Supporting Information

Emans et al. 10.1073/pnas.0907774107

SI Text

Methods. ***Preparation of liposomes.*** Multi-walled liposomes composed of dioleyl-phosphatidylcholine (DOPC), cholesterol (C), cardiolipin (CL), and triolein (T) were prepared as described elsewhere with some minor modifications (1, 2). Briefly, 8.9 μM DOPC, 8 μM C, 1.5 μM CL, and 0.1 μM T, all purchased from Sigma Chemicals, were dissolved in 0.5 mL chloroform and then mixed with equal volume of anhydrous ethyl ether in a scintillation vial. 1 mL of 0.15 M aqueous sucrose solution containing ~200 ng of TGFβ1 (R&D Sciences) and 0.4 M Suramin was then added to the organic phase over 5 s under an argon atmosphere with constant agitation. The contents of the scintillation vial were then gently vortexed for 5–10 min to create a milky-white water/lipid emulsion. The emulsion was then drawn through a 25-gauge needle using a 5 mL syringe few times to size the liposomes and then rapidly introduced into 2.5 mL of 0.2 M sucrose solution placed in a scintillation vial. The contents of the scintillation vial were then transferred to a 250 mL Erlenmeyer flask and the organic phase was evaporated under constant agitation by using repeated cycles of vacuum followed by argon flushing until the solution became clear. The liposomes were then pelletized by centrifugation after dilution with 1X PBS (500 g for 5 min) and then re-suspended in 3 mL PBS. The final liposome preparation contained 60 ng/mL of TGFβ1, 0.4 M Suramin, and had a mean diameter of ~50 nm. Two Hundred Fifty μL of this solution was mixed with 1 mL of Hyaluronic Gel (HA Gel) (Sepragel-ENT). As a control HA gelHa Gel without TGFβ1/Suramin was used.

Specifications of ultrapure agarose. Ultrapure low-melting agarose was purchased from Invitrogen (Invitrogen, Cat no: 10975035, Lot No: MO91807) and used as received. The agarose was chemically unmodified, with granule size ranging from (250–380 μm in diameter) and had a number average molecular weight of 120 KDa. The gelling temperature was between 34.5–37.5 °C, with a gel-strength of ~500 g/cm^2. The sulfate content in the gel was ≤0.11% and the gel was free of DNase and RNase activity.

Preparation of platelet-rich plasma (PRP) and agarose-PRP gels. PRP was prepared as described elsewhere (3). In brief, 10 mL of autologous blood was drawn from each rabbit was prevented from coagulation by 1 mL citrate-phosphate-dextrose-adenine (BD Sciences VacutrainerTM, citrate tube). The blood was centrifuged at $150 \times g$ for 20 min. The blood was thus separated into its three basic components: red blood cells, PRP (buffy coat), and platelet-poor plasma. The buffy coat and 0.5 ml of the upper pellet were collected and centrifuged at $500 \times g$ for 20 min. After centrifugation, all but 0.5 mL of the supernatant were removed and the remaining supernatant was used to resuspend the pellet that together formed the PRP. PRP-supplemented agarose was prepared as follows: Agarose gel (4-wt percentage) was prepared by dissolving 4 g of low-melting agarose (Invitrogen) in 100 mL of 0.9% NaCl and steam sterilized. Consequently, the agarose solution was warmed to 45 °C in a water bath to liquefy it and after cooling until 37 °C 50 μL of 4% low-melting agarose (30 °C, Invitrogen) 550 μL of PRP was mixed with 550 μL 4% Agarose.

Screening study for harvesting IVB cartilage. The first five rabbits of this study were used to determine the onset of cartilage formation in the IVB after injection of HA-Liposome (TGF-β1/Suramin) -Gel or Agarose-Gel. Identically to previous studies, periosteum derived neo-tissue formation could easily be monitored by inspection and palpation; during the first 7 d no neo-tissue formation was detected in both groups by both inspection and palpation, after 14 d neo-tissue formation was observed by inspection/palpation. At this time, hardly any IVB derived neo-tissue formation was detected by palpation/inspection after Agarose gel injection. One wklater (a total follow-up of 21 d) neo-tissue formation after Agarose gel injection was observed by inspection/palpation (Fig 2*A*). Using this information, we determined to harvest EAC 14 d after HA-TGF-β1/Suramin -Gel injection and 21 d after Agarose-Gel injection.

Staining Protocols. After decalcification in EDTA, the condyles and upper tibia were dehydrated in a series of increasing concentrations of ethanol solutions and embedded in glycidylmethacrylate (GMA) (Technovit 7100). Five micrometer sections were cut and the GMA sections were stained with thionine or with Alizarin red and counterstained with hematoxylin to reveal calcified tissue/deposits. The O'Driscoll score (max score of 24) for cartilage repair was used to evaluate the quality of cartilage repair (4). The sections were scored by two blinded observers, who had no prior knowledge of the sample codes.

For immunohistochemical staining of Collagen Type II, sections were de-paraffinized and hydrated using standard protocols. Then the sections were pretreated with 1% Hyaluronidase (30 min, 37 °C) and 0.2% pronase (30 min, 37 °C) and a 1:50 dilution in PBS of the primary antibody, mouse-monoclonal anti Collagen Type II (II-II6B3, DSHB), solution was applied. After overnight incubation with the primary antibody at room temperature, sections were washed with PBS and incubated with the secondary antibody rabbit anti-mouse IgG-HRP (ITK diagnostics). Subsequently, sections were washed with PBS and incubated with the avidin-biotin complex conjugated with Horseradish Peroxidase. Diaminobenzidine was used as a substrate for peroxidase.

RNA Isolation and Quantitative Real Time PCR (RT-qPCR). Immediately after harvest, the tissue from the IVB was frozen in liquid nitrogen, pulverized, and the resulting powder collected in TriZol reagent. Similarly, for the in vitro hypoxia experiment, differentiated periosteal cells were collected in TriZol reagent under the same oxygen levels as they were allowed to differentiate. Total RNA was extracted, cDNA was synthesized, and RT-qPCR was performed in triplicate for Collagen Type I (COL1), II (COL2), and X (COLX), Aggrecan, VEGF, Hypoxia Inducible Factor (HIF)-1α, and Sox9 using previously published primer sequences (5) and normalized to 28S rRNA. The mRNA quantification was carried out by using an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems). Serially diluted standard curves were included to quantify the samples. PCR conditions were: 2 min at 50 °C, 10 min at 95 °C, 40 cycles for 15 s at 95 °C, and 1 min at 60 °C followed by a melt curve 15 s at 95 °C ramp to 60 °C for 1 min, followed by 20 s at 95 °C. Data were analyzed by using Sequence Detection Software version 1.7 (Applied Biosystems).

Western Analysis. Cells cultured under hypoxic or normoxic conditions were lysed using standard RIPA buffer and the proteins were isolated by homogenizing the cell lysate, followed by sonication and centrifugation ($13.000 \times g$, 4 °C) to remove insoluble material. Total protein concentration in the purified lysate was determined by using the BCA (Sigma–Aldrich). Ten μg of total protein was loaded per sample and separated by SDS-PAGE, and the protein bands were transferred to nitrocellulose membranes by electroblotting. Immunodetection of Collagen

type II and Tubulin was achieved by using primary antibodies towards type II collagen (goat polyclonal, Southern Biotechnology Associates, Inc.) and α-Tubulin (mouse monoclonal, Sigma–Aldrich), resp., followed by HRP-conjugated polyclonal rabbit-anti-goat and rabbit-anti-mouse secondary antibodies, resp. (Dako). Bound antibodies were visualized by enhanced chemiluminescence using hyper film (GE Healthcare).

Statistical Analysis. The Mann–Whitney U test with a Hochberg post hoc correction test (6) were used for statistical analysis of the histological O'Driscoll scores for cartilage repair that takes into account nine parameters resulting in a score of 0–24 points (4). The Wilcoxon signed-ranks test was used to calculate statistical significance of the fold-increase in mRNA that was observed under hypoxic conditions compared to normoxic conditions. The significance level was set at $P < 0.05$.

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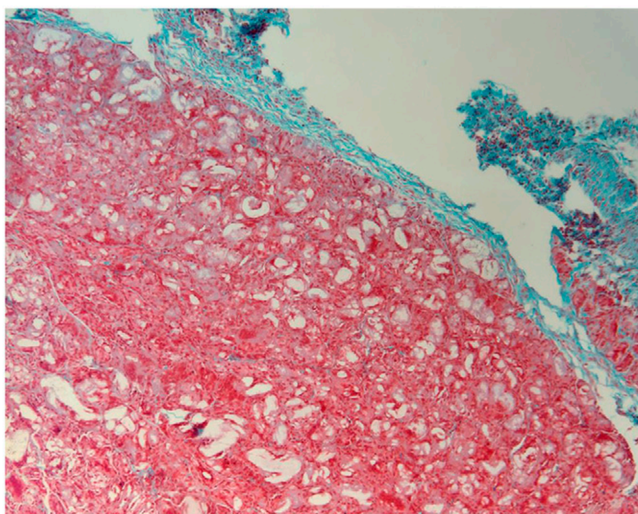

Fig. S1. Representative Safarin-O stained tissue section from IVB 21 d postinjection with Agarose-PRP. Note that no cartilage was formed in the IVB. Original magnification: X100.

Table S1. Outcomes in IVB sites as a function of biomaterial-gel composition.

Biomaterial in IVB	# IVB sites (*n*)	Cartilage (%) and Cross-sectional Area (mm^2)	
HA Gel	6	0/6 (0)	-
HA-TGF-β1/Suramin -Gel	20	13/20 (65)	5.2 ± 0.8
Agarose-Gel	19	12/19 (63)	6.4 ± 2.1
Agarose + PRP-Gel	6	0/6 (0)	-