TISSUE ENGINEERING: Part C
Volume 25, Number 3, 2019

DOI: 10.1089/ten.tec.2018.0295

termis
Tissue Engineering
& Regenerative Medicine
International Society

METHODS ARTICLE

Specific, Sensitive, and Stable Reporting of Human Mesenchymal Stromal Cell Chondrogenesis

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Chondrogenesis is critical to the development and repair of not only articular cartilage but also bone. Preclinical studies suggest that defects in both tissues can be repaired using culture-expanded chondroprogenitor cells, such as mesenchymal stem/stromal cells (MSCs), but directing efficient chondrogenesis by candidate cell populations is an ongoing bottleneck to their clinical application. The goal of this study was to describe a method for the molecular reporting of chondrogenic activity by primary stem/progenitor cells that can complement more labor-intensive destructive measures. A chondrogenesis-responsive promoter was generated, consisting of four repeats of a SOX9-binding enhancer sequence from the first intron of *COL2A1* positioned upstream of the core *COL2A1* promoter. This promoter was inserted into a lentiviral expression plasmid containing reporter genes copepod green fluorescent protein (copGFP) and firefly luciferase (fLuc), and the resulting lentiviral vector (LV) was used to transduce human MSCs derived from intramedullary reamings. To determine the specificity and stability of reporter expression, MSCs were differentiated in pellet culture for up to 4 weeks. To assess the sensitivity of reporter detection *in vivo*, undifferentiated and predifferentiated MSC pellets were implanted into osteochondral defects made in immune-suppressed rats. Chondrogenic differentiation of LV-transduced MSCs in pellet culture led to a strong upregulation of both copGFP and fLuc. Robust reporter activity was achieved using LV doses that did not compromise MSC chondrogenesis. Specific reporter induction was sustained over several passages post-transduction. Reporter expression levels were dependent on both pellet culture duration and TGF-β1 dose. When predifferentiated pellets were implanted into rat osteochondral defects, reporter activity was initially diminished but recovered over the following 2 weeks, suggesting acute postsurgical inflammation suppressed reporter expression. This hypothesis was supported by potent cytokine inhibition of reporter levels and glycosaminoglycan deposition within additional pellets *in vitro*. When combined with lentiviral transgene integration, the *COL2A1*-based promoter allowed specific, sensitive, and stable reporting of MSC chondrogenic activity. This promoter can be used with the extensive selection of reporter vectors now available to study different chondroprogenitor cells with promise for cartilage and bone tissue engineering and regenerative medicine.

Keywords: chondrogenesis, mesenchymal stromal cells, lentiviral vectors, endochondral ossification, osteochondral defect, bioluminescence imaging

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Impact Statement

The promoter characterized in this study has been made accessible as a resource for the skeletal tissue engineering and regenerative medicine community. When combined with suitable reporter vectors, the resulting tools can be used for noninvasive and/or high-throughput screening of test conditions for stimulating chondrogenesis by candidate stem/progenitor cells. As demonstrated in this study, they can also be used with small animal imaging platforms to monitor the chondrogenic activity of implanted progenitors within orthotopic models of bone and cartilage repair.

Introduction

Chondrogenesis is a critical process for both skeletal development and repair.[1,2] Within synovial joints, the formation and maintenance of hyaline cartilage are necessary for joint articulation and load distribution.[3] The long bones of the appendicular skeleton develop, in part, through the process of endochondral ossification, which initially involves formation of a cartilage anlage[4]; moreover, fractures to these bones can be successfully repaired through the formation and osseous remodeling of a cartilaginous callus.[5] These examples of cartilage formation all involve the condensation of mesenchymal cells that then differentiate into chondrocytes, as regulated by the transcription factor sex-determining region Y like high mobility group box (SOX)9.[6] SOX9 controls associated expression of cartilage matrix proteins like type II collagen.[7,8] One key difference between the cartilages formed during endochondral ossification versus articular cartilage development involves the hypertrophic maturation of chondrocytes during the former process.[2]

Clinical options for treating bone and cartilage defects remain inadequate, prompting ongoing research into stimulating chondrogenesis for the repair of both defect types.[9,10] Because chondrocyte sources are limited, much of these efforts are focused on applying culture-expanded chondroprogenitor cells, including adult mesenchymal stem/stromal cells (MSCs) from various tissue sources, embryonic stem cells, and induced pluripotent stem cells (iPSCs). A current bottleneck to this strategy concerns how to direct these candidate chondroprogenitor cells to form functional cartilage, either *in vitro* or directly within skeletal defects. For cartilage engineering *in vitro*, essential cues must be determined for efficient commitment of progenitors to the chondrogenic lineage and timely deposition of a functional matrix. To direct chondrogenesis *in situ*, which could bypass the complexity and cost of *in vitro* cartilage engineering,[11] a better understanding is required of how the defect environment influences the chondrogenic activity of implanted chondroprogenitors. For example, pro-inflammatory cytokines known to be upregulated after joint injury and posttraumatic osteoarthritis have been shown to directly inhibit the chondrogenic differentiation of MSCs.[12,13]

The study of cell-based skeletal tissue repair *in vivo* has been advanced by the ability to genetically modify progenitor cells to express molecular reporters.[14] Among the available reporters, the luciferases (e.g., firefly, *Renilla*, *Gaussia*, and *Metridia*) have been particularly valuable tools, catalyzing strong bioluminescent reactions that can be measured quantitatively. Commercial imaging platforms have been developed to detect bioluminescence noninvasively in rodents; these platforms are now a standard tool at research institutions. Bioluminescence has been used to study candidate stem cells within a variety of skeletal tissue repair models.[15–19] This typically involves the genetic modification of candidate stem cell populations *ex vivo* before their delivery within skeletal defects. Recombinant lentiviral vectors (LVs) are commonly used for this purpose, due to their broad tropism and efficient integration of transgenic DNA into the genome of primary cells, including MSCs.[20]

Exploiting the postnatal specificity of type II collagen expression within cells of the chondrogenic lineage, regulatory sequences from *COL2A1*, the gene encoding the alpha 1 chain of type II collagen, were used to develop a reporter construct for nondestructive monitoring of chondrogenic commitment by candidate repair cells. Although the reporting strategy described in this study can be applied to multiple chondroprogenitor cell populations, this study focused on human MSCs from bone marrow stroma, one of the best characterized populations with chondrogenic potential.[21,22] Specific, sensitive, and stable reporter expression by LV-modified MSCs was demonstrated in tissue culture and within rat osteochondral defects.

Materials and Methods

Promoter design and cloning

A chondrogenesis-responsive promoter (4eCOL2A1; Fig. 1A) was made based on previously described constructs[7,8,23–25]—particularly one used to generate an ATDC5-based reporter line.[26] The promoter sequence consisted of four repeats of a SOX9 binding motif from the first intron of *COL2A1* (+2126 to +2174) placed upstream of the core *COL2A1* promoter (−164 to +37). These regulatory sequences were flanked by restriction sites for *Spe*I (5′ end) and *Bam*HI (3′ end). In addition, an *Nhe*I site was placed between the four enhancer repeats and the core promoter, allowing for substitution of either the enhancer or promoter sequences. For example, longer 5′ sequences of the *COL2A1* promoter could be used in place of the 200-bp sequence described in this study, to more closely reflect endogenous *COL2A1* regulation.[27] Alternatively, reporter signal strength could potentially be boosted by doubling the number of enhancer repeats[26]: the four repeats could be removed from a donor vector by *Spe*I and *Nhe*I digestion and inserted into a destination vector containing the basal promoter, following a single cut with *Nhe*I (note that *Spe*I and *Nhe*I digestions produce compatible overhanging ends).

To generate a plasmid for efficient cloning of the 4eCOL2A1 sequence and transferring it to different expression vectors, the 4eCOL2A1 sequence was synthesized by GenScript (Piscataway, NJ) with protective bases added to both ends and then inserted into the *Eco*RV site of cloning vector pUC57. The resulting construct, pUC57-4eCOL2A1, has since been sequenced and deposited with Addgene (ID# 97211).

The 4eCOL2A1 promoter was next inserted in place of a truncated, ''minimal'' cytomegalovirus (mCMV) promoter

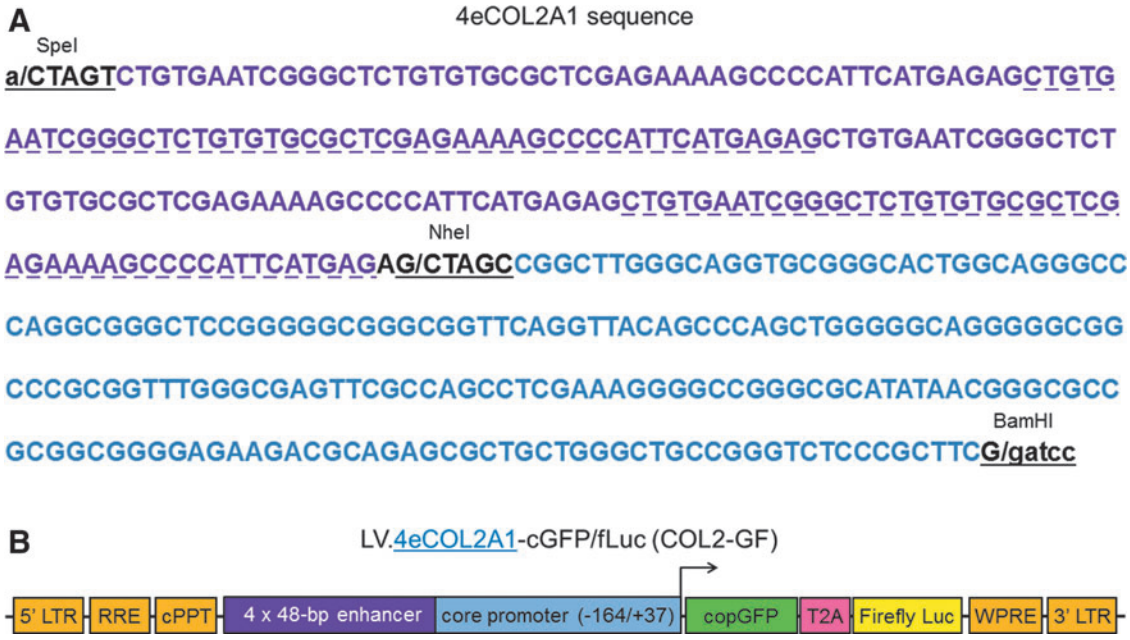

FIG. 1. Chondrogenic reporter construct. **(A)** The chondrogenic promoter sequence, consisting of four repeats of a sox9 binding motif from the first intron of *COL2A1* placed upstream of the core *COL2A1* promoter. These regulatory sequences were flanked by restriction sites for *Spe*I (5′ end) and *Bam*HI (3′ end). An *Nhe*I site was placed between the four enhancer repeats and the core COL2A1 promoter, to allow for substitution of either the enhancer or promoter sequences. **(B)** The promoter sequence from **(A)** was subcloned into a third-generation, self-inactivating lentiviral expression plasmid. In the resulting reporter construct, pGF-4eCOL2A1, copGFP, and firefly luciferase are coexpressed using a T2A sequence. This construct was used to produce lentivector LV.COL2-GF. copGFP, copepod green fluorescent protein; T2A, *Thosea asigna* virus 2A.

within the third-generation lentiviral reporter construct pGreenFire1-mCMV (pTRH1-mCMV-dscGFP-T2A-Fluc; System Biosciences, Mountain View, CA). The mCMV sequence was removed from pGreenFire1-mCMV by *Spe*I and *Bam*HI digestion, and the 4eCOL2A1 sequence (removed from pUC57-4eCOL2A1 by the same restriction digests) was ligated into this expression vector. For the resulting reporter construct (pGF-4eCOL2A1; Fig. 1B), copepod green fluorescent protein (copGFP) and firefly luciferase (fLuc) are coexpressed using the *Thosea asigna* virus 2A (T2A) self-cleaving peptide.[28] The sequence of pGF-4eCOL2A1 has been confirmed and the construct deposited with Addgene (ID# 97210).

LV generation

Replication-deficient lentivirus (LV.COL2-GF) was generated using pGF-4eCOL2A1 and a third-generation packaging system from the Didier Trono laboratory.[29] Packaging plasmids pMDLg/pRRE (ID# 12251), pRSV-Rev (ID# 12253), and pMD2.G (ID# 12259) were all acquired from Addgene. The expression vector and three packaging plasmids were cotransfected into 293T cells using TurboFect reagent (Thermo Fisher Scientific). For each 100-mm dish containing subconfluent 293T cells (i.e., seeding 2.8×10^6 cells/dish the prior day), 7.5 μg pGF-4eCOL2A1 was combined with 6.5 μg pMDLg/pRRE, 2.5 μg pRSV-Rev, and 3.5 μg pMD2.G in serum-free Dulbecco's modified Eagle's medium (DMEM), and the TurboFect manufacturer's protocol was followed. The plasmid/TurboFect mixture (2 mL) was then added dropwise to each dish of cells in 10 mL fresh high-glucose DMEM (Gibco) with 10% fetal bovine serum (FBS; HyClone, Logan, UT) and 1% antibiotic/antimycotic (Ab/Am) cocktail (Gibco). The next morning, 293T monolayers were gently rinsed with phosphate-buffered saline (PBS; Gibco) to remove excess extracellular plasmid, and conditioned media were harvested at 48 and 72 h post-transfection. Media from multiple dishes were pooled at each collection timepoint, detached cells were removed from the media by centrifugation at 400 *g* for 10 min, and the media were filtered using polyethersulfone 0.45 μm filters (Whatman) and aliquoted for long-term storage at −80°C. In addition to LV.COL2-GF, additional control lentivectors for nonspecific (LV.mCMV-GF) and constitutive (LV.CMV-GF) reporter expression were generated using System Biosciences vectors pGF1-mCMV and pGF1-CMV, respectively. Total titers were estimated by detection of HIV p24 capsid protein in conditioned media lysates using the Lenti-X p24 Rapid Titer Kit (Clontech). Titers of $1.1–2.5\times10^5$ pg p24 per milliliter 293T supernatant were obtained. Viral doses are reported as viral particles (vp) per cell using a conversion factor of 12,500 vp/pg p24.

MSC isolation and transduction

In accordance with a protocol approved by the local institutional review board, MSCs were isolated from intramedullary reamings collected from four patients (one male, three female; ages 71–78) that underwent hip hemiarthroplasty. As previously described,[30,31] intramedullary reamings generated using a Reamer Irrigator Aspirator (Synthes, Paoli, PA) were filtered of osseous particles, and the saline-diluted filtrate was collected in a sterile vessel. Stromal cells within the red blood cell-depleted fraction of

this filtrate were isolated by plastic adherence in low-glucose DMEM supplemented with 10% FBS and 1% Ab/Am. The lot of FBS used was preselected for enhanced proliferation and osteochondral differentiation of human MSCs. Nonadherent cells were removed after 4 days. After 2 weeks in primary culture, adherent colonies were recovered using 0.05% trypsin and 0.5 mM EDTA buffer (Invitrogen), and MSCs were further expanded in growth medium containing 5 ng/mL recombinant human fibroblast growth factor-2 (PeproTech, Rocky Hill, NJ).[32] The immunophenotype and multipotency of MSCs isolated using this approach—including data from some of the same donors used in this study—have been previously reported.[31,33]

For lentiviral transduction, MSCs were seeded into six-well plates, and the culture media was replaced with 3 mL/well growth medium containing 293T supernatants (LV dose range of 500–10,000 vp/cell, with specific doses detailed in individual figure legends) and 8 μg/mL hexadimethrine bromide (Polybrene; Sigma). To enhance transduction efficiency, the cells were then ''spinoculated'' with the virus at 1200 *g* for 45 min at 32°C.[34,35] The media was changed the following day, and the cells were passaged for pellet culture within 48–72 h of transduction. For some experiments, 24 h following lentiviral transduction, MSCs were additionally transduced with an adenoviral vector encoding human TGF-β1 (Ad.TGF-β1; 100 vp/cell) as previously described.[36,37] Unless stated otherwise, passage 4–6 MSCs were used for chondrogenesis experiments.

Pellet culture

MSCs were cultured as pellets and induced along the chondrogenic lineage as previously described.[13,22,38] MSCs were seeded at 2×10^5 cells per well into polypropylene, v-bottom 96-well plates (Corning). The plates were spun at 400 *g* for 5 min, and the supernatant was aspirated and replaced with chondrogenic medium consisting of high-glucose DMEM (containing L-glutamine and sodium pyruvate; Corning) with 1% Ab/Am cocktail, 1% ITS+Premix (BD Biosciences), 40 μg/mL L-proline, 50 μg/mL ascorbic acid-2-phosphate, and 100 nM dexamethasone (all from Sigma). Subgroups of pellets received recombinant human growth factors (TGF-β1) and/or cytokines (IL-1, TNF-α), all from PeproTech, at the concentrations specified in the figure legends. Within the first 24 h of culture, the cells formed free-floating aggregates (Fig. 2A). Media were changed the next morning and every other day thereafter. Pellets or pellet-conditioned media were collected at those timepoints detailed in the figure legends. For pellets transduced with Ad.TGF-β1, transgene levels in conditioned media were measured using a human TGF-β1 DuoSet Kit (R&D Systems).

Luciferase activity

To control for treatment effects on cell viability, luciferase activities from LV.COL2-GF pellets were normalized by either (i) the activity from pellets transduced in parallel with LV.CMV-GF and cultured under the same treatment conditions or (ii) the DNA content of the pellet lysate.

At the timepoints indicated in the figure legends, pellets were rinsed with PBS. When pellet DNA content was not to be measured, the pellets were directly transferred to white, round-bottom 96-well plates (Corning) containing 50 μL/well Glo Lysis buffer (Promega). The well plates were agitated on an orbital plate shaker for 30 min at room temperature to release luciferase from the pellets. Luciferase activities were determined using the Bright-Glo Luciferase Assay System (Promega), adding 50 μL of Bright-Glo

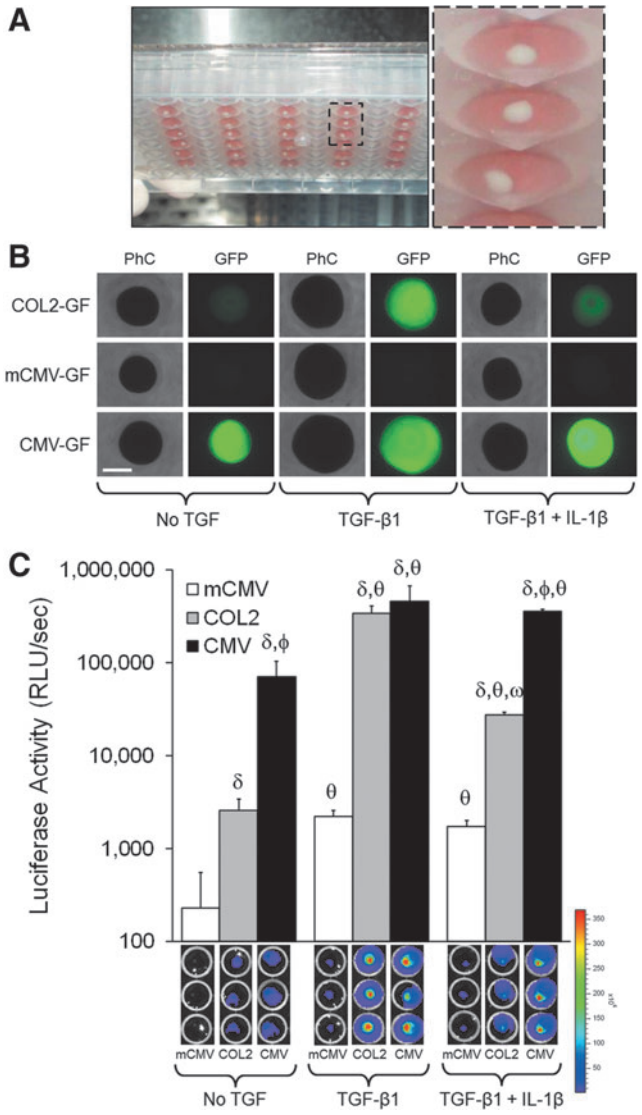

FIG. 2. Reporter activity in 4-week MSC pellets. **(A)** When seeded into polypropylene 96-well plates, MSCs aggregate into spherical pellets (200,000 cells). *Inset* shows individual pellets the day after seeding, by which time they had detached from the nonadherent plastic surface. **(B)** The translucent v-bottom wells permit noninvasive monitoring of copGFP expression with time during pellet culture. Representative fluorescence or PhC micrographs are shown after 4 weeks of culture without TGF-β1, with 10 ng/mL TGF-β1 alone, or co-stimulation with TGF-β1 and 1 ng/mL IL-1β (the latter added during the final 14 days of culture). Scale bar = 1 mm. **(C)** *Top:* After fluorescent imaging, pellets were lysed and luciferase levels measured. Significance notations: δ, compared to mCMV-GF; ϕ, compared to COL2-GF; ω, compared to no-TGF control; θ, compared to TGF-β1 alone. *Bottom*: Additional pellets were incubated in 0.5 mg/mL D-luciferin, and bioluminescence flux was measured using an IVIS station. The heat map represents radiance values from 10 to 375×10^6 photons/s/cm^2/sr. MSC, mesenchymal stem/stromal cell; PhC, phase contrast.

substrate to 50 μL of pellet lysate. After 5 min incubation, luminescence was measured (integration time = 0.8 s/well) on a Synergy MT microplate reader (BioTek).

For those pellets where DNA content was also to be measured, each rinsed pellet was transferred to 100 μL Glo Lysis buffer in a 2 mL round bottom, snap cap microcentrifuge tube (Qiagen) containing a 5-mm stainless steel bead (Qiagen). The pellet was then homogenized by shaking the tube at 25 Hz for 30 s using a TissueLyser II (Qiagen). A 50-μL aliquot of homogenate was transferred to the white, round bottom plates for luciferase assay, as described above. The remaining homogenate was used for DNA assay, as described below.

DNA and glycosaminoglycan content

PBS-rinsed pellets were either homogenized in Glo Lysis buffer, as described above, or digested overnight at 60°C using 0.3 mg/mL papain in 50 mM sodium phosphate (pH 6.8), 5 mM EDTA, and 2 mM dithiothreitol (all from Sigma). Pellet DNA content was determined using a Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes). Samples were diluted 10-fold in Tris-EDTA buffer and combined with an equal volume of PicoGreen dye in Tris-EDTA buffer within black 96-well plates (Corning). After 5 min, fluorescence intensities were measured on the Synergy MT microplate reader. DNA concentrations were determined from a standard curve of calf thymus DNA.

Papain digests were analyzed for glycosaminoglycan (GAG) content using the dimethylmethylene blue (DMMB) dye binding assay.[39] Aliquots of pellet digests, serially diluted in digest buffer without papain, were combined with DMMB solution (pH 3), and sample absorbance was measured at 595 nm. Sample GAG concentrations were interpolated from a standard curve of chondroitin sulfate from bovine trachea (Sigma), and pellet GAG content was normalized by DNA content in the papain digests.

Osteochondral defect model

Animal studies were conducted as preapproved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center. For implantation and imaging of human MSCs *in vivo*, 14-week-old male Fischer-344 rats were used that received an immune-suppressive cocktail of 3.3 mg/mL FK-506 and 1.7 mg/mL SEW2871 (both from Cayman Chemicals), to prevent rejection of the xenograft.[40] As a modification to this protocol, the cocktail (200 μL) was delivered by a model 2002 ALZET osmotic pump (Durect Corp.) that releases its reservoir content at a continuous rate of 0.5 μL/h over 14 days, resulting in continuous delivery of FK-506 (~0.15 mg/kg/day) and SEW2871 (~0.075 mg/kg/day). The pump was implanted subcutaneously within a dorsal flank during osteochondral defect generation. These rats received bilateral osteochondral defects in the patellar grooves of both knees, based on previously described models.[41,42] After medial arthrotomy and lateral dislocation of the patella, 1.0 mm defects were made using a sterile biopsy punch (Robbins Instruments, Chatham, NJ) to a depth of ~1.0 mm. After flushing the defect with saline, MSC pellets (cultured for either 3 or 28 days) were press fit into each defect, randomly assigning one knee a COL2-GF pellet and the other knee a CMV-GF pellet.

Bioluminescence imaging

At days 2, 7, and 14 postimplantation, rats underwent bioluminescence imaging (BLI) at the Longwood Small Animal Imaging Facility. Rats were anesthetized with isoflurane, the fur over the knee joint capsule was shaved, and the injection site swabbed with ethanol. Each knee was injected with 50 μL of 0.5 mg/mL D-luciferin (Caliper Life Sciences, Hopkinton, MA) in PBS (total dose = 25 μg/joint) using an insulin syringe, inserting the needle through the patellar tendon into the intra-articular space.[43] After flexing and extending the knees several times to help distribute the luciferin, rats were positioned in an IVIS-50 imaging station (Caliper Life Sciences) so that the knees faced a charge-coupled device camera. Five minutes after injection, photon emissions from both knees were collected for an additional 5 min, after which the rats were returned to their cages to recover from anesthesia. Bioluminescent signal was superimposed over a photograph of the rat, and the flux within a circular region of interest encircling each knee was quantified using Living Image software (Caliper Life Sciences). At day 14, rats were euthanized at least 2 h after *in vivo* imaging. The distal femora with defects were quickly explanted, rinsed in PBS, and bathed in 12-well plates containing 0.5 mg/mL D-luciferin for 10 min. The plate was then imaged in the IVIS-50 station, using an exposure time of 30 s. After imaging, the femora were rinsed again in PBS and fixed for 2 days with 4% paraformaldehyde (PFA) in PBS (pH 7.4).

Histology and immunohistochemistry

Pellets were fixed for 30 min with 4% PFA, encapsulated in 0.5% agarose in PBS for better handling, and then processed for embedding in paraffin. For detection of proteoglycans in the extracellular matrix, 5-μm sections through the center of MSC pellets were stained with 1.0% Toluidine blue. Distal femora explants were decalcified with 15% EDTA in PBS (pH 7.4) before paraffin embedding. Transverse sections (5 μm) through the center of osteochondral defects were stained with 1.0% Safranin O, 0.05% Fast Green, and Weigert's iron hematoxylin (all dyes from Sigma).[44]

For immunohistochemistry, endogenous peroxidases were quenched with 3% hydrogen peroxide for 10 min. Antigen retrieval for detecting type II collagen was achieved using 0.1% hyaluronidase (Sigma) and 0.1% pronase (Sigma) in PBS for 30 min at 37°C, while heat-induced epitope retrieval in 0.1 M sodium citrate buffer (pH 6) overnight at 60°C was used for detecting human mitochondria. After blocking with 1% BSA and 10% normal horse serum (Vector Laboratories, Burlingame, CA) in PBS for 1 h, slides were incubated overnight (4°C) with 1 μg/mL mouse anti-type II collagen IgG1 (clone II-II6B3; Developmental Studies Hybridoma Bank, University of Iowa) or 5 μg/mL mouse anti-human mitochondria IgG1 (ab92824; Abcam) in blocking buffer. Antigens were visualized using a VECTASHIELD Elite ABC Kit (Vector Laboratories), using 3,3′-diaminobenzidine (ImmPACT DAB; Vector Laboratories) as the substrate. Immunostained slides were then rinsed and counterstained with Gill No. 2 hematoxylin (type II collagen) or 0.5% Methyl Green (human mitochondria).

Statistical analysis

In vitro experiments were repeated at least thrice using cells from different donors. While the reported trends were reproduced among all donors, variation in chondrogenic response to test factors did not permit pooling of data from multiple donors. Accordingly, representative data from one donor are shown in each figure (mean ± standard deviation). For *in vitro* pellet culture assays, statistical significance between individual treatment groups (3-5 pellets per group) was determined by two-tailed unpaired *t*-tests, while differences in BLI signal (5-6 knees per group) within the rat model were determined by Mann–Whitney *U* test. Within the figures, statistical significance is denoted for p-values <0.05.

Results

Initial reporter characterization

Lentiviral doses used for MSC transduction were based on total particle titer, as determined by p24 content of 293T conditioned media. Because the lentiviral expression constructs used here did not include constitutive expression of a selectable marker, reporter activity represents a subpopulation of MSCs in pellet culture. For initial reporter characterization, a dose of LV.CMV-GF was used that resulted in >70% of MSCs transduced, as determined by fluorescence microscopy and flow cytometry (data not shown). Similar total titers of LV.COL2-GF and LV.mCMV-GF were used for this initial characterization.

Chondrogenic differentiation of human MSCs was performed using a well-established pellet culture model,[22] forming and cultivating pellets within nonadherent, v-bottom plates (Fig. 2A).[38] While these well plates are not amenable to phase contrast imaging, their translucence allows assessment of copGFP expression during culture using a fluorescence microscope. As shown in Figure 2B, MSCs cultured for 28 days in chondrogenic medium containing 10 ng/mL TGF-β1 produced larger pellets compared to those cultured in basal chondrogenic medium. For MSCs transduced with LV.COL2-GF, TGF-β1 treatment enhanced pellet fluorescence captured with a FITC filter, indicative of copGFP expression. Some background fluorescence was observed in COL2-GF pellets cultured in no-TGF-β1 medium relative to similarly-treated mCMV pellets. In contrast, CMV-GF pellets displayed robust GFP fluorescence, regardless of TGF-β1 supplementation. For each transduction group, some of the TGF-β1-treated pellets received 1 ng/mL IL-1β during the last 2 weeks of culture, after MSCs would have committed to the chondrogenic lineage[45,46]: this cytokine has been shown to inhibit reporter activity in human chondrocytes transfected with a plasmid containing ~4 kb of the 5′ flanking sequence of *COL2A1*.[47,48] In the presence of IL-1β, COL2-GF pellets displayed reduced fluorescence compared to TGF-β1 alone; in contrast, IL-1β did not alter visibly the fluorescence of CMV-GF pellets.

After evaluating GFP expression, some pellets were placed in lysis buffer with agitation to release fLuc, and luciferase activity was measured by commercial assay. As shown in Figure 2C, quantitative measures of luciferase activity reflected the qualitative trends in copGFP fluorescence. In basal chondrogenic medium, COL2-GF reporter levels were significantly higher than in mCMV-GF pellets but lower than in CMV-GF pellets. Addition of TGF-β1 to the differentiation medium increased total luciferase activity for COL2-GF pellets by more than 100-fold at the day 28 endpoint. When IL-1β was added over the final 14 days, COL2-GF activity was about 10-fold lower than in TGF-β1-only pellets, whereas CMV-GF levels were not significantly altered.

A final set of pellets were incubated in 0.5 mg/mL D-luciferin and then imaged on an IVIS bioluminescence station. Bioluminescence signal was readily detected, particularly from COL2-GF and CMV-GF pellets, with trends in flux similar to that shown for the destructive luciferase assay and GFP fluorescence.

Transgene silencing of the CMV promoter has been reported with extended culture following stable transgene integration.[49,50] To assess the stability of reporter activity with passage, transduced MSCs were cultured for up to 12 total passages—well beyond the passage in which they are typically used for study—then cultured as pellets for 28 days with TGF-β1 and/or IL-1β. Pellet reporter levels were generally stable over several passages, as was the inhibition of COL2-GF levels by IL-1β. For the MSCs shown in Supplementary Figure S1, COL2-GF but not CMV-GF luciferase activity decreased at passage 12, such that the ratio of COL2:CMV levels was significantly reduced (Supplementary Fig. S1D).

Detailed in vitro *characterization*

The influence of lentiviral dose on luciferase activity and chondrogenic induction was examined. Detectable COL2-GF luciferase activity was measured at day 28 using viral doses as low as 500 vp/cell (Fig. 3A). As expected, LV.CMV-GF transduction at the same approximate dose as LV.COL2-GF produced significantly higher luciferase activity. When normalized by DNA content, COL2-fLuc activity increased viral dose dependently up to 5000 vp/cell, but did not increase further at 10,000 vp/cell. To assess relative chondrogenic induction, GAG and DNA levels were measured in additional pellets made from the same transduction batches. GAG content was significantly reduced at 5000 and 10,000 vp/cell LV.COL2-GF compared to lower doses (Fig. 3B). These quantitative changes were associated with slightly smaller pellets that displayed reduced proteoglycan staining by Toluidine Blue (Fig. 3D). DNA content was also reduced with more than 2500 vp/cell LV.COL2-GF (Fig. 3C). At lower virus doses, DNA levels were not significantly different between COL2-GF and CMV-GF pellets.

The time course of chondrogenic gene expression for human MSC pellets induced by TGF-β is well established.[45,46] Because *COL2A1* is a highly specific marker of chondrogenic commitment, the 4eCOL2A1 promoter (Fig. 1) was predicted to be time-dependently activated for MSC pellets cultured in the presence of TGF-β1. To test this prediction, pellets were analyzed at multiple timepoints during the course of pellet maturation (Fig. 4). To better examine the pellets under fluorescence microscopy, they were briefly transferred to 48-well plates for GFP imaging (Fig. 4A). Little signal was observed throughout the 4-week culture period in COL2-GF pellets cultured without TGF-β1. In the presence of 10 ng/mL TGF-β1, by day 4 there was a significant increase in COL2-GF reporter levels; these levels further increased between weeks 1 and 2 after TGF-β1 induction. In contrast, CMV-GF levels were initially strong

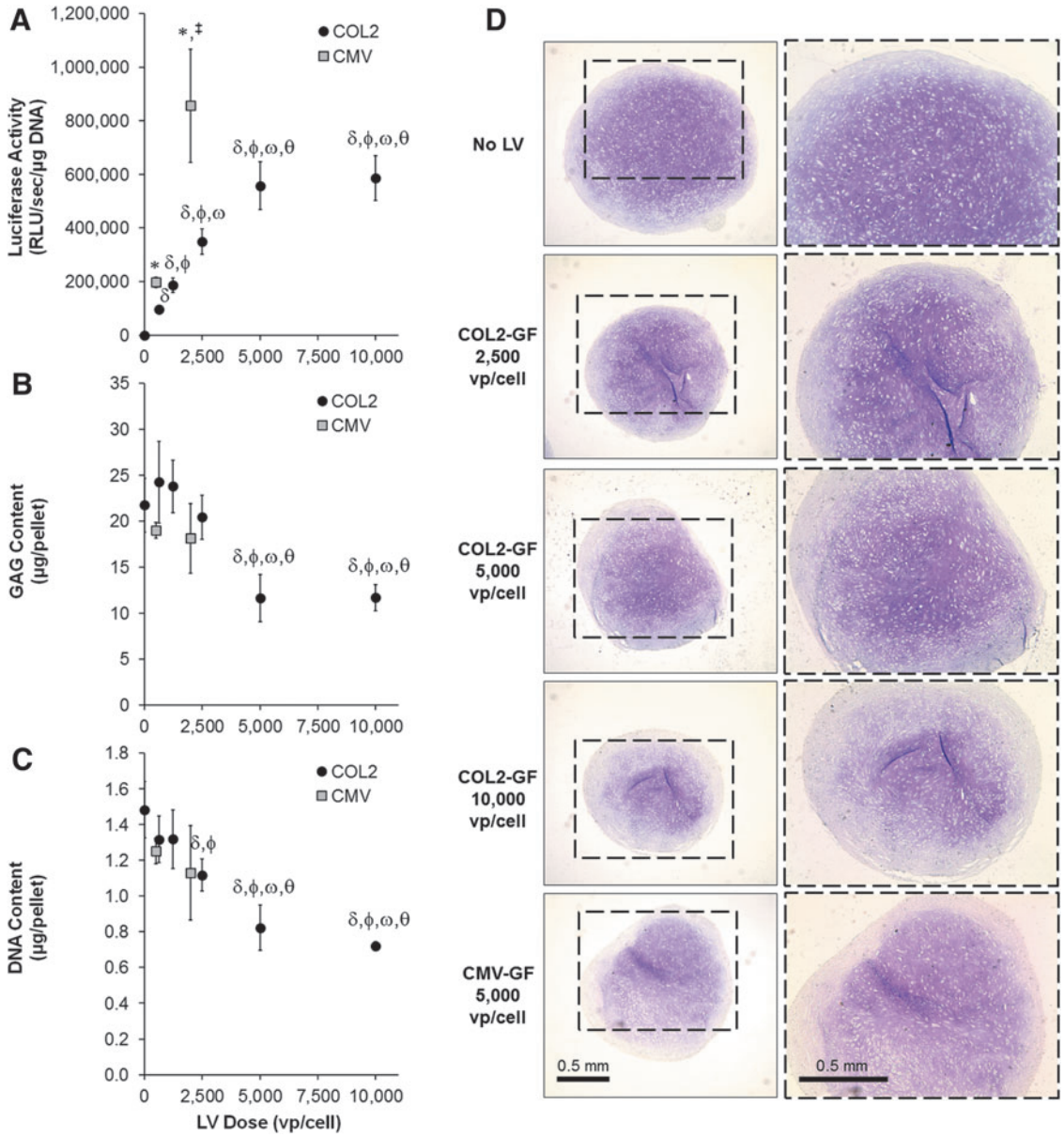

FIG. 3. Lentiviral dose response. **(A)** Luciferase activities, normalized by DNA content, of 4 week pellets are shown for MSCs transduced with variable doses of LV.COL2-GF or LV.CMV-GF. (**B, C**) Total GAG and DNA contents (µg/pellet) of additional pellets for the dose groups tested in **(A)**. Significance notations: δ, compared to 0 vp/cell; ϕ, compared to 625 vp/cell; ω, compared to 1250 vp/cell; θ, compared to 2500 vp/cell; *, compared to 625 vp/cell COL2-GF; ‡, compared to 625 vp/cell CMV-GF. **(D)** Toluidine Blue staining of sections from representative pellets for select test doses shown in **(A–C)**. Scale bars = 500 µm. GAG, glycosaminoglycan; vp, viral particles.

but declined with time in pellet culture, both in the presence or absence of TGF-β1. This general decline can be partly explained by the overall decline in pellet cell number with time (data not shown), as observed previously with TGF-β1-treated human MSC pellets.[46,51] Of note, CMV-GF levels were uniformly increased in the presence of TGF-β1, which is partly explained by increased pellet cell number in the presence of TGF-β1 stimulation (data not shown). Total luciferase activities (Fig. 4B) reflected observed trends in GFP fluorescence (Fig. 4A). A slight reduction in COL2-GF activity was measured between weeks 2 and 4 in the presence of TGF-β1: while this reduction might be explained by declining pellet cell number, it is also possible that SOX9 activity declined as MSC-derived chondrocytes underwent hypertrophic maturation.[22,52] When luciferase levels were reported as fold inductions over no-TGF-β1 controls, it became apparent that the induction of CMV-GF by TGF-β1 treatment (~5-fold) was not time dependent, while induction of COL2-GF increased to about 25-fold by 2 weeks of culture and remained steady thereafter (Fig. 4C). The ratio of COL2-GF to CMV-GF reporter levels sharply increased with TGF-β1 stimulation after the first week of culture, continuing to increase throughout the culture period studied (Fig. 4D). This ratio also significantly increased for no-TGF-β1 cultures—although it remained substantially lower than for TGF-stimulated cultures.

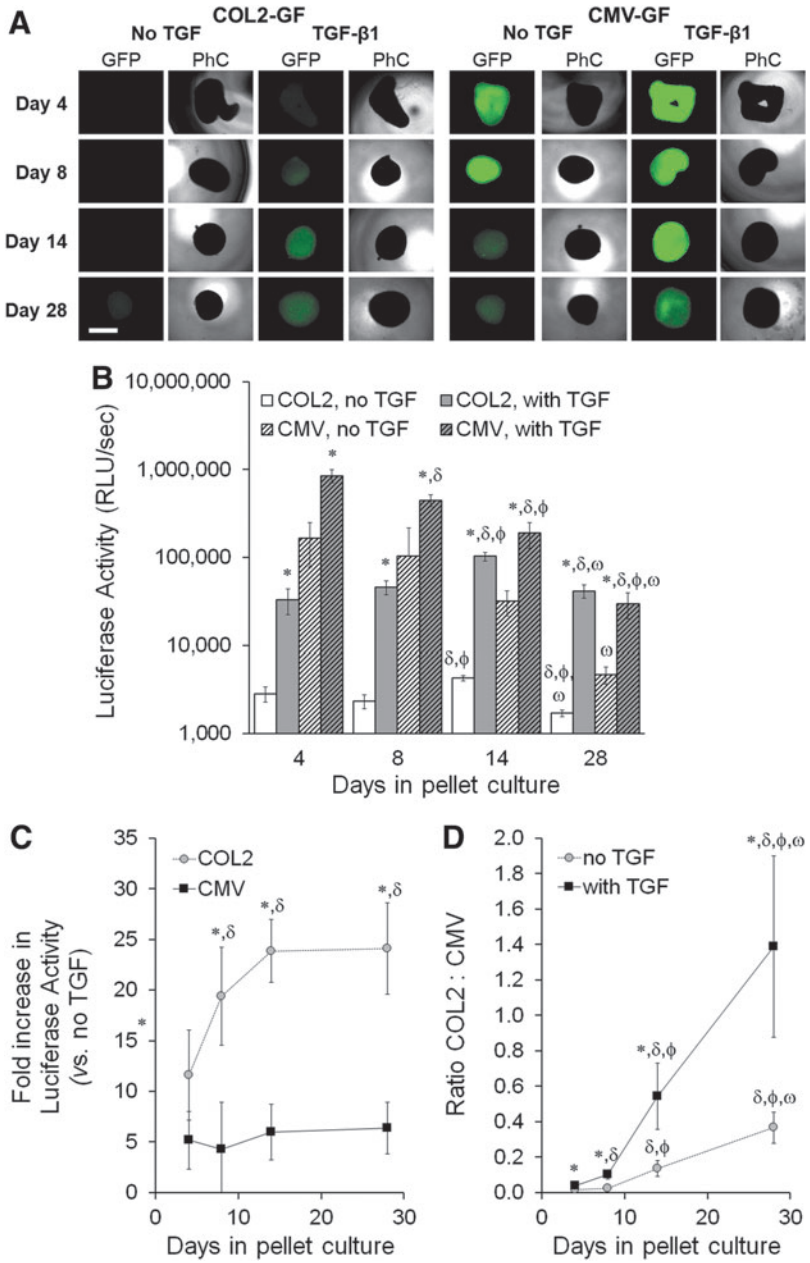

FIG. 4. Time course of reporter activity during pellet culture. **(A)** During culture, pellets were periodically transferred to 48-well culture plates (for more clear imaging compared to their home v-bottom plates). Fluorescence micrographs are shown next to the pellet silhouette captured by phase contrast imaging. Scale bar = 1 mm. **(B)** Luciferase activities were measured in lysed pellets collected at multiple timepoints during pellet culture. Significance notations: *, compared to no-TGF condition; δ, compared to day 4; ϕ, compared to day 8; ω, compared to day 14. **(C)** Fold increases in luciferase activity for TGF-β1-treated pellets over no-TGF controls are shown for each reporter vector at different pellet culture timepoints. Significance notations: *, compared to CMV-GF; δ, compared to day 4. **(D)** COL2:CMV luciferase activity ratios are shown as a function of time. Significance notations: *, compared to no-TGF control; δ, compared to day 4; ϕ, compared to day 8; ω, compared to day 14.

The induction of human MSC chondrogenesis by TGF-β1 is dose dependent. To determine whether the COL2-GF reporter would reflect this TGF-β1 dose response, transduced pellets were cultured for 4 weeks with various doses of TGF and then harvested for either luciferase or GAG/DNA measurements (Fig. 5A, B). Both luciferase activity and GAG content were enhanced by TGF-β1 concentrations of 2.5 ng/mL, but not 1.25 ng/mL; both measures dose-dependently increased with TGF-β1 concentrations up to 10 ng/mL. At the day 28 endpoint, there was a significant linear correlation ($R^2 = 0.92$; $p = 0.010$) between COL2-GF activity and GAG/DNA content (Fig. 5C).

Evaluation of in vivo *reporting*

In addition to monitoring chondrogenesis *in vitro*, the selection of luciferase as a reporter gene presents the opportunity to monitor chondrogenesis noninvasively in rodents using BLI. One preclinical model for evaluating treatment strategies for promoting chondrogenesis *in vivo* is the rat osteochondral defect model.[53] To determine whether a LV.COL2-GF dose shown not to interfere with chondrogenesis (Fig. 3) was sufficient for noninvasive detection by BLI, MSCs were transduced with 1250 vp/cell LV.COL2-GF or LV.CMV-GF. To stimulate chondrogenesis both before and after pellet implantation into defects, MSCs were additionally transduced with an adenoviral vector encoding human TGF-β1.[36] The cells were then formed into pellets and cultured for either 3 (undifferentiated) or 28 (predifferentiated) days (Fig. 6A). On the day of their implantation, pellets were imaged for GFP expression by fluorescence microscopy, confirming the activation of COL2-GF reporter activity in predifferentiated but not undifferentiated pellets. Pellets were then implanted into 1-mm defects made in the

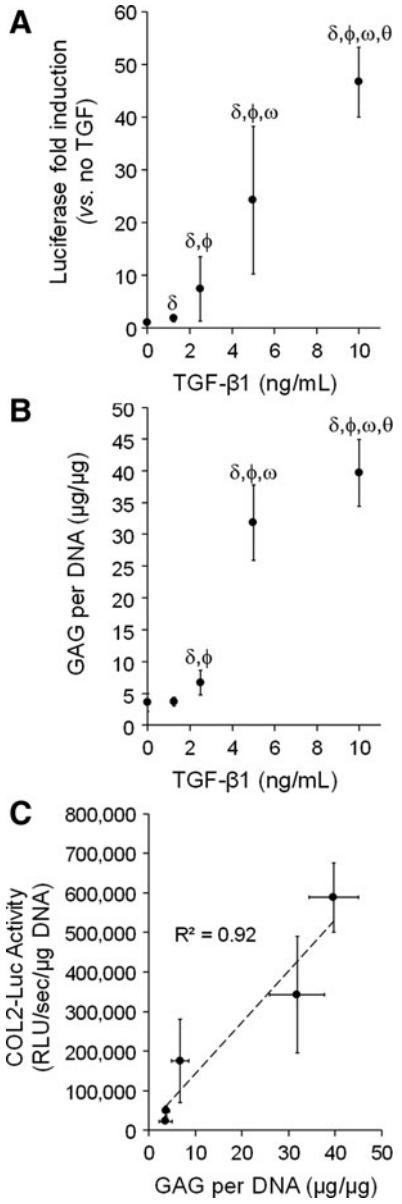

FIG. 5. TGF-β1 dose response. **(A)** Fold increases in luciferase activity for TGF-treated pellets over no-TGF-β1 controls are shown as a function of TGF-β1 dose given throughout the 4-week culture. **(B)** GAG content, normalized by DNA content, is shown for the same TGF-β1 doses tested in **(A)**. Significance notations: δ, compared to 0 ng/mL; ϕ, compared to 1.25 ng/mL; ω, compared to 2.5 ng/mL; θ, compared to 5 ng/mL. **(C)** For the doses tested in **(A, B)**, luciferase reporter activities are plotted versus GAG per DNA levels. Regression analysis was performed to test for a linear relationship (*dotted trend line*; $R^2 = 0.92$; $p = 0.010$) between luciferase activity and GAG content.

patellar groove of immune-compromised rats (Fig. 6B, left), implanting one CMV-GF and one COL2-GF pellet into the contralateral knees of each animal.

BLI was performed on the rats at days 2, 7, and 14 after defect implantation (Fig. 6B). Signal from CMV-GF knees was initially much stronger than for COL2-GF knees, regardless of differentiation status; this corresponded with much stronger GFP expression at the time of implantation (Fig. 6A). Unlike with preimplantation expression, however, at day 2 there was not a significant difference in COL2-GF signal between predifferentiated and undifferentiated pellets (Fig. 6E). From day 2 to 7, there was a trend toward increased COL2-GF signal from predifferentiated pellets, while there was no change in undifferentiated pellets. During this time there was a trend toward decreased signal in CMV-GF knees for both predifferentiated and undifferentiated pellets (Fig. 6E). From day 7 to 14, there was a trend toward decreased COL2-GF signal for both differentiation groups and significant decreases in CMV-GF signal for both groups. At day 14, the distal femora with patellar defects were removed from the rats, and those explants underwent BLI for more sensitive measurement of reporter activity (Fig. 6C). Specific signal was detected in nearly all explants, including those from COL2-GF knees that did not yield signal when measured noninvasively just hours earlier. The ratios of COL2-GF to CMV-GF signal were calculated for pellets immediately before implantation, during noninvasive BLI, and for distal femur explants (Fig. 6F). A significant decrease in the ratio of COL2:CMV activities was observed for day 2 BLI compared to pellets just before implantation. This ratio significantly increased by day 7 compared to day 2. There was a further increase by day 14 that was not significantly different from day 7. While the ratio for day 14 explants was lower compared with knees measured noninvasively the same day, this difference was not statistically significant ($p = 0.15$).

After imaging, the distal femora were sectioned in the transverse plane for assessment of the repair tissue at the day 14 endpoint (Fig. 6D). Safranin O/Fast Green staining revealed low GAG content within defects implanted with either undifferentiated or predifferentiated pellets: repair tissue stained with less intensity than intact articular cartilage proximal to the defect. In contrast, immunostaining for type II collagen was stronger in those defects implanted with predifferentiated pellets. Type II collagen immunostaining generally correlated with the higher COL2-GF signal for predifferentiated pellets and was focused within the core of the defect, suggesting that it was of donor cell origin. This was confirmed by colocalized immunostaining for human mitochondria (Fig. 6D). Significant numbers of donor cells were present in both defect groups at the endpoint. In two knees, type II collagen immunostaining was associated with calcified cartilage or bone throughout the full depth of repair tissue (Supplementary Fig. S2A), suggesting the predifferentiated pellet implant stimulated endochondral bone formation. For the defect that was implanted with a COL2-GF pellet, there was no luminescent signal from the femoral explant (Supplementary Fig. S2B).

Because there is an acute inflammatory response to the type of injury induced by an osteochondral defect, it is possible that pro-inflammatory cytokines upregulated during the first week after pellet implantation dampened MSC chondrogenesis—even in the presence of local TGF-β1 overexpression. To determine the influence of pro-inflammatory cytokine challenge on TGF-β1-overexpressing MSCs, additional cells were genetically modified as done for the rat study and placed in pellet culture. The adenoviral vector used encodes for latent human TGF-β1, such that most of the secreted transgene in the conditioned media were in latent form; activated TGF-β1 levels between 2 and 8 ng/mL were measured throughout the

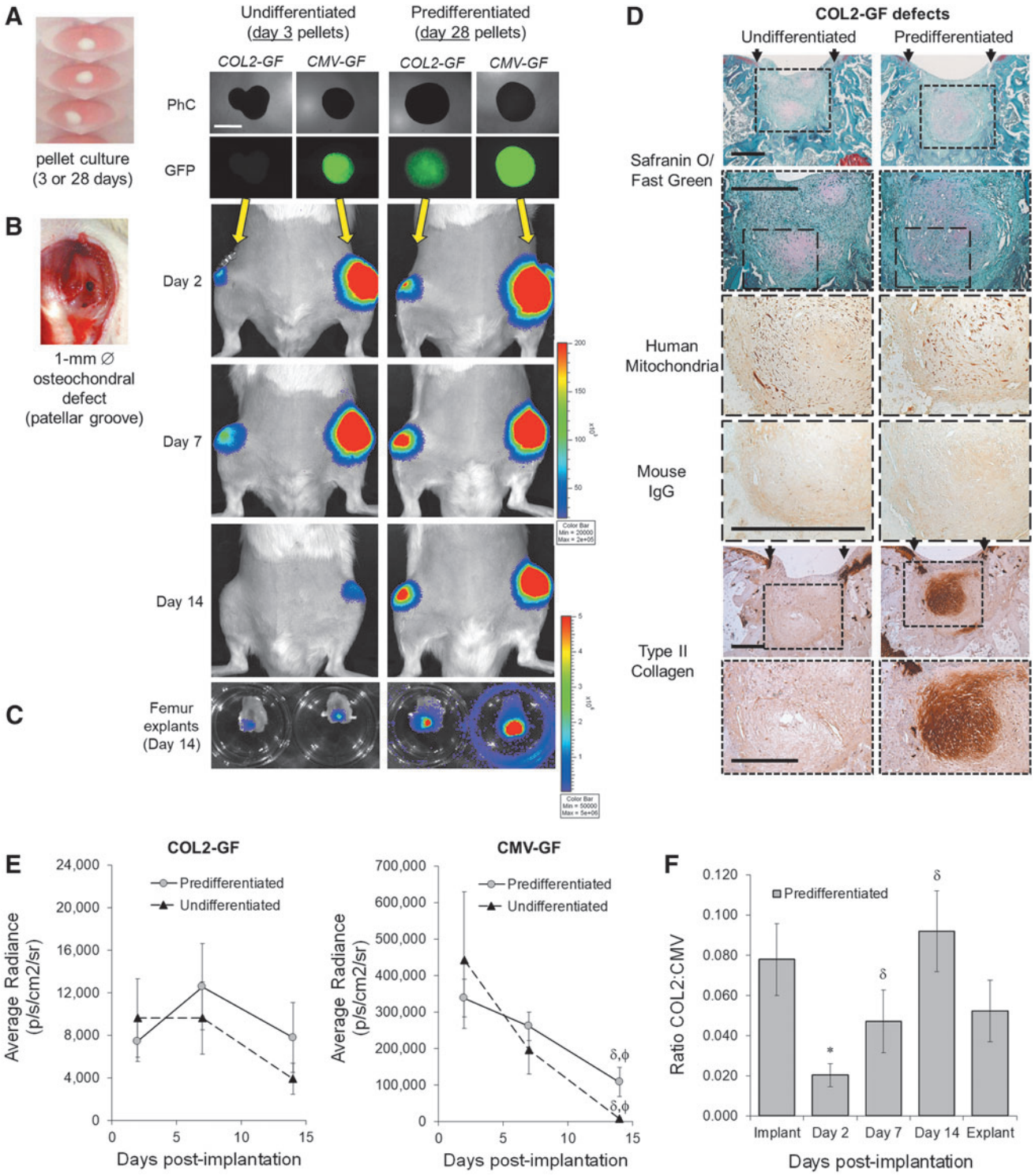

FIG. 6. Bioluminescence imaging of MSC pellets within rat osteochondral defects. **(A)** MSCs were transduced with LV.COL2-GF or LV.CMV-GF followed by Ad.TGF-β1 and cultured as pellets for either 3 (undifferentiated) or 28 days (predifferentiated). *Right:* On the day of implantation, representative pellets were imaged by fluorescence microscopy for copGFP expression. Scale bar = 1 mm. **(B)** Pellets were implanted into 1-mm osteochondral defects made in the patellar groove of immune-compromised rats (*left*). Luciferase expression in the defects was imaged noninvasively using bioluminescence imaging at days 2, 7, and 14 after pellet implantation. The heat map represents a radiance range of $2–20\times10^4$ photons/s/cm^2/sr. **(C)** After noninvasive imaging, the distal femora were removed and imaged in a bath of luciferin substrate. The heat map represents a radiance range from 5 to 500×10^4 photons/s/cm^2/sr. **(D)** Histology/immunohistochemistry of day 14 defects. Transverse sections were either stained with Safranin O/Fast Green or immunostained for human mitochondria or type II collagen. All scale bars = 500 μm. **(E)** Luminescent flux measurements (5-6 knees/group; mean ± standard error of the mean) from defects implanted within either COL2-GF or CMV-GF reporter cells. **(F)** COL2:CMV activity ratios are shown for predifferentiated pellets on the day of implantation (Implant), during noninvasive imaging of knees at days 2–14, or for imaging of day 14 explants (Explant). Significance notations: δ, compared to day 2; ϕ, compared to day 7; *, compared to implant.

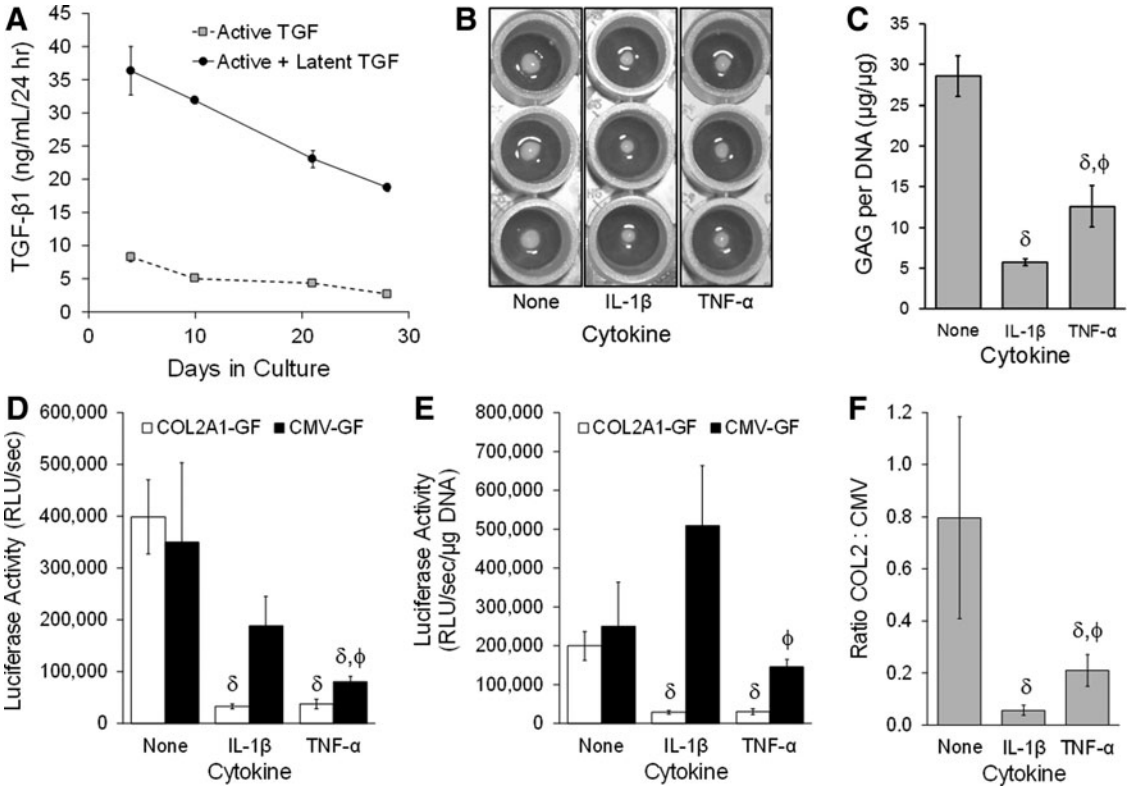

FIG. 7. COL2 reporter inhibition in the presence of pro-inflammatory cytokines. **(A)** MSC pellets were modified to overexpress the latent form of TGF-β1. ELISA of pellet-conditioned media demonstrated that a fraction of the expressed TGF-β1 was converted to the active form, resulting in about 5–10 ng/mL/24 h active TGF-β1 during the culture period. **(B)** Images of MSC pellets after 4 weeks of TGF-β1 overexpression without cytokines or in the continuous presence of 1 ng/mL IL-1β or 5 ng/mL TNF-α. **(C)** GAG content, normalized by DNA content, for the same groups shown in **(B)**. **(D)** Total luciferase activities (RLU/s) for pellets from COL2-GF or CMV-GF reporter cells. **(E)** Luciferase activities normalized to pellet DNA content. **(F)** COL2:CMV activity ratios are shown for the groups shown in **(D, E)**. Significance notations: δ, compared to TGF-β1 alone; ϕ, compared to IL-1β1.

duration of culture (Fig. 7A). Similar to the prior experiments using recombinant TGF-β1 (Supplementary Fig. S1A), in the presence of TGF-β1 overexpression, IL-1β reduced both pellet size and GAG content compared to no-cytokine controls. TNF-α also reduced pellet size and GAG content, but not to the same extent as IL-1β (Fig. 7B, C). Both cytokines reduced total COL2-GF and CMV-GF reporter levels compared to no-cytokine controls. However, the degree of reduction in total luciferase activity was greater for the COL2-GF reporter. TNF-α led to a greater reduction in total CMV-GF activity than did IL-1β (Fig. 7D). To account for differences in day 28 pellet cell numbers with treatment, luciferase activity was normalized by pellet DNA content; while normalized COL2-GF activity was reduced by both cytokines, CMV-GF activity per cell was slightly increased in the presence of IL-1β but not TNF-α (Fig. 7E). The ratio of COL2:CMV activities was reduced more by IL-1β than TNF-α (Fig. 7F)—similar to the trends in GAG/DNA content (Fig. 7C).

Discussion

The goal of this study was to characterize a chondrogenesis-responsive promoter that can be combined with different molecular reporter constructs to provide a toolset for the skeletal tissue engineering and regenerative medicine (TERM) community. A challenge to the evaluation of chondrogenic inductive strategies for MSCs and other candidate cell involves the time required to get feedback on cell differentiation and matrix deposition. Specific markers of chondrogenic gene expression, such as *COL2A1*, are not upregulated until several days after MSC induction.[45,46] Analysis of gene expression can take one or more additional days. The promoter described in this study, which is sensitive to activation by the master chondrogenic transcription factor SOX9, resulted in specific induction of copGFP and fLuc within a few days after TGF-β stimulation of human MSC pellets. Both reporters can be measured nondestructively within minutes or, upon cell lysis, more quantitatively within an hour. Luciferase levels can also be measured nondestructively for those investigators with access to a BLI platform. Consequently, this approach can provide rapid feedback on the efficiency of test regimens, allowing earlier initiation of follow-up tests while waiting on detailed molecular analysis of tissue constructs. When used with animal models of defect repair, the reporter can provide noninvasive insights into repair activity by donor cells, complementing destructive endpoint measures.

Because type II collagen expression is specific to chondrocytes within the appendicular skeleton, various promoter and/or enhancer elements from *COL2A1* have been utilized for reporting chondrogenesis both *in vitro* and *in vivo*.

Col2-GFP transgenic mice were generated nearly 20 years ago, demonstrating specificity in cells of the chondrocyte lineage[54]; more recently, Diekman *et al.* derived iPSCs from these mice and used GFP expression to purify iPSC subpopulations that had been predifferentiated successfully into chondroprogenitor cells.[55] Many of the reporter vectors that have been used *in vitro* were derived from those plasmids used to work out control of *COL2A1* expression by SOX9 and other transcriptional regulators.[7,8,47] Oberbauer *et al.* used a 600-bp sequence of the proximal *COL2A1* promoter without enhancer elements in a plasmid encoding *Metridia* secreted luciferase, providing a rapid screening approach for relatively chondrogenic populations of human adipose-derived stem cells (ADSCs).[56] The authors were able to identify donor samples with enhanced chondrogenic potential within 3 days of inducing differentiation *in vitro*. The specific promoter design used in this study, consisting of 4 repeats of a conserved enhancer sequence (containing a SOX9 binding motif) placed upstream of a relatively short sequence encompassing the *COL2A1* basal promoter, was first validated in transfected ATDC5 cells, a murine chondroprogenitor line, using fLuc and red fluorescent protein reporters.[26] The present study indicates similar reporter activation in human cells.

Chondrogenesis-responsive promoters have also been used with lentiviral reporter vectors for characterizing the chondrogenic activity of human iPSCs, ADSCs, and bone marrow-derived MSCs.[17,19,25] Due to differences in promoter sequence, cell source, and protocols for isolation, culture, and lentiviral transduction, a direct comparison cannot be made with those previously described promoters. The lentiviral construct described in this study is the first to combine *COL2A1* enhancer repeats with the basal promoter, potentially balancing signal strength (from multiple enhancer repeats) with responsiveness to endogenous transcriptional regulators that act on the core *COL2A1* promoter.[27,47,48] The relative induction of luciferase activity shown above suggests a specific and sensitive option for reporting chondrogenic activity by stem/progenitor cells with promise for cartilage TERM. Importantly, the constructs described in this study have been fully sequenced and deposited with the nonprofit Addgene for immediate use by the research community. In addition to primary human chondroprogenitors, the 4eCOL2A1 promoter can likely be used with other mammalian cells of relevance for preclinical evaluation. For example, similar COL2-GF activity has been observed for equine MSCs differentiated in pellet culture.[57] Because a very similar sequence was used successfully in murine ATDC5 cells,[26] it is likely that the 4eCOL2A1 promoter can also be used with cells of rodent origin; however, to date this has not been explicitly tested.

The chondrogenic potential of human MSCs was described 20 years ago.[21,22] Since that time, they have been tested extensively as a cell source for bone and cartilage tissue engineering.[9,10] However, MSCs cultured under chondrogenic differentiation conditions *in vitro* tend to terminally differentiate into hypertrophic chondrocytes, as opposed to forming stable articular chondrocytes.[58,59] This cell fate is associated with the ossification of MSC-derived cartilage after implantation *in vivo*.[52] Until strategies for preventing this differentiation pathway in MSCs can be identified, other chondroprogenitor cell populations may be more suitable for the repair of articular cartilage. Instead, MSCs may be more effective in directing endochondral bone repair, through the formation of callus-like cartilage tissue within bone defects.[60] Recent evidence has supported this possibility, demonstrating that conditions which promote hypertrophic maturation of MSC-derived chondrocytes[21,61] can produce tissue constructs that act as a template for the formation of bone *de novo* at ectopic and orthotopic implantation sites.[62,63] The osteochondral defect study reported here shows that TGF-β1-overexpressing, predifferentiated MSC pellets stimulated boney remodeling in the osseous phase of the osteochondral defect; in one case, calcified cartilage and bone were present in the full depth of the repair tissue by 2 weeks postimplantation (Supplementary Fig. S2).

Acute and chronic inflammation is detrimental to chondrogenesis. Pro-inflammatory cytokines and other toll-like receptor (TLR) ligands associated with osteoarthritis, rheumatoid arthritis, or defect infection are known to potently inhibit not only the anabolic activity of chondrocytes[64] but also chondrogenesis of MSCs and other chondroprogenitor cells.[12,13,65–67] The present study supports these past findings by demonstrating the potent inhibition of COL2-GF reporter activity *in vitro* for MSC pellets cultured in the presence of IL-1β or TNF-α (Figs. 2 and 7). Both cytokines are known to be upregulated during the acute inflammation phase following a joint injury or following surgical intervention associated with articular cartilage and bone defect repair.[68] Pro-inflammatory cytokine challenge may explain the transient downregulation in bioluminescent signal during the first week after COL2-GF pellet implantation into rat osteochondral defects (Fig. 6F). Moreover, cytokine-mediated GAG loss may explain the weak Safranin O staining within defects that received predifferentiated pellets, despite the residual pellets still staining for type II collagen (Fig. 6D). This apparent discrepancy is consistent with a prior study demonstrating GAG loss that preceded collagen loss by nearly 2 weeks in bovine cartilage stimulated with IL-1 *in vitro*.[69] Early dampening of chondrogenic activity may prevent or delay articular cartilage or bone defect repair. These data support the delivery of anti-inflammatory factors during the period when a pro-inflammatory challenge would be expected; indeed, previous studies have showed efficacy for anti-cytokine treatment to promote chondrogenesis of MSCs in tissue constructs.[70,71] Additional work is required to test whether such therapies would improve MSC-based cartilage formation *in vivo*.

The methodology reported in this study has limitations that can be addressed in future study. The expression vector chosen as a test case, while coexpressing fluorescent and luminescent reporters under control of the same promoter, does not include constitutive expression of a selectable marker for enriching transduced cells; consequently, not all of the pellet MSCs were represented in reporter assays. Moreover, by not controlling for variability in transgene copy number, this approach did not allow for an assessment of heterogeneity in reporter activity among individual MSCs. To overcome these limitations, the 407 kb 4eCOL2A1 promoter can be inserted into alternative constructs that incorporate selectable markers.[57] A related limitation to this approach is that it is likely more adaptable to studying culture-expanded cells than minimally-manipulated cells

(i.e., without culture expansion) that are also being examined for tissue repair, such as bone marrow concentrate[72] or the stromal vascular fraction of adipose tissue.[73] Finally, the use of an immune-compromised rat may have limited the physiological relevance of the defect microenvironment on chondrogenic activity by implanted MSCs. The results shown in Figure 6 should be validated using syngeneic reporter cells, such as MSCs derived from a COL2-fLuc transgenic rat; however, no such rat model currently exists.

Acknowledgments

The authors are grateful to Dr. Mark Vrahas (current affiliation: Cedars-Sinai Medical Center, Los Angeles, CA) for recovering the intramedullary reamings used to isolate MSCs. Special thanks to Dr. Mary Goldring (Hospital for Special Surgery, New York, NY) for providing valuable insights into *COL2A1* gene regulation and prior *COL2A1*-based reporter constructs. This work was supported by grants from the National Institute of Arthritis Musculoskeletal and Skin Diseases (AR057105, AR069253), the National Institute of General Medical Sciences (GM125503), and the Klarman Family Foundation. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2

References

1. Ferguson, C.M., Miclau, T., Hu, D., Alpern, E., and Helms, J.A. Common molecular pathways in skeletal morphogenesis and repair. Ann N Y Acad Sci **857,** 33, 1998.
2. Goldring, M.B., Tsuchimochi, K., and Ijiri, K. The control of chondrogenesis. J Cell Biochem **97,** 33, 2006.
3. Shum, L., Coleman, C.M., Hatakeyama, Y., and Tuan, R.S. Morphogenesis and dysmorphogenesis of the appendicular skeleton. Birth Defects Res C Embryo Today **69,** 102, 2003.
4. Kronenberg, H.M. Developmental regulation of the growth plate. Nature **423,** 332, 2003.
5. Einhorn, T.A., and Gerstenfeld, L.C. Fracture healing: mechanisms and interventions. Nat Rev **11,** 45, 2015.
6. Bi, W., Deng, J.M., Zhang, Z., Behringer, R.R., and de Crombrugghe, B. Sox9 is required for cartilage formation. Nat Genet **22,** 85, 1999.
7. Lefebvre, V., Zhou, G., Mukhopadhyay, K., *et al*. An 18-base-pair sequence in the mouse proalpha1(II) collagen gene is sufficient for expression in cartilage and binds nuclear proteins that are selectively expressed in chondrocytes. Mol Cell Biol **16,** 4512, 1996.
8. Zhou, G., Lefebvre, V., Zhang, Z., Eberspaecher, H., and de Crombrugghe, B. Three high mobility group-like sequences within a 48-base pair enhancer of the Col2a1 gene are required for cartilage-specific expression in vivo. J Biol Chem **273,** 14989, 1998.
9. Johnstone, B., Alini, M., Cucchiarini, M., *et al*. Tissue engineering for articular cartilage repair—the state of the art. Eur Cells Mater **25,** 248, 2013.
10. Verrier, S., Alini, M., Alsberg, E., *et al*. Tissue engineering and regenerative approaches to improving the healing of large bone defects. Eur Cells Mater **32,** 87, 2016.
11. Evans, C.H., Palmer, G.D., Pascher, A., *et al*. Facilitated endogenous repair: making tissue engineering simple, practical, and economical. Tissue Eng **13,** 1987, 2007.
12. Majumdar, M.K., Wang, E., and Morris, E.A. BMP-2 and BMP-9 promotes chondrogenic differentiation of human multipotential mesenchymal cells and overcomes the inhibitory effect of IL-1. J Cell Physiol **189,** 275, 2001.
13. Wehling, N., Palmer, G.D., Pilapil, C., *et al.* Interleukin-1beta and tumor necrosis factor alpha inhibit chondrogenesis by human mesenchymal stem cells through NF-kappaB-dependent pathways. Arthritis Rheum **60,** 801, 2009.
14. Mayer-Kuckuk, P., and Boskey, A.L. Molecular imaging promotes progress in orthopedic research. Bone **39,** 965, 2006.
15. Horie, M., Sekiya, I., Muneta, T., *et al*. Intra-articular Injected synovial stem cells differentiate into meniscal cells directly and promote meniscal regeneration without mobilization to distant organs in rat massive meniscal defect. Stem Cells **27,** 878, 2009.
16. Feeley, B.T., Conduah, A.H., Sugiyama, O., Krenek, L., Chen, I.S., and Lieberman, J.R. In vivo molecular imaging of adenoviral versus lentiviral gene therapy in two bone formation models. J Orthop Res **24,** 1709, 2006.
17. Vilalta, M., Jorgensen, C., Degano, I.R., *et al*. Dual luciferase labelling for non-invasive bioluminescence imaging of mesenchymal stromal cell chondrogenic differentiation in demineralized bone matrix scaffolds. Biomaterials **30,** 4986, 2009.
18. Takaku, Y., Murai, K., Ukai, T., *et al*. In vivo cell tracking by bioluminescence imaging after transplantation of bioengineered cell sheets to the knee joint. Biomaterials **35,** 2199, 2014.
19. Correa, D., Somoza, R.A., and Caplan, A.I. Nondestructive/noninvasive imaging evaluation of cellular differentiation progression during in vitro mesenchymal stem cell-derived chondrogenesis. Tissue Eng Part A **24,** 662, 2018.
20. Lin, P., Lin, Y., Lennon, D.P., Correa, D., Schluchter, M., and Caplan, A.I. Efficient lentiviral transduction of human mesenchymal stem cells that preserves proliferation and differentiation capabilities. Stem Cells Transl Med **1,** 886, 2012.
21. Mackay, A.M., Beck, S.C., Murphy, J.M., Barry, F.P., Chichester, C.O., and Pittenger, M.F. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng **4,** 415, 1998.
22. Johnstone, B., Hering, T.M., Caplan, A.I., Goldberg, V.M., and Yoo, J.U. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res **238,** 265, 1998.
23. Muramatsu, S., Wakabayashi, M., Ohno, T., *et al*. Functional gene screening system identified TRPV4 as a regulator of chondrogenic differentiation. J Biol Chem **282,** 32158, 2007.
24. Kawato, Y., Hirao, M., Ebina, K., *et al.* Nkx3.2 promotes primary chondrogenic differentiation by upregulating Col2a1 transcription. PLoS One **7,** e34703, 2012.
25. Yang, S.L., Harnish, E., Leeuw, T., *et al*. Compound screening platform using human induced pluripotent stem

cells to identify small molecules that promote chondrogenesis. Protein Cell **3,** 934, 2012.
26. Kan, A., Ikeda, T., Saito, T., *et al*. Screening of chondrogenic factors with a real-time fluorescence-monitoring cell line ATDC5-C2ER: identification of sorting nexin 19 as a novel factor. Arthritis Rheum **60,** 3314, 2009.
27. Osaki, M., Tan, L., Choy, B.K., *et al*. The TATA-containing core promoter of the type II collagen gene (COL2A1) is the target of interferon-gamma-mediated inhibition in human chondrocytes: requirement for Stat1 alpha, Jak1 and Jak2. Biochem J **369,** 103, 2003.
28. Kim, J.H., Lee, S.R., Li, L.H., *et al*. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. PLoS One **6,** e18556, 2011.
29. Dull, T., Zufferey, R., Kelly, M., *et al*. A third-generation lentivirus vector with a conditional packaging system. J Virol **72,** 8463, 1998.
30. Porter, R.M., Liu, F., Pilapil, C., *et al*. Osteogenic potential of reamer irrigator aspirator (RIA) aspirate collected from patients undergoing hip arthroplasty. J Orthop Res **27,** 42, 2009.
31. Ferreira, E., Porter, R.M., Wehling, N., *et al*. Inflammatory cytokines induce a unique mineralizing phenotype in mesenchymal stem cells derived from human bone marrow. J Biol Chem **288,** 29494, 2013.
32. Solchaga, L.A., Penick, K., Porter, J.D., Goldberg, V.M., Caplan, A.I., and Welter, J.F. FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. J Cell Physiol **203,** 398, 2005.
33. Steinert, A.F., Kunz, M., Prager, P., *et al*. Mesenchymal stem cell characteristics of human anterior cruciate ligament outgrowth cells. Tissue Eng Part A **17,** 1375, 2011.
34. Bahnson, A.B., Dunigan, J.T., Baysal, B.E., *et al*. Centrifugal enhancement of retroviral mediated gene transfer. J Virol Methods **54,** 131, 1995.
35. Del Vecchio, M.A., Georgescu, H.I., McCormack, J.E., Robbins, P.D., and Evans, C.H. Approaches to enhancing the retroviral transduction of human synoviocytes. Arthritis Res **3,** 259, 2001.
36. Palmer, G.D., Steinert, A., Pascher, A., *et al*. Gene-induced chondrogenesis of primary mesenchymal stem cells in vitro. Mol Ther **12,** 219, 2005.
37. Palmer, G.D., Stoddart, M.J., Gouze, E., *et al*. A simple, lanthanide-based method to enhance the transduction efficiency of adenovirus vectors. Gene Ther **15,** 357, 2008.
38. Penick, K.J., Solchaga, L.A., and Welter, J.F. High-throughput aggregate culture system to assess the chondrogenic potential of mesenchymal stem cells. Biotechniques **39,** 687, 2005.
39. Farndale, R.W., Buttle, D.J., and Barrett, A.J. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta **883,** 173, 1986.
40. Liu, F., Ferreira, E., Porter, R.M., *et al*. Rapid and reliable healing of critical size bone defects with genetically modified sheep muscle. Eur Cells Mater **30,** 118, 2015.
41. Kuroda, R., Usas, A., Kubo, S., *et al*. Cartilage repair using bone morphogenetic protein 4 and muscle-derived stem cells. Arthritis Rheum **54,** 433, 2006.
42. Pagnotto, M.R., Wang, Z., Karpie, J.C., Ferretti, M., Xiao, X., and Chu, C.R. Adeno-associated viral gene transfer of transforming growth factor-beta1 to human mesenchymal stem cells improves cartilage repair. Gene Ther **14,** 804, 2007.
43. Lee, H.H., O'Malley, M.J., Friel, N.A., *et al*. Persistence, localization, and external control of transgene expression after single injection of adeno-associated virus into injured joints. Hum Gene Ther **24,** 457, 2013.
44. Schmitz, N., Laverty, S., Kraus, V.B., and Aigner, T. Basic methods in histopathology of joint tissues. Osteoarthritis Cartilage **18(Suppl 3),** S113, 2010.
45. Barry, F., Boynton, R.E., Liu, B., and Murphy, J.M. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res **268,** 189, 2001.
46. Sekiya, I., Vuoristo, J.T., Larson, B.L., and Prockop, D.J. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc Natl Acad Sci U S A **99,** 4397, 2002.
47. Goldring, M.B., Fukuo, K., Birkhead, J.R., Dudek, E., and Sandell, L.J. Transcriptional suppression by interleukin-1 and interferon-gamma of type II collagen gene expression in human chondrocytes. J Cell Biochem **54,** 85, 1994.
48. Peng, H., Tan, L., Osaki, M., *et al*. ESE-1 is a potent repressor of type II collagen gene (COL2A1) transcription in human chondrocytes. J Cell Physiol **215,** 562, 2008.
49. Krishnan, M., Park, J.M., Cao, F., *et al*. Effects of epigenetic modulation on reporter gene expression: implications for stem cell imaging. FASEB J **20,** 106, 2006.
50. Wen, S., Zhang, H., Li, Y., *et al*. Characterization of constitutive promoters for piggyBac transposon-mediated stable transgene expression in mesenchymal stem cells (MSCs). PLoS One **9,** e94397, 2014.
51. Dexheimer, V., Frank, S., and Richter, W. Proliferation as a requirement for in vitro chondrogenesis of human mesenchymal stem cells. Stem Cells Dev **21,** 2160, 2012.
52. Pelttari, K., Winter, A., Steck, E., *et al*. Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. Arthritis Rheum **54,** 3254, 2006.
53. Chu, C.R., Szczodry, M., and Bruno, S. Animal models for cartilage regeneration and repair. Tissue Eng Part B Rev **16,** 105, 2010.
54. Grant, T.D., Cho, J., Ariail, K.S., Weksler, N.B., Smith, R.W., and Horton, W.A. Col2-GFP reporter marks chondrocyte lineage and chondrogenesis during mouse skeletal development. Dev Dyn **218,** 394, 2000.
55. Diekman, B.O., Christoforou, N., Willard, V.P., *et al*. Cartilage tissue engineering using differentiated and purified induced pluripotent stem cells. Proc Natl Acad Sci U S A **109,** 19172, 2012.
56. Oberbauer, E., Steffenhagen, C., Feichtinger, G., *et al*. A luciferase-based quick potency assay to predict chondrogenic differentiation. Tissue Eng **22,** 487, 2016.
57. Martin-Pena, A., Porter, R.M., Plumton, G., *et al*. Lentiviral-based reporter constructs for profiling chondrogenic activity in primary equine cell populations. Eur Cells Mater **36,** 156, 2018.
58. Mueller, M.B., and Tuan, R.S. Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. Arthritis Rheum **58,** 1377, 2008.
59. Fahy, N., Gardner, O.F.W., Alini, M., and Stoddart, M.J. Parathyroid hormone-related protein gradients affect the

progression of mesenchymal stem cell chondrogenesis and hypertrophy. Tissue Eng Part A **24,** 849, 2018.
60. Farrell, E., van der Jagt, O.P., Koevoet, W., *et al*. Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair? Tissue Eng **15,** 285, 2009.
61. Muraglia, A., Corsi, A., Riminucci, M., *et al*. Formation of a chondro-osseous rudiment in micromass cultures of human bone-marrow stromal cells. J Cell Sci **116,** 2949, 2003.
62. Scotti, C., Tonnarelli, B., Papadimitropoulos, A., *et al*. Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. Proc Natl Acad Sci U S A **107,** 7251, 2010.
63. Scotti, C., Piccinini, E., Takizawa, H., *et al*. Engineering of a functional bone organ through endochondral ossification. Proc Natl Acad Sci U S A **110,** 3997, 2013.
64. Goldring, M.B., and Goldring, S.R. Osteoarthritis. J Cell Physiol **213,** 626, 2007.
65. Sitcheran, R., Cogswell, P.C., and Baldwin, A.S., Jr. NF-kappaB mediates inhibition of mesenchymal cell differentiation through a posttranscriptional gene silencing mechanism. Genes Dev **17,** 2368, 2003.
66. Pevsner-Fischer, M., Morad, V., Cohen-Sfady, M., *et al*. Toll-like receptors and their ligands control mesenchymal stem cell functions. Blood **109,** 1422, 2007.
67. Heldens, G.T., Blaney Davidson, E.N., Vitters, E.L., *et al*. Catabolic factors and osteoarthritis-conditioned medium inhibit chondrogenesis of human mesenchymal stem cells. Tissue Eng Part A **18,** 45, 2012.
68. Lotz, M. Cytokines in cartilage injury and repair. Clin Orthop Relat Res **391 Suppl,** S108, 2001.
69. Li, Y., Wang, Y., Chubinskaya, S., *et al*. Effects of insulin-like growth factor-1 and dexamethasone on cytokine-challenged cartilage: relevance to post-traumatic osteoarthritis. Osteoarthritis Cartilage **23,** 266, 2015.
70. Glass, K.A., Link, J.M., Brunger, J.M., Moutos, F.T., Gersbach, C.A., and Guilak, F. Tissue-engineered cartilage with inducible and tunable immunomodulatory properties. Biomaterials **35,** 5921, 2014.
71. Moutos, F.T., Glass, K.A., Compton, S.A., *et al*. Anatomically shaped tissue-engineered cartilage with tunable and inducible anticytokine delivery for biological joint resurfacing. Proc Natl Acad Sci U S A **113,** E4513, 2016.
72. Chu, C.R., Fortier, L.A., Williams, A., *et al*. Minimally manipulated bone marrow concentrate compared with microfracture treatment of full-thickness chondral defects: a one-year study in an equine model. J Bone Joint Surg Am **100,** 138, 2018.
73. Jurgens, W.J., van Dijk, A., Doulabi, B.Z., *et al*. Freshly isolated stromal cells from the infrapatellar fat pad are suitable for a one-step surgical procedure to regenerate cartilage tissue. Cytotherapy **11,** 1052, 2009.

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Received: October 12, 2018
Accepted: February 5, 2019
Online Publication Date: March 15, 2019