Sonoporation Increases Therapeutic Efficacy of Inducible and Constitutive *BMP2/7 In Vivo* Gene Delivery

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Abstract

An ideal novel treatment for bone defects should provide regeneration without autologous or allogeneous grafting, exogenous cells, growth factors, or biomaterials while ensuring spatial and temporal control as well as safety. Therefore, a novel osteoinductive nonviral *in vivo* gene therapy approach using sonoporation was investigated in ectopic and orthotopic models. Constitutive or regulated, doxycycline-inducible, bone morphogenetic protein 2 and 7 coexpression plasmids were repeatedly applied for 5 days. Ectopic and orthotopic gene transfer efficacy was monitored by coapplication of a luciferase plasmid and bioluminescence imaging. Orthotopic plasmid DNA distribution was investigated using a novel plasmid-labeling method. Luciferase imaging demonstrated an increased trend (61% vs. 100%) of gene transfer efficacy, and micro-computed tomography evaluation showed significantly enhanced frequency of ectopic bone formation for sonoporation compared with passive gene delivery (46% vs. 100%) dependent on applied ultrasound power. Bone formation by the inducible system (83%) was stringently controlled by doxycycline *in vivo*, and no ectopic bone formation was observed without induction or with passive gene transfer without sonoporation. Orthotopic evaluation in a rat femur segmental defect model demonstrated an increased trend of gene transfer efficacy using sonoporation. Investigation of DNA distribution demonstrated extensive binding of plasmid DNA to bone tissue. Sonoporated animals displayed a potentially increased union rate (33%) without extensive callus formation or heterotopic ossification. We conclude that sonoporation of *BMP2/7* coexpression plasmids is a feasible, minimally invasive method for osteoinduction and that improvement of bone regeneration by sonoporative gene delivery is superior to passive gene delivery.

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 (Stannard *et al.*, 2007). These nonunion fractures, also called pseudoarthroses, are characterized by a failure to heal within 8–9 months (Stannard *et al.*, 2007). 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 out-

comes, although at the cost of an invasive procedure requiring surgical intervention causing donor-site morbidity (Jones, 2005; Sen, 2007). Other experimental treatment modalities include combinations of autologous stem cell treatment, biomaterials, and growth factors (Bruder *et al.*, 1998; Axelrad *et al.*, 2007; Kanakaris and Giannoudis, 2008; Schmidmaier *et al.*, 2008). Therapies that rely on stem cell treatment require invasive sampling and *in vitro* expansion of these cells before implantation, which again can lead to donor-site morbidity and furthermore require expensive good manufacturing practice regulations facilities for safe cell enrichment and

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expansion (Verbeek, 2012). 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 to produce (Vaibhav *et al.*, 2007) and need to be applied in potentially dangerous supraphysiological doses (Gamradt, 2004), which can lead to adverse effects such as heterotopic ossification (Benglis *et al.*, 2008) or immune responses (Hwang *et al.*, 2009). A novel therapeutic approach should therefore enable a cost-effective, efficient, and minimally invasive treatment of nonunion fractures. It should preferentially be applied in a one-step procedure without the need for autologous or allogeneic implantation material. Several studies have demonstrated the feasibility of transient gene therapies for osteoinduction either through *ex vivo* (Gamradt, 2004) or *in vivo* viral (Bleiziffer *et al.*, 2007) or nonviral gene transfer (Luo *et al.*, 2005; Bleiziffer *et al.*, 2007). 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 posttranslational modifications and conformation of the factor, thereby mediating higher bioactivity at lower concentrations compared with the application of recombinant factors (Brooks, 2006). Since nonviral *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 (sonoporation), a relatively novel nonviral strategy that relies on neutral microbubble contrast agent-mediated cell permeabilization *in situ* to trigger uptake of plasmid DNA (Mehier-Humbert, 2005; Li *et al.*, 2009), appears to be superior in terms of reduced invasiveness and clinical translation compared with other nonviral methods such as electroporation (Cemazar *et al.*, 2006). To compensate for the lower efficacy of this nonviral gene transfer method, a highly osteoinductive coexpression strategy for bone morphogenetic protein (BMP) 2 and 7 (BMP2/7), which has been shown to potently mediate osteogenic differentiation *in vitro* (Kawai *et al.*, 2006) and *in vivo* (Zhao *et al.*, 2005; Kawai *et al.*, 2006), was selected to be investigated for its regenerative potential in a femur nonunion model in rats. The work presented herein was aimed at demonstrating the feasibility of an ultrasound-mediated (i.e., sonoporation), constitutive or regulated, doxycycline-inducible BMP2/7 coexpression strategy for bone regeneration. This approach for enhanced fracture regeneration solely relies on minimally invasive injection of a BMP2/7 coexpression plasmid DNA–microbubble mixture in conjunction with a transcutaneously applied ultrasound trigger to mediate deep within-tissue *in vivo* nonviral 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 Institutes of Health. Female Hsd:ATHymic Nude-Foxn1^{nu} nude mice ($n=36$; Harlan Laboratories, Bresso, Italy) of ~12 weeks of age and weighing ~30 g were used for ectopic testing in this study.

Male Sprague-Dawley rats ($n=43$; Charles River, Wilmington, MA) weighing ~450 g were used for orthotopic testing in this study.

Plasmids

The inducible and constitutive single-vector BMP2/7 coexpression plasmids, pTetON-BMP2/7 and pVAX1-BMP2/7– (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/hgtb), are described elsewhere (Feichtinger *et al.*, under review). Briefly, the constitutive pVAX1-BMP2/7– system was created by a multiexpression cassette strategy with separate CMV-promoter-driven BMP2 and BMP7 expression cassettes in divergent orientation on the FDA's DNA vaccine guideline-compliant plasmid backbone of pVAX1 (Life Technologies, Palo Alto, CA). The single-vector TetON-inducible BMP2/7 coexpression system pTetON-BMP2/7 was derived from the Tet-regulated coexpression 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) to pTREtightBI, generating a TetON-inducible expression plasmid with three transcriptional entities. All plasmid preps were carried out by Endo-free plasmid Maxi or Giga kits (Qiagen, Hilden, Germany), or plasmids were produced as endo-free preps by Plasmid Factory (Bielefeld, Germany).

Sonoporation protocols

An SP100 Sonoprotector (Sonidel Ltd., Dublin, Ireland) emitting 1 MHz ultrasound was employed for sonoporative gene transfer in this study. The columnar beam was set at different watt/cm² (W/cm²) spatial average temporal peak settings with an effective radiating area of 0.8 cm² at a pulse frequency of 100 Hz/100% duty cycle.

2 W/cm² treatment protocol. This protocol was a modified protocol derived from the ultrasound parameters applied in previous studies by Li *et al.* (2009). Daily treatment, which was repeated for 5 days, consisted of application of 2 W/cm² 1 MHz ultrasound at a duty cycle of 25% for 3 min after injection of a plasmid DNA–microbubble contrast agent mixture. Total energy delivered to the target site per daily treatment was 90 J/cm².

4 W/cm² treatment protocol. This protocol was a modified protocol derived from Osawa *et al.* (2009). Daily treatment, repeated for 5 days, consisted of application of 4 W/cm² 1 MHz ultrasound at a duty cycle of 50% for 1 min followed by a pause of 1 min to prevent tissue overheating and was repeated for a total of 5 times (5 × 1 min treatment at 1 min intervals). Total energy delivered to the target site per day was 600 J/cm².

Animals were placed on a neoprene adsorber pad (Sonidel Ltd.) with ultrasound contact gel (Aquasonic 100; Parker Laboratories, Fairfield, NJ) 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 coexpression *in vivo*. About 20 µg constitutive coexpression plasmid pVAX1-BMP2/7– was codelivered with 20 µg of the internal luciferase control plasmid pCBR (Promega GmbH, Mannheim, Germany).

Sonoporation was carried out by the SP100 device and neutral, lipid-based microbubbles (MB101; Sonidel Ltd.) at a final concentration of 4×10^8 /ml (50% v/v). About 50 μ l of this plasmid-microbubble solution was injected into gastrocnemius muscle in nude mice and immediately thereafter sonoporated. Anesthesia was initialized in an inhalation box provided with isoflurane (Forane; Abbott Gesm.b.H., Vienna, Austria) (3 vol.% isoflurane-oxygen mixture) and maintained by inhalation of 1.5–2% isoflurane-oxygen mixture. This treatment was repeated for 5 subsequent days (total therapeutic DNA dose 100 μ g/animal; ~ 3.3 mg/kg) using the same injection site. Sonoporation was carried out by either 2 W/cm² (25% duty cycle) or 4 W/cm² (50% duty cycle) protocols in order to investigate the influence of ultrasound power settings on gene transfer efficacy. Hindlimbs were sonoporated for 1 min 5 times using the described power settings. Gene transfer efficacies of sonoporated hindlimbs were compared with passive gene transfer in control hindlimbs without sonoporation. To investigate the potential competitive influence of an internal control plasmid on overall bone formation efficacy, 2 W/cm² sonoporations were additionally carried out without pCBR addition. Four weeks after the surgery, the mice were euthanized with inhalation anesthesia (see above) and an intracardiac overdose of thiopentalsodium (Thiopental; Sandoz GmbH, Vienna, Austria) (120 mg/kg) and gastrocnemius muscles were explanted for histology.

Inducible BMP2/7 coexpression *in vivo*. The doxycycline-inducible coexpression system for BMP2/7, TetON-BMP2/7, was sonoporated *in vivo* using the 2 W/cm² sonoporation protocol. 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 2 mg/day doxycycline in 300 μ l Ringer's solution intraperitoneally for 7 days for induction of BMP2/7 coexpression after sonoporative or passive gene transfer. Sonoporated control animals were not treated with doxycycline.

Orthotopic BMP2/7 sonoporation

Femur defect model. Anesthesia was induced via inhalation of 2% isoflurane and maintained through an intraperitoneal injection of a mixture of ketamine hydrochloride (110 mg/kg; Ketamidol; Richter Pharma AG, Wels, Austria) and xylazine (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 hr 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, four-hole titanium plate (Stryker, Duisburg, Germany) was fixed onto the anterolateral surface of the femur using four cortical 7 mm titanium screws (Synthes, Oberdorf, Switzerland). Subsequently, a 4 mm segmental bone defect was inflicted by

two 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 euthanized via an intracardiac injection of thiopentalsodium (Thiopental; Sandoz GmbH; 120 mg/kg). Micro-computed tomography (μ CT)-based detection of plate and/or screw dislocation at 4 or 8 weeks postsurgery led to exclusion of the animal from μ CT bone regeneration evaluation (8 of 32 animals, 25% drop out).

Constitutive BMP2/7 coexpression *in vivo*. About 50 μ g of the constitutive coexpression plasmid pVAX1-BMP2/7 – was codelivered 3 days postsurgery with 50 μ g of the internal luciferase control plasmid pCBR using sonoporation in the active gene transfer treatment group (total therapeutic DNA dose 250 μ g; 0.5 mg/kg). The luciferase control group consisted of animals sonoporated with 50 μ g of the internal luciferase control plasmid pCBR and 50 μ g of an empty pDNA backbone (pUK21; Plasmid Factory) to normalize the DNA content according to the therapy group.

The efficacy of passive gene delivery to the defect was investigated using either injections of therapeutic plasmid codelivered with the internal luciferase control plasmid (50 μ g pVAX1-BMP2/7 + 50 μ g pCBR) or injections of the internal luciferase control plasmid codelivered with a non-expressing empty plasmid (50 μ g pCBR + 50 μ g pUK21) without ultrasound treatment. Furthermore, we investigated the endogenous regenerative potential and union rate in an empty control group, which did not receive any treatment. Sonoporation was carried out using the SP100 sonoporation and neutral, lipid-based microbubbles (MB101; Sonidel Ltd.) at a final concentration of 8×10^8 /ml (75% v/v). 200 μ l of the plasmid-microbubble solution was injected under X-ray guidance and immediately after sonoporated into the femur defect site using the 4 W/cm² sonoporation protocol. This treatment was repeated for 5 days, resulting in a total therapeutic DNA dose of 250 μ g/animal (~ 0.5 mg/kg).

Plasmid DNA labeling and orthotopic detection

To enable therapeutic DNA detection at the defect site for biodistribution monitoring, we applied a novel metabolic DNA labeling method in *Escherichia coli* based on the nucleoside (2'S)-2'-deoxy-2'-fluoro-5-ethynyluridine (F-ara-EdU) (Neef and Luedtke, 2011). pCBR plasmid DNA was labeled with F-ara-EdU using a thymidine-auxotrophic *E. coli* strain (Chi1776; DSMZ, Braunschweig, Germany) and supplementing with 394 medium (Jerome *et al.*, 2009) containing 20 mg/ml thymidine + 20 mg/ml F-ara-EdU. Plasmids were isolated by the Endo-free Plasmid Maxi Kit (Qiagen).

A separate cohort of rats ($n=7$) was allocated for imaging of *in situ* orthotopic luciferase expression and subsequent plasmid DNA detection 24 hr after the last gene transfer. Detection was carried out on decalcified paraffin sections of the femora by an Alexa Fluor 680-labeled azide (Life Technologies) for the Cu⁺-catalyzed click reaction described by Neef and Luedtke (2011). AF680 signals specific for F-ara-EdU-labeled plasmid DNA were detected using an Odyssey near-infrared scanner (LI-COR Biosciences, Lincoln, NE) and confocal laser scanning microscopy (CLSM). In CLSM, a separate 488 nm channel was used in addition to the 680 nm 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-labeled plasmid DNA served as a negative control.

Bioluminescence imaging

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

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

A separate group of rats was examined for gene transfer localization at the defect site 24 hr after the last gene transfer using bioluminescence imaging *in vivo* after surgically opening the defect site (30 min after luciferin administration) under general anesthesia (see section Femur defect model above). Additionally, femora were imaged *ex vivo* after the rats were euthanized by general anesthesia (see above) by an overdose of thiopentalsodium (Thiopental; Sandoz GmbH) (120 mg/kg) by intracardiac injection. Luciferase imaging was carried out after explantation of the femur and adjacent muscles 45 min after luciferin administration.

In vivo μ CT

In vivo μ CT images of nude mouse hindlimbs were obtained using a VivaCT 75 (Scanco Medical AG, Brütisellen, Switzerland) 14 and 28 days after the last gene transfer under short inhalation anesthesia. Bone volume (mm^3) and bone mineral density (mg hydroxyapatite per cm^3 [$\text{mg HA}/\text{cm}^3$]) for 2 and 28 days were calculated using Scanco software and a standard density calibration phantom.

In vivo μ CT images of rat femora at 0, 4, and 8 weeks after surgery were exported into MATLAB (MATLAB version 7.12.0; The MathWorks Inc., Natick, MA) for processing. To facilitate analysis, the volumes corresponding to all three time points were rigidly registered onto each other using a three-step approach.

First, the supporting titanium plate was segmented using a manually determined gray-level threshold. When using such a simple segmentation procedure, image artifacts 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 ($>3,000$ voxels).

In a second step, the major directions of the titanium plate were extracted from the processed binary segmentations using principal component analysis (PCA) (Pearson, 1901). Rotating the volumes according to these major axes served as an initialization to the final matching step. For this, we employed the iterative closest point (ICP) (Besl, 1992) algorithm.

While reliable in practice, it is well known to be susceptible to its initialization. Alignment using PCA assured 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 were combined into a rigid deformation matrix. This transformation was then applied to each volume in the sequence except the reference, ensuring correct correspondences of each voxel between acquisition times.

All volumes were then represented as color-coded overlays of the three time points. New bone at the defect site was segmented manually and quantified as bone volume/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 for 24 hr, 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 hematoxylin and eosin 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) primary antibody in a 1:50 dilution overnight and the appropriate secondary peroxidase-conjugated antibody ImmPRESS Anti-Goat Ig Peroxidase (Vector Labs, Peterborough, United Kingdom) for 30 min after blocking of endogenous peroxidase activity with 3% H_2O_2 in Tris-buffered saline. Nuclear counterstaining was carried out using Mayer's hematoxylin solution for 1 min.

Statistical analysis

Results are presented as median $\pm$ interquartile range, unless otherwise stated. Statistical testing was carried out using the nonparametric Kruskal-Wallis test in conjunction with Dunn's multiple comparison test as posttest 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 nonparametric Kruskal-Wallis test in conjunction with Dunn's multiple comparison test as posttest. Orthotopic passive luciferase expression data passed Kolmogorov-Smirnov normality testing and therefore was tested with a two-tailed *t*-test for significance. Gene transfer efficacy data (frequency of successful gene transfer) based on luciferase imaging and bone formation efficacy data (frequency of successful bone formation) were tested for improvement by sonoporation using a one-tailed Fisher's exact test. Bone volume per tissue volume 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 (* $p < 0.05$, ** $p < 0.01$).

Results

Ectopic BMP2/7 sonoporation

Gene transfer efficacies and luciferase gene expression. Evaluation of gene transfer efficacy 24 hr after the last

gene transfer via bioluminescence activity in nude mice confirmed successful gene transfer in 8 of 13 animals (61% efficacy) for passive gene delivery, 6 of 7 animals (85%) for 2 W/cm² sonoporation, and 7/7 animals (100%) for 4 W/cm² sonoporation (Fig. 1A). Bioluminescence imaging of the internal luciferase control demonstrated variable luciferase activity in all animals (Fig. 1B) 24 hr after the last treatment. No significant difference in bioluminescence levels was observed among passive gene transfer, 2 W/cm² sonoporation, and 4 W/cm² sonoporation. Sonoporation with 4 W/cm² caused considerable skin burns at the ultrasound exit sites in contrast to the 2 W/cm² protocol.

Ectopic bone formation using constitutive *BMP2/7* coexpression *in vivo*. Passive gene delivery of the constitutive *BMP2/7* coexpression plasmid pVAX1-BMP2/7– successfully induced ectopic bone formation in 6 of 13 animals (46%) (Fig. 2A). Sonoporative gene delivery with either 2 or 4 W/cm² protocols successfully induced ectopic bone formation in 5 of 7 (71%) or 7 of 7 animals (100%), respectively (Fig. 2A). The 2 W/cm² sonoporation of pVAX1-BMP2/7– without the luciferase plasmid led to bone formation in 6 of 7 animals (85%) (Fig. 2A). Bone volumes were variable and did not exhibit significant differences (Fig. 3G and H). Medians of bone volumes were 0.09 mm³ at 14 days and 0.04 mm³ at 28 days posttreatment using the constitutive coexpression systems with passive gene transfer: 0.002 mm³ (0.03 mm³ without pCBR addition) at 14 days and 0.17 mm³ (0.01 without CBR addition) at 28 days posttreatment using the constitutive coexpression system with the 2 W/cm² sonoporation protocol and 0.02 mm³ at 14 days and 0.01 mm³ at 28 days posttreatment using the constitutive coexpression system with the 4 W/cm² sonoporation protocol. Bone mineral densities did not show significant differences among treat-

ment groups and different time points posttreatment. Medians of bone mineral densities were 179.4 mg HA/cm³ at 14 days and 238 mg HA/cm³ at 28 days posttreatment using the constitutive coexpression systems with passive gene transfer; 160 mg HA/cm³ (201.8 mg HA/cm³ without pCBR addition) at 14 days and 243.6 mg HA/cm³ (230.4 mg HA/cm³ without CBR addition) at 28 days posttreatment using the constitutive coexpression system with the 2 W/cm² sonoporation protocol and 177.4 mg HA/cm³ at 14 days and 220.7 mg HA/cm³ at 28 days posttreatment using the constitutive coexpression system with the 4 W/cm² sonoporation protocol. Ectopic bones displayed variable morphology and irregular shape in all observed treatment groups (representative images in Fig. 3A–F). Direct comparison of 14-day with 28-day *in vivo* μ CT images of the same ossicles (Fig. 3A–F) showed moderate bone remodeling and resorption. Histological examination of ectopic bones at 4 weeks by hematoxylin and eosin staining revealed fully developed ossicles with a compact layer of bone surrounding a bone marrow cavity (Fig. 4A) populated by a heterogeneous cell population consisting of adipocytes, hematopoietic myeloid progenitor cells, megakaryocytes, and erythrocytes (Fig. 4D). Immunohistochemical detection of the internal luciferase control showed diffuse staining in muscle fibers surrounding the ectopic bone structure and strong staining of bone lining and bone marrow cells (Fig. 4B). Von Kossa staining for mineralization confirmed the observed structures in hematoxylin and eosin staining as mineralized bone tissue (Fig. 4C).

Ectopic bone formation using inducible *BMP2/7* coexpression *in vivo*. Sonoporation of the inducible single-vector *BMP2/7* coexpression system pTetON-BMP2/7 using the 2 W/cm² sonoporation protocol successfully induced

FIG. 1. Influence of sonoporation on orthotopic and ectopic gene transfer efficacies. Ectopic (A) and orthotopic (C) gene transfer efficacies based on luciferase activity. Ectopic luciferase activity (*BMP2/7* and luciferase codelivery) (B) and orthotopic luciferase activity (only luciferase: “pCBR” vs. luciferase and *BMP2/7* codelivery: “pCBR+pVAX1-BMP2/7–”) (D) 24 hr after last gene transfer for passive and sonoporative gene delivery. Median $\pm$ interquartile range. * $p < 0.05$.

FIG. 2. Enhancement of ectopic bone formation efficacy by sonoporation and effect of doxycycline on controlled bone formation (inducible BMP2/7 coexpression system). Ectopic bone formation efficacies (frequency of successful bone formation after gene delivery) for constitutive pVAX1-BMP2/7-based coexpression experiments (A) with and without luciferase plasmid addition (+CBR Luc/-CBR Luc). Ectopic bone formation efficacies (frequency of successful bone formation after gene delivery) for inducible pTetON-BMP2/7-based coexpression experiments (B) without luciferase plasmid addition and with or without doxycycline (DOX) application. Results represented for passive gene transfer and 2 and 4 W/cm² sonoporation protocols. **p* < 0.05; ***p* < 0.01.

ectopic bone formation in 5 of 6 animals (83%) only when induced with 2 mg of doxycycline per 48 hr for 7 days as observed by *in vivo* μ CT at 14 and 28 days posttreatment. No bone formation was observed without application of doxycycline (0 of 6 animals) (Fig. 2B). Furthermore, no bone formation was observed when using passive gene transfer and induction with 2 mg of doxycycline per 48 hr for 7 days (Fig. 2B). Ectopic bones did not show any significant differences in bone volumes (Fig. 3G and H) or bone mineral densities compared with ectopic bones generated using the constitutive coexpression system pVAX1-BMP2/7- at 14 and 28 days posttreatment. Medians of bone volumes were 0.1 mm³ at 14 days and 0.01 mm³ at 28 days posttreatment using the inducible coexpression system with the 2 W/cm² sonoporation protocol. Medians of bone mineral densities were 149.7 mg HA/cm³ at 14 days and 249.6 mg HA/cm³ at 28 days posttreatment.

Orthotopic BMP2/7 sonoporation

Orthotopic luciferase gene expression and gene transfer efficacies. Orthotopic bioluminescence imaging demonstrated strong luciferase expression limited to the defect site (Fig. 5B-D) and no activity in adjacent muscle tissue (Fig. 5C). No difference in localization and gene expression levels was observed between sonoporation and passive gene transfer (Fig. 1D). A significant reduction of bioluminescence was observed for both passive gene transfer and sonoporation when the luciferase plasmid was coadministered with pVAX1-BMP2/7- (Fig. 1D), indicating potential competitive expression. Gene transfer efficacies estimated via bioluminescence imaging were 66% (6 of 9 animals) for passive gene transfer and 85% (12 of 14 animals) for the 4 W/cm² sonoporation group (Fig. 1C).

Orthotopic F-ara-EdU-labeled luciferase plasmid DNA distribution. Detection of luciferase plasmid DNA biodistribution 24 hr after last gene transfer at the defect site through near-infrared scanning (Fig. 6A) revealed extensive signals in bone tissue. In-depth examination through CLSM demonstrated the confined presence of plasmid DNA within cells of the bone marrow (Fig. 6B), at the defect-site granulation tissue (Fig. 6C), diffuse distribution of plasmid DNA within intact bone tissue (Fig. 6D), and weak diffuse signals in surrounding muscle fibers (Fig. 6E) with confined signals in satellite cells (Fig. 6E, arrows). No differences in biodistribution were observed between passive gene transfer and sonoporation groups.

Orthotopic bone regeneration using constitutive BMP2/7 coexpression *in vivo*. *In vivo* μ CT images of defects at 4 and 8 weeks postsurgery showed 3 potential unions with substantial bone growth out of 6 animals of the sonoporation treatment group (Fig. 7A), 1 potential union in the passive gene transfer treatment group (Fig. 7B), 1 potential union in the luciferase control group (Fig. 7C), and 1 potential union in the empty control group (Fig. 7D) at 8 weeks postsurgery. In-depth examination of the μ CT images at different angles and cut planes confirmed 2 unions out of 6 animals (33% union rate; Fig. 8A) in the treatment group (Fig. 7A, asterisks) and 1 union (16% union rate; Fig. 8A) in the passive gene transfer treatment group (Fig. 7B, asterisk) at 8 weeks postsurgery. No unions were confirmed (Fig. 8A) in the luciferase control and empty control groups 8 weeks postsurgery (Fig. 7C and D). Quantification of bone volume/tissue volume at the initial margins of the defect did not show any significant differences between treatment groups and controls at both 4 weeks (Fig. 8B) and 8 weeks (Fig. 8C) postsurgery.

FIG. 3. *BMP2/7* coexpression induces ectopic bone formation *in vivo*. Representative images of ectopic bones at 14 and 28 days posttreatment for 4 W/cm² constitutive *BMP2/7* coexpression system sonoporation ("4 Watt/cm²") (A), 2 W/cm² constitutive *BMP2/7* coexpression system sonoporation ("2 Watt/cm²") (B), 2 W/cm² constitutive *BMP2/7* coexpression system sonoporation without luciferase internal control ["2 Watt/cm² (-CBR)"] (C), passive gene transfer of the constitutive *BMP2/7* coexpression system ("PASSIVE") (D), passive gene transfer of the constitutive *BMP2/7* coexpression system without luciferase ["PASSIVE (-CBR)"] (E), and inducible *BMP2/7* coexpression system without luciferase with 2 W/cm² sonoporation protocol ("TetON 2 Watt/cm²") (F). Scale bars represent 1 mm. Quantitative *in vivo* micro-computed tomography (μ CT) data for ectopic bones 2 weeks (G) and 8 weeks (H) posttreatment. Median $\pm$ interquartile range. Color images available online at www.liebertpub.com/hgtb

FIG. 4. Formation of ectopic bone and a stem cell niche by *BMP2/7* coexpression *in vivo*. Histologies of ectopic bone (representative image of $4\text{W}/\text{cm}^2$ sonoporation). Hematoxylin and 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 hematoxylin and eosin (**D**) showing hematopoietic bone marrow with hematopoietic stem cells (HSC) and adipocytes (AC). Scale bars: (**A**–**C**) $50\ \mu\text{m}$; (**D**) $20\ \mu\text{m}$. Color images available online at www.liebertpub.com/hgtb

Discussion

The aim of this study was to evaluate constitutive or regulated, doxycycline-inducible, nonviral sonoporative *BMP2/7* gene codelivery *in vivo* for bone regeneration. It has been demonstrated that the repeated codelivery of a highly bioactive *BMP2/7* gene combination effectively mediates bone formation *in vivo* and that gene expression can be precisely controlled. Direct comparison of sonoporation with passive gene delivery demonstrated an increased probability of gene expression and bone formation dependent on total ultrasound energy applied, whereas when successful gene transfer was accomplished, gene expression levels and bone

volumes were not increased via sonoporation. In orthotopic models, a beneficial effect of sonoporation on gene transfer probabilities was indicated and that localized orthotopic gene expression was achieved. DNA tracking revealed that a large fraction of plasmid DNA binds to intact bone and is thus absorbed from the defect site. *In vivo* μCT data suggest 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 that the rate of successful gene transfer can be increased

FIG. 5. Spatially controlled orthotopic gene delivery *in vivo*. Luciferase expression after orthotopic gene delivery. Bioluminescence activity in rat femurs depicted as false-color images of average photons/sec/cm²/steradian (**A**, *in vivo*; **B**, *in situ*; **C**, *ex vivo*, femur and adjacent muscle; **D**, *ex vivo*). Color images available online at www.liebertpub.com/hgtb

FIG. 6. Plasmid DNA biodistribution and binding to intact bone in the defect area. Orthotopic luciferase plasmid tracking using (2'S)-2'-deoxy-2'-fluoro-5-ethynyluridine (F-ara-EdU) metabolically labeled pCBR luciferase plasmid DNA detected with Alexa Fluor 680 azide 24 hr after last gene transfer. Odyssey near-infrared scan of F-ara-EdU signals in a representative femur sample (**A**). Scale bar represents 2 mm. 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 100 μ m. Color images available online at www.liebertpub.com/hgtb

FIG. 7. Bone regeneration in femur fractures by orthotopic BMP2/7 sonoporation. Registered *in vivo* μ CT images of potential unions each from different viewing angles and sagittal cut plane: 0 weeks (white), 4 weeks (light blue), and 8 weeks postfracture (dark blue) of potential unions in the sonoporation therapy group (SONOBMP) (A), passive gene transfer therapy group (VECBMP) (B), luciferase sonoporation control group (SONOLUC) (C), and empty control group (EMPTY) (D). Unions confirmed by in-depth examination are marked with an asterisk. Scale bars represent 2 mm. Color images available online at www.liebertpub.com/hgtb

compared with passive gene delivery when applying ultrasound of increasing power (Fig. 1A). It was possible to reach gene transfer efficacies of 100% using the 4 W/cm^2 (600 J/cm^2) sonoporation protocol; however, there were no significant differences in expression levels observed between passive gene transfer and sonoporation (Fig. 1B). Therefore, we conclude that sonoporation increases gene transfer efficacy compared with passive gene delivery and that the efficacy depends on the applied ultrasound power. This is in line with the findings of Li *et al.* (2009), showing 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 4 W/cm^2 protocol in nude mice, which has not been reported by Osawa *et al.* (2009), who used an outbred mice strain in which skin damage is less obvious. This phenomenon disappeared when ultrasound power was reduced from 600 to 90 J/cm^2 using the 2 W/cm^2 protocol. Bone formation efficacies of the different gene transfer protocols (Fig. 2) generally paralleled gene transfer efficacies observed by luciferase monitoring (Fig. 1A), improving significantly with increasing ultrasound power.

FIG. 8. Effect of orthotopic BMP2/7 sonoporation on union rate. Observed union rates based on in-depth μ CT examination (A). Orthotopic bone volumes per defect tissue volumes based on quantitative *in vivo* μ CT evaluation 4 weeks (B) and 8 weeks (C) postfracture. Empty control group (Empty), luciferase control group (pCBR), and treatment groups (pCBR+pVAX1-BMP2/7-). Data depicted for passive gene transfer (PASSIVE), and 4 W/cm² sonoporation (4 Watt SONO). $n=6$, median $\pm$ interquartile range.

BMP2/7 coexpression is considered highly effective as reported by several *in vitro* and *in vivo* studies (Kawai *et al.*, 2006, 2009) because of the formation of a highly bioactive BMP2/7 heterodimeric growth factor (Israel *et al.*, 1996; Kawai *et al.*, 2009), which is less prone to inhibition by endogenous inhibitors (Zhu *et al.*, 2006) and induces endogenous BMP4 expression (Kawai *et al.*, 2006). 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 by Osawa *et al.* (2009) with BMP2; 1.5 $\times$ less of the dose used by Sheyn *et al.* (2008) with BMP9], lower number of repetitive treatments [5 $\times$ vs. 7 $\times$ by Osawa *et al.* (2009)], or lower total applied ultrasound power [600 J/cm² in 24 hr vs. 1,500 J/cm² in 24 hr by Sheyn *et al.* (2008)] compared with similar studies using only single BMP gene transfer (Sheyn *et al.*, 2008; Osawa *et al.*, 2009, 2010). Therefore, we conclude that the selected coexpression strategy is superior to single-factor expression in the case of BMPs and that this allows higher therapeutic efficacy while using an actual low-efficacy non-viral gene transfer method. It might be feasible in the future to enable less invasive sonoporative gene transfer to compete with current electroporation approaches (Pelled *et al.*, 2005; Kawai *et al.*, 2006; Sheyn *et al.*, 2008), which are currently more effective than sonoporation (Sheyn *et al.*, 2008) but rely on invasive insertion of electrodes in contrast to minimally invasive transcutaneous application of ultrasound.

This study employed an internal luciferase control for gene transfer monitoring, which did not exhibit a significant detrimental effect on bone formation efficacy (Fig. 2A) in the ectopic model. Therefore, we conclude that there is no substantial detrimental effect of adding a separate internal control plasmid in this setup. The authors would like to note, however, that the luciferase levels obtained in this study were lower than those of previously unpublished data for single luciferase sonoporation (Hofmann *et al.*, in preparation), indicating competitive expression between internal control plasmids and therapeutic plasmids. *In vivo* quantitative μ CT data showed no significant difference in bone volumes or bone mineral densities for passive gene transfer or sonoporation protocols in contrast to bone formation efficacy, which is in line with the findings on gene transfer efficacies described above. Bone volumes achieved by BMP2/7 gene delivery (passive or sonoporative) were comparable to ectopic bone volumes achieved with BMP9 sonoporation by Sheyn *et al.* (2008). Average bone mineral densities were within the range of ectopic bone mineral densities expected in ectopic bone formation models (Usas *et al.*, 2009; Wijdicks *et al.*, 2009), but lower when compared with BMP9 sonoporative gene transfer (Sheyn *et al.*, 2008). Average bone volumes, determined in this study and in Sheyn *et al.*'s (2008) study in contrast to other BMP gene delivery work, however, are still limited, if compared with

approaches that use biomaterials in conjunction with gene delivery or growth factor delivery (Bessa *et al.*, 2010; Wegman *et al.*, 2012). This is probably because of the templating function of biomaterials, which provides a templating environment in contrast to the solution injection approach used in this study. Therefore, it might be of interest if ectopic bone volumes could be increased by sonoporation in conjunction with biomaterials, such as hydrogels. Such an approach for matrix-assisted sonoporation has already been demonstrated *in vitro* by our group (Nomikou *et al.*, 2013) and therefore shall be investigated in future studies.

The observed ectopic bone structures were irregular in shape with multiple centers of ossification per animal (Fig. 3A–F) representing individual foci of gene delivery, expected to be formed because of the chosen multiple-injection strategy adapted from Osawa *et al.* (2009) based on the fact that Osawa *et al.*'s (2009) and our own unpublished data showed that it was impossible to generate ectopic bones by single sonoporation, which indicates that gene expression levels obtained are still relatively low and not sufficient for osteoinduction via a single treatment. It is thus of great importance to modify the approach to a single gene delivery protocol by using more effective therapeutic transgenes in combination with a biomaterial as mentioned above in order to improve and spatially control bone formation *in vivo*. Histological examination of the ectopic centers of ossification (Fig. 4A, C, and D) showed functional ectopic bone tissue with calcified compact bone (Fig. 4C) surrounding a bone marrow cavity (Fig. 4D), which hosted hematopoietic bone marrow. This formation of a stem cell niche after BMP gene delivery has been reported before (Sheyn *et al.*, 2008; Osawa *et al.*, 2009) and demonstrates that simple gene delivery without biomaterials, stem cells, or recombinant growth factors can induce the formation of complex tissues *in vivo*. Furthermore, immunohistochemical staining for luciferase (Fig. 4B) proved that luciferase recipient cells are participating in ectopic bone formation and present in the bone marrow cavity, indicating that endogenous transgene receptive cells are contributing to ectopic bone and bone marrow formation. These findings demonstrate that direct *in vivo* codelivery of bone morphogenetic protein-encoding plasmids can provide a viable alternative to stem cell or recombinant protein-based therapies. Furthermore, the data presented herein clearly indicate that sonoporative gene delivery is superior in direct comparison with passive gene delivery with regard 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 coexpression *in vivo*. Doxycycline-inducible BMP2/7 coexpression from a modified (Feichtinger *et al.*, under review) bidirectional TetON system (Baron *et al.*, 1995) showed effective (83% 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 (Fig. 2B). This clearly showed tight coexpression control of two individual transgenes *in vivo* by systemic application of an inductor. By narrowing *in vivo* BMP2/7 coexpression down to ~7 days, it was possible to demonstrate that even this short time window of *in vivo* BMP expression, recapitulating endogenous expression patterns (Yu *et al.*, 2010), is sufficient for *in vivo* bone formation. This

reduction of therapeutic gene dose and therapeutic gene expression time compared with the constitutive coexpression systems enabled us to clearly demonstrate the effect of sonoporation on bone formation efficacy (Fig. 2B). Notably, potentially because of the reduced gene dose and/or expression time compared with the constitutive system, it was not possible to induce bone formation by passive gene transfer even in animals that received doxycycline treatment. Therefore, we conclude that sonoporation, in general, increases gene transfer efficacy and enables reduction of therapeutic gene dose and therapeutic gene expression time because of higher overall gene transfer efficacies and expression, which was not observed as dramatically with the constitutive coexpression systems, because overall gene expression levels were probably already saturated in contrast to the inducible system.

Orthotopic BMP2/7 sonoporation

Using X-ray-guided repeated DNA injection to the defect site, it was possible to achieve spatially controlled gene transfer to the target site *in vivo* without off-target expression in adjacent tissues (Fig. 5A–D). Gene transfer efficacies were higher when using sonoporation compared with passive gene transfer (Fig. 1C), demonstrating the feasibility of using ultrasound for orthotopic gene delivery. Interestingly, in contrast to the ectopic findings, there was a significant decrease in luciferase activity when the luciferase plasmid was coapplied with the constitutive BMP2/7 coexpression plasmid in passive and sonoporative gene transfer (Fig. 1D). This indicates that competitive expression might reduce overall gene expression efficacy if multiple independent expression entities are applied *in vivo*. Therefore, to rule out false-negative signals caused by competitive expression in animals that received both plasmids, we performed gene transfer efficacy calculations only for the groups that exclusively received the luciferase plasmid. Tracking of plasmid DNA biodistribution at the defect site (Fig. 6) using a novel metabolic DNA labeling method based on the work of Neef and Luedtke (2011) showed a different picture from tracking of luciferase gene expression at the target site. 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 because of 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 (Coelho-Castelo *et al.*, 2006), and this 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 the extensive binding of DNA to intact bone, indicating that the applied DNA gets absorbed by bone tissue. Binding of nucleic acids to ceramic hydroxyapatite has been well known already for some time (Kothari and Shankar, 1974; Mazin, 1977) and harnessed for DNA purification (Shan *et al.*, 2012) and delivery (Choi and Murphy, 2010; Zhang *et al.*, 2011). This study, however, is the first study to demonstrate this effect *in vivo* with biological hydroxyapatite and naked exogenous DNA in orthopedic gene delivery. This absorption might be considered as another caveat in *in vivo* naked DNA transfer,

additionally to nuclease digestion (Houk *et al.*, 1999; Ribeiro *et al.*, 2004) and should be considered in future studies as a parameter potentially responsible for low therapeutic efficacy of these approaches in bone regeneration at relatively high doses of DNA (Bonadio *et al.*, 1999; Schwabe *et al.*, 2012). *In vivo* quantitative and qualitative μ CT data, although no significant difference could be found within this study design, indicate a potential beneficial effect of BMP2/7 gene delivery on regeneration. The 4 mm femur segmental defect model used in this study had an overall nonunion rate of 83% at 8 weeks postsurgery. After in-depth μ CT evaluation of the empty control group, nonunion rate of the model was determined to be 100% (0% union rate) and was therefore performing comparably to previous studies in rats by our group (Schmidhammer *et al.*, 2006; Schutzenberger *et al.*, 2012). It has been demonstrated that orthotopic BMP2/7 sonoporation leads to 2/6 confirmed unions (33% union rate) and that passive gene delivery could achieve at least 1/6 unions (16% union rate) compared with 0/6 unions in both the negative luciferase control and empty defect control groups, respectively, when carefully evaluated by μ CT, which has been shown to be the only reliable method in determining union rate (Schmidhammer *et al.*, 2006). Therefore, and in conjunction with luciferase data, we conclude that sonoporative gene transfer enables orthotopic gene delivery and that orthotopic BMP2/7 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* before gene delivery as demonstrated by Kimelman-Bleich *et al.* (2011) or by combining our approach with the mentioned matrix-assisted sonoporation technology (Nomikou *et al.*, 2013). 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, our data indicated that application of an internal luciferase control reduces gene expression at the defect site because of competitive expression. Therefore, therapeutic efficacy could be enhanced in future studies by applying only the therapeutic coexpression plasmid without the internal luciferase control.

Nonspecific influence of ultrasound on bone regeneration *in vivo*. Ultrasound stimulation is a well-known physical method to enhance osteogenic differentiation and bone regeneration *in vivo*. Specifically low-intensity ultrasound stimulation (LIPUS) has successfully been applied for this purpose *in vitro* (Ikeda *et al.*, 2006), *in vivo* at ectopic sites (Watanuki *et al.*, 2009), and for clinical treatment of nonunion fractures (Griffin *et al.*, 2008; Romano *et al.*, 2009). Furthermore, it has been documented successfully that ultrasound stimulation *in vivo* increases the volume of ectopic bone formation with recombinant human BMPs *in vivo* (Wijedicks *et al.*, 2009), thus enabling a reduction in dose using LIPUS. Therefore, it is important to take into account potential nonspecific effects of ultrasound stimulation on bone for the study described herein. Although nonspecific effects of ultrasound on bone

formation unrelated to gene transfer cannot be ruled out, it is unlikely that substantial effects of ultrasound occurred in this study. The ultrasound trigger used for gene delivery is of a different frequency (1 MHz vs. 1.5 MHz for LIPUS) and uses substantially higher power intensities (90–600 J/cm²/day compared with ~7 J/cm²/day in LIPUS) and is considered a high-intensity ultrasound stimulus that can lead to tissue heating and damage not occurring in LIPUS treatment (Ikeda *et al.*, 2006). LIPUS itself has, however, been shown to increase the therapeutic efficacy of nonvirally delivered BMP expression plasmids (Watanuki *et al.*, 2009) *in vivo* and could therefore be additionally employed in future studies to further increase therapeutic efficacy of gene therapeutic approaches. The luciferase control groups employed in this study did not show increased bone volumes compared with empty controls; therefore, it is unlikely that the employed ultrasound had a significant impact on bone regeneration. While it is important to discuss implications of ultrasound stimulation on tissue regeneration, we did not observe such effects in this study.

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Author Disclosure Statement

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