Electronic Supporting Information

Clot-entrapped blood cells in synergy with human mesenchymal stem cells create a pro-angiogenic healing response

Melanie A. Burkhardt, Isabel Gerber, Cameron Moshfegh, Miriam S. Lucas, Jasmin Waser, Maximilian Y. Emmert, Simon P. Hoerstrup, Falko Schlottig, Viola Vogel

Supporting Figures

Figure S1

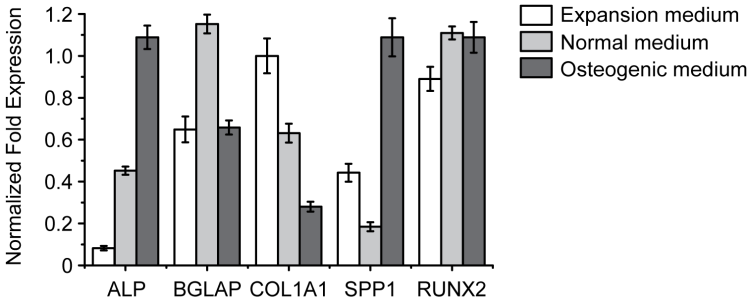

Figure S1: Osteogenic gene expression of hMSCs cultivated for 7 days under expansion, normal or osteogenic medium conditions on tissue culture plastic. Gene expression was normalized to average expression of *RPL13A* and *TBP* reference genes and represented as mean normalized fold expression ± standard error of the mean of technical triplicates of duplicate samples. *ALP* and *SPP1* show an upregulation in osteogenic medium compared to expansion or normal medium. *COL1A1* is downregulated under osteogenic compared to normal medium conditions.

Figure S2

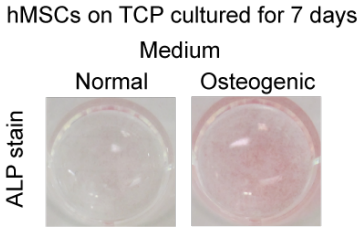

Figure S2: Osteogenic medium increases ALP protein activity compared to normal medium. hMSCs were cultured for 7 days in 24-well plates in normal or osteogenic medium and subsequently stained for ALP activity (red). Shown are representative macroscopic images of hMSC cultures in wells with weak or no ALP staining for normal medium and with positive ALP staining (red) for osteogenic medium. Here, the ALP activity represents the upregulation of *ALP* observed in RT-PCR.

Figure S3

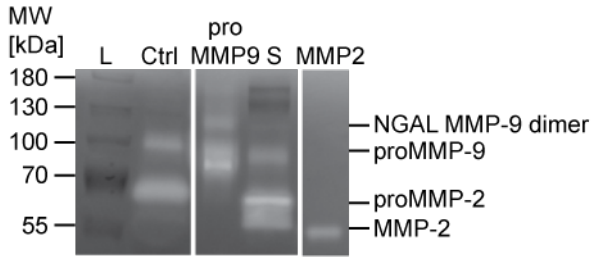

Figure S3: Gelatin zymography gel representing control bands for proMMP-9 and MMP-2. Gelatin zymography was performed with molecular weight ladder (L), control supernatant (Ctrl), an actual sample (S) in combination with native human proMMP-9 (proMMP9) and recombinant human MMP-2 (MMP2) to verify band specificities. Native and recombinant MMPs were loaded at 0.5 ng/lane.

Figure S4

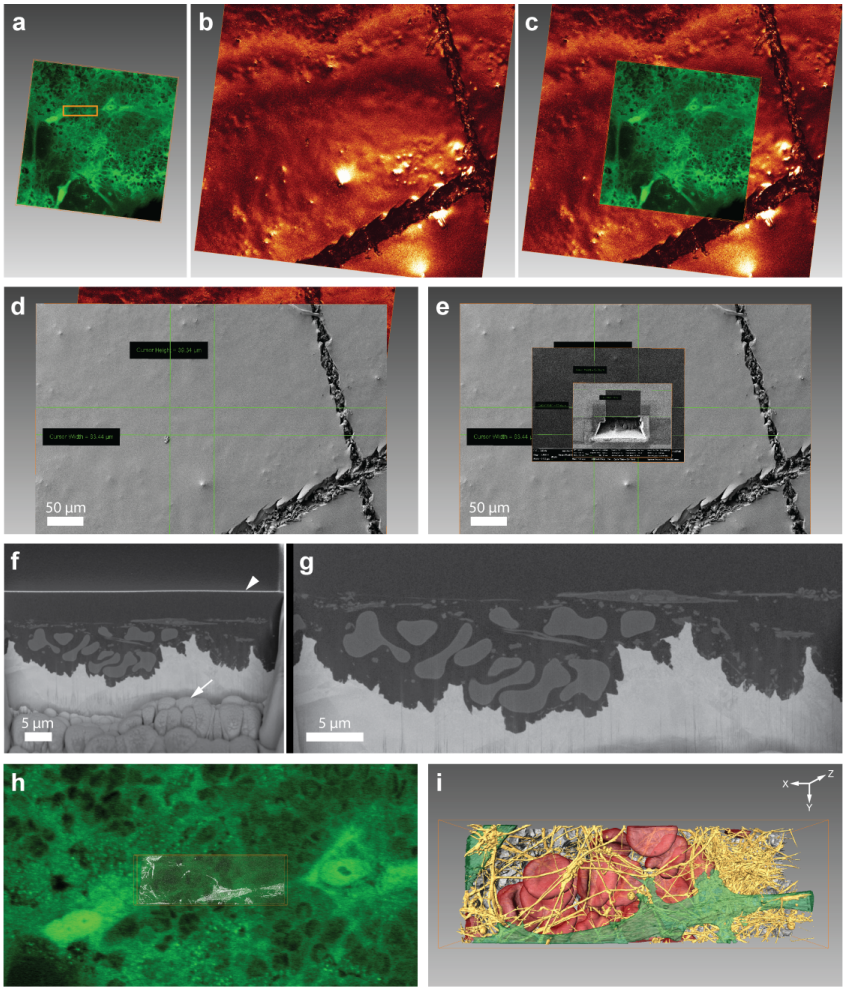

Figure S4: Correlative light and electron microscopy to allocate region of interest (ROI) for FIB-SEM. hMSCs were cultured for 7 days on blood-exposed alkali-treated Ti surfaces in osteogenic medium. (a) CLSM maximum intensity projection of Nile blue staining in Epon-

embedded sample. Nile blue dye located to hMSC cell bodies and was excluded from RBCs (dark spots). (b) CLSM surface reflection of Epon-embedded sample with marks to help relocate the ROI. (c) Overlay of Nile blue fluorescence image and reflection image of Epon-embedded sample. (d) SEM image of ROI relocated by correlation of surface features. (e) Allocated ROI, deposited carbon layer on Epon (dark rectangle above the trench) and milled trench prepared for FIB-SEM stack acquisition. (f) SEM image of cut sample surface with Epon edge (white arrowhead) and redeposition of material in the trench (white arrow). (g) Single slice of the FIB-SEM volume. (h) Correlation of FIB-SEM volume with CLSM Nile blue fluorescence image. (i) Top view of 3D model of segmented FIB-SEM volume. Ti surface is represented in grey, RBCs in red, hMSCs in green and matrix structures in yellow. Total volume represented by orange bounding box measures (x, y, z) 48.9 x 16.2 x 18.4 μm^3.

Supporting Tables

Table S1

A hematogram with automated leukocyte differentiation, coagulation status and fibrinogen concentration, as well as concentration of Haemoglobin A1c (HbA1c) was performed for blood samples of all 3 blood donors to exclude non-diagnosed diabetic conditions. Table S1 presents measured parameter values per donor, as well as mean values and standard deviations (SD) for all 3 donors. The blood donors used in this study were all classified as healthy, as all analyzed parameters were within normal range.

Parameter	**Unit**	**Normal range**	**Donor values**			**Mean**	**SD**
Donor			1	2	3		
Gender	Male (m)/ Female (f)		m	f	m		
Haemoglobin	g/l	117-170	160	130	155	148	16
Haematocrit	l/l	0.35-0.5	0.49	0.39	0.47	0.45	0.05
Erythrocytes	10^{12}/l	3.9-5.7	5.58	4.37	5.07	5.01	0.61
Platelets	10^9/l	143-400	335	312	187	278	80
Leukocytes	10^9/l	3.0-9.6	7.39	5.92	4.38	5.90	1.51
Neutrophils	10^9/l	1.4-8.0	4.38	4.02	2.4	3.60	1.05
Monocytes	10^9/l	0.16-0.95	0.62	0.38	0.33	0.44	0.16
Eosinophils	10^9/l	0.0-0.7	0.17	0.05	0.18	0.13	0.07
Basophils	10^9/l	0.0-0.14	0.02	0.02	0.03	0.02	0.01
Lymphocytes	10^9/l	1.5-4.0	2.2	1.45	1.44	1.70	0.44
Quick	%	>70	108	94	77	93	16
INR	-	<1.2	1	1.1	1.2	1.1	0.1
aPTT	s	24-36	24	25	26	25	1
Fibrinogen	g/l	1.5-4.0	2.5	2	2	2.2	0.3
HbA1c	%	4.8-5.9	5.3	4.8	5.1	5.1	0.3

Table S2

RT-PCR Primers used to investigate changes in gene expression of osteogenic markers of hMSCs when cultured in T75cm^2 TCP flasks for 7 days with expansion, normal or osteogenic medium. Table S2 shows gene names, NCBI accession numbers of mRNA sequences used for Primer Sequence design with LaserGene Software, employed forward and reverse primer sequences and resulting amplicon sizes. RPL13A and TBP were verified as the most stable reference genes across the investigated medium conditions. The genes analyzed consisted of RPL13A, ribosomal protein 13A; TBP, tata box binding protein; ALP, alkaline phosphatase; BGLAP, bone γ-carboxyglutamate protein, also known as osteocalcin; COL1A1, collagen type I alpha 1; SPP1, secreted phosphoprotein 1, also known as osteopontin and RUNX2, runt-related transcription factor 2.

Gene	mRNA ID (NCBI Accession No.)	Amplicon Size (bp)	Primer Sequence (5'-3')
RPL13A Forward	NM_012423.3	144	TGGGCCGGAAGGTGGTGGT
RPL13A Reverse			TGGGGGCCCGGAAGTGGTA
TBP Forward	NM_003194.4	146	ACCGCAGCTGCAAAATATTGTATCCA
TBP Reverse			CCGTGGTTCGTGGCTCTCTTATCC
ALP Forward	NM_000478.4	255	GCCGGAAATACATGTACCCCAAGAAT
ALP Reverse			GGTCCGTCACGTTGTTCCTGTTCA
BGLAP Forward	NM_199173.4	266	CAGGAGGGCAGCGAGGTAGTGAA
BGLAP Reverse			AAGGGCAAGGGGAAGAGGAAAGAA
COL1A1 Forward	NM_000088.3	240	GAAGGGCCACGACAAAGCAGAAAC
COL1A1 Reverse			CCCCACCCCACCCATCACATAG
SPP1 Forward	NM_001251830.1	278	GATGGCCGAGGTGATAGTGTGGTTTA
SPP1 Reverse			TTCCGCTTATATAATCTGGACTGCTTGTG
RUNX2 Forward	NM_001024630.3	124	TCCGGAATGCCTCTGCTGTTATGA
RUNX2 Reverse			TGGGGAGGATTTGTGAAGACGGTTAT

Supporting Movies

Movie S1

3D model of hMSCs interacting with blood clot on alkali-treated Ti in culture for 7 days. hMSCs were cultured for 7 days on blood-exposed alkali-treated Ti surfaces in osteogenic medium. FIB-SEM tomography was used to study the interaction of hMSCs and blood clot with rough Ti surface. The movie shows electron-density based grey value images while sliding through the imaged volume. A 3D model was received after segmentation of Ti surface (grey), RBCs (red), hMSCs (green) and matrix structures (yellow), see also Figure 9. Total volume represented by orange bounding box measures (x, y, z) 48.9 x 16.2 x 18.4 μm^3.

Supporting Experimental

Isolation and culture of human mesenchymal stem cells

For hMSC isolation, bone marrow was diluted, cells were pelleted, resuspended in expansion medium and hMSCs selected by cell adhesion to tissue culture plastic (TCP). The mesenchymal character of the cells was confirmed previously by their proliferative potential, the characteristic expression of surface antigens and the ability to selectively differentiate into osteogenic and chondrogenic lineages upon stimulation [1]. hMSCs were expanded in expansion medium (Dulbecco's Modified Eagle Medium (DMEM, low glucose, GlutaMAX™ Supplement, pyruvate; 21885, Life Technologies) supplemented with 10% FBS, MSC qualified (Invitrogen, 12662-029), 1% non-essential amino acids (Invitrogen, 11140-035), 1ng/ml human recombinant basic fibroblast growth factor (bFGF) (Invitrogen, 13256-029)) in Triple cell culture flasks (Nunc, culture area 500 cm^2) at a seeding density of 2500 cells/cm^2 at 37°C and 5% CO_2 in a humidified atmosphere. Near confluent hMSCs were frozen in FBS, MSC qualified, + 10% DMSO (Sigma, D2438). Before an experiment, passage 4 hMSCs were thawed in expansion medium, cultured in TCP flasks (T75 cm^2, TPP) and medium was exchanged every two to three days. Near-confluent hMSCs were released from TCP with Accutase (A6964, Sigma), centrifuged and resuspended in normal or osteogenic culture medium. Normal culture medium consisted of DMEM supplemented with 2% (v/v) Human serum (converted) type AB (C11-002, Lot No. C00209-2663, PAA, Pasching, Austria) without iso-agglutinins to eliminate blood type-dependent immune reactions. Osteogenic medium consisted of normal medium, freshly supplemented with (+)-sodium L-ascorbate (50 μg/ml, Sigma, A4034), β-glycerophosphate (1mM, Sigma, G9422) and dexamethasone (10 nM, Sigma, D4902). hMSCs were seeded onto bare or blood-exposed native and alkali-treated Ti surfaces in 24-well plates at a seeding density of 5000 cells/cm^2. Normal and osteogenic medium was collected and replaced by fresh medium after 3 days of culture and cells were further maintained in culture up to 7 days.

Scanning electron microscopy

Glutaraldehyde-fixed samples were further treated with osmium tetroxid (OsO_4, 0.5% (v/v) in ddH_2O diluted from 2% solution, Polysciences, 23310-10) for 1h and followed by dehydration in a graded series of ethanol (from 50% to 100% in ddH_2O). Subsequently, Ti surfaces were dried over the critical point of CO_2 (Tk: 31°C, Pk: 73.8 bar) using a critical-point dryer (Tousimis CDP 931, Rockville, USA). After sputter coating with 5 nm platinum (MED010, Balzers), images were recorded with a Zeiss Leo 1530 field-emission scanning electron microscope at 3 kV acceleration voltage detecting secondary electron signals.

Correlative light and electron microscopy (CLEM), FIB-SEM

Glutaraldehyde fixed samples were stored in PBS containing 0.05% sodium azide at 4°C. Samples were washed in 0.2M HEPES buffer pH 7.4 and postfixed for 40 min in 1.5% potassium hexacyanoferrate(II) and 1% OsO_4 solution, followed by 1h in 1% OsO_4 alone,

each in 0.2M HEPES pH 7.4, and en bloc staining with 1% aqueous uranyl acetate for 40 min with washing steps in-between with the same buffer [2,3]. For correlative definition of the ROI for FIB tomography, the samples were stained with saturated aqueous Nile blue sulphate for 1h [4]. Subsequently, the samples were washed with ddH_2O, dehydrated in a graded series of 30, 50, 70, 90 and 100% ethanol, followed by two water-free 100% ethanol incubation steps, 20 min each. Samples were impregnated first with 33% Epoxy resin (Epoxy embedding Kit, Fluka, 45359), then with 66% resin in water-free ethanol for 1h each, followed by two submersion steps for 1h in 100% resin. All impregnation steps were performed at room temperature in small plastic dishes filled with the resin mix. Completely resin-submerged samples were removed from the resin bath, excess resin at the sample edge and on the backside was removed by placing the samples upright on filter paper [3]. Subsequently, samples were transferred to an oven and heated to 60°C. Polymerization was allowed to take place for 1 up to 3 days at 60°C [3]. CLEM was performed as previously described [4]. Briefly, surface marks were created on thin layer embedded samples by thin scalpel cuts into the resin layer, to enable relocation of the region of interest (ROI). To choose a ROI for subsequent electron microscopy, Nile blue fluorescence and surface reflection signals showing the surface marks were acquired with a confocal laser scanning microscope (Zeiss, LSM Meta 510) using a 40x NA1.2 water-immersion objective. FIB-SEM volume data was recorded with a FIB-SEM CrossBeam workstation NVision 40 (Zeiss) at 2 kV acceleration voltage, using an energy selective backscattered (EsB) detector. Image registration and partial segmentation was done with Fiji [5]. Further segmentation, image correlation and creation of a 3D model was achieved with Amira 6.0 (FEI).

Immunofluorescence

Samples fixed for 20min with 4% PFA in PBS and rinsed three times with PBS were blocked by incubation of 2% (w/v) bovine serum albumin (BSA) in PBS for 1h. All antibodies were incubated at room temperature and diluted in 2% (w/v) BSA in PBS. For staining of Fn and fibrin, 1:100 diluted sheep anti-human fibronectin (AHP08, AbD Serotec) and mouse monoclonal anti-human fibrin(ogen) (F9902, Sigma) primary antibodies were incubated for 1h, followed by 3 rinses with PBS and one rinse with 2% (w/v) BSA in PBS for 5 min each. Incubation of secondary antibodies donkey anti-sheep IgG Alexa 488 (1:200, Molecular Probes) and goat anti-mouse IgG Alexa 555 (1:200, Molecular Probes) for 1h followed. For ALP and CD14 staining, duplicate samples per condition were rinsed once with PBS, fixed with 4% PFA in PBS for 1min, stained for ALP by incubating in 1mg/ml FastRed (Fast Red TR Salt hemi(zinc chloride) salt, F8764, Sigma) in 0.1 M Tris buffer (pH 8.5) with freshly added 10 mg/ml aqueous Naphthol AS-MX phosphate (N4875, Sigma, final concentration 200 μg/ml) for 30 min in the dark. Samples were washed with PBS and fixed in 4% PFA in PBS for 20 min. Samples were subsequently stained for CD14 monocyte/macrophage marker by incubation with biotinylated primary antibody (R&D, BAF183, 1:50), washed as described before, incubation with streptavidin-Alexa 488 (S11223, Invitrogen, 1:200). Subsequently all

stained Ti surfaces were washed with PBS, stained for cell nuclei with 5 µg/ml 4',6-diamidino-2-phenylindole, dilactate (DAPI, D3571, Invitrogen) in PBS for 10 min and rinsed twice with PBS and mounted on glass coverslips (24 x 50 mm^2, Carl Roth) using Prolong® Gold Antifade Mountant (P36934, Molecular Probes). Stained Ti surfaces analysed with an Olympus FV1000 laser scanning confocal microscope using a 60x 1.45 NA oil immersion objective and a 10x 0.4 NA air objective with a field of view of 0.718 mm^2 and a pixel size of 0.83 x 0.83 µm. SHG of collagen was imaged with a Leica TCS SP8 MP (multiphoton) microscope by detecting backward scattered signals of blood clots and hMSCs on mounted Ti discs.

Image processing, analysis and quantification

High-resolution confocal z-stacks stained for Fn, fibrin and cell nuclei were deconvolved with Huygens Essential Deconvolution software (SVI, Hilversum, Netherlands). A theoretical point spread function (PSF) was calculated based on the imaging and sample properties, using a time-dependent refractive index of the embedding medium ProLong Gold Antifade according to the manufacturer's characterization. The calculated PSF was used in the iterative deconvolution process with a signal to noise ratio of 5, and termination criteria of maximum 100 iterations and quality threshold 0.01. Accurate deconvolution was verified by comparison of deconvolved images with the original images. Confocal z-stack images of ECM (fibronectin and fibrin) components on Ti surfaces were quantified using Fiji [5]. Briefly, separate channels of z-stacks have been thresholded with a fixed lower limit of 300. Voxels above the threshold were taken as positive for the respective channel, quantified and converted to volume. Colocalized voxels of fibrin and fibronectin were quantified on logical AND combined masks of the two separate channels. Analyses included 7 stacks of different field of views per surface for duplicate Ti surfaces per condition repeated for 3 different blood donors, resulting in n=42 analysed stacks per condition. Number of leukocytes and hMSCs were analysed from low-magnification images of DAPI stained nuclei. Masks of cell nuclei were created after background subtraction, applying local threshold (Bernsen method) and analysis of particles with size of 15-4000 pixels and circularity of 0.2-1.0. Created masks were applied to original images to get measures of total nuclei number as well as size and grey level intensity for single nuclei. For images from blood and hMSC conditions, measured area, mean grey and maximum intensity values of all nuclei were used as input parameters for a k-Means clustering algorithm applied in MATLAB R2014b (The MathWorks, Inc., Natick, Massachusetts, USA) resulting in separation of hMSC and leukocyte nuclei into 2 clusters. hMSC and leukocyte quantification represents 6-12 images (area = 0.718 mm^2) for 2 Ti surfaces per condition repeated for 3 blood donors. Number of CD14-positive cells were analysed from low intensity images after applying a fixed threshold of 300 and subsequent analysis of particles with size of 60-Infinity. ALP positive area fraction was quantified on low magnification images, defined as the area fraction above a fixed threshold of 300. ALP and CD14 quantifications represent 12 images for duplicate Ti surfaces per condition repeated for

2 blood donors.

ELISAs and generic MMP assays

Pooled supernatants were analysed with Elisa assays for CICP pro-collagen type I (Quidel, MicroVue CICP EIA, 8003), which is cleaved off before collagen triple helix assembly; VEGF (Invitrogen, VEGF Human Antibody Pair, No. CHG0113, with Antibody Pair Buffer Kit (CNB0011)), Osteopontin (R&D Systems, Human Osteopontin Quantikine Elisa Kit, DOST00), BMP-2 (PeproTech, Human BMP-2 Standard TMB Elisa Development Kit) and D-Dimer (Thermo Scientific, D-Dimer Elisa Kit, Human, EHDDIMER). Active matrix metalloproteinases (MMPs) and total MMPs, by activation of proMMPs for 2h before the assay, were quantified using a fluorometric MMP generic assay (Anaspec, SensoLyte 520 generic MMP assay kit, No. 71158). All assays were performed according to the manufacturer's instructions measuring supernatant samples in duplicates.

Zymography

Gelatine and casein zymography were performed with pooled supernatants thawed on ice. 20 μl of each supernatant was mixed with 5x sample buffer and loaded to 10% Tris-Glycine gels with 0.1% gelatin (EC6175BOX, Invitrogen) or 12% Tris-Glycine gels with 0.05% beta-casein (EC6405BOX). As protein size makers PageRuler^{TM} Plus Prestained Protein Ladder (10-250 kDa, 26619, Invitrogen) and PageRuler^{TM} Prestained Protein Ladder (10-180 kDa, 26616, Invitrogen) were included. Gels were run in Novex Tris-Glycine SDS running buffer (LC2675, Invitrogen) on a XCell Surelock Mini-Cell system (EI0001, Invitrogen) at a constant voltage of 125 V for 90 min. Subsequently gels were gently shaked in renaturing buffer (LC2670, Invitrogen) for 30 min, followed by incubation overnight (gelatin gels) or 48h (casein gels) at 37°C in development buffer (LC2671, Invitrogen). Gels were stained with Coomassie R-250 solution (B3194, TCI America) and imaged with a MultiImage™ Light Cabinet (Alpha Innotech Corporation). Intensity of bands was analysed with Fiji [5] and band intensity was normalized to a control sample run on all gels. Protein concentrations of samples were measured at λ = 280 nm with a UV-Vis spectrophotometer (NanoDrop 2000, Thermo Scientific).

Real-Time PCR

hMSCs were cultured in T75 cm^2 flasks (TPP) for 7 days in expansion (FBS medium), normal or osteogenic medium. RNA isolation from the cells was performed according to the protocol from Qiagen, with RNeasy Mini Kit using a QiaShredder and including DNA digestion. RNA purity was verified by spectroscopic analysis of absorbance ratio A_{260nm}/A_{280nm} (>2) and salt contaminations by absorbance ratio A_{260nm}/A_{230nm} (<1.8) with a UV-Vis spectrophotometer (NanoDrop 2000, Thermo Scientific). RNA was reverse-transcribed into cDNA using iScript Advanced cDNA Synthesis Kit (BioRad, 170-8842). cDNA, primers and SsoAdvanced™ Universal SYBR® Green Supermix (172-5272, Bio-Rad) were mixed and genes amplified on a CFX Connect™ Real-Time PCR System (Bio-Rad, 185-5200). Primers were designed with

the Lasergene Software PrimerSelect (DNASTAR, Madison, WI) and synthesized by Microsynth (Balgach, Switzerland) (for Primer sequences see Supplementary Table S2).

Supporting References

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