after application of a single dose of doxycycline (Doxy), 0ng/ml Doxy control (0ng Doxy/ml), 250ng/ml Doxy induction (250ng Doxy/ml), 1000ng/ml Doxy induction (1000ng Doxy/ml). Scale bars represent 200µm.

Parts of the data in this graph is have already been established in G. A. Feichtinger's Master Thesis in 2008 (http://othes.univie.ac.at/744/, AC# AC07147280).



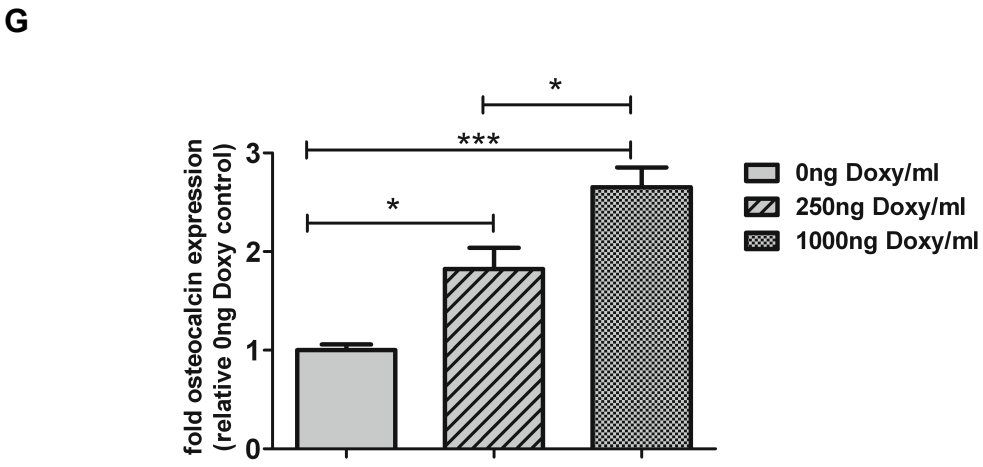


Figure 6: (A-C) Morphology of C2C12 cells transfected with the inducible response/activator BMP2/7 co-expression plasmid (pTetON-BMP2/7), 6 days post transfection. Control cells without doxycycline (A, 0ng Doxy/ml), cells induced with 250ng/ml doxycycline (B, 250ng Doxy/ml), cells induced with 1000ng/ml doxycycline (C, 1000ng Doxy/ml). Scale bars represent 200µm.

(D-F): Co-culture experiment with pCMVE/mOCP-EYFPHis osteocalcin reporter transfected cells and inducible response/activator BMP2/7 co-expression plasmid (pTetON-BMP2/7) transfected cells (1:1).

Reporter signal in co-cultures without induction of BMP2/7 co-expression (D, 0ng Doxy/ml), reporter signal in co-cultures after induction with 250ng/ml doxycycline (E, 250ng Doxy/ml), reporter signal in co-cultures after induction with 1000ng/ml doxycycline (F, 1000ng Doxy/ml). Arrowheads mark cell clusters with reporter gene expression. Scale bar represents 200µm.

(G) Endogenous osteocalcin expression 6 days after transfection with the inducible response/activator BMP2/7 co-expression plasmid (pTetON-BMP2/7). Fold induction of endogenous osteocalcin expression depicted as fold changes relative to control cells (no doxycycline addition) normalized to hypoxanthine-guanine phosphoribosyltransferase housekeeping gene expression. Fold expression in control cells (0ng DOXY) Fold expression after induction with 250ng/ml doxycycline (250ng DOXY) and fold expression after induction with 1000ng/ml doxycycline (1000ng DOXY). n=6, Average±standard error or the mean (AVG±SEM) p<0,05 (*), p<0,001(***).

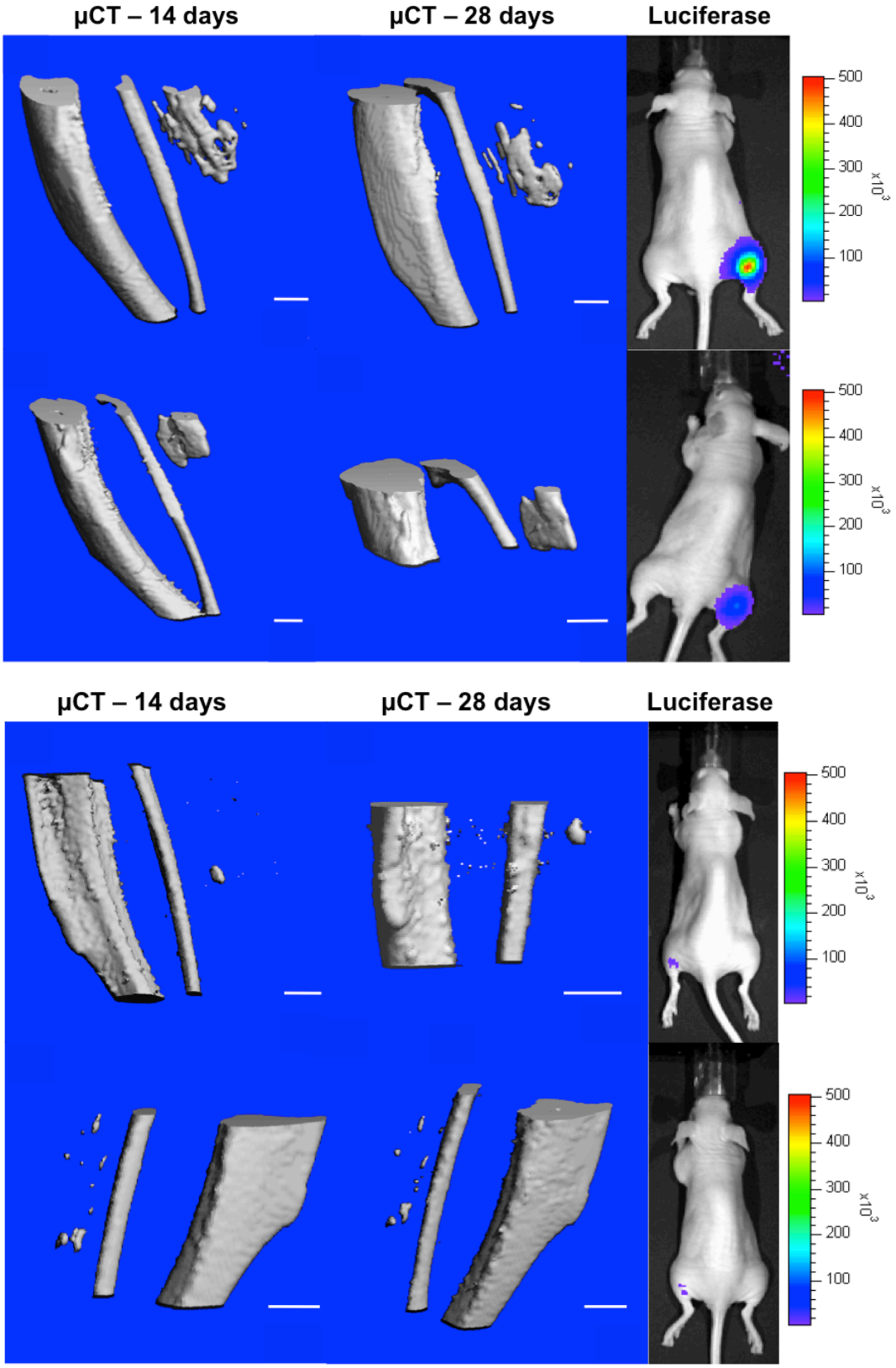


Figure 7: Representative *in vivo* µCTscans of ectopic bone formation at 14 days and 28 days of 4 representative cases after non-viral in vivo gene transfer of the constitutive BMP2/7 co-expression plasmid pVAX1-BMP2/7-. Scale bar represents 1mm.

Representative luciferase activity as observed by *in vivo* bioluminescence imaging 24h post last injection (day 6 post first injection). Colour scale represents emitted photons/sec.

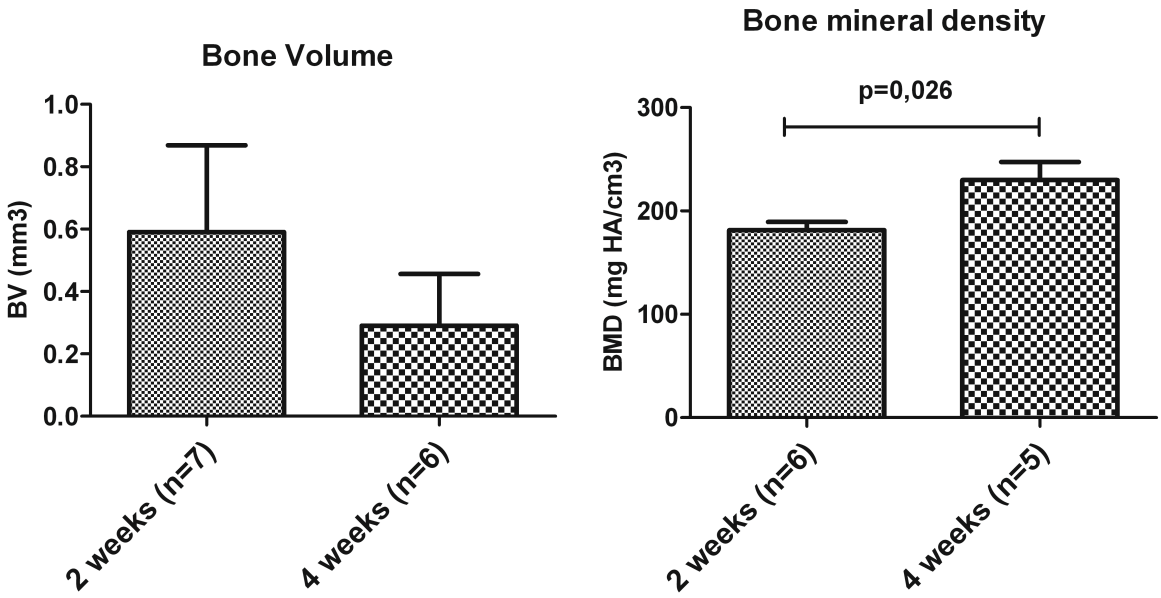


Figure 8: Bone volume (BV, mm3) and Bone mineral density (BMD, mg hydroxyapatite/cm3) values for ectopic bones, 18 days and 28 days post treatment. Average±standard error of the mean (AVG±SEM).

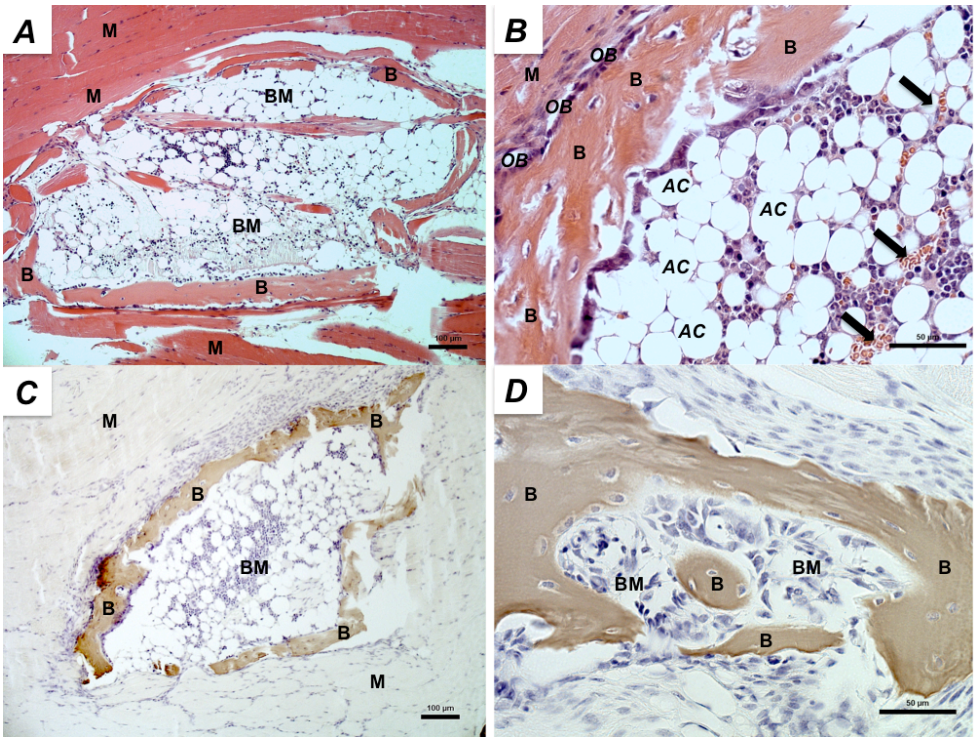


Figure 9: Histology of ectopic bone in *gastrocnemius* muscles.

Haematoxylin/Eosin (HE) staining (A, B): Muscle tissue (M), Bone tissue (B), Bone marrow (BM), Adipocytes (AC), Haematopoiesis is depicted by black arrows (B). Scale bars represent 100µm (A) or 50µm (B) respectively. Von Kossa staining for mineralization (C, D): Muscle tissue (M), Bone tissue (B), Bone marrow (BM). Nuclei counterstained with haematoxylin (Mayer). Scale bars represent 100µm (C) or 50µm (D) respectively.

Chapter III - Evaluation of non-viral BMP2/7 *in vivo* gene transfer for ectopic and orthotopic osteoinduction

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Short title: Evaluation of BMP2/7 gene therapy for bone regeneration

Abstract

An ideal treatment for critical size bone defects should provide regeneration without autologous or allogenous grafting, exogenous cells, growth factors or biomaterials. Therefore, a novel osteoinductive non-viral sonoporation gene therapy was investigated in ectopic and orthotopic models. Constitutive or doxycycline-inducible bone morphogenetic protein 2 and 7 co-expression plasmids were repeatedly applied for 5 days. Ectopic and orthotopic gene transfer efficacy was monitored by co-application of a luciferase plasmid and bioluminescence imaging. Luciferase imaging and ectopic bone formation assays demonstrated increased gene transfer and bone formation efficacy for sonoporative gene transfer compared to passive gene delivery. Inducible therapeutic gene expression was stringently controlled by doxycycline and no ectopic bone formation was observed without induction. Orthotopic gene transfer was demonstrated using bioluminescence imaging and DNA distrubution was investigated using a novel plasmid labeling method. Orthotopic evaluation in a rat femur non-union model demonstrated increased gene transfer efficacy using sonoporation. Investigation of DNA distribution demonstrated extensive binding of plasmid DNA to bone tissue. Sonoporated animals displayed an increased fracture union rate (33%) without extensive callus formation or heterotopic ossification. We conclude that sonoporation of BMP2/7 co-expression plasmids is a feasible, minimally invasive method for osteoinduction & improvement of orthotopic bone regeneration.

Keywords: Sonoporation, non-viral, gene therapy, heterodimer, bone morphogenetic protein, TetON, inducible, ectopic, orthotopic

Introduction

Traumatic fractures can lead to defects of critical size that can fail to regenerate completely, depending on the fracture site and general clinical situation of the patient [1]. These non-union fractures, also termed pseudoarthroses, are characterized by a failure to heal within 8-9 months [1, 2]. Such a situation represents a major clinical challenge, which is still not addressed adequately by conventional treatment. Autologous bone grafting from the iliac crest can provide satisfying regenerative outcome, although at the cost of an invasive procedure requiring surgical intervention causing donor site morbidity [3-5]. Other experimental treatment modalities include combinations of autologous stem cell treatment, biomaterials and growth factors [2, 6-11]. Therapies that rely on stem cell treatment require invasive sampling and *in vitro* expansion of these cells prior to implantation, which again can lead to donor site morbidity and furthermore require expensive GMP facilities for safe cell enrichment and expansion [12]. Furthermore, these procedures hold the inherent drawback of currently failing to be applied in a minimally invasive one-step approach. Recombinant growth factors on the other hand, are expensive in production [13, 14] and interestingly need to be applied in potentially dangerous high supraphysiological dosing [15], which can lead to adverse effects such as heterotopic ossification [16] or immune responses [17]. A novel therapeutic approach should therefore enable a cost-effective, efficient and minimally invasive treatment of non-union fractures. It should preferentially be applied in a one-step procedure without the need for autologous or allogenous implantation material. Several studies have demonstrated the feasibility of transient gene therapies for osteoinduction either through *ex vivo* [15, 18, 19] or *in vivo* viral [20-22] or non-viral gene transfer [21, 23-26]. The main advantage of this approach is that endogenous cells are forced to express an

osteoinductive factor *in situ* directly at the fracture site, leading to correct post-translational modifications and conformation of the factor, thereby mediating higher bioactivity at lower concentrations compared to application of recombinant factors [27, 28]. Since non-viral *in situ* gene transfer has a much better safety profile than viral modalities, it was selected as gene transfer modality within this study. Ultrasound mediated gene transfer, a relative novel non-viral strategy that relies on neutral microbubble contrast agent mediated cell permeabilisation *in situ* to mediate uptake of plasmid DNA [29-32], appears to be superior in terms of reduced invasiveness and clinical translation as compared to other non-viral methods such as electroporation [33, 34]. In order to compensate for lower efficacy of this non-viral gene transfer method, a highly osteoinductive co-expression strategy for bone morphogenetic protein (BMP) 2 and 7 (BMP2/7), which has been shown to potently mediate osteogenic differentiation *in vitro* [25, 35] and *in vivo* [25, 36, 37], was selected to be investigated for its regenerative potential in a femur non-union model in rats. The work presented herein, aimed at demonstrating the feasibility of an ultrasound mediated orthotopic BMP2/7 co-expression strategy for enhanced fracture regeneration, solely relying on minimally invasive injection of a BMP2/7 co-expression plasmid DNA/microbubble mixture in conjunction with a transcutaneously applied ultrasound trigger to mediate deep within tissue *in vivo* non-viral transient gene transfer.

Materials and Methods

Animals

The animal protocol review board of the City Government of Vienna, Austria approved all experimental procedures in accordance with Austrian law and the Guide for the Care and Use of Laboratory Animals as defined by the National Institute of Health. Female Hsd:Athymic Nude-Foxn1^{nu} nude mice (Harlan Laboratories, Bresso, Italy) of approx. 12 weeks age weighing approx. 30g were used for ectopic testing in this study.

Male Sprague-Dawley rats (Charles River, Wilmington, MA, US) weighing approx. 450g were used for orthotopic testing in this study.

Plasmids

The inducible and constitutive single-vector BMP2/7 co-expression plasmids, pTetON-BMP2/7 and pVAX1-BMP2/7-, are described elsewhere (Feichtinger *et al.* 2012, in submission). Briefly, the constitutive pVAX1-BMP2/7- system was created by using a multi-expression cassette strategy with separate CMV-promoter driven BMP2 and BMP7 expression cassettes in divergent orientation on the plasmid backbone of pVAX1 (Life Technologies, Palo Alto, CA, US). The single-vector TetON inducible BMP2/7 co-expression system pTetON-BMP2/7 was derived from the Tet-regulated co-expression system pTREtight-BI (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) by cloning BMP2 and BMP7 cDNAs into the multiple cloning sites and additionally transferring the entire Tet-transactivator expression cassette from pTetON-Advanced (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) to

pTREtightBI, generating a TetON inducible expression plasmid with 3 transcriptional entities. All plasmid preps were carried out using Endo-free plasmid Maxi or Giga kits (Qiagen, Hilden, Germany) and verified by restriction digests (data not shown) prior to *in vivo* application.

Sonoporator

A SP100 Sonoporator (Sonidel Ltd, Dublin, Ireland) emitting 1MHz ultrasound was employed for sonoporative gene transfer in this study.

The columnar beam was set at different Watt/cm^2 spatial average temporal peak settings with an effective radiating area of 0.8 cm^2 at a pulse frequency of 100Hz/100% duty cycle.

Sonoporation protocols

2 Watt/cm^2 treatment protocol: This protocol was a modified protocol derived from the ultrasound parameters applied in [32]. Daily treatment, which was repeated for 5 days consisted of application of 2 Watt/cm^2 1MHz ultrasound at a duty cycle of 25% for 3 minutes after injection of a plasmid DNA/microbubble contrast agent mixture. Total energy delivered to the target site per daily treatment was 90 Joule/cm^2.

4 Watt/cm^2 treatment protocol: This protocol was a modified protocol derived from [38]. Daily treatment, which was repeated for 5 days consisted of application of 4 Watt/cm^2 1MHz ultrasound at a duty cycle of 50% for 1min followed by a pause of 1min to prevent tissue overheating and was repeated for a total of 5 times (5x 1min treatment at 1 min intervals). Total energy delivered to the target site per daily treatment was 300 Joule/cm^2.

Animals were placed on a neoprene adsorber pad (Sonidel Ltd, Dublin, Ireland) with ultrasound contact gel (Aquasonic 100, Parker Laboratories Inc. Fairfield, New

Jersey) to prevent reflection of ultrasound from the exit site back into the tissue and subsequent potential constructive interference with passing ultrasound, which could cause tissue overheating and damage.

Ectopic BMP2/7 sonoporation

Constitutive BMP2/7 co-expression in vivo

20µg constitutive co-expression plasmid pVAX1-BMP2/7- was co-delivered with 20µg of the internal luciferase control plasmid pCBR (Promega GmbH, Mannheim, Germany) using sonoporation. Sonoporation was carried out using the SP100 device and neutral, lipid-based microbubbles (MB101, Sonidel Ltd, Dublin, Ireland) at a final concentration of $4x10^8$/ml (50% v/v). 50µl of this plasmid-microbubble solution was injected into *m. gastrocnemius* in nude mice and immediately thereafter sonoporated. This treatment was repeated for 5 subsequent days (total therapeutic DNA dose 100µg; ~3.3mg/kg). Sonoporation was carried out using either 2 Watt/cm^2 (25% duty cycle) or 4 Watt/cm^2 (50% duty cycle) protocols in order to investigate the influence of ultrasound power settings on gene transfer efficacy. Hindlimbs were sonoporated for 1min 5 times using the described power settings. Gene transfer efficacies of sonoporated hindlimbs were compared to passive gene transfer in control hindlimbs without sonoporation. In order to investigate the potential competetive influence of an internal control plasmid on overall bone formation efficacy, 2 Watt/cm^2 sonoporations were additionally carried out without pCBR addition. 4 weeks after the surgery the mice were sacrificed in general anaesthesia (see above) by an overdose of thiopentalsodium (Thiopental sandoz, Sandoz GmbH, Vienna, Austria) (120

mg/kg) by intracardiac injection and *gastrocnemius* muscles were explanted for histology.

Inducible BMP2/7 co-expression in vivo

The doxycycline inducible co-expression system for BMP2/7, TetON-BMP2/7, was sonoporated *in vivo* using 2 Watt/cm^2 (25% duty) and the protocol described for the constitutive system. The inducible systems were applied without an internal pCBR luciferase control plasmid. Controls for passive gene delivery were set up by intramuscular injection of the plasmids. Animals in the induction group received 2mg/day doxycycline in 300µl Ringer's solution intraperitoneally for 7days for induction of BMP2/7 co-expresssion after sonporative or passive gene transfer. Sonoporated control animals were not treated with doxycycline.

Orthotopic BMP2/7 sonoporation

Femur-defect model

Anaesthesia was induced via inhalation of 2% isoflurane and maintained through an intraperitoneal injection of a mixture of ketaminhydrochlorid, 110 mg/kg, (Ketamidor, Richter Pharma AG, Wels, Austria) and xylazin, 12 mg/kg (Rompun 2%, Bayer AG, Vienna, Austria). Preoperatively, the animals received a 2 ml liquid depot of Ringer's solution (Mayerhofer Pharmazeutika GmbH, Linz, Austria) mixed with 0.3 ml butafosan (Catosal, Bayer Health Care Austria GmbH, Vienna, Austria). Analgesia was provided via a daily subcutaneous injection of carprofen, 4 mg/kg (Rimadyl, Pfizer Corporation GmbH, Vienna, Austria) over the course of 4 days and a

subcutaneous injection of buprenorphin, 0.05 mg/kg, every 12 hours during the first 2 days.

During surgical procedure the rats were placed on a thermostatic plate in a lateral decubitus position. A lateral approach was used and the femur exposed. A straight, 4-hole titan plate (Stryker, Duisburg, Germany) was fixed onto the anterolateral surface of the femur using four cortical 7mm titan screws (Synthes, Oberdorf, Switzerland). Subsequently, a 4 mm segmental bone defect was inflicted by 2 parallel osteotomies in the femur's midshaft using a gigli saw and a template. The operation wound was finally closed in two layers using sutures. The animals were allowed free movement for 8 weeks and were then sacrificed via an intracardiac injection of thiopentalsodium (Thiopental sandoz, Sandoz GmbH, Vienna, Austria) (120 mg/kg). Plate and screw dislocation led to exclusion of the animal from the study.

Constitutive BMP2/7 co-expression in vivo

50µg constitutive co-expression plasmid pVAX1-BMP2/7- was co-delivered 3 days post fracture with 50µg of the internal luciferase control plasmid pCBR using sonoporation in the active gene transfer treatment group (total therapeutic DNA dose: 250µg; 0.5mg/kg). The luciferase control group consisted of animals sonoporated with 50µg of the internal luciferase control plasmid pCBR and 50µg of an empty pDNA backbone (pUK21, Plasmid Factory, Bielefeld, Germany) to normalize the DNA content according to the therapy group.

The efficacy of passive gene delivery to the fracture gap was investigated using either treatment injections (50µg pVAX1-BMP2/7- + 50µg pCBR) or luciferase control injections (50µg pCBR + 50µg pUK21) without ultrasound treatment. Furthermore, we investigated the endogenous regenerative potential & union rate in an empty control

group, which did not receive any treatment. Sonoporation was carried out using the SP100 sonoporator and neutral, lipid-based microbubbles (MB101, Sonidel Ltd, Dublin, Ireland) at a concentration of $8x10^8$/ml (75% v/v). 200µl of this plasmid-microbubble solution was injected under x-ray guidance and immediately after sonoporated into the femur defect site.

Plasmid DNA labelling & orthotopic detection

In order to enable therapeutic DNA detection at the defect site for biodistribution monitoring, we applied a novel metabolic DNA labelling method in E.coli based on the nucleoside (2'S)-2'-deoxy-2'-fluoro-5-ethynyluridine (F-ARA-EdU) [39]. pCBR plasmid DNA was labelled with F-ARA-EdU using a thymidine-auxotrophic E.coli strain (Chi1776, DSMZ, Braunschweig, Germany) and supplementing 394 medium [40] containing 20mg/ml thymidine + 20mg/ml F-ARA-EdU. Plasmids were prepped using the Endo-free Plasmid Maxi Kit (Qiagen, Hilden, Germany).

A separate cohort of animals was allocated for imaging of *in situ* orthotopic luciferase expression and subsequent plasmid DNA detection 24h post last gene transfer. Detection was carried out on decalcified paraffin sections of the femora using an Alexa Fluor 680nm labelled azid (Life Technologies, Palo Alto, CA, US) for the Cu2+ catalized click reaction described in [39]. AF680 signals, specific for F-ARA-EdU labelled plasmid DNA were detected using an Odyssey near infrared scanner (LI-COR Biosciences, Lincoln, NE, US) and confocal laser scanning microscopy (CLSM). In CLSM, a separate 488nm channel was used in addition to the 680nm channel in order to use tissue autofluorescence to enable the generation of overlay images with specific AF680 signals and general tissue architecture. A decalcified femur control sample without F-ARA-EdU labelled plasmid DNA served as a negative control.

Bioluminescence imaging

All nude mice that received the internal pCBR luciferase control plasmid were imaged 24h post last gene transfer in order to determine gene transfer efficacies. Imaging was carried out under short inhalation anaesthesia using a Xenogen IVIS100 Imaging system (Caliper Life Sciences GmbH, Mainz, Germany). Mice received 5mg D-luciferin potassium salt (Caliper Life Sciences GmbH, Mainz, Germany) in 300µl Ringer's solution (Mayerhofer Pharmazeutika GmbH, Linz, Austria) intraperitoneally before imaging. 20 minutes post D-luciferin administration, the mice were imaged for 2 minutes.

Sprague-Dawley rats were imaged 24h post last gene transfer in order to determine gene transfer efficacies. Imaging was carried out under short inhalation anaesthesia a Xenogen IVIS100 Imaging system. Rats received 90mg D-luciferin potassium salt in 3ml Ringer's solution intraperitoneally before imaging. 30 minutes post D-luciferin administration, the rats were imaged for 2 minutes.

A separate group of rats was examined for gene transfer localization at the fracture site 24h post last gene transfer using bioluminescence imaging *in vivo* after surgically opening the defect site (30min post luciferin administration) under general anaesthesia (see "femur-defect model" above).

Additionally, femora were imaged *ex vivo* after rats were sacrificed with in general anaesthesia (see above) by an overdose of thiopentalsodium (Thiopental, Sandoz GmbH, Vienna, Austria) (120 mg/kg) by intracardiac injection. Luciferase imaging was carried out after explantation of the femur and adjacent muscles 45min post luciferin administration.

In vivo micro Computer Tomography (µCT) analysis

In vivo µCT images of nude mouse hindlimbs were obtained using a VivaCT 75 (Scanco Medical AG, Brütisellen, Switzerland) 14 days and 28 days post last gene transfer under short inhalation anaesthesia. Bone volume (mm^3) and bone mineral density (mg hydroxyapatite per cm^3) for 2 and 28 days were calculated using Scanco software and a standard density calibration phantom.

In vivo µCT images of rat femora at 0, 4 and 8 weeks post fracture were exported into MATLAB (MATLAB version 7.12.0. The MathWorks Inc., 2012, Natick, MA, US) for processing. In order to facilitate analysis, the volumes corresponding to all 3 time points were rigidly registered onto each other using a three-step approach.

First, the supporting titanium plate was segmented using a manually determined grey-level threshold. When using such a simple segmentation procedure, image artefacts can lead to the erroneous segmentation of parts of the image as well as to the splitting of the titanium plate. To limit the influence of this onto subsequent steps of the analysis, only large elements of the segmentation were retained (> 3000 voxels).

In a second step, the major directions of the titanium plate were extracted from the processed binary segmentations using Principal Component Analysis (PCA, [41]). Rotating the volumes according to these major axes serves as an initialization to the final matching step. For this, we employ the Iterative Closest Point (ICP, [42]) algorithm. While reliable in practice, it is well known to be susceptible to its initialization. Alignment using PCA assures the convergences of ICP to the correct matching between volumes.

The rotation computed using PCA and the deformation obtained after convergence of the ICP-process are combined into a rigid deformation matrix. This transformation

was then applied to each volume in the sequence except the reference, ensuring the correct correspondences of each voxel between acquisition times.

All volumes were then represented as colour-coded overlays of the 3 time points. New bone at the defect site was segmented manually and quantified as bone volume/tissue (defect) volume.

Histology

Gastrocnemius muscles from nude mice were excised and fixed with 4% buffered paraformaldehyde (Sigma Aldrich GmbH, Vienna, Austria) in phosphate-buffered saline (PBS) for 24h, transferred in 50% ethanol and stored in 70% ethanol at 4°C. Samples were embedded in paraffin without decalcification, and several sections of the same sample were stained with haematoxylin and eosin (H&E) and von Kossa staining for mineralization according to standard histology protocols. Localization of the internal luciferase control expression was detected using the G7451 Goat Anti-Luciferase (Promega GmbH, Mannheim, Germany) primary antibody in a 1:50 dilution overnight and an appropriate secondary peroxidase conjugated antibody ImmPRESS Anti-Goat Ig Peroxidase (Vector Labs, Peterborough, UK) for 30min after blocking of endogenous peroxidase activity with 3% H_2O_2 in Tris-buffered saline. Nuclear counter-staining was carried out using Mayer's haematoxylin solution for 1min.

Statistical analysis

Results are represented as median±interquartile range unless otherwise stated. Statistical testing was carried out using the non-parametric Kruskal-Wallis test in conjunction with Dunn's multiple comparison test as post-test for luciferase expression, ectopic bone volume and bone mineral density data.

Orthotopic sonoporation luciferase expression data did not pass normality testing (Kolmogorov-Smirnov test) and was therefore tested with the non-parametric Kruskal-Wallis test in conjunction with Dunn's multiple comparison test as post-test. Orthotopic passive luciferase expression data passed Kolmogorov-Smirnov normality testing and therefore was tested with a two-tailed t-test for significance. Bone volume per tissue volume (BV/TV) data from orthotopic models was confirmed to follow a Gaussian distribution and therefore tested with parametric ANOVA and Tukey's test for significance. $p<0.05$ was considered statistically significant.

Results

Ectopic BMP2/7 sonoporation

Gene transfer efficacies & luciferase gene expression

Evaluation of gene transfer efficacy 24 hours post last gene transfer via bioluminescence activity confirmed successful gene transfer in 8 out of 13 animals (61.5% efficacy) for passive gene delivery, 6 out of 7 animals (85.7%) for 2 Watt/cm^2 sonoporation and 7/7 animals (100%) for 4 Watt/cm^2 sonoporation (Figure 1A). Bioluminescence imaging of the internal luciferase control activity 24h post the 5-day gene transfer protocol demonstrated variable luciferase activity in all animals (Figure 1B). No significant difference in bioluminescence levels was observed between passive gene transfer, 2 Watt/cm^2 or 4 Watt/cm^2 sonoporation. Sonoporation with 4 Watt/cm^2 was observed to cause considerable skin burns at the ultrasound exit sites in contrast to the 2 Watt/cm^2 protocol (data not shown).

Ectopic bone formation using constitutive BMP2/7 co-expression in vivo

Passive gene delivery of the constitutive BMP2/7 co-expression plasmid pVAX1-BMP2/7- to *gastrocnemius* muscles successfully induced ectopic bone formation in 6 out of 13 animals (46.2%) (Figure 2A). Sonoporative gene delivery with either 2 Watt/cm2 or 4 Watt/cm^2 protocols successfully induced ectopic bone formation in 5 out of 7 (71.4%) or 7 out of 7 animals (100%) respectively (Figure 12A). The potential detrimental influence of incorporating an internal luciferase control plasmid on bone formation efficacy was investigated separately for passive gene delivery and 2

Watt/cm^2 sonoporation protcols by exclusively delivering the osteogenic pVAX1-BMP2/7- without addition of pCBR. 2 Watt/cm^2 sonoporation of pVAX1-BMP2/7 without the luciferase plasmid led to an increased bone formation in 6 out of 7 animals (85.7%) (Figure 2A).

Bone volumes of ectopic bone were variable and did not exhibit significant differences among the different treatment groups and time points as observed by *in vivo* µCT (Figure 3G, H). Furthermore, no significant differences in bone volumes were observed for treatments with or without the internal luciferase control plasmid pCBR. Medians of bone volumes were 0.094 mm^3 at 14 days and 0.044 mm^3 at 28 days post treatment using the constitutive co-expression systems with passive gene transfer. Medians of bone volumes were 0.0025 mm^3 (0.03 mm^3 without pCBR addition) at 14 days and 0.171 mm^3 (0.0105 without CBR addition) at 28 days post treatment using the constitutive co-expression system with the 2 Watt/cm^2 sonoporation protocol. Medians of bone volumes were 0.02 mm^3 at 14 days and 0.013 mm^3 at 28 days post treatment using the constitutive co-expression system with the 4 Watt/cm^2 sonoporation protocol.

Bone mineral densities of ectopic bones did not show significant differences among treatment groups and different time points post treatment as observed by *in vivo* µCT. No significant differences in bone mineral densities were observed for treatments with or without the internal luciferase control plasmid pCBR. Medians of bone mineral densities were 179.4 mg HA/cm^3 at 14 days and 238 mg HA/cm^3 at 28 days post treatment using the constitutive co-expression systems with passive gene transfer. Medians of bone mineral densities were 160 mg HA/cm^3 (201.8 mg HA/cm^3 without pCBR addition) at 14 days and 243.6 mg HA/cm^3 (230.4 mg HA/cm^3 without CBR

addition) at 28 days post treatment using the constitutive co-expression system with the 2 Watt/cm^2 sonoporation protocol. Medians of bone mineral densities were 177.4 mg HA/cm^3 at 14 days and 220.7 mg HA/cm^3 at 28 days post treatment using the constitutive co-expression system with the 4 Watt/cm^2 sonoporation protocol.

Ectopic bones displayed variable morphology ranging from relatively large to multiple small ossicles of irregular shape in all observed treatment groups (representative images in Figure 3 A-F). Direct comparison of 14 day with 28 day *in vivo* µCT images of the same ossicles (Figure 3 A-F) showed moderate bone remodeling and resorption.

Histological examination of ectopic bones at 4 weeks by HE staining revealed fully developed ossicles in *gastrocnemius* muscles, with a compact layer of bone surrounding a bone marrow cavity (Figure 4A) with a heterogenous cell population consisting of adipocytes, haematopoietic myeloid progenitor cells megakaryocytes and erythrocytes (Figure 4D). Immunohistochemical detection of the internal control marker luciferase showed diffuse staining in muscle fibers surrounding the ectopic bone structure and strong staining of bone lining and bone marrow cells (Figure 4B). Von Kossa staining for mineralization furthermore confirmed the observed structures in HE staining as mineralized bone tissue (Figure 4C). These observations were made for ectopic bones generated by passive and sonoporative BMP2/7 gene transfer.

Ectopic bone formation using inducible BMP2/7 co-expression in vivo

Sonoporation of the inducible single-vector BMP2/7 co-expression system pTetON-BMP2/7 (without luciferase control plasmid) using the 2 Watt/cm^2 sonoporation protocol successfully induced ectopic bone formation in 5 out of 6 animals (83.3%) only when induced with 2mg of doxycycline per 48h for 7 days as observed by *in vivo*

µCT at 14 days and 28 days post treatment. No bone formation was observed without application of doxycycline (0 out of 6 animals) (Figure 2B). Furthermore, no bone formation was observed when using passive gene transfer and induction with 2mg of doxycycline per 48h for 7 days (Figure 2B). The generated ectopic bones did not show any significant differences in bone volumes (Figure 3G, H) or bone mineral densities when compared to ectopic bones generated using the constitutive co-expression system pVAX1-BMP2/7 at 14 and 28 days post treatment. Medians of bone volumes were 0.01188 mm^3 at 14 days and 0.0186 mm^3 at 28 days post treatment using the inducible co-expression system with sonoporation. Medians of bone mineral densities were 149.7 mg HA/cm^3 at 14 days and 249.6 mg HA/cm^3 at 28 days post treatment using the constitutive co-expression system with sonoporation.

Orthotopic BMP2/7 sonoporation

Orthotopic luciferase gene expression & gene transfer efficacies

Monitoring of orthotopic luciferase gene expression with bioluminescence imaging demonstrated strong luciferase expression limited to the defect site (Figure 5B, C, D) and not associated with adjacent muscle tissue (Figure 5C). No difference in localization was observed between sonoporation and passive gene transfer (data not shown). There was no significant difference in luciferase gene expression levels after sonoporation or passive gene transfer (Figure 1D). A significant reduction of bioluminescence was observed for both passive gene transfer and sonoporation when the luciferase plasmid was administered with the therapeutic pVAX1-BMP2/7

co-expression plasmid (Figure 1D), indicating potential competitive expression limiting the reliability of luciferase gene expression monitoring in the therapy group. Therefore, orthotopic gene transfer efficacies for passive and sonoporative gene transfer were determined using the luciferase groups, which received only luciferase plasmid. Gene transfer efficacies estimated via bioluminescence imaging were 66.67% (6 out of 9 animals) for passive gene transfer and 85.71% (12 out of 14 animals) for the 4 Watt/cm^2 sonoporation group (Figure 1C).

Orthotopic F-ARA-EdU labeled luciferase plasmid DNA distribution

Detection of luciferase plasmid DNA biodistribution 24h post last gene transfer at the defect site through near infrared scanning of histology slides (Figure 6A) revealed extensive signals in bone tissue of femurs. In depth examination of these signals through CLSM demonstrated the confined presence of plasmid DNA within cells of the bone marrow (Figure 6B), at the defect site granulation tissue (Figure 6C), diffuse distribution of plasmid DNA within intact bone tissue (Figure 6D) and weak diffuse signals in surrounding muscle fibers (Figure 6E) with confined signals in satellite cells (Figure 6E, arrows). No differences in biodistribution were observed between passive gene transfer and sonoporation groups (data not shown).

Orthotopic bone regeneration using constitutive BMP2/7 co-expression in vivo

In vivo µCT images of fractures at 4 and 8 weeks post fracture showed 3 potential unions out of 6 animals of the sonoporation treatment group (Figure 7A), 1 potential union in the passive gene transfer treatment group (Figure 7B), 1 potential union in

the luciferase control group (Figure 7C) and 1 potential union in the empty control group (Figure 7D) at 8 weeks post treatment. In depth examination of the μCT images at different angles and cut planes confirmed 2 unions out of 6 animals (33.3% union rate, Figure 8A) in the treatment group (Figure 7A, asterisks) and 1 union (16.7% union rate. Figure 8A) in the passive gene transfer treatment group (Figure 7B, asterisk) at 8 weeks post fracture. No unions were confirmed (Figure 8A) in the luciferase control and empty control groups 8 weeks post fracture (Figure 7C, D).

Quantification of bone volume/tissue volume at the initial margins of the fracture gap did not show any significant differences between treatment groups and controls at both 4 weeks (Figure 8B) and 8 weeks (Figure 8C) post fracture.

Discussion

The aim of this study was to evaluate the feasibility of controlled non-viral sonoporative gene delivery *in vivo* for application in bone regeneration. It has been demonstrated that the repeated co-delivery of the highly bioactive gene-combination of BMP2/7 effectively mediates bone formation *in vivo* in ectopic models and that gene expression can be precisely controlled using inducible co-expression systems. Direct comparison of sonoporation with passive gene delivery demonstrated an increased probability of gene expression and bone formation for sonoporation increasing with total ultrasound energy applied to the target site, whereas when successful gene transfer was accomplished, gene expression levels and bone volumes were not increased via sonoporation. In orthotopic models, it was possible to replicate the beneficial effect of sonoporation on gene transfer probabilities and to demonstrate localized orthotopic gene expression. DNA tracking revealed that a large fraction of plasmid DNA binds to the matrix of the intact bone and is thus absorbed from the defect site. *In* vivo µCT data suggests enhanced orthotopic bone regeneration after active sonoporative gene transfer of BMP2/7 to the target site.

Ectopic BMP2/7 sonoporation

Ectopic gene transfer monitoring with luciferase showed no significant differences in expression strength between passive gene transfer and sonoporation (Figure 1B). The probability of successful gene transfer, however, was increased when ultrasound was applied for gene transfer with increasing power (Figure 1A). It was possible to reach gene transfer efficacies of 100% using the 4 Watt/cm^2 (300 Joule/cm^2)

sonoporation protocol. Therefore, we conclude that sonoporation increases gene transfer efficacy compared to passive gene delivery and that the efficacy depends on the applied ultrasound power. This is in line with findings demonstrated in [32], which showed that *in vitro* there is a delicate balance between ultrasound power, cell viability and sonoporation efficacy. We observed skin burning at the exit sites of ultrasound in the more effective 4 Watt/cm^2 protocol in nude mice, which has not been reported in [38], in which an outbred mice strain was used in which skin damage is less obvious. This phenomenon disappeared when ultrasound power was reduced from 300 Joule/cm^2 to 90 Joule/cm^2 using the 2 Watt/cm^2 protocol. Bone formation efficacies of the different gene transfer protocols (Figure 2) generally paralleled gene transfer efficacies observed by luciferase monitoring (Figure 1A). The only group, which resulted in 100% ectopic bone formation efficacy, was using the 4 Watt/cm^2 sonoporation protocol.

BMP2/7 co-expression is considered more effective than BMP2 expression alone as reported by several *in vitro* and *in vivo* studies [35-37, 43] due to the formation of a very highly bioactive BMP2/7 heterodimeric growth factor [35, 44, 45], which is less prone to inhibition by endogenous inhibitors [46] and induces endogenous BMP4 expression [43]. By applying this co-expression strategy, we were able to achieve 100% bone formation efficacy at lower DNA doses (1/5 of the dose used in [38] with BMP2; 1.5x less of the dose used in [26] with BMP9), lower number of repetitive treatments (5x vs 7x in [38]) or lower total applied ultrasound power (300 Joule/cm^2 in 24h vs 1500 Joule/cm^2 in 24h in [26]) when compared to similar studies using only single BMP gene transfer [26, 38, 47]. Therefore, we conclude that the selected co-expression strategy is superior to single factor expression in the case of BMPs and that this allows higher therapeutic efficacy using an actual low-efficacy non-viral gene transfer method. By using this approach it might be feasible in the future to enable

the less invasive sonoporative gene transfer method to compete with current electroporation approaches used for bone regeneration [26, 43, 48], which have been shown to be more effective than sonoporation due to higher transfection efficacy as reported in [26] but rely on invasive insertion of electrodes in contrast to minimally invasive transcutaneous application of ultrasound.

Addition of the internal luciferase control plasmid pCBR to the gene transfer protocol did not exhibit a significant detrimental effect on bone formation efficacy (Figure 2A). Therefore, we conclude that there is no substantial detrimental effect of adding a separate internal control plasmid for gene expression monitoring on bone formation efficacy in the ectopic model. The authors would like to note, however, that the luciferase levels obtained in this study, were lower than in previously unpublished data for single luciferase sonoporation only (Hofmann *et al.* in submission), indicating competitive expression between internal control plasmids and therapeutic plasmids.

In vivo quantitative µCT data showed no significant difference in bone volumes or bone mineral densities for passive gene transfer or sonoporation protocols, which is in line with the luciferase gene expression levels and kinetics which did not show significant differences either as described above. Overall bone volumes achieved by BMP2/7 gene delivery either passive or via sonoporation were comparable to ectopic bone volumes achieved with BMP9 sonoporation in [26]. The average bone mineral densities were within the range of ectopic bone mineral densities expected in ectopic bone formation models and comparable to ectopic bones formed via delivery of genetically modified stem cells or recombinant BMPs [49, 50] but lower when compared to BMP9 sonoporative gene transfer [26].

The obtained average bone volumes, exclusively demonstrated in this study and in [26] in contrast to other BMP gene delivery work, however, are still limited, if

compared to approaches which use biomaterials in conjunction with gene delivery or growth factor delivery [51-53]. This is probably due to the template function of an implanted biomaterial, which provides a template and environment for efficient ossification of a larger volume than the DNA injection only approach used in this study. Therefore, it might be of interest if ectopic bone volumes and bone formation efficacies could be increased by using a sonoporation approach in conjunction with biomaterials, such as hydrogels. The feasibility of such an approach for matrix-assisted sonoporation has already been demonstrated *in vitro* and functional protocols were developed (Nomikou *et al.*, in submission) and therefore shall be investigated in future studies.

Bone structures were irregular in shape with multiple centres of ossification per animal (Figure 3A-F). This represents individual foci of gene delivery, expected to be formed due to the chosen multiple injection strategy adapted from [38]. Data from Osawa *et al.* [38] and own unpublished data showed that it was impossible to generate ectopic bones via a single injection of DNA and single sonoporation, which indicates that gene expression levels obtained by sonoporation are still relatively low and not sufficient for osteoinduction when applied in a singular fashion. Therapeutic feasibility of such an approach might be limited if bone formation cannot be limited to one specific focus and shape. Therefore, it is of great importance to either modify the approach to a single gene delivery protocol by using more effective therapeutic transgenes preferable in combination with a biomaterial as mentioned above in order to spatially control and limit bone formation to one specific site *in vivo*.

Histological examination of ectopic centres of ossification (Figure 4A, C, D) showed functional ectopic bone tissue with calcified compact bone (Figure 4C) surrounding a bone marrow cavity (Figure 4D), which hosted haematopoietic bone marrow. This formation of a stem cell niche after BMP gene delivery has been reported by other

groups as well [26, 38] and proved that by simple gene delivery without biomaterials, stem cells or recombinant growth factors can induce the formation of complex tissues *in vivo*, a prerequisite for demonstration of the feasibility of this approach for functional tissue regenerative therapies. Furthermore, immunohistochemical staining for the internal luciferase transgene (Figure 4B) proved that luciferase recipient cells are participating in ectopic bone formation and present in the bone marrow cavity, thus indicating that endogenous transgene receptive cells are directly differentiating and contributing to ectopic bone formation and bone marrow.

In general, the ectopic findings for application of the constitutive BMP2/7 co-expression system demonstrate that direct *in vivo* co-delivery of bone morphogenetic protein encoding plasmid DNA could provide a viable alternative to stem cell or recombinant protein based therapies. Furthermore, the data presented herein, clearly indicates that sonoporative gene delivery is superior in direct comparison with passive gene delivery with regards to gene transfer probability and that gene transfer efficacies are dependent on the total amount of ultrasound energy applied to the target site.

Inducible BMP2/7 co-expression in vivo

Doxycycline inducible BMP2/7 co-expression from a modified (Feichtinger *et al.* in submission) bidirectional TetON system [54] showed effective (83.3% efficacy) bone formation only when applied with ultrasound and only if gene expression from the system was activated by systemic application of doxycycline for 7 days (Figure 2B). This clearly showed tight control of the co-expression of 2 individual transgenes *in vivo* by systemic application of an inductor. By narrowing *in vivo* BMP2/7 co-expression down to approximately 7 days, it was possible to demonstrate that even

this short time window of *in vivo* BMP expression, potentially recapitulating endogenous BMP expression patterns shorter than 14 days [55], is sufficient for *in vivo* bone formation. This reduction of therapeutic gene dose and the therapeutic gene expression time window compared to the constitutive co-expression systems enabled to demonstrate an even pronounced effect of sonoporation on bone formation efficacy in the case of the inducible system (Figure 2B). Notably, in conjunction with the assumed reduced gene dose, it was not possible to induce bone formation by passive gene transfer even in animals that received doxycycline treatment when expression was limited to 7 days. Therefore, we conclude that sonoporation in general increases gene transfer efficacy and enable reduction of therapeutic gene dose and therapeutic gene expression time windows due to higher overall gene transfer efficacies & expression, which was not observed as dramatically with the constitutive co-expression systems, because overall gene expression levels were probably already saturated and therefore it was not possible to observe these subtle differences with these systems.

Orthotopic BMP2/7 sonoporation

Using X-ray guided repeated DNA injection to the fracture site it was possible to achieve local, spatially controlled gene transfer to the target site *in vivo* without substantial off-target expression in adjacent tissues (luciferase imaging, Figure 5A-D). Gene transfer efficacies, as paralleled by the ectopic findings, were higher when using sonoporation as compared to passive gene transfer (Figure 1C), demonstrating the feasibility of using ultrasound for orthotopic gene delivery. Interestingly, in

contrast to the ectopic findings, there was a clear, significant drop of luciferase activity when the luciferase plasmid was co-applied with the constitutive BMP2/7 co-expression plasmid in passive and sonoporative gene transfer (Figure 1D). This indicates that competitive expression might reduce overall gene expression efficacy if multiple independent expression entities are applied *in vivo.* Therefore we based gene transfer efficacy calculation only on the groups, which exclusively received the luciferase plasmid to rule out false negative signals observed due to competitive expression in animals that received both luciferase and BMP2/7 co-expression plasmids.

Tracking of luciferase plasmid DNA biodistribution at the defect site (Figure 6) using a novel metabolic DNA labelling method based on the work of Neef *et al.* [39] showed a different picture than tracking of luciferase gene expression at the target site. At the relatively high plasmid DNA dose used in this study, it has been observed that the DNA spreads from the site of application to unintended tissues such as bone marrow, intact bone and surrounding muscles. Whereas expression at these sites could not be evaluated due to the limitations of luciferase imaging, it has been shown by other studies that the presence of plasmid DNA at off-target sites does not automatically trigger off-target transgene expression [56] and should therefore be carefully investigated in future studies before conclusions about off-target expression can be made. The most striking finding of plasmid DNA biodistribution monitoring was extensive, diffuse binding of the plasmid DNA to intact bone at the defect site and at bone tissue lining the bone marrow lumen, indicating that a large fraction of the applied DNA gets absorbed by mineralized bone tissue already 24 h post application. Binding of nucleic acids to ceramic hydroxyapatite has been well known already for some time [57, 58] and harnessed for DNA purification [59] and delivery [60, 61]. This study, however, at least to the knowledge of the authors, is the first study to

demonstrate this effect *in vivo* with biological hydroxyapatite and naked exogenous DNA in orthopaedic gene therapy. The absorption of DNA from the defect site might be considered as another caveat in *in vivo* naked DNA transfer, additionally to nuclease digestion [62, 63], which might be specific for orthopaedic applications and should be considered in future studies as parameter potentially responsible for low therapeutic efficacy of these approaches in bone regeneration at relatively high doses of DNA [64, 65].

In vivo quantitative and qualitative µCT data, although no significant difference could be found within this study design, indicate a potential beneficial effect of BMP2/7 gene delivery on fracture regeneration. The 4mm femur fracture model used in this study had an overall approximate 83.3% non-union rate at 8 weeks post fracture, when taking potential unions into account prior to in depth µCT evaluation which led to 100% non-union rate, and was therefore performing comparably to previous studies in rats by our group [66, 67]. It has been demonstrated that active orthotopic BMP2/7 co-delivery via sonoporation leads to 2/6 unions in the therapy group (union rate 33.3%) and passive gene delivery could achieve at least 1/6 unions (16.6% union rate) compared to 0/6 unions in both the luciferase negative control and empty defect control groups respectively when carefully evaluated by µCT, which has been shown to be the only reliable method in determining fracture union rate [66]. Therefore, and in conjunction with luciferase data, we conclude that sonoporative gene transfer enables orthotopic gene delivery and that orthotopic BMP2/7 gene delivery by sonoporation enhances bone regeneration at orthotopic sites. Thus, it might be possible to definitely prove therapeutic efficacy in future studies if the therapeutic effect is enhanced significantly either by additional recruitment of cells to a biomaterial *in situ* prior to gene delivery as demonstrated in [68] or by combining our approach with the mentioned matrix-assisted sonoporation technology (Nomikou

et al., in submission). These potential modifications of the current protocol address different aspects, which potentially limited the therapeutic efficacy in this study, such as the initial lack of expression-capable cells at the defect site and DNA absorption from the target site by the surrounding intact bone tissue, given that an additional biomaterial could recruit endogenous cells and retain the therapeutic DNA at the site of action. Furthermore, the data presented herein indicates that application of an internal luciferase control plasmid can reduce transgene expression at the defect site due to competitive expression. Therefore, a simple means of enhancing therapeutic efficacy in future studies aiming exclusively at the evaluation of bone regeneration could be to apply only the therapeutic co-expression plasmid without the internal luciferase control.

Conflicts of interest & Acknowledgements

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Figures

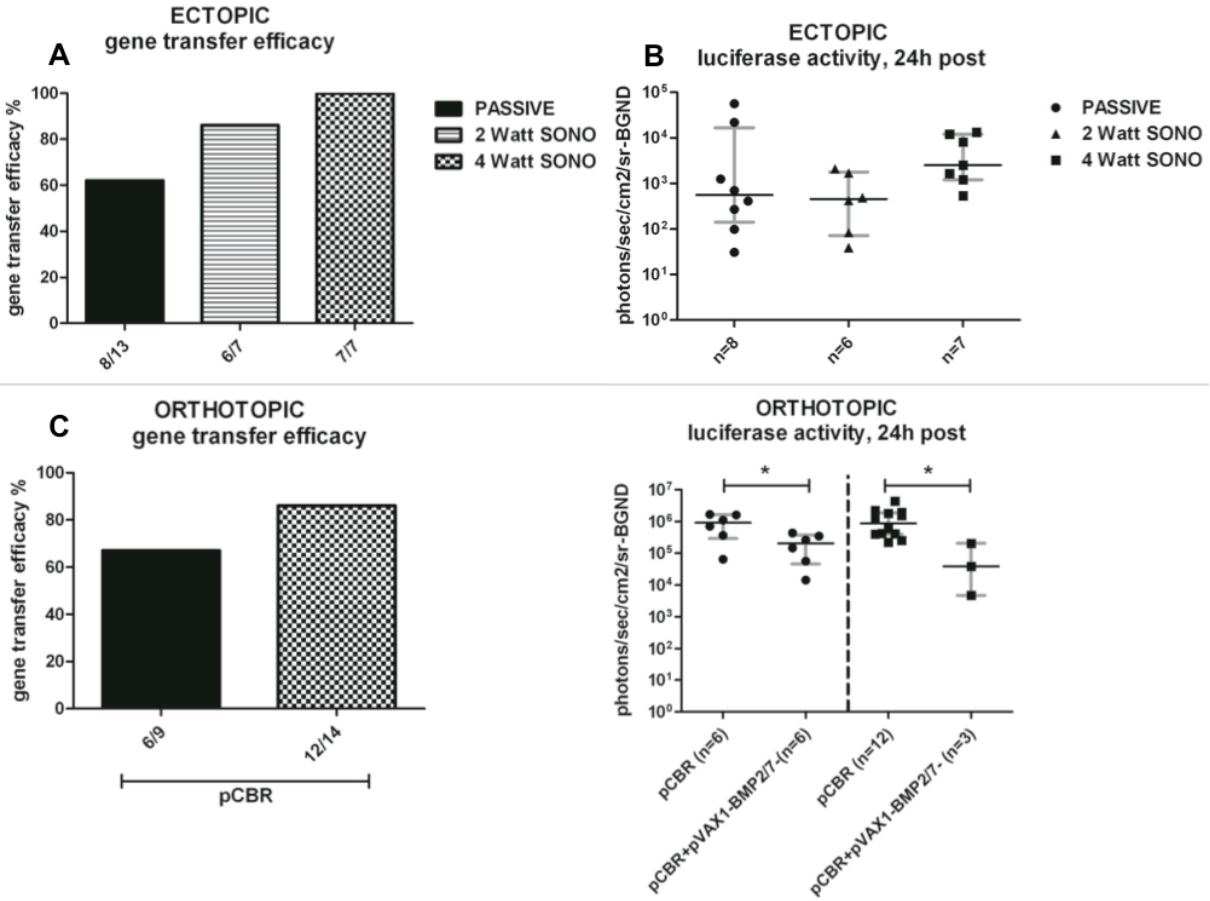


Figure 1: Ectopic (A) and orthotopic (C) gene transfer efficacies based on luciferase activity. Ectopic luciferase activity (BMP2/7 and luciferase co-delivery) (B) and orthotopic luciferase activity (only luciferase: „pCBR“ vs. luciferase and BMP2/7 co-delivery: „pCBR+pVAX1-BMP2/7-„)(D) 24 hours post last gene transfer for passive and sonoporative gene delivery. Median ± interquartile range.

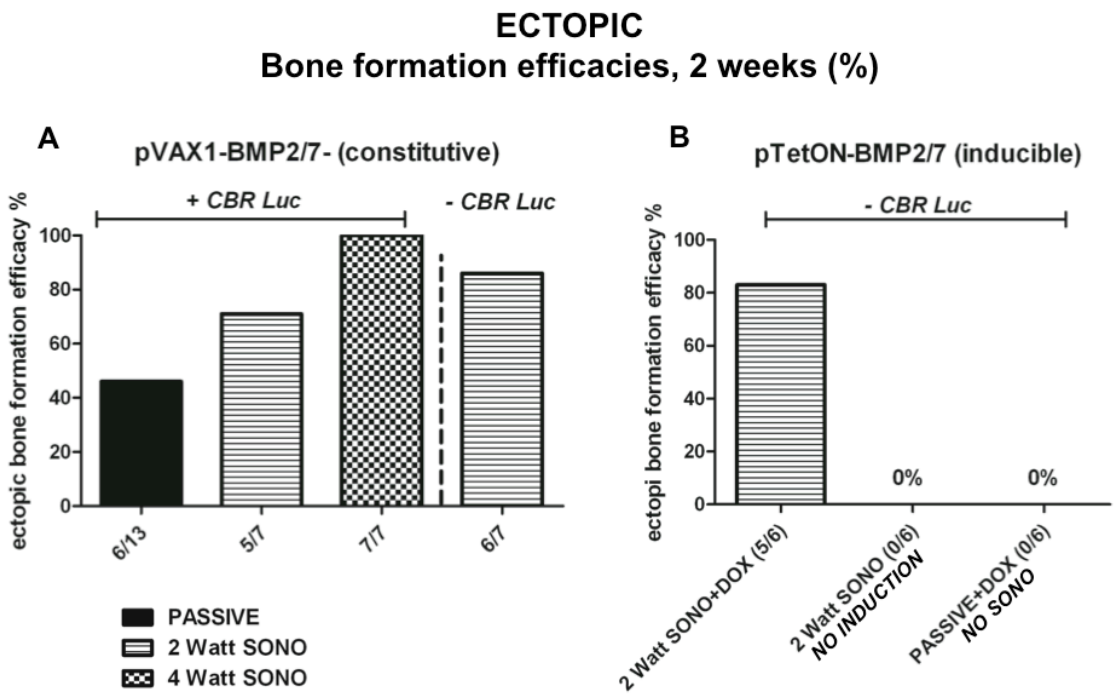
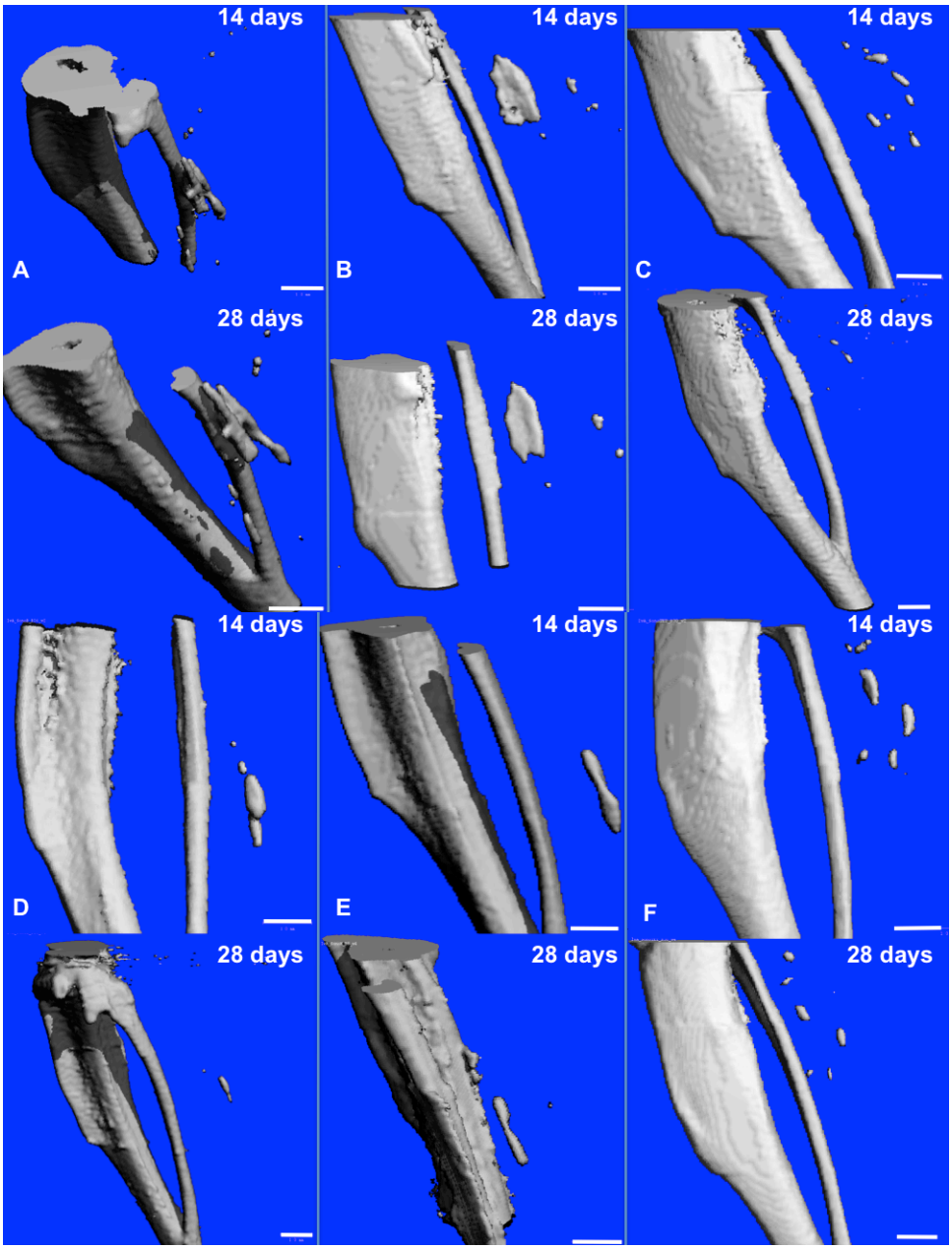


Figure 2: Ectopic bone formation efficacies for constitutive pVAX1-BMP2/7- based co-expression experiments (A) with and without luciferase plasmid addition (+CBR Luc/-CBR Luc). Ectopic bone formation efficacies for inducible pTetON-BMP2/7 based co-expression experiments (B) without luciferase plasmid addition and with or without doxycycline (DOX) application. Results represented for passive gene transfer and 2 Watt/cm^2 and 4 Watt/cm^2 sonoporation protocols.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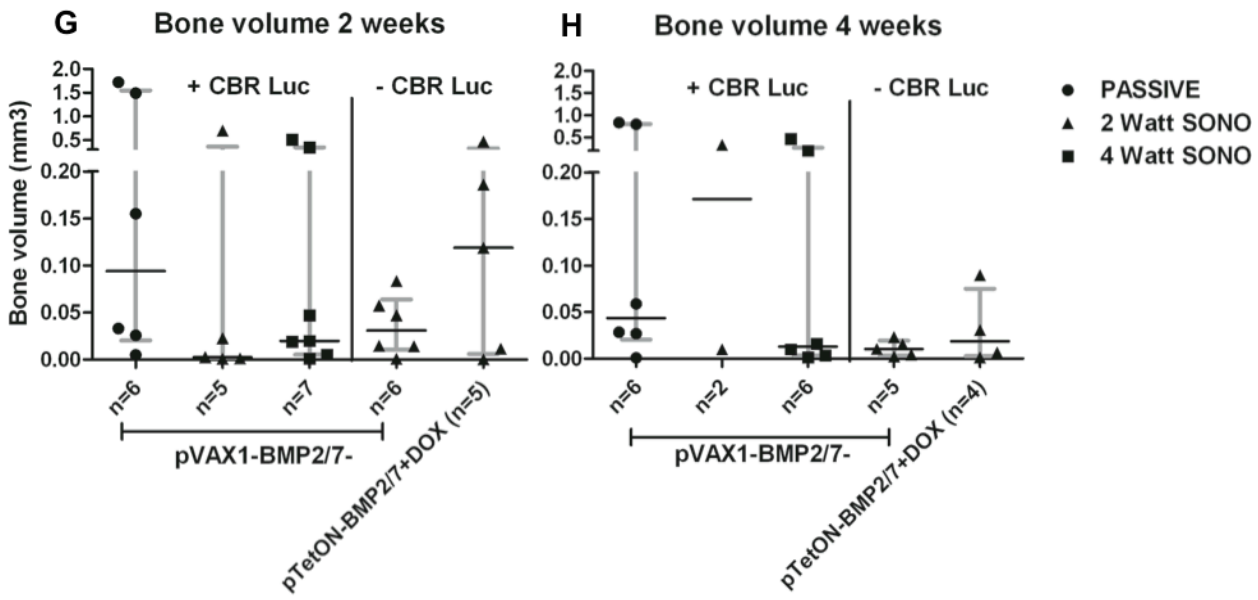

Figure 3: Representative images of ectopic bones for 4 Watt/cm2 constitutive BMP2/7 co-expression system sonoporation (A), 2 Watt/cm2 constitutive BMP2/7 co-expression system sonoporation (B), 2 Watt/cm2 constitutive BMP2/7 co-expression system sonoporation without luciferase internal control (C), passive gene transfer of the constitutive BMP2/7 co-expression system (D), passive gene transfer of the constitutive BMP2/7 co-expression system without luciferase (E) and inducible BMP2/7 co-expression system without luciferase with 2 Watt/cm2 sonoporation protocol. Scale bars represent 1mm. Quantitative *in vivo* µCT data for ectopic bones 2 weeks (G) and 8 weeks (H) post treatment. Median ± interquartile range.

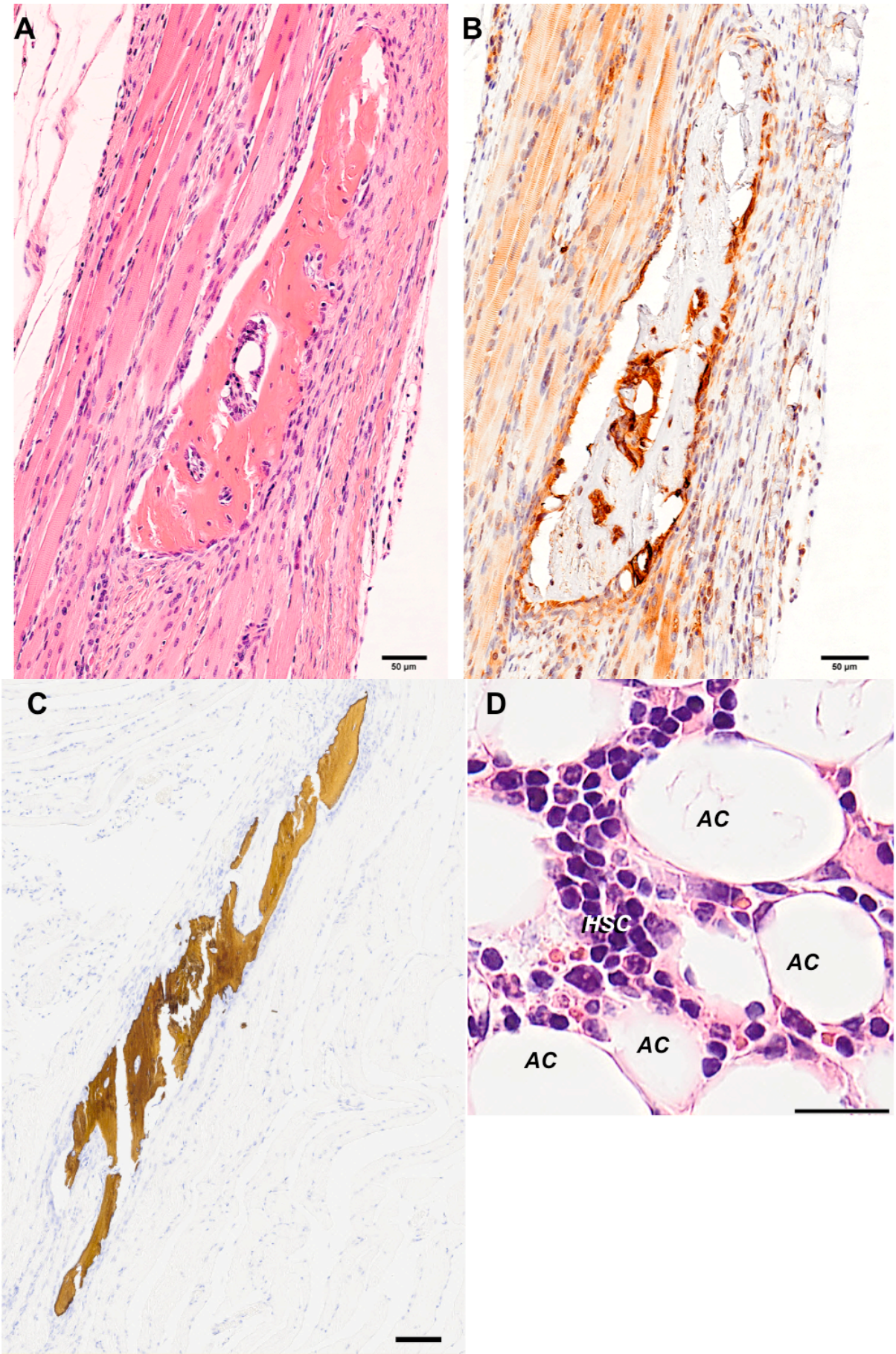
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Figure 4: Histologies of ectopic bone (representative image of 4 Watt/cm2 sonoporation). Haematoxylin/Eosin staining (A), Immunohistochemical detection of luciferase (B), von Kossa staining for mineralization (C) and close-up of bone marrow of ectopic bones stained with haematoxylin/eosin showing haematopoietic bone marrow with adipocytes („AC“) and haematopoietic stem cells („HSC“). Scale bars represent 50µm in (A) and (B), 100µm in (C) and 20µm in (D).

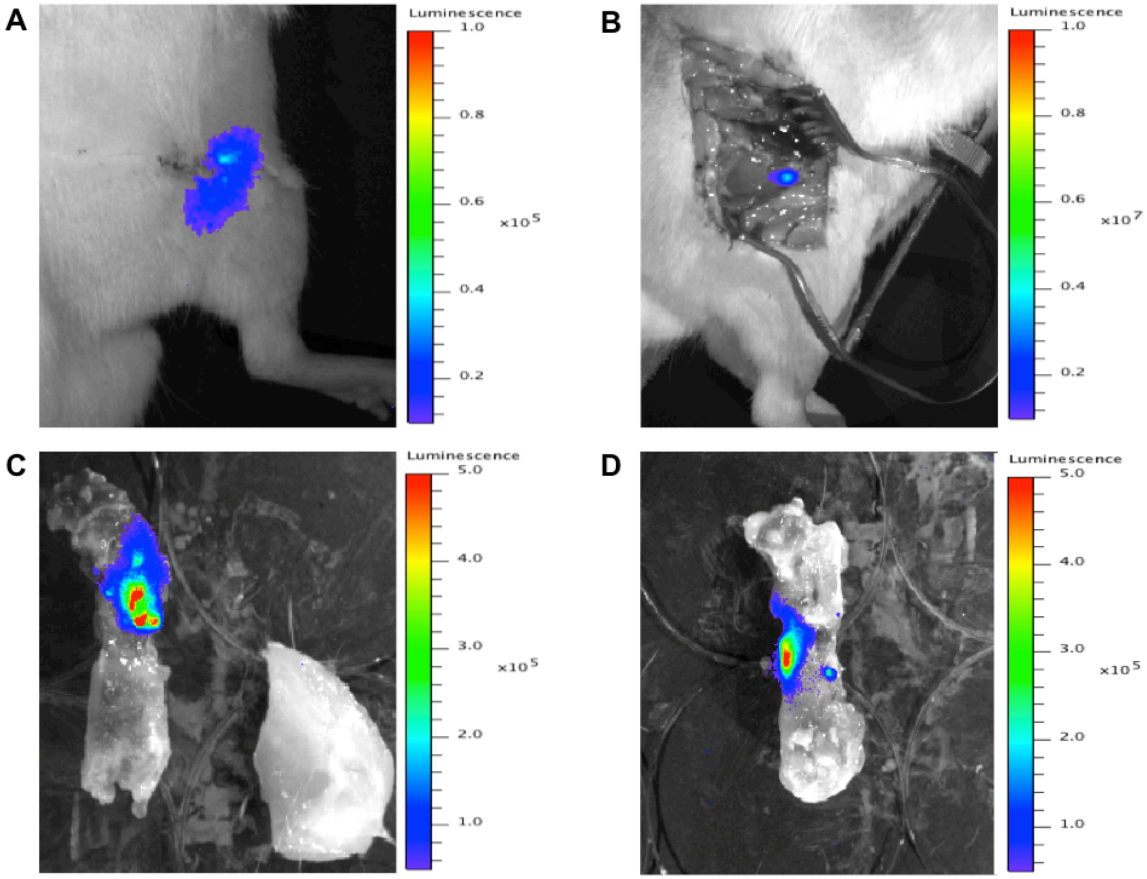

Figure 5: Orthotopic luciferase expression. Bioluminescence activity in rat femurs depicted as false colour images of average photons/sec/cm^2/steradian (A: *in vivo*; B: *in situ*; C: *ex vivo*, femur & adjacent muscle; D: *ex vivo*).

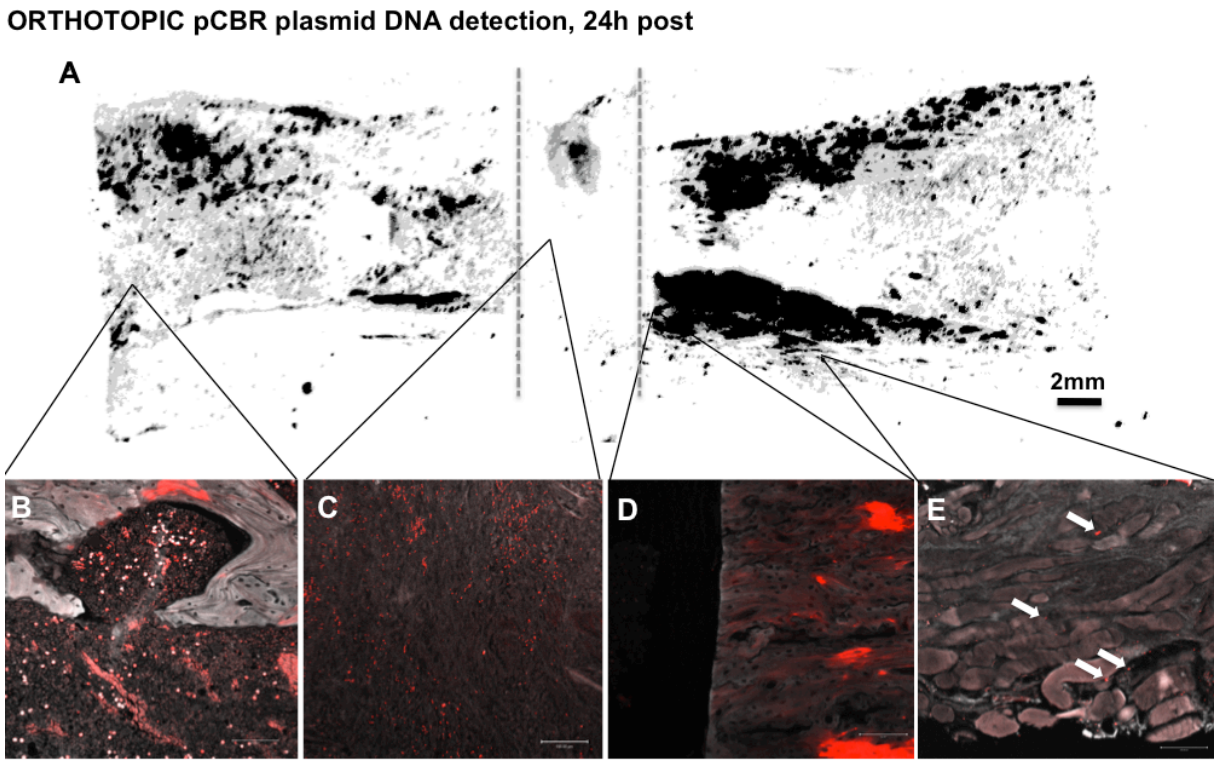

Figure 6: Orthotopic luciferase plasmid tracking using (2'S)-2'-deoxy-2'-fluoro-5-ethynyluridine (F-ARA-EdU) metabolically labelled pCBR luciferase plasmid DNA detected with Alexa Fluor 680 24 hours post last gene transfer. Odyssey near infrared scan of F-ARA-EdU signals in a representative femur sample (A), scale bar represents 2mm. In-depth examination of selected areas using confocal laser scanning microscopy: femoral bone marrow cavity (B), defect granulation tissue (C), intact bone tissue of remaining femur stump (D) and surrounding muscle tissues (E). Scale bars represent 100µm.

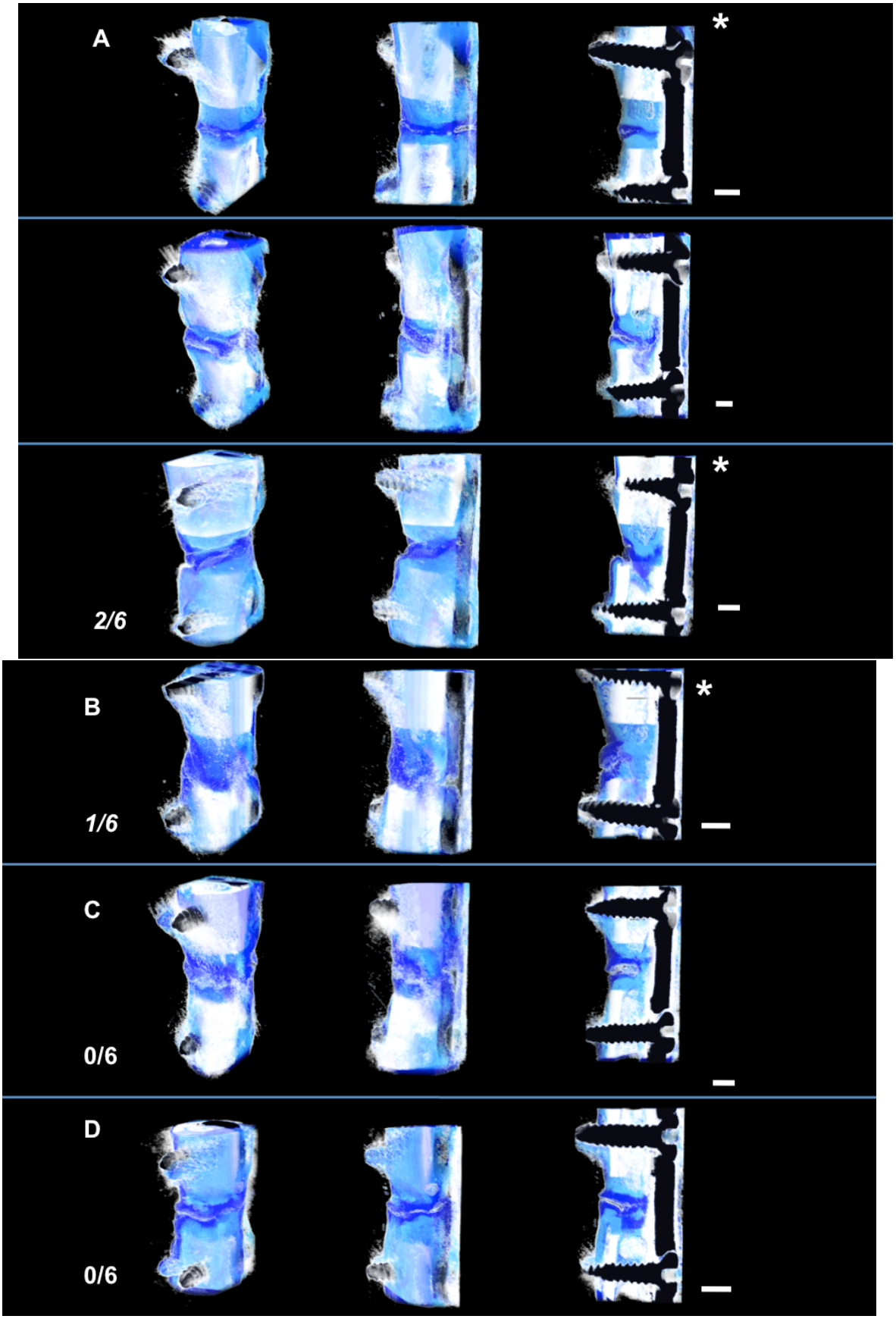

Figure 7: Registered *in vivo* µCT images of potential unions: 0 weeks (white), 4 weeks (light blue) and 8 weeks post fracture (dark blue) of potential unions in the sonoporation therapy group (A), passive

gene transfer therapy group (B), luciferase sonoporation control group (C) and empty control group (D). Unions confirmed by in depth examination are marked with an asterisk. Scale bars represent 2mm.

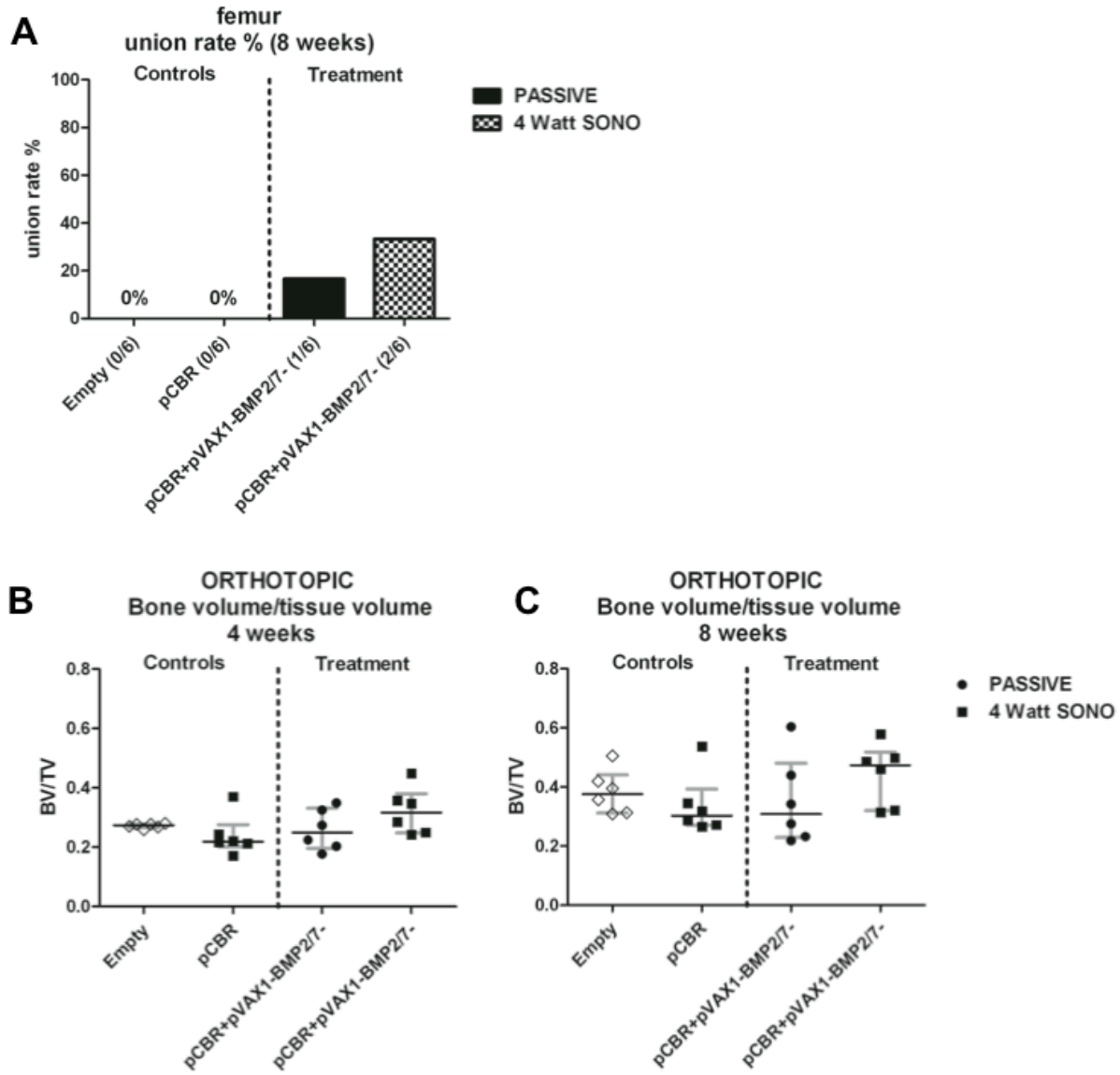

Figure 8: Observed union rates based on in-depth µCT examination (A). Orthotopic bone volumes per defect tissue volumes based on quantitative *in vivo* µCT evaluation 4 weeks (B) and 8 weeks (C) post fracture. Empty control group („Empty"), luciferase control group („pCBR") and treatment groups („pCBR+pVAX1-BMP2/7-"). Data depicted for passive gene transfer („PASSIVE") and 4 Watt/cm2 sonoporation („4 Watt SONO"). n=6, Median±interquartile range.

General Discussion

The overall aim of this work is to provide a novel non-viral transient gene therapy for bone tissue regeneration *in vivo*. Requirements for an ideal novel treatment strategy for bone tissue regeneration are: effective support of the endogenous regenerative capacity at lower manufacturing cost than recombinant growth factors, minimally invasively, preferably in the form of an injectable therapeutic and applicable as a one-step procedure. The approach presented in this thesis is based on injectable plasmid DNA solutions that are tailored for maximum bioactivity and minimal-invasiveness to effectively support endogenous bone regeneration without use of exogenous cells, recombinant growth factors or biomaterials. Target gene selection wais aimed at providing a factor with high specific bioactivity for osteoinduction by *in vivo* co-expression in order to compensate for potentially low transfection efficacy of non-viral gene delivery. Active ultrasound-mediated minimally invasive non-viral *in vivo* gene transfer was investigated to enhance gene transfer efficacy. Additionally, to control spatial gene delivery *in vivo* by sonoporation, specific control over non-viral therapeutic transgene co-expression with TetON inducible co-expression plasmid has been study *in vivo* in this study for the first time. An enhanced non-sample destructive *in vitro* reporter gene assay for highly sensitive on-line detection of osteogenic differentiation was established (Chapter I, pages 44-70) and subsequently employed in screening for therapeutic bone morphogenetic candidate genes & gene combinations along with testing of different constitutive and inducible co-expression plasmid configurations (Chapter II, pages 78-114). This led to the selection of a co-expression strategy for BMP2 and BMP7, which displayed the highest bioactivity for further *in vivo* testing (Chapter III, pages 125-192) and the design of constitutive and

inducible therapeutic BMP2/7 co-expression plasmids (Chapter II, pages 78-114). Evaluation of constitutive multi-cassette co-expression plasmids with different expression cassette topologies *in vitro* (Chapter II, pages 78-114), indicated potential transcriptional interference phenomena impairing transcription when compared to multi-vector delivery where effects were topology dependent. Tandem organisation of expression cassettes displayed lower induction of differentiation as compared to divergent promoter organisation. *In vivo* evaluation of the therapeutic potential of the designed co-expression systems in conjunction with passive and sonoporative gene transfer (*in vivo* in ectopic and orthotopic models) for osteoinduction (Chapter III, pages 125-192) demonstrated potent osteoinduction after repeated administration of therapeutic DNA.

Results obtained reveal that non-viral BMP2/7 co-expression by passive gene transfer or sonoporation *in vivo* leads to ectopic bone formation including a haematopoietic stem cell niche in ectopic models. Sonoporative gene transfer enabled higher success rates in gene delivery when compared to passive gene delivery in intramuscular ectopic studies and orthotopic studies in a femur fracture model. Detection of reporter gene expression in ectopic and orthotopic settings demonstrated successful local and spatially controlled gene expression. Plasmid bio-distribution monitoring using a novel metabolic labelling method showed binding of the plasmid DNA to intact bone at the defect site and at bone tissue lining the bone marrow lumen, indicating absorption of DNA by mineralized bone tissue. Orthotopic over-expression of BMP2/7 enhanced bone regeneration in the femur fracture model, although its efficacy is still limited.

In conclusion, the *in vitro* and *in vivo* results of this study demonstrates that BMP2/7 co-expression is superior to single BMP gene delivery and that the designed expression systems in conjunction with the established treatment protocols for non-

viral sonoporative gene delivery effectively mediates bone formation *in vivo* as well as enhanced endogenous orthotopic bone regeneration *in vivo.* Whilst improvements in terms of therapeutic efficacy and DNA dosing are still necessary, the therapeutic approach described in this study provides a viable alternative to recombinant growth factor or stem cell delivery. There is a need for further investigation of this approach in large animal studies with the future goal of clinical translation.

A novel artificial *cis*-acting sequence for osteoblast specific expression by adding a viral enhancer element to a specific minimal murine osteocalcin promoter element is presented in Chapter I, . Signal amplification (approx. up to 250-fold) of the CMVE-enhanced osteoblast specific reporter gene expression was achieved via 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. The cytomegaloviral enhancer has been used to amplify expression from several tissue specific promoters with fair results concerning the maintenance of tissue specificity. Until now, this has been successfully demonstrated with neuronal [1], cardiac and lung tissue [2] specific constructs but not for bone tissue specific signal amplification. The work in Chapter I represents another CMV-enhanced version of a tissue specific promoter suitable for restricted expression to be added to the collection of chimeric promoter elements that retain their specificity.

There is general consensus regarding possible risks of impairing or even abolishing the tissue specificity of the employed tissue specific promoter by using this strategy for signal amplification [2] depending on the employed promoter and the cellular context. 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. With

regards to the bone specific hybrid CMVE and osteocalcin promoter system it is unlikely that there might be non-specific activation by other growth factors, as no activation of the system above background levels was observed in the unrelated growth factor controls. 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 enabled real-time monitoring of osteogenic differentiation thereby, reducing sample numbers and hands-on time.

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 this novel CMV-enhanced reporter gene system, it was possible to detect osteocalcin expression activating amounts of recombinant BMP2 down to concentrations of 2nM with a strong linear correlation observed within a range of 2-10 nM. Whilst reporter gene approaches such as the BRE-Luc system [3] t are more sensitive for BMP growth factor testing than the approach demonstrated in this study, ours is however 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, our system allows bioactivity and osteoinductivity monitoring of many different osteoinductive substances not limited to a growth factor family since osteocalcin is the downstream target of Runx2, the osteogenic regulatory transcription factor, where many osteoinductive signalling pathways converge [4]. The activation of our assay is differentiation specific and not signalling cascade specific.

Higher osteoinductive potential of constitutive BMP2/7 co-delivery in comparison with single gene delivery has been confirmed by osteocalcin reporter gene assays *in vitro* and presented in Chapter II. It has been observed that the co-transfection of individual BMP2 and BMP7 encoding plasmids leads to a higher osteoinductive stimulus *in vitro* when compared to single co-expression plasmid delivery. Higher sensitivity of the fluorescent reporter gene assay in comparison to ALP assays has been demonstrated by clearly depicting higher osteogenic activity for BMP2 over BMP7 single gene expression, which was not observable in ALP testing. Application of the reporter gene assay with the inducible BMP2/7 co-expression system *in vitro* clearly demonstrated that extent of osteogenic differentiation was directly linked to inductor dose controlled BMP2/7 co-expression.

The low background activity observed in myoblastic differentiated C2C12 cells, is independent of osteoblastic differentiation and could be attributed to the employed CMV-enhancer element, which is capable of binding multiple general transcriptional activators present in CMV permissive cells [5]. Since the observed results suggest that the background expression in the developed assay only reached very low levels and expression was specifically enriched in cellular clusters undergoing differentiation (dependent on the applied amount of osteoinductive growth factor), it can be concluded that the addition of the CMV-enhancer to the reporter plasmid does not impair the specificity of the system in this particular combination and cellular context yet enables sensitive detection of osteogenic differentiation *in vitro*, applicable for both, recombinant growth factor and therapeutic gene testing.

Chapter II details the study aimed at designing and evaluating co-expression systems for the constitutive and inducible co-delivery of 2 different BMP genes for induction of osteogenic differentiation via non-viral gene therapy. The potency of the differentiation induced by expression from constitutive single molecule co-expression plasmids was dependent on expression cassette topology and associated potential transcriptional interference.

It was possible to demonstrate the feasibility of applying this BMP2/7 co-expression strategy in an inducible fashion, triggering the expression of 2 transgenes simultaneously in a doxycycline dependent manner. This system enabled tight control of BMP expression and induction of differentiation *in vitro*, being silent in the non-induced state as confirmed by multiple assays *in vitro*. *In vivo* ectopic evaluation of the constitutive co-expression system with convergent orientation of expression cassettes clearly demonstrates an osteoinductive potential. Repeated injections into mouse hindlimb muscles led to formation of ectopic bone in 46% of treated animals after 5 injections of only 20µg therapeutic DNA per day, displaying similar efficacy at lower daily and total doses when compared to approaches using BMP2 gene delivery only [6].

Higher osteoinductive potential of BMP2/7 co-delivery in comparison with single gene delivery has been confirmed by ALP assays and osteocalcin reporter gene assays *in vitro* as part of the study. It has been observed that co-transfection of individual BMP2 and BMP7 encoding plasmids leads to a higher osteoinductive stimulus *in vitro* when compared to single co-expression plasmid delivery. Lower activity of the single plasmid BMP2/7 co-expression system compared to higher activity of the co-transfection approach of individual BMP2 and 7 expression plasmids *in vitro* strongly suggested interference phenomena impairing expression in the constitutive CMV-based co-expression systems. It is therefore necessary that the influence of

transcriptional interference phenomena [7] be taken into account when working with multiple expression cassettes.

CMV-enhancer elements were shown to compete for transcription initiation complex formation in plasmids. This was through interference of their respective enhancer elements [8], which might partially account for the lower expression efficacy and osteoinductive potential of both constitutive co-expression systems *in vitro.* Enhancedosteoinduction was observed with the divergent promoter system compared to tandem organization by *in vitro* testing demonstrating that such effects have to be elucidated empirically [9] with different cassette arrangements. Given that BMP-heterodimer formation relies on BMP dimerization within the endoplasmatic reticulum of the cell [10], it is anticipated that co-transfection of a single molecule is still preferable, when aiming at heterodimer production for maximum bioactivity. Co-transfection efficacy and balanced expression of 2 transgenes *in vitro* has been demonstrated to be highly variable [11] with limited number of cells positive for both transgenes when using individual plasmids for co-transfection, thus indicating a potential advantage of delivering 2 transgenes via single vector strategies [12].

Regulation of therapeutic gene expression levels and kinetics allows controlling of optimal dosing and can help mimic physiological gene expression patterns. Furthermore, bi-directional inducible systems [13] can deliver 2 transgenes simultaneously under the control of an inducible bidirectional promoter.

Co-delivery of BMP2 and BMP7 with this expression strategy enabled tight inductor controlled expression, which lead to subsequent differentiation. The extent of osteogenic differentiation, as determined by microscopy, ALP assays, osteocalcin reporter gene assay and quantitative real-time PCR clearly indicates a direct link between doxycycline dose and differentiation outcome. 1000ng/ml induced cultures

showed the most potent osteoinduction of all tested systems *in vitro* and no induction of osteogenic differentiation was observed without induction with doxycycline. Tightly regulated gene co-expression was observed after transfection of a singular plasmid unit *in vitro*, narrowing the effective time window of BMP2/7 co-expression down to approximately 4 days for C2C12 cells. This system will provides a useful tool in studies requiring to demonstrate control over *in vivo* bone formation via doxycycline triggered gene expression and to determine minimally required therapeutic BMP-expression time windows for bone induction.

The developed, inducible and constitutive BMP2/7 co-expression plasmids are useful tools for investigating the potential of non-viral gene therapeutics and the results obtained with these systems herein provide further evidence of the feasibility for developing co-expression based gene therapeutics, paving the way for further investigation in ? studies in Chapter III.

Following the identification of a feasible therapeutic gene combination and the design of suitable expression plasmids, the feasibility of controlled non-viral sonoporative gene delivery *in vivo* for application in bone regeneration has been evaluated in Chapter III. It has been demonstrated that the repeated co-delivery of the highly bioactive gene-combination of BMP2/7 effectively mediates bone formation *in vivo* in ectopic models and that gene expression can be precisely controlled using inducible co-expression systems. Direct comparison of sonoporation with passive gene delivery demonstrated an increased probability of gene expression and bone formation with sonoporation increasing proportionately with total ultrasound energy applied to the target site. However, when successful gene transfer was accomplished, gene expression levels and bone volumes were not observed to increase via sonoporation. In orthotopic models, it was possible to replicate the beneficial effect of sonoporation on gene transfer probabilities and to demonstrate localized orthotopic gene expression. DNA tracking revealed that a large fraction of plasmid DNA binds to the matrix of the intact bone and is thus absorbed from the defect site. *In* vivo µCT data suggests enhanced orthotopic bone regeneration after active sonoporative gene transfer of BMP2/7 to the target site.

Ectopic gene transfer monitoring with luciferase showed no significant differences in expression strength between passive gene transfer and sonoporation. The probability of successful gene transfer, however, was increased when ultrasound was applied for gene transfer with increasing power. It was possible to reach gene transfer efficacies of 100% using the 4 Watt/cm^2 (300 Joule/cm^2) sonoporation protocol. Therefore, we conclude that sonoporation increases gene transfer efficacy compared to passive gene delivery and that the efficacy depends on the applied ultrasound power. This is in line with previous studies [14] which showed that, *in vitro* there is a delicate balance between ultrasound power, cell viability and sonoporation efficacy.

We observed skin burning at the exit sites of ultrasound in the more effective 4 Watt/cm^2 protocol in nude mice, which was not reported in previous studies [15]. This phenomenon disappeared when ultrasound power was reduced from 300 Joule/cm^2 to 90 Joule/cm^2 using the 2 Watt/cm^2 protocol. 100% ectopic bone formation efficacy was only observed when using the 4 Watt/cm^2 sonoporation protocol.

BMP2/7 co-expression is considered more effective than BMP2 expression alone as reported in Chapter II and by several *in vitro* and *in vivo* studies [16-19] due to the formation of a very highly bioactive BMP2/7 heterodimeric growth factor [18, 20, 21], which is less prone to inhibition by endogenous inhibitors [22] and induces endogenous BMP4 expression [19]. By applying this co-expression strategy, we were able to achieve 100% bone formation efficacy at lower DNA doses (1/5 of the dose used in [15] with BMP2; 1.5x less of the dose used in [23] with BMP9), lower number of repetitive treatments (5x vs 7x in [15]) or lower total applied ultrasound power (300 Joule/cm^2 in 24h vs 1500 Joule/cm^2 in 24h in [23]) when compared to similar studies using only single BMP gene transfer [6, 15, 23]. Therefore, we conclude that the selected co-expression strategy is superior to single factor expression in the case of BMPs and that this allows for higher therapeutic efficacy using an actual low-efficacy non-viral gene transfer method. By using this approach it might be feasible in the future to enable the less invasive sonoporative gene transfer method to compete with current electroporation approaches used for bone regeneration [19, 23, 24], which have been shown to be more effective than sonoporation due to higher transfection efficacy as reported in [23] but rely on invasive insertion of electrodes in contrast to minimally invasive transcutaneous application of ultrasound.

Ectopic application of the constitutive BMP2/7 co-expression system demonstrates that direct *in vivo* co-delivery of bone morphogenetic protein encoding plasmid DNA could provide a viable alternative to stem cell or recombinant protein based

therapies. Furthermore, the data presented in this study, clearly indicates that sonoporative gene delivery is superior in direct comparison with passive gene delivery with regards to gene transfer probability and that gene transfer efficacies are dependent on the total amount of ultrasound energy applied to the target site.

Doxycycline inducible BMP2/7 co-expression from a modified bidirectional TetON system [13] showed effective (83.3% efficacy) bone formation only when applied with ultrasound and only if gene expression from the system was activated by systemic application of doxycycline for 7 days. Tight control of the co-expression of 2 individual transgenes *in vivo* by systemic application of an inductor was observed. By narrowing *in vivo* BMP2/7 co-expression down to approximately 7 days, it was possible to demonstrate *in vivo* BMP expressionto potentially recapitulate endogenous BMP expression patterns (shorter than 14 days) [25], sufficient for *in vivo* bone formation. This reduction of therapeutic gene dose and gene expression time window compared to the constitutive co-expression systems demonstrates a even pronounced effect of sonoporation on bone formation efficacy in the case of the inducible system. Notably, in conjunction with the assumed reduced gene dose, it was not possible to induce bone formation by passive gene transfer even in animals that received doxycycline treatment when expression was limited to 7 days. Sonoporation thus, in general increases gene transfer efficacy, enables reduction of therapeutic gene dose and therapeutic gene expression time windows due to higher overall gene transfer efficacies & expression. This was not observed as dramatically with the constitutive co-expression systems, because overall gene expression levels were probably already saturated and therefore it was not possible to observe these subtle differences with these systems.

Orthotopic gene transfer efficacies, as paralleled by the ectopic findings, were higher when using sonoporation as compared to passive gene transfer, demonstrating the

feasibility of using ultrasound for orthotopic gene delivery. Interestingly, in contrast to the ectopic findings, there was a clear, significant drop of luciferase activity when the luciferase plasmid was co-applied with the constitutive BMP2/7 co-expression plasmid in passive and sonoporative gene transfer. This indicates that competitive expression might reduce overall gene expression efficacy if multiple independent expression entities are applied *in vivo.*

Tracking of luciferase plasmid DNA biodistribution at the defect site using a novel metabolic DNA labelling method based on the work of Neef *et al.* [26] provided a different outcome than tracking of luciferase gene expression at the target site. At the relatively high plasmid DNA dose used in this study, it has been observed that the DNA spreads from the site of application to unintended tissues such as bone marrow, intact bone and surrounding muscles. Whereas expression at these sites could not be evaluated due to the limitations of luciferase imaging, it has been shown by other studies that the presence of plasmid DNA at off-target sites does not automatically trigger off-target transgene expression [27] and should therefore be carefully investigated in future studies when making conclusions about off-target expression. An unusual finding of plasmid DNA bio-distribution monitoring was, extensive diffuse binding of the plasmid DNA to intact bone at the defect site and at bone tissue lining the bone marrow lumen, indicating that a large fraction of the applied DNA gets absorbed by mineralized bone tissue within 24 h post application. Binding of nucleic acids to ceramic hydroxyapatite has been well known already for some time [28, 29] and harnessed for DNA purification [30] and delivery [31, 32]. This study, to the knowledge of the authors, is the first study to demonstrate this effect *in vivo* with biological hydroxyapatite and naked exogenous DNA in orthopaedic gene therapy. The absorption of DNA from the defect site might be considered as another caveat in *in vivo* naked DNA transfer, additionally to nuclease digestion [33, 34], which might

be specific for orthopaedic applications and should be considered in future studies as parameter potentially responsible for low therapeutic efficacy of these approaches in bone regeneration at relatively high doses of DNA [35, 36].

In vivo quantitative and qualitative µCT data(although no significant difference could be found within this study design) revealed a potential beneficial effect of BMP2/7 gene delivery on fracture regeneration. The 4mm femur fracture model used in this study had an overall approximate 83.3% non-union rate at 8 weeks post fracture, when taking potential unions into account prior to in depth µCT evaluation which led to 100% non-union rate, and was therefore performing comparably to previous studies in rats by our group [37, 38]. It has been demonstrated that active orthotopic BMP2/7 co-delivery via sonoporation leads to 2/6 unions in the therapy group (union rate 33.3%) and passive gene delivery could achieve at least 1/6 unions (16.6% union rate) compared to 0/6 unions in both the luciferase negative control and empty defect control groups respectively when evaluated by µCT- the only reliable method in determining fracture union rate [37]. Therefore, in conjunction with luciferase data, it is concluded that sonoporative gene transfer enables orthotopic gene delivery and that orthotopic BMP2/7 gene delivery by sonoporation enhances bone regeneration at orthotopic sites. Thus, it might be possible to definitely prove therapeutic efficacy in future studies if the therapeutic effect is enhanced significantly either by additional recruitment of cells to a biomaterial *in situ* prior to gene delivery as demonstrated in [39] or by combining our approach with the mentioned matrix-assisted sonoporation technology (Nomikou *et al.*, in submission). These potential modifications of the current protocol address different aspects, which potentially limited the therapeutic efficacy in this study, such as the initial lack of expression-capable cells at the defect site and DNA absorption from the target site by the surrounding intact bone tissue, given that an additional biomaterial could recruit endogenous cells and retain the

therapeutic DNA at the site of action. Furthermore, the data presented in this study indicates that application of an internal luciferase control plasmid can reduce transgene expression at the defect site due to competitive expression. Therefore, a simple means of enhancing therapeutic efficacy in future studies aiming exclusively at the evaluation of bone regeneration could be, to apply only the therapeutic co-expression plasmid without the internal luciferase control. Gene transfer efficacy, dosing, consistency and expression vector design are variables critically influencing success of such strategies and l require further improvement in future studies.

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List of Abbreviations

µCT	Micro Computed Tomography
2D	Two dimesional
3D	Three dimensional
ALP	Alkaline Phosphatase Activity
AP-1	Activator Protein 1
ATMP	Advanced Therapy Medicinal Products
BMP2	Bone Morphogenteic Protein 2
BMP7	Bone Morphogenteic Protein 7
BV	Bone Volume
C/EBP	CCAAT/enhancer binding protein
cDNA	Complementary Deoxyribonucleic acid
CHO	Chinese Hamster Ovary
CLSM	Confocal Laser Scanning Microscopy
CMV	Cytomegalovirus
CMVE	Cxytomegalovirus immediately early enhancer
Ct	Cycle Threshold
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid

EMA	European Medicinces Agency
EMBs	Encapsulated Micro Bubbles
F-ARA-Edu	(2'S)-2'deoxy-2'-fluoro-5-ethynyluridine
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
FGF2	Fibroblast Growth Factor 2
GMP	Good Manufacturing Process
H&E	Haematoxylin and Eosin
HA	Hydroxyapatite
ICP	Iterative Closest Point
IRES	Intra-ribosomal Entry Sites
MetLuc	*Metridia longa* luciferase
mOCP	Murine Osteocalcin Promoter
mOG2P	Murine Osteocalcin 2 Promoter Element
NaOH	Sodium Hydroxide
OSE2	Osteoblast Specific Element 2
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pDNA	Plasmid Deoxyribonucelic acid

qPCR	Quantitative Polymerase Chain Reaction
rhBMP2	Recombinant Human Bone Morphogenetic Protein 2
RUNX2	Runt-related Transcription Factor 2
Tet	Teetracycline
TGFβ	Transforming Growth Factor- beta
TV	Tissue Volume
VEGF-A	Vascular Endothelial Growth Factor A

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Involved in numerous multidisciplinary projects in the realms of tissue engineering and shock / sepsis / intensive care related research: bone and cartilage regeneration, wound healing, neuroregeneration, *in vivo* imaging and cell tracking (bioluminescence and fluorescence), systemic RNAi *in vivo*, gene and growth factor delivery and cell therapy.

Officially responsible for ensuring safe usage of GM organisms at the institute.

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Involved in the successful submission of a Eurostars grant- UGen : Project no. E!5650 to fund my PhD degree.

Involved in numerous multidisciplinary international and national collaborative projects with colleagues at University of Ulster, University of Leeds, University of Twente, Hacettepe University, Vienna Medical University.

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Laboratory and Office Assistant

Involved in cryopreservation of gametes and cell culture.

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Extensive knowledge of Microsoft Office programs and have taken advanced courses in Excel, PowerPoint and

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Recipient of Best Oral Presentation award at Tissue Engineering and Regenerative Medicine Society (TERMIS-EU), Granada, Spain (2011)

Awarded certificate for ranking as one of the top fifty best abstract submissions at Tissue Engineering and Regenerative Medicine Society (TERMIS-EU), Galway, Ireland (2010)

Co-recipient of silver award for best poster at Bone Tec conference, Hannover, Germany (2009)

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In: *Biological Adhesive Systems: From Nature to Technical and Medical Application.* Eds. Byern, Janek von & Grunwald, Ingo; Springer

ORAL PRESENTATIONS (2006-2012)

ECTES (2012), Basel, Switzerland; “Enhancement of orthotopic bone regeneration by non-viral sonoporation gene therapy”

ESGCT (2011), Brighton, UK; “Non-viral sonoporation gene therapy for orthotopic bone regeneration”

Bone-Tec (2011), Hannover, Germany; “Non-viral sonoporation gene therapy for orthotopic bone regeneration”

Leeds Dental Institute (2011), Leeds, UK; Invited Speaker: “Non-viral sonoporation gene therapy for orthotopic bone regeneration”

TERMIS-AP Chapter meeting (2011), Singapore, “Non-viral sonoporation gene therapy for orthotopic bone regeneration”

TOPEA meeting (2011), Barcelona, Spain “Non-viral sonoporation gene therapy for orthotopic bone regeneration”

TERMIS-EU Chapter meeting (2011), Granada, Spain, “Non-viral sonoporation gene therapy for orthotopic bone regeneration”

Embryology conference (2011) Yekaterinburg, Russia; "Генная терапия в тканевой инженерии". (“Gene therapy for tissue engineering”)

Expertissues Winter School (2011) Salzburg, Austria; “Reporterconstructs for Monitoring”

4th Wiener Biomaterial Symposium (2010), Vienna, Austria, “Inducible BMP2/BMP7 co-expression system for osteoinduction”

Expertissues Scientific Meeting (2010), Guimarães, Portugal, “Signal enhanced reporter gene assay for osteogenesis: osteocalcin coupled expression monitoring of a bioluminescent secreted metridia luciferase” and “Inducible BMP2/BMP7 co-expression system for osteoinduction”

TOPEA meeting (2010), Barcelona, Spain (2010) “Inducible BMP2/BMP7 co-expression system for osteoinduction”

TERMIS-EU Chapter meeting (2010), Galway, Ireland, “Inducible BMP2/BMP7 co-expression system for osteoinduction”

Bone-Tec (2009), Hannover, Germany; Keynote lecture: “Induction and detection of osteogenic differentiation *in vitro*”

2nd TERMIS world congress (2009), Seoul, Korea; “Signal enhanced reporter gene assay for osteogenesis coupled expression monitoring of a bioluminescent secreted metridia luciferase”

7th international conference on bone morphogenetic proteins (2008), Lake Tahoe, USA; “*In vitro* characterization of therapeutic expression systems for bone regeneration”

14th Biomedical Science and Technology Symposium (2008), Marmaris, Turkey; “Induction and detection of osteogenic differentiation *in vitro*“

TERMIS-EU Chapter Meeting (2007), London, UK; “Reporter-vector systems to monitor osteogenic differentiation“

ECM VIII Bone Tissue Engineering Conference (2007), Davos, Switzerland; “Reporter-vector systems to monitor osteogenic differentiation“

6th International Conference on Bone morphogenetic proteins (2006), Dubrovnik, Croatia; “Reporter-vector systems for osteogenic differentiation“