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Supplemental Information

Healing of a Large Long-Bone Defect through Serum-Free In Vitro Priming of Human Periosteum-Derived Cells

Johanna Bolander, Wei Ji, Jeroen Leijten, Liliana Moreira Teixeira, Veerle Bloemen, Dennis Lambrechts, Malay Chaklader, and Frank P. Luyten

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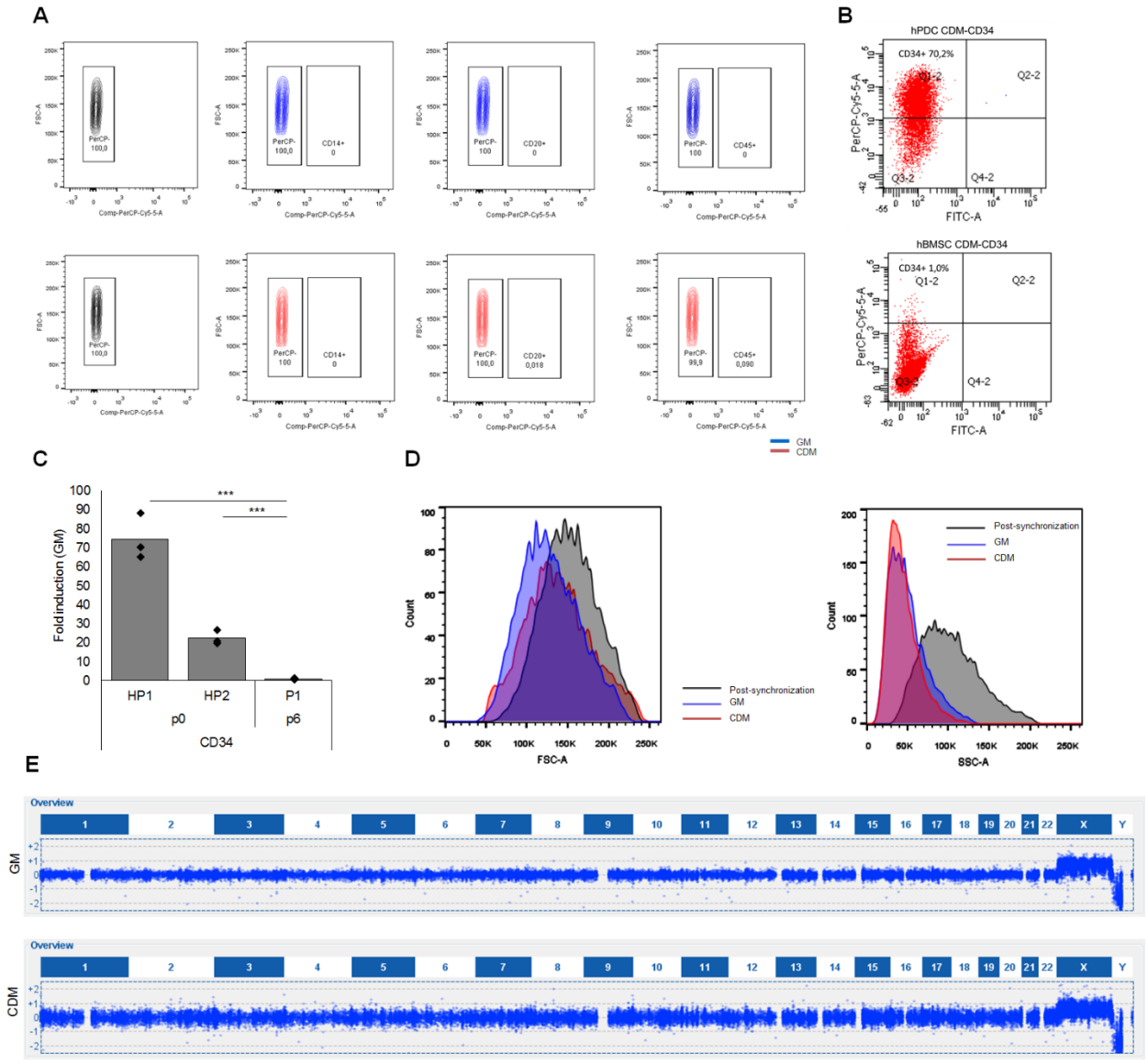

Figure S1. Serum free pre-conditioning affected hPDC phenotype. Related to Figure 1.

(**A**) Flow cytometry analysis of individual hematopoietic markers after six days of pre-conditioning in CDM or 10 % FBS. (**B**) The induced CD34 positivity by CDM pre-conditioning was specific for hPDCs and was not seen in hBMSCs. (**C**) mRNA transcript level of *CD34* in passage one (p1) hPDCs from individual donors (HP1 and HP2) and hPDCs expanded in GM to passage 6 (p6) pre-conditioned in CDM or GM. (**D**) Pre-conditioning in CDM affect cell size and granularity. (E) Karyotype analysis confirmed no significant aberrations.

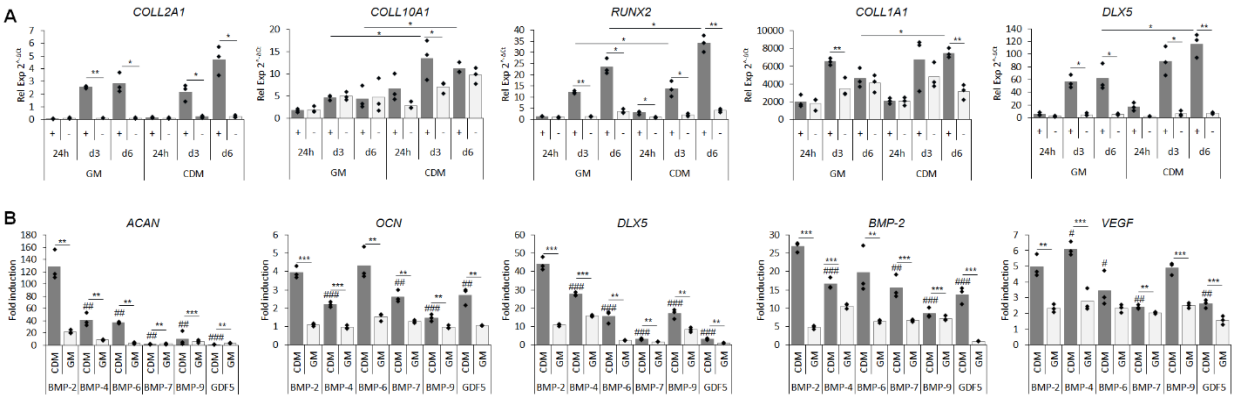

Figure S2. The serum free pre-conditioning enhanced both osteogenic and chondrogenic differentiation induced upon BMP-stimulation. Related to Figure 2.

(**A**) CDM pre-cultured cells followed by BMP-2 stimulation displayed enhanced expression of chondrogenic markers *COLL2A1*, *COLL10A1*and osteogenic markers *RUNX2* and *COLL1A1*. In addition elevated expression of transcriptional regulator *DLX5* was seen. The effect of BMP-2 in CDM pre-cultured cells was not only limited to BMP-2. (**B**) Increased expression of *ACAN*, *OCN*, *DLX5*, *BMP-2* and *VEGF* was seen in this pre-culture regimen followed by 2D stimulation of BMP-2, BMP-4, BMP-6, BMP-7, BMP-9 and GDF5. *(Statistical significance to: BMP-2: #: < 0.05, ##: < 0.01, ###: < 0.001).*

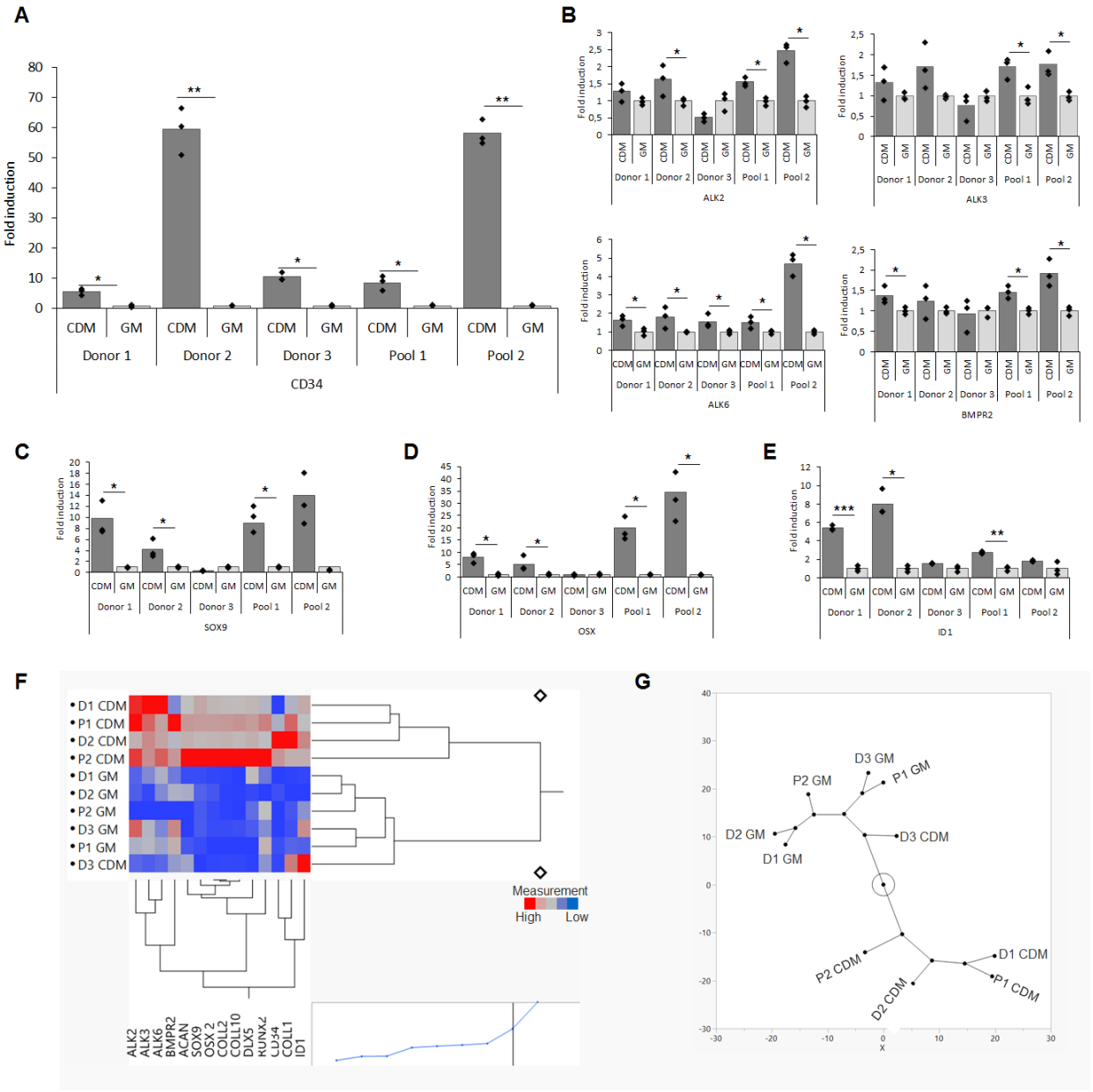

Figure S3. The improved pre-conditioning effect was confirmed in young and adult donors. Related to Figure 1 and Figure 2.

(**A**) Pre-conditioning of individual donors (D1-3) and two different pools (P1-2) of cells of different gender and ages displayed elevated CD34 expression, (**B**) as well as adapted expression level of BMP type 1 and type 3 receptors. Expression of (**C**) SOX9, (**D**) OSX and (**E**): ID1 following BMP-2 stimulation. (**F**) Cluster correlation displayed association of pre-conditioning in CDM and expression levels of marker genes. (**G**) Constellation plot over the clustered data displayed grouping of the majority of CDM pre-conditioned cell populations over individual characteristics from the specific cell populations.

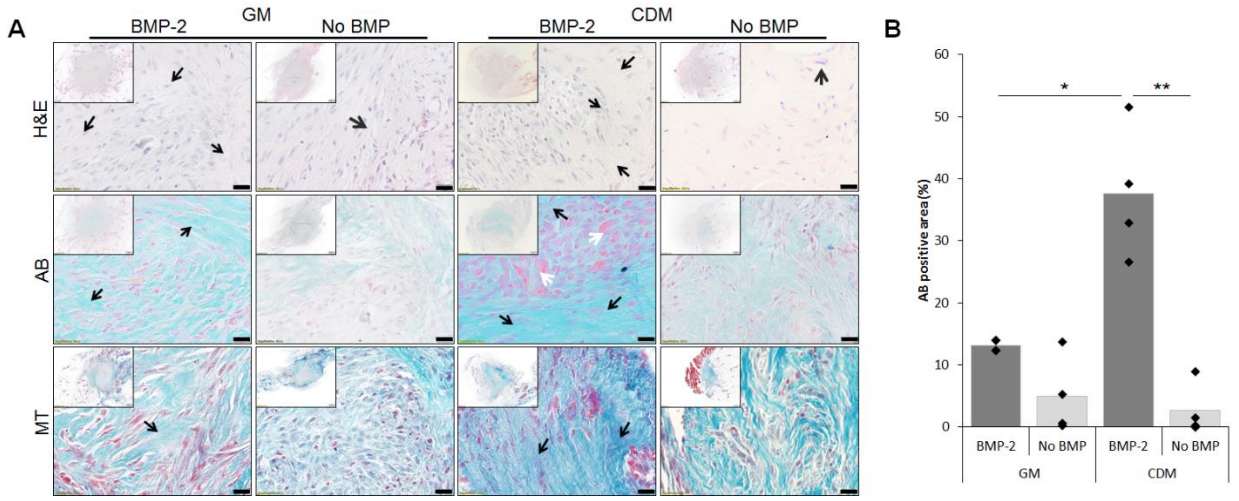

Figure S4. Serum free pre-conditioning led to enhanced GAG-production *in vivo*. Related to Figure 1 and Figure 2.

(**A**) Histology 3 weeks post subcutaneous implantation by H&E staining of *in vivo* samples displayed denser matrix formation in BMP-2 stimulated cells (black arrows) whereas more fibrous tissue were depicted in non-stimu;ated conditions (grey arrows), Alcian blue (AB) displayed cellular condensations (white arrows) surrounded by a GAG production in BMP-2 stimulated cells (black arrows) which was confirmed by Masson's Trichrome (MT) stain (black arrows). (**B**) Upon quantification of percentage of AB positive area, CDM pre-conditioning followed by BMP-2 stimulation led to elevated cartilage formation. *Scale bar: 20 µm.*

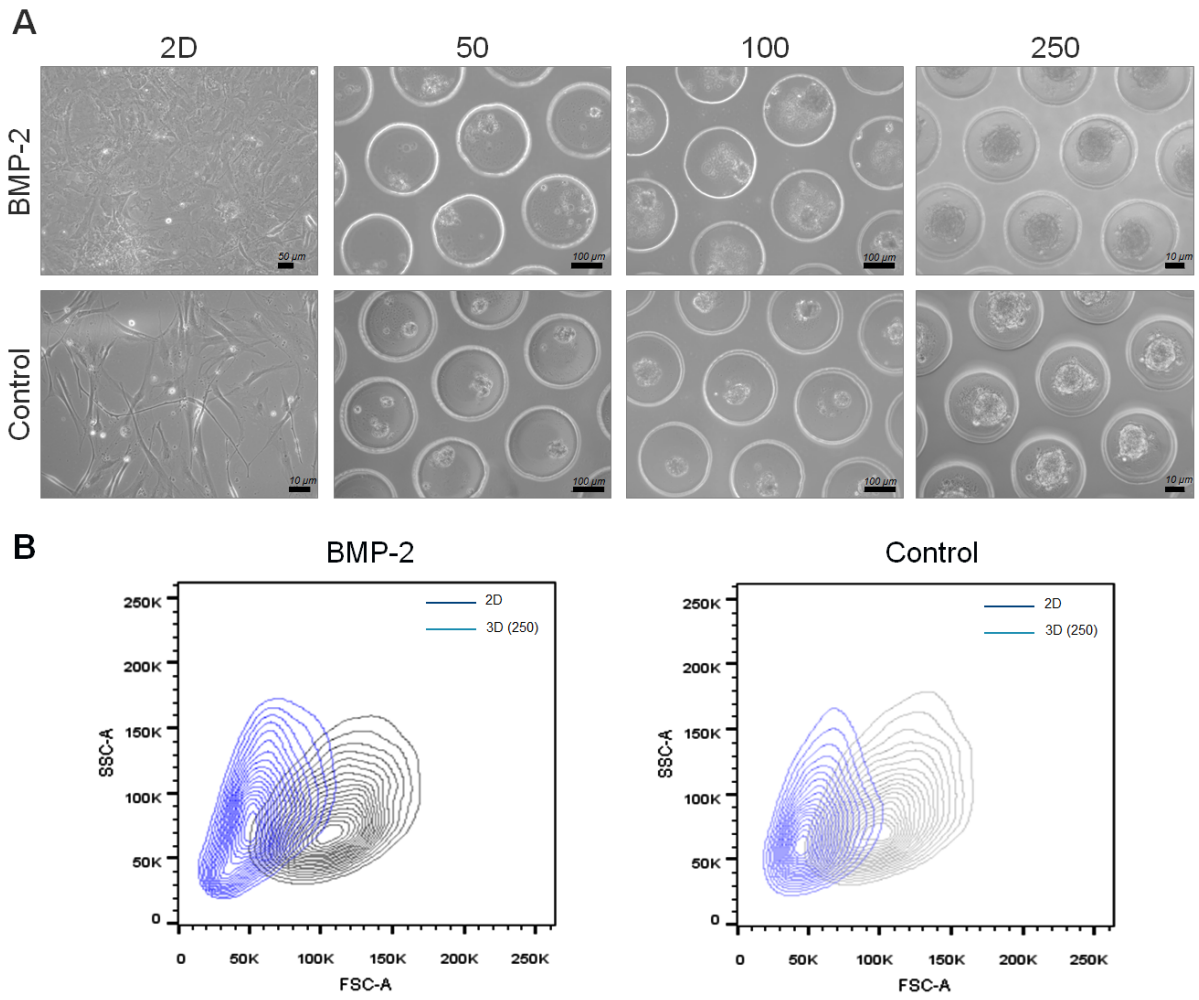

Figure S5. The number of cells per aggregate affected aggregate formation. Related to Figure 4 and Figure 5.

(**A**) Bright field images after 6 days of aggregation in cell densities of 50, 100 or 250 cells/ aggregate in the presence of or without BMP-2 displayed stable formation of aggregates of 100 and 250 cells. (**B**) Aggregation induced a decrease in cell size after disassembly of the aggregates in the presence of or without BMP-2 stimulation.

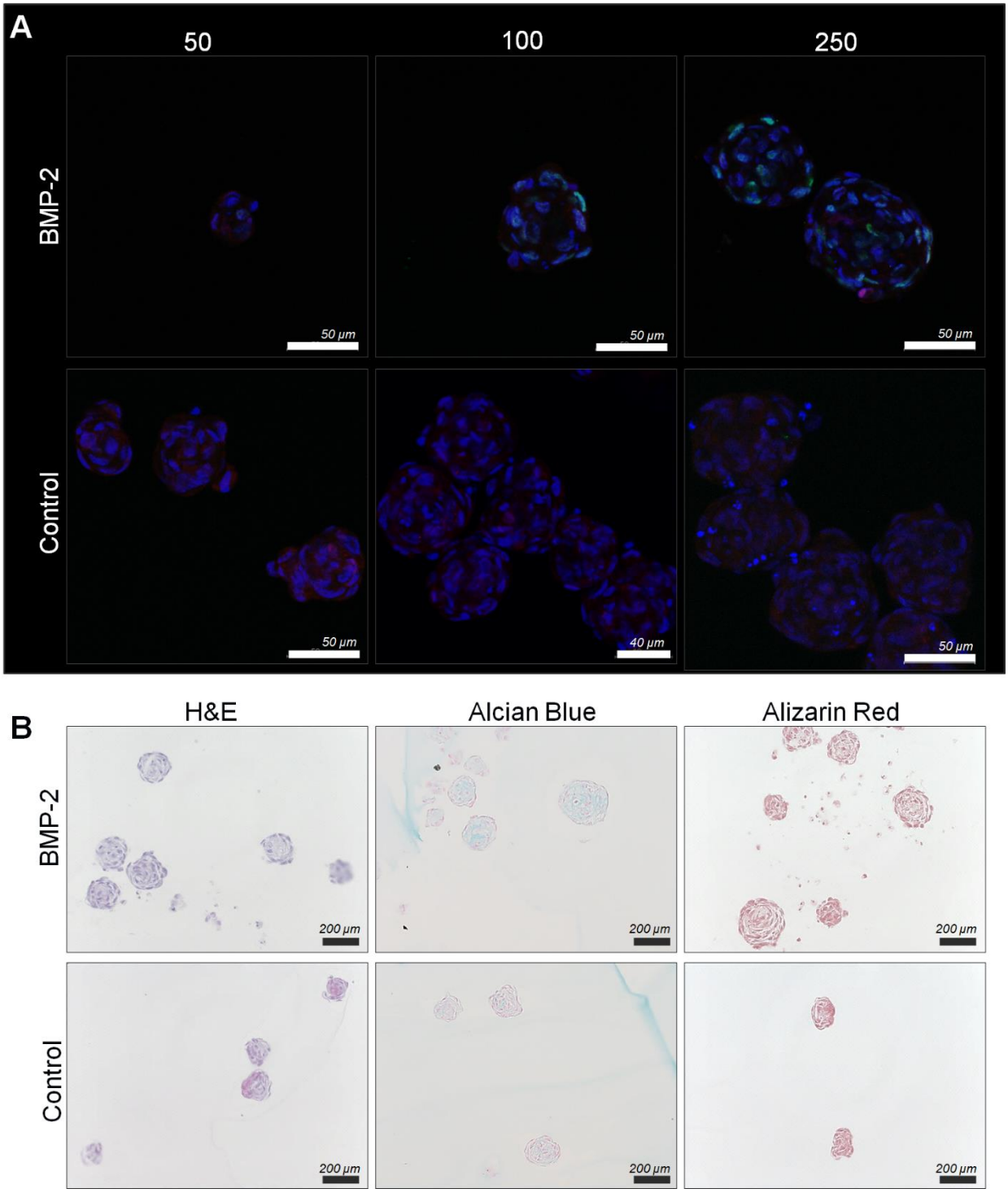

Figure S6. Aggregate size steered in vitro primed lineage specification. Related to Figure 4 and Figure 5.

(**A**) IHC for SOX9 (red), OSX (green) and DAPI (blue) on aggregates of 50, 100 or 250 hPDCs/aggregate cultured in the presence with or without BMP-2, confirm gene expression data. The results are representative of two or more independent experiments. (**B**) Histology for H&E, Alcian blue and Alizarin red on 6 days aggregated cells with and without BMP-2 stimulation displayed moderate matrix formation *in vitro*. *Unlabelled scale bar: 200 µm.*

Table S1. Primer sequences used for mRNA transcript analysis.

Gene	Forward sequence	Reverse sequence
SOX9	TGGAGACTTCTGAACGAGAGC	CGTTCTTCACCCACTTCCTC
ACAN	GTCTCACTGCCCAACTAC	GGAACACGATGCCTTTCAC
COLL2A1	GGCTTCCATTTCAGCTATGG	AGCTGCTTCGTCCAGATAGC
COLL10A1	ACGATACCAAATGCCCACAG	GTGGACCAGGAGTACCTTGC
RUNX2	CGCATTCCTCATCCCAGTAT	GCCTGGGGTCTGTAATCTGA
OSX	AGTGACCTTTCAGCCTCCAA	GGGAAAAGGGAGGGTAATCA
COLL1A1	GACGAAGACATCCCACCAAT	AGATCACGTCATCGCACAAC
OCN	GTGCAGCCTTTGTGTCCAA	GCTCACACACCTCCCTCCT
DLX5	CAGCCAAAGCTTATGCCGAC	CGGTCACTTCTTTCTCTGGCT
ID1	GGCTGTTACTCACGCCTCAAG	CCAACTGAAGGTCCCTGATGTAG
VEGF	TGCAGATTATGCGGATCAAACC	TGCATTCACATTTGTTGTGCTGTAG
BMP-2	GTATCGCAGGCACTCAGGTC	TTTTCCCACTCGTTTCTGGT
FGF2	AAAAACGGGGGCTTCTTCCT	TGTAGCTTGATGTGAGGGTCG
ALK1	ATCTGAGCAGGGCGACAC	ACTCCCTGTGGTGCAGTCA
ALK2	ATGTGACCAAGAGCCTGCAT	CGCAGGAGAGACCTTCACAC
ALK3	CAGGGCACTGTCCAGATGAT	AGCTGGGCTTTTGGAGAATC
ALK6	ATGACTCTGGGTTGCCTGTG	TCAATGGAGGCAGTGTAGGG
BMPR2	GGTTGGAAACCATCCCACTT	TGGTCCCAACAGTCTTCGAT
ACVR2a	CCGGAGATGGAAGTCACACAGCCC	TGGGGGTGGTCCTGGGTCTTGA
ACVR2b	GCTGCAGACGGACCCGTGGA	GGCCAGGCCCGGGTGTTTCA
CCNE1	GCCAGCCTTGGGACAATAATG	CTTGCACGTTGAGTTTGGGT
BIRC5	ACCAGGTGAGAAGTGAGGGA	AACAGTAGAGGAGCCAGGGA
CDK1	GGATGTGCTTATGCAGGATTCC	CATGTACTGACCAGGAGGGATAG
CD105	CACTAGCCAGGTCTCGAAGG	CTGAGGACCAGAAGCACCTC
CD34	AATGAGGCCACAACAAACATCACA	CTGTCCTTCTTAACCTCCGCACAGC

Supplemental experimental procedures

Cell culture

Isolation and *in vitro* expansion of hPDCs were carried out as previously described (De Bari et al., 2001). Cells from individual donors: donor1 (D1): 10 years female; D2: 23 years male; D3: 47 years female and two pooled populations: pool 1 (P1): 13±4.5 years and pool 2 (P2): 29±16 years, were expanded to passage six (p6) in medium containing 10 % fetal bovine serum (FBS), further referred to as (GM) as previously described (De Bari et al., 2006, Bolander et al., 2016). Cells isolated from a 43 years male (HP1) and a 16 years female (HP2) were plated and RNA was isolated after confluency was reached at the first seeding, representing p0. The P1 population was used as a representative when nothing else stated. For pre-conditioning, cells were cultured for six days in serum free chemically defined media with the removal of the growth factor cocktail, β-Glycerophosphate and lineolic acid (patent US20010039050)(Gomez-Barrena et al., 2015), further defined as serum free chemically defined media (CDM) (see below for component specification) or in GM as standard control. DNA content was measured using Quant-iT^{TM} PicoGreen$^{®}$ dsDNA assay (Invitrogen, Merelbeke, BE) according to the manufacturer's instructions (n=3). To evaluate the effect of pre-conditioning prior to differentiation, BMP-stimulation and/or aggregation was carried out for six days directly following pre-conditioning.

Detailed components in the chemically defined medium

The serum free chemically defined medium (CDM) consisted equal parts of Dulbecco's Modified Eagle Medium and HAM's F-12 (Invitrogen) supplemented with: antibiotics-antimyocotics solution (100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B; Invitrogen, Merelbeke, BE), 5 µg/ml ITS^{TM} PREMIX (BD Biosciences), 2 µg/ml L-α-Phosphatidylethanolamine (Jena Bioscience), 1x10^{-4} M α-ketoglutaric acid, 1.25 µg/ml Taurine, 0,25 U/ml Cerulosplasmin, 5 µg/ml Cholesterol, 10 µg/ml L-gluthatione reduced, 1,25 µg/ml Taurine, 9x10^{-7} M D-α-tochopherol succinate, 50 µg/ml L-ascorbic acid 2 sulphate, 1,6x10^{-9} M 3,3',5-Triiodo-L-thyronine sodium salt, 1x10^{-9} M Hydrocortisone and 5x10^{-10} M Parathyroid hormone (Sigma-Aldrich).

Flow cytometry

Flow cytometry was performed to characterize the expression of stemness markers on human periosteal cells by using human MSC Phenotyping kit (130-095-198, Miltenyi Biotec, Leiden, NL). Individual marker expression on hPDCs was characterized by staining the cells with human specific CD14-PerCP, CD20-PerCP, CD-34 PerCP-Vio700, CD45-PerCP, CD105-PE (130-098-072, 130-098-077, 130-097-915, 130-098-145, 130-098-906, Miltenyi Biotec). The extracellular staining was performed according to manufacturer's instructions. In brief, cells were mixed with MSC Phenotyping Cocktail or individual antibodies and incubated in dark at 4°C. After staining, the cells were washed and analysed using BD FACS Canto^{TM}cell analyser (BD Biosciences, Erembodegem, BE) with FlowJo V10 software. The number of cell surface receptors expressed per cell was quantified using BD QuantiBRITE^{TM}-Phycoerythrin beads (BD Biosciences, San Jose, CA),) as previously described (Pannu et al., 2001).

Cell separation

Separation of CD34^{+} cells was performed with a COLiso Magnetic Separation Kit-Human CD34^{+}Cells according to the manufacturers' instructions (K10134, ProMab Biotechnologies, Richmond, US). Upon separation, 94% purity of CD34^{+} cells of which 100% were CD14^{-}, CD20^{-} and CD45^{-}, as confirmed by flow cytometry.

Karyotype analysis

Genomic DNA was extracted using the QIAamp DNA Blood Mini QIAcube Kit on the Qiacube robotic workstation according to the manufacturer's instructions. Array analysis was performed using the 8x 60K CytoSure ISCA v2 microarray (AMADID 020040, Oxford Gene Technology, OGT, Oxford, UK). Genomic DNA was labeled for 2 hours using the CytoSure Labelling Kit (Oxford Gene Technology). The sample was labeled with Cy5 and hybridized versus Cy3-labeled sex-matched reference DNA. Hybridization was performed for minimum 16 hours in a rotator oven (SciGene, CA, US) at 65 °C. Washing of arrays was performed using Agilent wash solutions with a Little Dipper Microarray Processor (SciGene). Arrays were scanned using an Agilent microarray scanner at 2-µm resolution, followed by calculation of signal intensities using Feature Extraction software (Agilent Technologies).Visualization of results and data analysis were performed using the CytoSure Interpret Software (Oxford Gene Technology) and the circular binary segmentation algorithm. Quality control metrics are monitored with CytoSure Interpret software (Oxford Gene Technology).

In vitro BMP-stimulation and evaluation

Pre-conditioned cells were seeded at a cell seeding density of 10 000 cells/cm^2 followed by stimulation in CDM or GM containing 10% FBS supplemented with 100ng/ml BMP-2 (Medtronics, Minneapolis, Minnesota, US), BMP-4, -6, -7, -9 or GDF6 (Peprotech, London, UK). To investigate *in vitro* differentiation, samples were collected for DNA quantification and Alkaline phosphatase (ALP) activity was measured using a commercial kit (Kirkegaard & Perry, Guilford, UK) normalized to DNA content. mRNA analysis were performed as previously described (Bolander et al., 2016), primer sequences are listed in supplementary table 1. To investigate nuclear translocation of downstream signalling molecules, Western blot analysis was performed. After 6 days of BMP-2 stimulation, cells were homogenised in Cell extraction buffer containing 0.3 M Phenylmethanesulfonyl fluoride and Protease Inhibition Cocktail (Sigma-Aldrich, Bornem, BE). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) according to manufacturer's instructions. Equal quantities of protein was loaded on NuPAGE 4-12 % Bis-Tris gels (Invitrogen) and electrophoresed to separate proteins according to size. These proteins were subsequently transferred to a Polyvinylidene fluoride membrane by wet transfer for further analysis. Primary antibodies were diluted according to the manufacturer`s instructions: rabbit monoclonal Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2)(43775), rabbit polyclonal p44/42 MAPK (Erk1/2)(9102), rabbit monoclonal Phospho-p38 MAPK (Thr180/Tyr182) (12F8)(4631), rabbit polyclonal p38 MAPK(9212), rabbit polyclonal Phospho-SMAD1 (Ser463/465)/SMAD5 (Ser463/465)/SMAD8 (Ser426/428)(9516), rabbit polyclonal SMAD1(6944), rabbit polyclonal SMAD2/3 (5678)(Cell Signalling Technology- BIOKÉ, Leiden, NL), mouse monoclonal Anti-Active-β-Catenin (05-665)(EMD Millipore, Overijse, BE), goat polyconal Phospho-SMAD2/3 (Ser423/425) (sc-11769)(Santa Cruz Biotechnology, Inc, Heidelberg, DE). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse monoclonal (Abcam) was used to assess equal loading of proteins. HRP-conjugated secondary antibodies were used at a dilution of 1:2000 (Jackson, Pennsylvania, US) and images were developed by a LAS3000 Imaging System (FUJI) following the application of SuperSignal® West Femto reagent (Thermo Scientific, Illnois, US). Densitometry analysis was performed using Biorad Quantity One software.

3D Microwell fabrication and cell aggregation

Fabrication of a microwell platform allowing for high throughput production of microaggregates with controlled cell density per aggregate was performed as previously described (Leijten et al., 2016). To seed hPDCs in aggregates containing 50, 100 or 250 cells/aggregate, the microwells were covered in 2 ml of CDM containing 100.000, 200.000 or 250.000 cells, respectively, allowing for the formation of 1000 aggregates/well. The stimulatory effect of aggregation in combination with or without BMP-2 was investigated by flowcytometry, mRNA transcript analysis and IHC.

In vivo implantation

Pre-conditioned and 2D stimulated cells or aggregates were washed and collected and 1*10^6 cells were encapsulated in a 100 µl Collagen type 1 gel, (5 mg/mL, BD Biosciences) and implanted subcutaneously in NMRI$^{nu/nu}$ mice. In addition, BMP-2 stimulated monolayer cultures of hPDCs followed by 24h aggregation in the absence of BMP-2 were included to study the effect of separate stimulation of BMP-2 and aggregation. Explants were harvested at 1, 3 and 6 weeks post implantation and qualitatively analysed by histology and IHC, for all ectopic conditions n=4. For orthotopic evaluation, CDM pre-conditioned hPDC were stimulated as aggregates in the presence of BMP-2 for 6 days. Subsequently after washing, the aggregates were collected and implants of 30 µl collagen type 1 gels (5 mg/mL, BD Biosciences) containing 1200 aggregates/gel were prepared. Subsequently, a critical size 4 mm long bone defect was created in the right hind tibiae of NMRI$^{nu/nu}$ mice, allowing to examine the regenerative bone-forming potential of the *in vitro* prepared construct as previously described (van Gastel et al., 2014). In total, 6 constructs were implanted, 4 for week 8, 2 for week 4 and 2 for week 2. As control for the critical defect, 5 fractures were made were one was excluded from the study due to surgical error with remnants of bone spicules in the fracture. At harvest, samples were fixated using 2% paraformaldehyde for 12 h. Explants from the orthotopic model were analysed by *ex vivo* nano-CT as previously described (Bolander et al., 2016), and samples were processed for IHC and histology.

Immunohistochemistry

To further identify cell differentiation, IHC was performed by dual immunohistochemistry (IHC) for SOX9 (NB100-304, NovusBiologicals, Abingdon, UK) and OSX (MAB7547, R&D Systems, Abingdon, UK). As secondary antibodies: Alexa 488 anti-mouse, (1:500) together with Biotinylated SP-conjugated goat anti rabbit (1:500) were used followed by incubation with Streptavidin Alexa 555 (1:500) (Jackson ImmunoResearch, Philadelphia, US) and DAPI (1:2500). Microscopic analysis was performed by confocal imaging using a FluoView FV1000 setup (Olympus) and visualized by Z-stacking of images obtained with a UPLSAPO 60x oil objective at 0.265 um x 0.265 um pixel size, and images acquired in z-direction were separated by a distance of 1.83 um. To identify *in vivo* tissue development, a

rabbit Phospho-SMAD1 (Ser463/465)/ SMAD5 (Ser463/465)/ SMAD8 (Ser465/467) antibody (Cell signalling, Leiden, NL), mouse Aggrecan monoclonal antibody to N-terminal neoepitope (DIPEN) (clone BC4) (1042002, MD Bioscience, Switzerland), rabbit polyclonal Anti-S100 (Z0311, Dako, Heverlee, BE) and rabbit polyclonal Anti-Ihh antibody - N-terminal (ab80191, abcam, Cambridge, UK) were used according to the manufacturers' instructions. Contribution to *de novo* formed tissue by implanted cells were investigated using a human specific primary anti-OCN guinea pig antibody (a generous gift from Dr. E. Van Herck, Legendo, KU Leuven, BE) which does not cross-react with mouse OCN (De Bari et al., 2006). As secondary antibodies, peroxidase-conjugated goat anti-rabbit, -mouse, or -guinea pig (Jackson ImmunoResearch) were used. For non-fluorescent IHCs, 3,30-diaminobenzidine (Sigma-Aldrich) were used as a chromogenic substrate and hematoxylin as counterstain. Tissue sections were visualized with an inverted microscope (IX83-P22F, Olympus).

Histology

To visualize glycosaminoglycans, samples were stained with acidic Alcian Blue (pH=1, Merck, Damstadt, DE) and counterstained with nuclear fast red (Vector Laboratories, Brussels, Belgium). General matrix staining was visualized using hematoxyline (Sigma-Aldrich) and eosin staining (Klinipath, Duiven, NL). Masson's Trichrome staining was performed to visualize immature and mature bone tissue by immersing sections in Weigerts iron hematoxylin solution, subsequently stained with ponceau-acid fuchsin solution (0.5% ponceau BS, 0.5% acid fuchsin in 10% acetic acid) followed by differentiation in 5% phosphomolybdic acid 5% phosphotungstic acid solution. The sections were then transferred directly to Mason's green solution (Klinipath, Olen, BE) before differentiation in 1% acetic acid. TRAP stain was performed to localize osteoclast activity as previously described (Bolander et al., 2016). Histological sections were visualized using an inverted imaging setup (IX83-P22F, Olympus).

Histomorphometry

For semi-quantitative analysis, stained tissue sections were imaged for an overview or at four defined locations per section and used as a representative. ImageJ software was used for quantification of positive staining and scanned images from 3 sections per explants were utilized for an average value. The color threshold was adjusted to depict the positive areas by defining minimum and maximum values for each color on control samples and an in-house developed MatLab code was used to quantify the positive area normalised to the total area.

Investigation of endogenous BMP production

Endogenous production of BMP-2 was analysed by a human BMP-2 ELISA development kit (Peprotech) in conditioned media from 6 days *in vitro* stimulated conditions. Fresh stimulation media was used as baseline. The ELISA was performed according to the manufacturer's instructions.

Statistical analysis

Data are expressed as individual data points with bars representing the average value. Statistical evaluation was determined using a non-paired unequal variance student t-test to compare between independent groups. Statistical significance is indicated on all graphs as follows between conditions: *:$p < 0.05$, **: $p < 0.01$, ***: $p< 0.001$ if nothing else is stated. Results are conducted from at least 3 independent experiments, n=3 for *in vitro* data and n=/>4 for *in vivo* experiments. Hierarchical clustering was performed using Ward's method in JMP® 10 (SAS Institute Inc., Cary, NC).

Supplemental results

The improved effect of serum free pre-conditioning was not age or gender dependent

The effect of the serum free pre-conditioning was confirmed in young and adult donors (D1-3) as well as in two cell pools with a different age average (P1-2) in which an elevated CD34 expression was correlated to enhanced expression of BMP-receptors (Figure S3A and B). In addition, BMP-2 stimulation induced elevated differentiation confirmed by *SOX9*, *OSX* and *ID1* expression (Figure S3C-E). Importantly, with ID1 expression as an example, the coefficient of variance was a 5-, 2-, 8-, 6- and 14-fold higher in serum conditions for the D1-P2 populations, respectively. Interestingly, the effect of the pre-conditioning led to a specific cluster correlation depending on the pre-conditioning (Figure S3F). A constellation plot displayed that pooled cell populations can function as a representative for individual donors, since the improved potential was independent of gender and age, but potentially affected by combined donor characteristics (Figure S3G).

Enhanced in vitro differentiation leads to elevated cartilaginous matrix deposition in vivo

To investigate the *in vivo* effect of the pre-conditioning regimen, BMP-2 stimulated cells were encapsulated in a COLL1 gel and implanted ectopically for three weeks to evaluate the potency of the cells to produce cartilage. H&E staining displayed denser staining in BMP-2 stimulated explants, which was further elevated in CDM pre-conditioned cells (black arrows) (Figure S4A). Fibrous tissue was mainly depicted in non-stimulated cells (grey arrows). The deposition of a glycosaminoglycan (GAG)-rich matrix by Alcian Blue (AB) staining demonstrated the presence of GAGs in BMP-2 treated implants (black arrows). Image quantification corroborated denser stain in CDM pre-conditioned cells followed by BMP-2 stimulation combined with cellular condensations (white arrows) (Figure S4B). The AB staining was further confirmed by Masson's Trichrome (MT) with denser areas indicated by black arrows (Figure S4B). These findings indicate that the potent response seen *in vitro* was also reflected *in vivo*.

Supplemental references

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