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Research Paper

A Fibrin Glue Composition as Carrier for Nucleic Acid Vectors

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Purpose. Gene delivery from biomaterials has become an important tool in tissue engineering. The purpose of this study was to generate a gene vector-doted fibrin glue as a versatile injectable implant to be used in gene therapy supported tissue regeneration.
Methods. Copolymer-protected polyethylenimine(PEI)-DNA vectors (*COPROGs*), naked DNA and PEI-DNA were formulated with the fibrinogen component of the fibrin glue TISSUCOL® and lyophilized. Clotting parameters upon rehydration and thrombin addition were measured, vector release from fibrin clots was determined. Structural characterizations were carried out by electron microscopy. Reporter and growth factor gene delivery to primary keratinocytes and chondrocytes *in vitro* was examined. Finally,chondrocyte colonized clots were tested for their potency in cartilage regeneration in a osteochondral defect model.
Results. The optimized glue is based on the fibrinogen component of TISSUCOL®, a fibrin glue widely used in the clinics, co-lyophilized with copolymer-protected polyethylenimine(PEI)- DNA vectors (*COPROGs*). This material, when rehydrated, forms vector-containing clots *in situ* upon thrombin addition and is suitable to mediate growth factor gene delivery to primary keratinocytes and primary chondrocytes admixed before clotting. Unprotected PEI-DNA in the same setup was comparatively unsuitable for clot formation while naked DNA was ineffective in transfection. Naked DNA was released rapidly from fibrin clots (>70% within the first seven days) in contrast to *COPROGs* which remained tightly immobilized over extended periods of time (0.29% release per day). Electron microscopy of chondrocytecolonized *COPROG*-clots revealed avid endocytotic vector uptake. *In situ* BMP-2 gene transfection and subsequent expression in chondrocytes grown in *COPROG* clots resulted in the upregulation of alkaline phosphatase expression and increased extracellular matrix formation *in vitro*. *COPROG*-fibrinogen preparations with admixed autologous chondrocytes when clotted *in situ* in osteochondral defects in the patellar grooves of rabbit femura gave rise to luciferase reporter gene expression detectable for two weeks (n=3 animals per group). However, no significant improvement in cartilage formation in osteochondral defects filled with autologous chondrocytes in BMP-2-*COPROG* clots was achieved in comparison to controls (n=8 animals per group).
Conclusions. *COPROGs* co-lyophilized with fibrinogen are a simple basis for an injectable fibrin gluebased gene-activated matrix. The preparation can be used is complete analogy to fibrin glue preparations that are used in the clinics. However, further improvements in transgene expression levels and persistence are required to yield cartilage regeneration in the osteochondral defect model chosen in this study.

KEY WORDS: gene-activated matrix fibrinogen; gene therapy; gene transfer; tissue adhesive; tissue engineering.

INTRODUCTION

The repair of critical size tissue defects and contact loss at implant surfaces is a major challenge in reconstructive surgery. Recombinant growth factors have been used with great success to promote and improve bone healing, for example, and first products are in clinical studies (1,2). However, the applicability of growth factors is restricted by limited commercial availability and by their limited half-lives *in vivo*, implying high and repeated dose requirements (in other words: limited bioavailability), and the difficulty of locally confining growth factor action, implying the risk of undesired systemic side effects (3). For these reasons, the development of sustained and controlled

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release technologies involving implantable biomaterials and biodegradable surface coatings has been a major focus of biomedical research during the recent years (4–10). A viable alternative to applying growth factors is transfecting or transducing their respective cDNAs under the control of suitable promoters in target cells *ex vivo* or *in vivo*. The gene-activated matrix (GAM) concept (11–13) is particularly appealing in this context as it combines gene therapeutic approaches to tissue regeneration with a sustained release concept which in addition provides target cells with a matrix to grow on. GAMs are biomaterial scaffolds comprising gene vectors. Cells growing on or into the matrix will get transfected/transduced by the immobilized or released vector and will consequently express the transfected (growth factor)gene, resulting in local autocrine and paracrine stimulation of a desired differentiation process. A variety of matrices and several nonviral and viral gene vectors have yielded promising results in models of tissue regeneration implying the GAM concept (11–13).

We have previously shown that copolymer-protected gene vectors ("*COPROGs*") developed in our laboratory were, in contrast to naked DNA, suitable to mediate sustained reporter gene expression upon subcutaneous implantation in a rat model when collagen sponges served as the implantable carrier matrix (14). *COPROGs* are polycation-DNA nanoparticles shielded with an electrostatically bound layer of an anionic PEG-peptide derivative copolymer (15) (Supplementary Fig. 1). The layer provides steric stabilization, protection from opsonization (15,16) and allows freeze drying of the vector with little loss of activity.

Several research groups have demonstrated the utility of fibrin glues for *in situ* immobilizing nonviral (17–21) and viral gene vector suspensions, mostly adenovirus or carrier materials for adenovirus (22–29), at target sites and in some cases have provided evidence of therapeutic benefit of fibrin matrix mediated gene delivery in models of wound healing, induction of angiogenesis or bone healing. Hence we were interested in the feasibility of generating a lyophilized fibrinogen component comprising *COPROGs* which was to be readily applicable by the user in complete analogy to fibrin glue which is widely used as a tissue sealant in clinical surgery. Here we report on the development of storable fibrinogen formulations co-lyophilized with *COPROGs* which, when rehydrated, serve as injectable components of *in situ* forming vector-loaded fibrin clots upon mixing with thrombin solution. To derive suitable formulations fulfilling the requirement of simple applicability, we examined the influences of naked DNA, PEI polyplexes and *COPROGs*, respectively, with and without lyophilization on fibrin clot formation. We characterized the release profiles of naked DNA and *COPROGs* from fibrin clots. We evaluated the transfecting potency of selected preparations *in vitro* with primary keratinocytes and primary rabbit chondrocytes. Finally, we used fibrinogen *COPROGs* preparations with suspended autologous chondrocytes to fill osteochondral defects in the patellar grooves of rabbit femura and evaluated cartilage regeneration.

MATERIALS AND METHODS

Reagents

If not otherwise stated, reagents including branched 25 kD polyethylenimine (PEI) were purchased from Sigma-Aldrich (Deisenhofen, Germany).

The protective copolymer P6YE5C was synthesized as described (15) and purified using a Superdex HighLoad XK 26/70 column (Amersham Biosciences, Freiburg, Germany) at a flow rate of 4.4 ml/min with Tris-buffered saline pH 7.4 as eluent. Pooled and concentrated product fractions were extensively dialyzed against water using Slide-A-Lyzer cassettes (Pierce, MWCO 3000 Da).

Plasmids were expanded and purified by PlasmidFactory GmbH & Co. KG, Bielefeld, Germany. The plasmid p55pCMV-IVS-luc+ coding for the firefly luciferase as a reporter gene under the control of the CMV promoter was kindly provided by Andrew Baker, Bayer Corp., USA. The cDNA of human bone morphogenetic protein 2 (BMP-2) was kindly provided by Genetics Institute, Inc., Cambridge, MA, USA. The plasmid pB-BMP2 was derived from p55pCMV-IVS-luc+ by removing the luciferase-encoding sequence using the Qiagen (Hilden, Germany) gel extraction kit after Hind III/Fse I digestion and by inserting the BMP-2 coding sequence which was PCR-amplified in order to introduce Hind III/Fse I restriction sites. The plasmid pB-GFP encoding enhanced green fluorescent protein was obtained in a similar manner. The plasmid pWRG-1630-hEGF encodes the human epidermal growth factor under the control of the CMV promoter and has been described by Andree *et al*. (30).

The fibrinogen and thrombin components of TISSUCOL®, a commercially available fibrin glue in clinical use, were kindly provided as lyophilized preparations by Baxter AG (Vienna, Austria).

Fibrin Glue Components

The fibrinogen component of TISSUCOL®-Kit is a lyophilized human plasma fraction containing fibrinogen, plasma fibronectin, factor XIII, and plasminogen in the ratio 35–55 mg/1–4.5 mg/5–25 Units/0.01–0.04 mg. Except for clotting experiments (see below), it was dissolved in bovine aprotinin solution (3,000 KIU/ml) to result in a fibrinogen concentration of 66 mg/ml. The lyophilized thrombin component of the kit was dissolved in 40 mM calcium chloride to result in a stock solution of 500 IU thrombin/ml. When required (such as for ROTEG analysis; see below), the fibrinogen and thrombin stock solutions were diluted with fibrinogen and thrombin dilution buffers, respectively, which are part of the TISSUCOL®-Kit.

Gene Vectors

All components (plasmid DNA, PEI, protective copolymer) were kept as stock solutions in deionized water. The concentrations of the individual components and the ionic strengths of vector preparations were adjusted to match the requirements of the various assays which were carried out (clotting analysis, release assays, gene delivery).

PEI-DNA polyplexes were used only for clotting analysis by rotation thromboelastography (ROTEG; see below) and were prepared by mixing equal volumes of DNA dilutions and corresponding PEI dilutions in 0.9% sodium chloride to result in an N/P ratio of 8 (the N/P ratio specifies the molar ratio of nitrogen atoms in the PEI component over phosphate atoms in the DNA component of the vector): 66.7/133.3/266.7/400 μg/ml DNA were added to 69.5/139/278/417 μg/ml PEI and mixed by pipetting.

Copolymer-protected PEI-DNA vectors were prepared in deionized water by adding one volume equivalent of DNA solution to one volume equivalent of PEI solution followed by mixing with another volume equivalent containing three charge equivalents of the protective copolymer P6YE5C (Supplementary Fig. 1). The charge equivalents refer to the ratio of negative charges in the copolymer to the negative charges of DNA (15). For ROTEG analysis (see below), such vector preparations were lyophilized and redissolved in 0.9% sodium chloride to result in the appropriate vector concentrations. For lyophilized fibrinogen-vector preparations to be used in gene delivery and release experiments, vectors were prepared in the same manner from DNA solutions of 120 μg/ml, and of PEI solutions of 125.1 μg/ml to result in a final DNA concentration after mixing with protective copolymer of 40 μg/ml in deionized water.

Fibrinogen-Vector Lyophilisates and Vector-Loaded Fibrin Clots for Cell Culture Experiments

TISSUCOL fibrin glue for clinical use, according to the manufacturer's instructions, is formed *in situ* by ejecting equal volumes of a fibrinogen solution and a thrombin solution through a two-channel syringe. Vector- and cell-loaded clots (see below) for cell culture experiments were prepared to result in similar final fibrinogen concentrations as suggested for clinical use by the manufacturer. However, thrombin and fibrinogen solutions were not mixed at 1:1 volume ratios. Rather, the volume fraction for thrombin was reduced to a few microliters (with correspondingly increased concentration), while the volume fraction of the fibrinogen solution was increased (with correspondingly lower concentration).

In general, naked DNA and vector preparations (40 μg/ml final DNA concentration, see above) were mixed fibrinogen stock solutions at appropriate *v/v* ratios and were lyophilized. Before use, the lyophilized vector-fibrinogen preparations were rehydrated with volumes of deionized water corresponding to the initial volume of the fibrinogen component, thus reconstituting the original fibrinogen concentration (Supplementary Fig. 2). In most examples presented below, the following standard protocol was used: Ten milliliters of a 40 μg/ml vector suspension was mixed with 2 ml of freshly dissolved TISSUCOL fibrinogen component (corresponding to 66 mg/ml fibrinogen) and lyophilized. Before use, the lyophilized vector-fibrinogen preparation was rehydrated with 2 ml deionized water. This resulted in vector suspensions with 200 μg/ml DNA concentration, 66 mg/ml fibrinogen concentration and a DNA: fibrinogen ratio of 3.03 μg/mg. If required, this setup was adjusted to fit the requirements of individual experiments.

Dependence of Clotting Parameters on Vector Loading—ROTEG (Rotation Thromboelastography) Analysis

A detailed protocol is given in Supplementary Materials. ROTEG analysis (Pentapharm GmbH, Munich, Germany) is essentially a rheometric measurement which is used in clinical laboratories to evaluate blood clotting parameters. Among these are the clotting time (CT), the clot formation time (CFT) and the maximum clot firmness (MCF). Physically, the latter is defined by the final viscosity reached during a measurement. The measurement output derived by the instrument is given as an amplitude in millimeters (based on the measurement principle and on historic reasons). CT is the time from measurement start (here: addition of thrombin) until onset of clot formation. CFT is the time from the onset of clot formation until an "amplitude" of 20 mm is reached which, by definition, is regarded to be representative of a firm clot.

Release of Gene Vectors from a Fibrin Matrix

Plasmid DNA was labeled with 125-iodine by the Commerford method as modified by Terebesi *et al.* (31) and subsequently by Mykhaylyk *et al.* (32). The labeling mixture was separated using a PD-10 gel filtration column (Amersham Biosciences, Freiburg, Germany) with water as eluent. Iodinated DNA was analyzed by gel electrophoresis and autoradiography. The labeled plasmid was mixed with unlabeled plasmid at a ratio of 1:4 (*w/w*). *COPROGs* were prepared as described above using this DNA mix (final DNA concentration 40 μg/ml). Aliquots of 2.5 ml of the uncomplexed naked DNA mix (labeled/unlabeled 1:4 *w/w*) or of the *COPROG* suspension, respectively, corresponding to 100 μg DNA each were mixed with 1 ml each of fibrinogen solutions of 66, 50 and 33 mg/ml, resulting in DNA/fibrinogen ratios (μg/mg) of 1.5, 2, and 3, respectively. After lyophilization and rehydration with 1 ml deionized water each, the bottoms of individual wells of a 96-well plate were covered with 50 μl each of reconstituted vector-fibrinogen solutions in triplicates (corresponding to 5 μg DNA per well or clot). Clotting was initiated by addition of 5 μl each of thrombin solution (0.2 IU each). After clotting, each well received 200 μl cell culture medium (DMEM; Biochrom, Berlin, Germany). The plate was positioned in a cell culture incubator throughout the release assay (37°C, 5% CO_2, 99% relative humidity). To measure the release, the supernatants of the wells were collected separately and replaced with fresh medium (DMEM, Biochrom, Berlin, Germany) after 1 h, 1, 4, 7, 11, 14 and 20 days. The radioactivities in the supernatants were detected using a gamma counter (Wallac 1480 Wizard 3″, PerkinElmer Wallac, Freiburg, Germany). The cumulative release was calculated as percentage of the applied dose.

Cell Culture and Transfections

Cells were grown at 37°C in an atmosphere of 5% CO_2 at 99% relative humidity.

Primary Keratinocytes

Skin samples were kindly provided by the dermatology and reconstructive surgery departments of the Technical University hospital (Munich) with informed consent of the patients. Fat and dermis were carefully removed. The samples were cut in bands of 2–3 cm length and treated for a maximum of 1 min with 70% ethanol followed by washing with PBS. The samples were then treated over night at 4°C in 0.5% dispase solution (Roche Diagnostics GmbH, Mannheim, Germany). Subsequently, the epidermis was carefully separated from residual dermis and was treated for 30 min with trypsin-EDTA (Invitrogen GmbH, Karlsruhe, Germany) at 37°C. The whole mixture was then transferred into fetal calf serum (PAA Laboratories, Linz, Austria) to result in a FCS concentration of 10% (*v/v*) and was resuspended vigorously. The mixture was then passed through a 70 μm cell strainer (BD Biosciences Discovery

Labware, Bedford, MA, USA) to obtain keratinocytes as single cell suspension. The cells were cultured in keratinocyte medium containing supplement (KSFM, Invitrogen) and 0.3% gentamicin (trade name Refobacin, Merck, Darmstadt, Germany) for two passages at 37°C in a 5% CO_2 atmosphere.

In a first set of experiments, primary keratinocytes were trypsinized and distributed in microcentrifuge tubes in aliquots of 175,000 cells. The cells were pelleted by centrifugation and the supernatants were discarded. The cells were then resuspended in 50 µl each of a reconstituted vector-fibrinogen preparation containing either naked DNA or *COPROGs* coding for firefly luciferase at 100 µg DNA/ml and fibrinogen at 33 mg/ml (corresponding to 5 µg DNA and 1.65 mg fibrinogen per 50 µl). The suspensions were transferred as drops to the centers of individual wells of a 24-well plate. Clotting was initialized by addition of 5 µl thrombin (500 IU/ml). After clot formation (30 min), 2 ml medium (KSFM including supplement) were added. Medium changes were performed every 2 days. The fibrin clots were resorbed by keratinocytes within 1 week. On days 1, 4, and 6 after clot formation three clots each were mechanically homogenized in 500 µl Reporter Lysis Buffer (Promega) using a Mini Bead Beater™ (Biospec Products, Bartlesville, USA). On days 8 and 13, the wells where the clots had been planted and which in the meantime were covered with cells, were washed with PBS followed by the addition of lysis buffer (250 mM Tris/HCl pH 7.8, 0.1% Triton X-100). Fifty µl each of the respective cell extracts were used for the luciferase assay which was carried out as described previously (14).

In a second set of experiments, 20,000, 50,000, 100,000 and 200,000 freshly trypsinized and pelleted keratinocytes were suspended in 17 µl keratinocyte medium each, mixed with 25 µl previously lyophilized and freshly rehydrated *COPROG*-fibrinogen preparation comprising the plasmid coding for hEGF. The *COPROG*-fibrinogen lyophilisate was prepared as described above with the exception that a 100 mg/ml fibrinogen component of Tissucol was used here. Consequently, 25 µl rehydrated preparation (as well as the fibrin clot formed in the subsequent step) contained 5 µg of DNA and 2.5 mg fibrinogen component). Different from the first set of experiments, the cell-*COPROG*-fibrinogen suspension was added to 8 µl of thrombin solution (500 I.U./ml) provided in the centers of the wells of a 24-well plate. After clot formation (30 min), 2 ml medium (KSFM including supplement and gentamicin) were added. Complete medium changes were performed every 24 h during 14 days, and EGF concentrations were determined in the supernatants by ELISA (Supplementary Materials).

Primary Chondrocytes

Primary rabbit chondrocytes were isolated from articular cartilage obtained from knee joints of New Zealand white rabbits. The cartilage was cut in small pieces and digested with 0.25% trypsin for 30 min and with 50 mg collagenase A (0.21 U/mg; Roche, Penzberg, Germany) in 20 ml DMEM (including supplements, see below) over night. The resulting cell suspension was centrifuged at 170×*g*. The cell pellet was washed once with DMEM, was then resuspended in DMEM (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and the cells were seeded in 25 cm^2 flasks and grown for 1 week without splitting before use in gene delivery assays.

After trypsinization, the cells were distributed in microcentrifuge tubes in aliquots of 5×10^5, 1×10^6, and 2×10^6 cells per tube, pelleted by centrifugation and resuspended in 340 µl autologous serum each which was collected just before and stored on ice until use. Lyophilized *COPROG*-fibrinogen formulations were prepared according to the general protocol described above (200 µg/ml DNA and 66 mg/ml fibrinogen after rehydration). The cell suspensions in autologous serum were mixed with 500 µl each reconstituted *COPROG*-fibrinogen preparation (corresponding to 100 µg DNA and 33 mg fibrinogen). Forty-two microliters of the resulting suspension were pipetted as drops directly into drops of 8 µl thrombin solution (500 IU/ml) provided in individual wells of 24-well plates. This resulted in 50 µl clots containing 5 µg DNA, 1.65 mg fibrinogen, 4 IU thrombin each, and 25,000, 50,000, and 100,000 cells, respectively. After clot formation (30 min), 1 ml medium (DMEM/10% FCS) were added. Medium changes were performed every 2 or 3 days. Luciferase assays were carried out in triplicates on days 1, 3, 7, 10, 14, and 21 after clot formation. Chondrocytes resorbed the clots more slowly than keratinocytes. On days 1, 3, 7 and 10 the assay was carried out with extracts derived from homogenized clots. On days 14 and 21, when the clots were already resorbed, the assay was carried out with lysates derived from the wells where the clots had been planted originally, similarly as described above for keratinocytes.

In another experiment, the expression kinetics of bone morphogenetic protein 2 (BMP-2) from chondrocyte-*COPROG* clots was evaluated. *COPROG*-fibrinogen lyophilisates were prepared with the BMP-2 expression plasmid in the same manner as described above for luciferase (3.03 µg DNA/mg fibrinogen). Clots were prepared with 50,000 cells per 50 µl final clot such as described above for the luciferase experiment (setup per single clot: 50,000 freshly trypsinized washed and pelleted cells resuspended in 17 µl autologous serum, mixed with 25 µl rehydrated *COPROG*-fibrinogen lyophilisate added to 8 µl thrombin, 500 IU/ml, in a 24 well plate. This results in clots comprising 5 µg DNA and 1.65 mg fibrinogen). Regular medium changes were performed every 2 or 3 days. In any case, 24 h before the sampling time points (days 1, 3, 6, 9, 14, 21, and 28 after clot formation), a complete medium change was performed. Medium samples as well as PBS-washed homogenized clots were shock-frozen in liquid nitrogen and stored at minus 80°C until evaluation by ELISA which was carried out according to the instructions of the manufacturer (human BMP-2 Quantikine, R&D Systems, Wiesbaden, Germany). For homogenization, clots were washed once with PBS, then covered with 500 µl PBS and shaken in the presence of zirconia beads using the Mini Bead Beater (Biospec Products, Bartlesville, USA). The samples were centrifuged and the BMP-2 ELISA was carried out with supernatants.

Photometric Determination of SEAP Expression

Alkaline phosphatase (ALPase) activity was measured using a biochemical assay from Greiner BioChemica (Flacht, Germany) based on conversion of p-nitrophenyl phosphate to p-nitrophenol, which was determined spectrophotometrically at 405 nm and 25°C according to the instructions of the manufacturer. Ten microliters of cell culture supernatant were used in a

1:50 dilution with the Greiner reagent kit. For each sample four measurements were performed and the mean was calculated. As a control for this assay Greiner Control I (Greiner BioChemica, Flacht, Germany) was used.

Alcian Blue and Toluidine Blue Staining of *COPROG*-Fibrin Clots Colonized with Primary Chondrocytes

After 3 weeks in culture, chondrocytes outgrown from plain fibrin clots or BMP-2-*COPROG* clots were rinsed twice with ice-cold phosphate-buffered-saline (PBS), fixed with methanol (2 min at −20°C), rinsed once with distilled water, and then stained overnight at room temperature with 0.1% alcian blue (Alcian Blue 8 GX; Sigma) in 0.1 N HCl. In addition, clots from an identical setups were embedded in tissue-TEC after 10 days in culture, histological sections were performed and toluidine blue stainings were carried out according to textbook protocols. Both dyes stain glycosamino glycans.

Electron Microscopy of *COPROG*-Fibrin Clots Colonized with Primary Chondrocytes

COPROG-fibrin clots were essentially prepared as described above with and without rabbit chondrocytes (setup per single clot: 50,000 freshly trypsinized washed and pelleted cells resuspended in 17 µl cell culture medium, mixed with 25 µl rehydrated *COPROG*-fibrinogen lyophilisate, incubated for 10 min, then added to 8 µl thrombin, 500 IU/ml, in a 24 well plate. This results in clots comprising 5 µg DNA and 1.65 mg fibrinogen. Cell-free clots were prepared in analogy with 17 µl of medium instead of cell suspension). In addition, plain fibrin clots without *COPROG* with embedded chondrocytes were prepared. For transmission electron microscopy, samples were fixed in 3% glutaraldehyde. Following the initial fixation step, the samples were post-fixed with 1% osmium tetroxide, dehydrated in series of alcohols, passed through propylene oxide and embedded in epoxy resin (Epon 812; Electron Microscopy Sciences, Hatfield, PA) according to routine procedures. Ultrathin sections were stained with uranyl acetate and lead citrate. Ultrathin sections were examined with an EM 10 CR transmission electron microscope (Zeiss, Jena, Germany).

Fluorescence Microscopy of *COPROG*-Fibrin Clots Colonized with Primary Chondrocytes

COPROG-fibrinogen lyophilisates were prepared with YOYO-1-labeled DNA and 6-TAMRA-SE (Invitrogen) labeled copolymer P6YE5C or with the PEI component FITC labeled and the other components unlabeled. The rehydrated preparations were mixed with chondrocytes at the same ratio as described above for electron microscopy. Aliquots of the mixtures were spread as smear on slides and observed under a fluorescence microscope (ZeissAxiovert). Other aliquots were clotted upon thrombin addition and observed at the time points indicated in Fig. 3.

Cell Viability and Proliferation in *COPROG*-Fibrin Clots. Determination of the Percentage of Transfected Cells

COPROG-fibrin clots (with the GFP reporter plasmid) comprising 50,000 chondrocytes per clot were prepared as described above for electron microscopy. Clots were removed from cell culture dishes, weighed and digested with collagenase as described above (Roche, Penzberg, Germany) for 3–4 h on days 1, 5, 8, 15, 22 after clot formation (n=6 for each time point). Cells were pelleted by centrifugation, resuspended in 1 ml FACS buffer (PBS/1% FCS) and counted. Cell viability was determined with trypan blue stain. The percentage of transfected cells was determined by FACS analysis.

Animal Model: Critical Size Osteochondral Defects and Implantation of Fibrin Clots

The procedures involving animal treatment and care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC council directive 86/609, OJ L 358, 1, Dec. 12, 1987; NIH guide for the care and use of laboratory animals, NIH publication no. 85–23, 1985; FELASA-guidelines). Female New Zealand White rabbits were obtained from Charles River (Sulzfeld, Germany) and kept under conventional housing conditions. Quarantine lasted 7 days. The animals were housed with appropriate bedding, provided with free access to drinking water and food. Rabbits were kept in V2A-cages under conditions of controlled temperature and light. By the time of treatment, the animals had reached a bodyweight between 3.5 and 4.0 kg.

The experiment started for every rabbit with a cartilage biopsy out of the right knee under general anesthesia, about 14 days prior setting an osteochondral defect and clot implantation in the left knee. After shaving and disinfection, the right knee joint was opened by a medial parapatellar arthrotomia under sterile conditions and the patella was laterally displaced. After that, cartilage pieces were peeled with a scalpel out of the patella groove and the joint was closed again. For postoperative analgesia the next 3 days 4 mg/kg carprofen was given once a day subcutaneously (s.c.) plus 0.025 mg/kg buprenorphine s.c. twice a day. A separate chondrocyte culture was established for every single animal. Sterile harvested cartilage was washed twice in PBS, cut in 1 mm^3 pieces, minced and treated on a shaker with 0.25% trypsin/EDTA for 30 min followed by digestion with 50 mg/20 ml DMEM complete collagenase A (0.21 U/mg; Roche, Penzberg, Germany) overnight. The resulting solutions were centrifuged at 900 rpm for 4 min. Cell pellets were resuspended in medium. The isolated chondrocytes were seeded in 25 cm^2 flasks, grown to a density of 80%, then seeded in 75 cm^2 flasks and splitted at a ratio of 1:3 every fifth day. Cells were cultured in Dulbecco's modified Eagles medium (Biochrom, Germany) containing 10% fetal bovine serum, 1% Pen/Strep and maintained at 37°C, 5% CO_2.

For clot implantation, the rabbits were anesthetized by i.m. injection of 0.25 mg/kg medetomidine and 17 mg/kg S-ketamine. After intubation, 30 mg/kg metamizole i.v. and 4 mg/kg carprofen s.c. were administered. Using sterile technique, a medial parapatellar arthrotomy was performed in the left knee and the patella was laterally displaced. A steel drill (3.6 mm in diameter) with a stop-device was used to create two corresponding osteochondral defects (3 mm deep) in the trochlear groove. The defects were cleaned and rinsed with sterile saline. Against bleeding, the base was sealed by an electrocauter. In parallel autologous chondrocyte cultures were trypsinized (0.25% trypsin-EDTA, 3 min, trypsinization stopped by addition

of DMEM complete), cells were washed with PBS and distributed in microcentrifuge tubes at 100,000 cells per tube (for use with luciferase *COPROG*-fibrinogen preparations) or 50,000 cells per tube (for use with BMP-2 *COPROG*-fibrinogen preparations). Cells were pelleted by centrifugation and were resuspended with 17 µl autologous serum each. Also in parallel, lyophilized *COPROG*-fibrinogen preparations comprising either the luciferase reporter plasmid or the plasmid coding for BMP-2 were rehydrated with water, resulting in a DNA concentration of 200 µg/ml and a fibrinogen concentration of 66 mg/ml. Just prior implantation into the osteochondral defects, 25 µl of the *COPROG*-fibrinogen suspension (corresponding to 5 µg DNA and 1.65 mg fibrinogen) were mixed with the 17 µl of autologous chondrocyte suspension in autologous serum. For *in situ* clot formation in the osteochondral defect, 8 µl thrombin solution (500 IU/ml) were inoculated into the defect, followed by immediate addition of the *COPROG*-fibrinogen-cell suspension. After clotting, the patella was repositioned within the trochlear groove. Postoperative analgesia consisted of 0.03 mg/kg buprenorphine on the surgery day and 4 mg/kg carprofen for 3 days.

In parallel to the animal experiments, *in vitro* clot cultures were established with surplus cells from every single animal with the same *COPROG*-fibrinogen preparations used for the animal experiments. This served as an *in vitro* control for the transfection competence of the *COPROG*-fibrinogen preparations.

Re-isolation of Implants and Determination of Transgene Expression

On days 4, 7, 14, 28 animals were sacrificed and implants from the luciferase group were recovered using a scalpel. After washing with PBS the samples were transferred to 2 ml screw cap tubes containing 500 µl Reporter Lysis Buffer (Promega) and 1 g of 2.5 mm zirconia beads (Biospec Products, Inc., Bartlesville, USA). Samples were homogenized using a Mini-BeadBeater (Biospec Products, Inc., Bartlesville, USA) and the luciferase assay was carried out as described above and elsewhere (14).

RNA Isolation, Reverse Transcription, Primer and Probes for TaqMan® PCR, and Quantitative RT-PCR. The regeneration tissue was extracted and then grinded under nitrogen liquid 12 weeks after clot implantation. RNA isolation of rabbit cartilage was performed using Qiazol and RNeasy Micro Kit according to the manufacturers protocol (Qiagen, Hilden, Germany). Isolated total RNA was reverse transcribed in 20-µl reactions using 2 µg of random hexamers and Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturers instructions. All primers and probes were obtained from Applied Biosystems (Darmstadt, Germany) gene expression assay by design service (BMP2: Hs00154192_m1 (Applied Biosystems); COL1A1: forward AACGGTGC TCCTGGTGAAG/ reverse GCGACCTGGAGGACCAT/ probe Fam-TCGTGATGGCAACCCT-NFQ; COL2A1: forward GGACGTTCAGGCGAAACTG/ reverse CAAAGGC GCACATGTCGAT/ probe Fam-CCCTGCTGGTCCTCC-NFQ; COL10A1, forward TGCCATAAAGAGTAAAGGTA TACCAGTGA/ reverse GGTCCAGAAGGACCTGGGT/ probe 1Fam-CCAGGAATACCTTGTTCTCC-NFQ; Sox9: forward CAAGACCCTCGGGAAGCT/ reverse CCGCCTCCT CCACGAA/ probe Fam-CTCGTTCAGCAGTCTCCA-NFQ; Cbfa1: forward TGGCCTTCCACTCTCAGTAAGAA/ reverse GGGATGAAATGCTTGGGAACTG/ probe Fam-CCAGG CAGGTGCTTCA-NFQ). Quantitative real time PCR was performed in 96-well optical plates in triplicates as described previously (33).

Preparation of Femoral Condyles and Histology

Rabbits were sacrificed after anesthesia with propofol by fast injection of 5 ml pentobarbital 4 and 12 weeks after implantation. The knee joints were harvested, assessed macroscopically and photographed. Femur condyles were fixed in 5% formaldehyde at 4°C in PBS for 48 h. For plastination sections, the condyles were dehydrated in increasing alcohol solutions and embedded in methyl-methacrylate (MMA). After hardening, 5 mm thick undecalcified sections were made using a Jung ultramicrotom. For histological evaluation May/Grünwald/Giemsa staining was performed. The other specimens were decalcified after fixation in 5% EDTA at room temperature and subsequently embedded in paraffin after dehydration. Serial sections were cut transversely, from the proximal to the distal aspect of the trochlear groove. HE and Alcian Blue staining were performed. Sections were evaluated using the ICRS score (34).

Immunohistochemistry

For detailed immunohistochemical analysis of hyaline cartilage components, paraffin sections were stained with monoclonal antibodies directed against collagen I and II, aggrecan and aggrecan link protein (Table I). For detailed description of immunohistological staining see reference (35). Briefly, all sections were deparaffinized, treated with 0.3% hydrogen

Table I. Antibodies Used, Sources and Enzyme Pretreatment

Antigen(s) recognized	Antibody	Dilution	Enzyme	Source
Aggrecan	12/21/1-C-6	1:5	Ch AC[a]	DSHB
Link protein	9/30/8A4	1:5	Ch AC[a]	DSHB
Collagen I	Col1	1:1500	Hyal/protease	Sigma
Collagen II	CIICI	1:6	Hyal/pronase	DSHB

All enzymes were purchased from Sigma Chemicals. Chondroitinase AC (37°C, 0.25 U/ml, 30 min); hyaluronidase (37°C, 3 U/ml in PBS pH 5, 60 min); protease XXIV (room temperature, 0.02 mg/ml in PBS pH 7.3, 60 min); pronase (room temperature, 2 mg/ml in PBS pH 7.3, 60 min)
[a] For aggrecan and link protein additional pretreatment with alkylating and reduction was necessary (see text for details)

peroxide in methanol for 30 min to block endogenous peroxidase activity. Enzyme pretreatment was necessary and differed for the different antigens (Table I). Non-specific binding of the primary antibodies was reduced by blocking with normal horse serum for 60 min. Control sections were incubated with normal mouse immunoglobulins (10 μg/ml) or were obtained by omitting the primary antibody and treating the sections with PBS alone. Antibody binding was detected with a Vectastain ABC 'Elite' avidin/biotin kit (Vector Labs, Burlingham, CA) and sections were counterstained with Mayer's hematoxylin. Staining intensity was graded semi quantitatively (no staining (−), weak staining (+) and strong staining (++) (36)).

Statistical Analysis

Results were evaluated using an unpaired *t*-test (Figs. 9 and 10).

RESULTS

Influence of Gene Vector Formulation on the Clotting Parameters of Fibrinogen

Naked DNA did not interfere with clot formation in the concentration range tested. Unprotected PEI-DNA did not significantly alter the clotting time CT (time span from thrombin addition until onset of clot formation; Fig. 3A Supplementary Materials), but had a major influence on maximum clot firmness (MCF; Fig. 3B, Supplementary Materials) and clot formation time (CFT; Fig. 3C, Supplementary Materials). In fact, above a ratio of 5 μg DNA/mg fibrinogen in PEI complex formulation, the MCF defined to represent a firm clot (amplitude of 20 mm, see "MATERIALS AND METHODS") was never reached when unprotected PEI-DNA complexes were incubated with fibrinogen (correspondingly, at these ratios the CFT for PEI-DNA was infinite). In practice, this means that above the 5 μg DNA/mg fibrinogen ratio no stable fibrin clots are formed with PEI-DNA complexes. Also *COPROGs* interfered with clot formation, however, concerning the most relevant parameters CFT and MCF to a much lesser degree than PEI-DNA complexes. Interestingly, *COPROGs* had a more pronounced impact on CT, meaning that the onset of clot formation was dose-dependently delayed, nevertheless firm clots formed at the DNA/fibrinogen ratios which we considered relevant for gene delivery applications (up to 20 μg DNA/mg fibrinogen; no firm clots formed at 30 μg/mg fibrinogen). As PEI-DNA interfered strongly with clot formation, the subsequent studies were carried out with naked DNA and *COPROGs* only. Co-lyophilization of naked DNA or *COPROGs* with the fibrinogen component of TISSUCOL, such as required for the preparations used in gene delivery, did not impair the clotting parameters (data not shown).

Release of Gene Vectors from Fibrin Clots

Gel electrophoresis and autoradiography showed that the labeled plasmid remained intact during the iodination procedure (data not shown). Clots of decreasing fibrinogen concentrations containing constant amounts of naked DNA or *COPROGs*, respectively, were prepared and covered with cell culture medium to mimic the conditions during gene delivery experiments. After 20 days, more than 80% of naked plasmid DNA was released, with an initial burst of 50% to 70% within the first 5 days, dependent on the density of the fibrin matrix (Fig. 1). The dilution of the fibrinogen component from 66 mg/ml to 50 mg/ml as well as to 33 mg/ml increased the total release of naked DNA by about 8% in both cases. In contrast, the release profile for *COPROGs* was approximately linear with only 0.29% release per day, resulting in less than 10% release within 20 days, independent of the three tested fibrinogen concentrations (Fig. 1; right). Similar as with naked DNA, there was a minor dependence of release on the fibrin concentration, i.e. slightly increasing release with decreasing fibrinogen content.

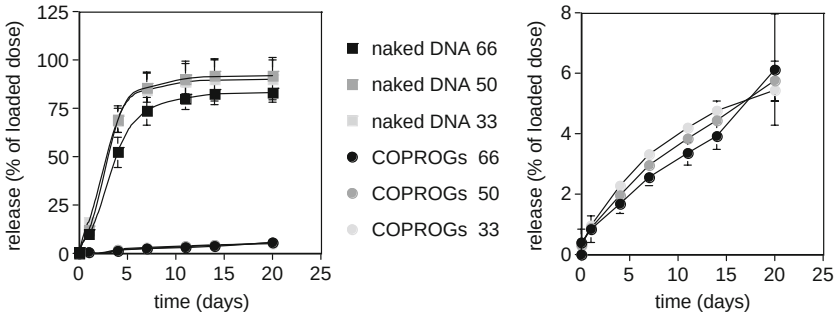

Fig. 1. Time-dependent release profiles of naked DNA and *COPROGs* from a fibrin matrix: 125-iodine was used to label the plasmid DNA. The radioactivity was measured by gamma counting. The fibrin-vector-matrix was produced with a vector dose of 100 μg DNA/ml fibrinogen solutions which were prepared at concentrations of 66, 50 and 33 mg/ml (symbolized in the figure as "naked DNA 66" etc.. Up to 70% of the naked DNA was released in an initial burst within the first 5 days. After 20 days, more than 80% of naked DNA was released from the fibrin clots. In contrast, *COPROGs* showed an approximately linear release profile with only 0.29% release per day, resulting in less than 10% release within 20 days, independent of the three tested fibrinogen concentrations. (n=3, ±S.D.). *Left*: comparison naked DNA and *COPROGs*. *Right*: Same data but Y-axis scaled to show the details for *COPROGs*.

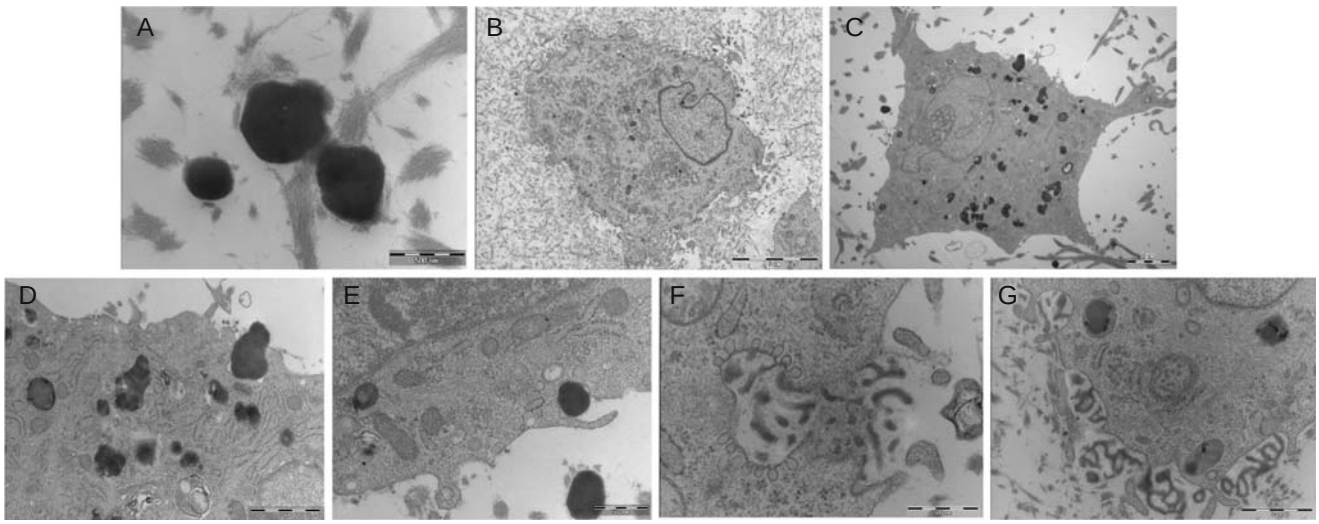

Fig. 2. Electron microscopy of *COPROG* clots. **A** *COPROG* clots without cells. *COPROGs* appear as electron dense potato shaped objects with diameters of 300–500 nm embedded in fibrin fibers. *Scale bar* 500 nm. **B** Chondrocytes embedded in plain fibrin clots. The cell in this micrograph is surrounded by dense fibrin network. *Scale bar* 5 μm. **C** Chondrocytes embedded in a *COPROG* clot. The fibrin network surrounding the cell in this micrograph is not as dense as in plain fibrin clots, suggesting that the presence of *COPROGs* in the preparation has an impact on the formation of the fibrin network, which is also the result of the ROTEG analysis (Supplementary Fig. 3). The cytoplasm is loaded with electron-dense objects, which we interpret as internalized *COPROGs*. *Scale bar* 2 μm. In higher magnification of the upper part of the same cell (**D**), *scale bar* 1 μm, it appears that not all of these objects reside in membrane-surrounded vesicles, suggestive of endosomal escape. The cell in the micrographs **C** and **D** is actively phagocytosing a *COPROG* particle. **E** Another cell from the same preparation apparently involved in macropinocytosis of a *COPROG* particle. *Scale bar* 500 nm. **F**, **G** In clots from the same preparation as in micrographs **C**–**E**, tubular structures can be observed which are likely *COPROGs* as well or associations of *COPROGs* with other components of the fibrin glue. The cell in micrograph **F** appears to embrace these tubular structures in an invagination comprising multiple caveolae. *Scale bar* 500 nm. **G** Another cell from the same preparation involved in phagocytosis and/or macropinocytosis. Such processes all over the plasma membrane were not observed in preparations of chondrocytes in plain fibrin clots without *COPROG* particles. *Scale bar* 1 μm.

Electron Microscopic and Fluorescence Microscopic Examination

Fibrin clots were prepared from plain fibrinogen and from *COPROG*-fibrinogen preparations without and with suspended chondrocytes, thin sections were derived from these clots and processed for electron microscopy (Fig. 2). The cell-free *COPROG*-clots display contrast-rich, potato-shaped objects which were absent in plain fibrin clots (not shown) representing *COPROGs* in various aggregate sizes ranging from 300 to 500 nm in diameter. *COPROG*-clots with suspended chondrocytes cultured for 1 day after clot formation until processing for microscopy display embedded chondrocytes having internalized multiple electron dense objects representing *COPROGs*. These objects appear to reside mostly in internal vesicles present in the cytoplasm, likely lysosomes (Fig. 2C). In some cases however, a limiting membrane enveloping the vector particles, can not be identified, suggesting endosomal release (Fig. 2D). Frequently, these objects are observed at invaginations and extensions of the plasma membrane, suggesting clathrin-independent uptake *via* phagocytosis and macropinocytosis (Fig. 2D–G; compare (37,38)). In some cell-containing *COPROG* clots, extended tubular rather than potato-shaped objects were observed in the direct vicinity of chondrocytes and during internalization (Fig. 2F, G). Compared with *COPROG*-free clots colonized with chondrocytes, apparently there is increased endocytotic activity with chondrocytes embedded in *COPROG* clots.

Fluorescence microscopy of chondrocyte-colonized clots prepared with *COPROGs* with differentially labeled components reveals early association of *COPROGs* with cells, probably right upon mixing with the rehydrated *COPROG*-fibrinogen preparation (Fig. 3). After 2 and more than 4 weeks, fluorescent spots are frequently associated with the cells (visible in large format overlays of brightfield and fluorescent pictures; Fig. 3f1). The YOYO (DNA) and TAMRA (protective copolymer) fluorescence are frequently found colocalized, but often green only fluorescence is found with cells, suggesting that the vector components dissociate, maybe upon cellular uptake.

Transfection of Primary Human Keratinocytes and Rabbit Articular Chondrocytes

The keratinocytes embedded in a fibrin-vector-matrix proliferated and degraded the fibrin clots irrespective of the vector formulation within 6 to 7 days. The more cells embedded in the clots, the faster was the degradation. After matrix preparation with *COPROGs*, transgene expression was observed on days 1 through 13 (which was the duration of the experiment) with a peak on day 1 and a sharp decline of expression at later time points (Fig. 4). In cells growing in the fibrin matrix loaded with naked plasmid DNA, no transgene expression was detectable except in one out of three samples on day 6 (Fig. 4A). A similar expression profile, with a rapid decline of expression was observed with keratinocytes growing

in clots comprising *COPROGs* coding for hEGF (Fig. 4B). Here, also the influence of cell density in the clot on transgene expression was examined. Highest expression levels were achieved with 100,000 keratinocytes initially embedded in the *COPROG* clot. In this case, hEGF production within the first 24 h was 840 pg per ml cell culture supernatant, and cumulative hEGF expression approached 1,750 pg hEGF per ml cell culture supernatant by day 14 (Fig. 4C).

Luciferase expression in rabbit articular chondrocytes growing in luciferase-*COPROG* clots persisted for at least 21 days (which was the duration of the experiment) with a peak on day 3 followed by a sharp decline until day 7 (Fig. 5A). There was no strong dependence of reporter gene expression on the cell density per clot, although on day 3, when peak levels of luciferase expression were observed, the lower cell densities (25,000 and 50,000 cells per clot) gave higher luciferase levels than 100,000 cells per clot. BMP-2 expression was persistent for at least 28 days with peak levels reached around day 6 (Fig. 5B; approximately 300 pg BMP-2 per clot and 200 pg per ml supernatant per 24 h). BMP-2 was detected in homogenized clots as well as in the medium samples taken, demonstrating that the growth factor readily

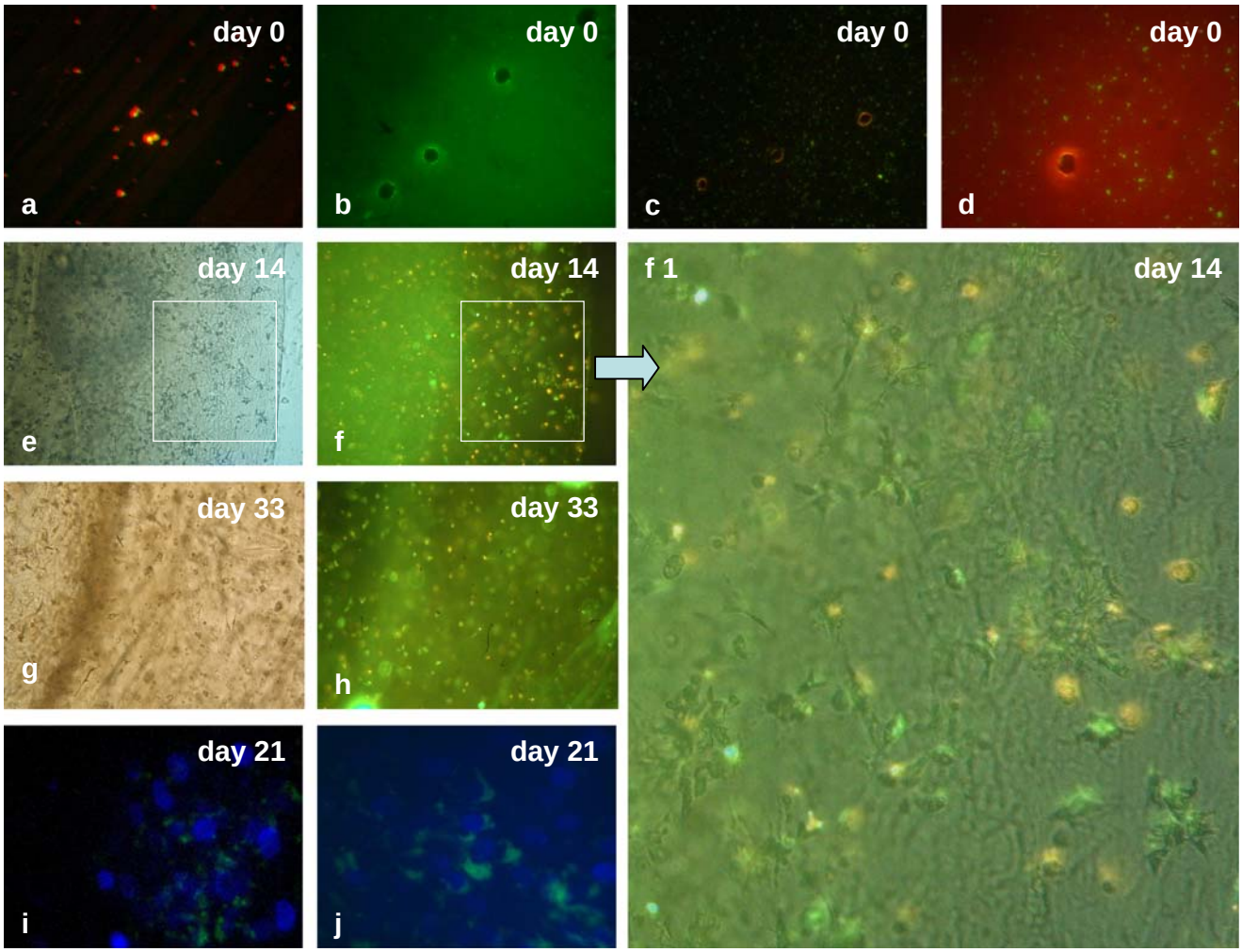

Fig. 3. Fluorescence microscopy. *COPROG*-fibrinogen lyophilisates were prepared with differentially fluorescence labelled components: DNA labeled with YOYO-1, copolymer P6YE5C labeled with TAMRA, PEI unlabeled (pictures **a**, **c**, **d**, **f**, **h**) or with DNA and P6YE5C unlabeled and PEI labeled with FITC (pictures **b**, **i**, **j**). After rehydration, smears were applied to glass slides followed by thrombin addition for clotting. *Bright field* (**e**, **g**) and fluorescence images were taken after different time periods, *red* and *green fluorescence* overlaid in **a** and **c**–**h**. **a** Smear performed from the plain preparation without cells. **b**–**j** The rehydrated preparations were mixed with primary rabbit chondrocytes before performing the smear. Picture **a** shows the vector distributed throughout the preparation, the protective copolymer always associated with the DNA component (*yellow fluorescence*). The vectors obviously become rapidly associated with cells (fluorescent corona surrounding the cells in pictures **b**–**d**. By day 14 and 33, respectively, YOYO and TAMRA staining is still detectable in the fibrin clot and in or associated with embedded cells (**e**–**h**). YOYO and TAMRA fluorescence are frequently colocalized, however, often green only fluorescence is associated with cells (picture **f1** which is an overlay and a magnetification of an area in pictures **e** and **f**). DAPI staining of preparations with FITC labeled PEI component suggest that vectors have been taken up by many cells 3 weeks after clot formation (**i**, **j**).

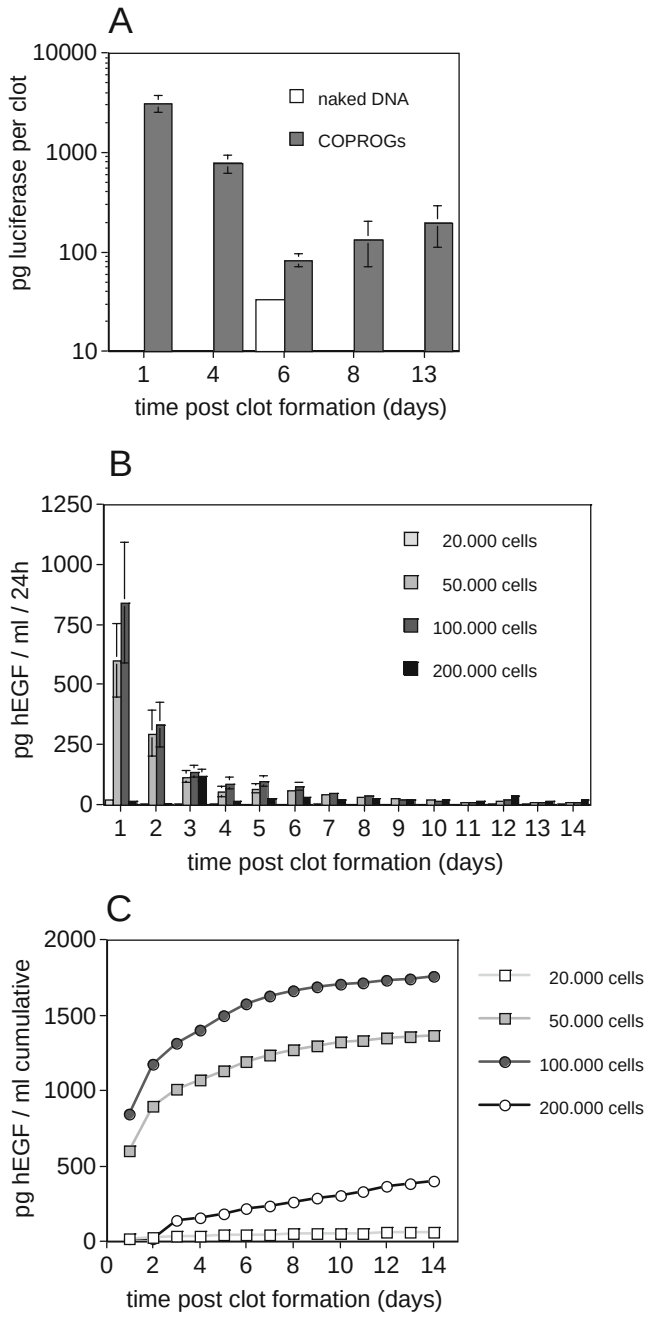

Fig. 4. Gene delivery to primary keratinocytes in vector-loaded fibrin clots. **A** After the second passage, human keratinocytes were embedded in a fibrin-vector-matrix with naked DNA and *COPROGs* carrying the firefly luciferase reporter gene. On days 1, 4, 6, 8 and 13 a luciferase assay was performed. *COPROGs* led to transgene expression persisting throughout the observation time with a peak on day 1. In contrast, naked DNA showed transgene expression only in one out of three samples on day 6. (n=3, ±S.D.). **B** Time course of hEGF expression by keratinocytes grown in hEGF-*COPROG* clots in dependence on cell density in the clot. Culture supernatants were replaced every 24 h and hEGF in the supernatant was determined by ELISA. There is peak expression within the first 24 h. Subsequently, expression declines in an exponential manner. An original density of 100,000 cell per clot yields highest expression levels as is best evident from cumulative expression in **C**.

diffused out of the clots. Background levels of BMP-2 expression from cells embedded in vector-free ("empty") clots were up to 35 pg per clot or ml supernatant per 24 h.

BMP-2 expression by chondrocytes growing in BMP-2-*COPROG*-clots had the expected physiological consequences in that alkaline phosphatase expression was up-regulated in good timely correlation with BMP-2 expression (Fig. 5B). Alkaline phosphatase levels in culture supernatants during the examination period were at least 1.5-fold above control levels (cells grown in GFP-*COPROG*-clots) by day 21 and three-fold above control levels by the time points of peak BMP-2 expression. When chondrocyte-colonized clots were cultured in medium supplemented with 50 µg/ml ascorbic acid, alcian blue staining of histological sections from BMP-2-*COPROG*-clots revealed cartilage-specific proteoglycan production which could not be detected in control clots (chondrocytes in plain fibrin clots and chondrocytes grown in luciferase-*COPROG*-clots) (Fig. 5C–E). However, increased extracellular matrix formation was absent irrespective of the transgene when chondrocyte-colonized clots were grown in the absence of supplement.

Cell Viability, Cell Growth and Percentage of Transfected Cells in Vector-Loaded Fibrin Clots

Viability, growth and percentage of transfected primary chondrocytes growing in GFP-*COPROG* clots was examined upon enzymatic digestion of clots at various time points after clot formation (Fig. 6). Cell viability was above 90% as determined by trypan blue staining and cell counting. The cell number per clot approximately doubled within the first week. Clot resorbtion and cell outgrowth into culture dishes becomes substantial after that. Due to this fact, the cell number per clot in what remains of the original clots declines such as do the weights of the clots (data not shown). At least until day 8, there was a steady increase in the percentage of transfected cells from ca. 1.3% on day 1 to more than 25% on day 8. Subsequently, there was a decline in the percentage of transfected cells, reaching ca. 17% by day 15 and 8% by day 22.

Animal Experiments

Luciferase Expression in Implants

Due to the ease and high sensitivity of the luciferase reporter assay, osteochondral defects of a first group of animals were filled with autologous chondrocytes embedded in fibrin clots comprising *COPROGs* coding for luciferase (Fig. 7). Implants were recovered on days 4, 7, 14 and 28 post implantation. Similarly as *in vitro*, there was a rapid decline of luciferase expression from around 39 pg luciferase per implant on day 4, to 13 pg on day 7, 1 pg by day 14 and undetectable levels by day 28.

Results with hBMP2/*COPROG* Implants

Histology

Paraffin and MMA sections were analysed after staining regarding the ICRS score (Fig. 8). The ICRS score includes

the quality of the surface, the matrix and the subchondral bone (34). In addition, the cell distribution, the cell viability and the cartilage mineralization are assessed. The hBMP2/*COPROG*-transfection did not increase the total ICRS score for cartilage remodelling after 4 and 12 weeks in comparison to controls (Fig. 9). There was no effect of hBMP2/*COPROG*-transfection regarding the surface, the matrix, the cell distribution and the subchondral bone. There was no negative

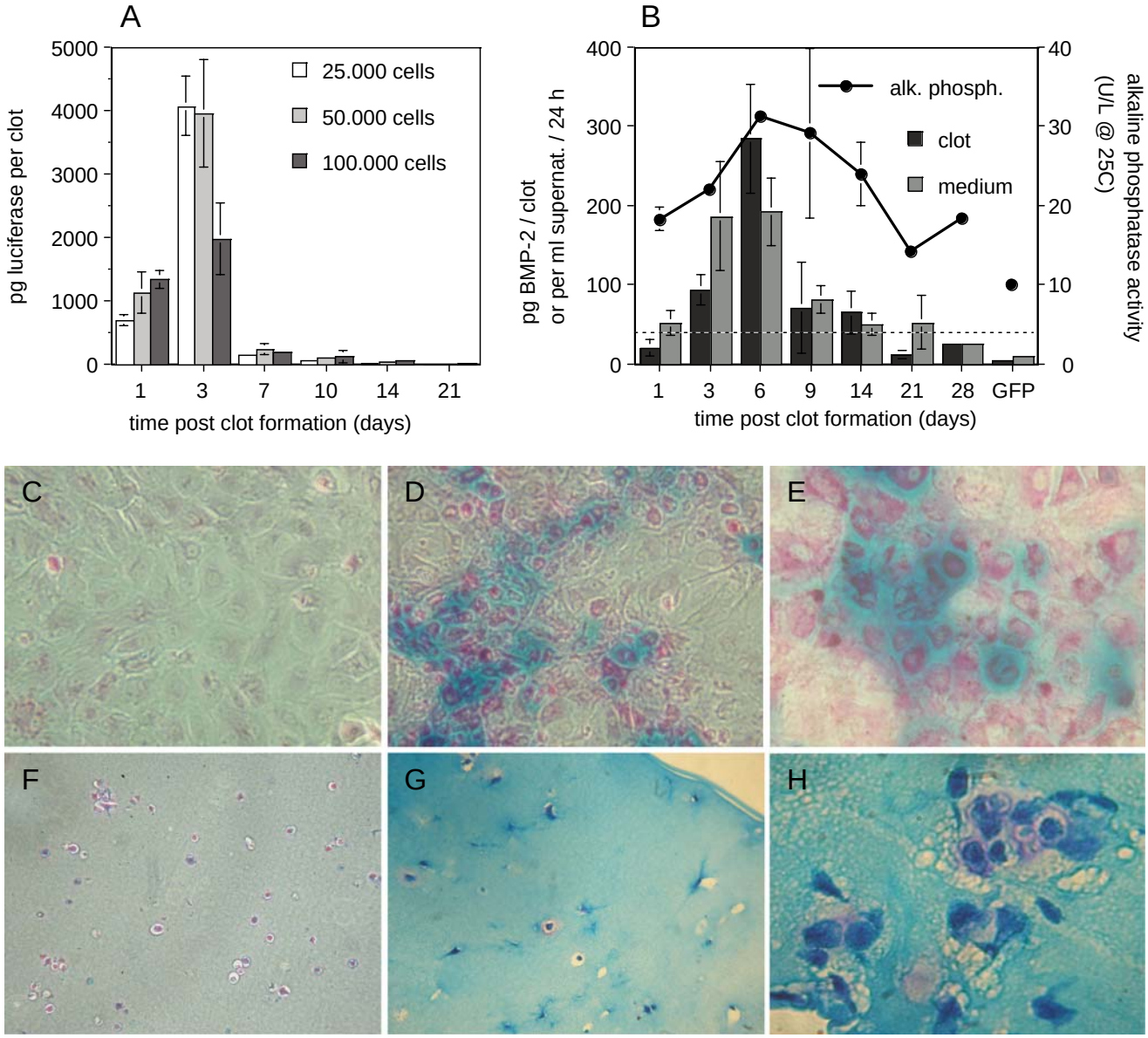

Fig. 5. Gene delivery to primary chondrocytes in vector-loaded fibrin clots. **A** Primary rabbit chondrocytes were embedded in a fibrin-vector-matrix with *COPROGs*, carrying the luciferase reporter gene, at increasing cell density per clot. The luciferase assay was performed on days 1, 3, 7, 10, 14 and 21. Peak expression was found on day 3. The cell density per clot did have some influence on the observed expression levels in the range tested, 50,000 cells per clot appeared to be the best choice. Mean value and standard deviations from triplicates. **B** Expression of the therapeutically relevant human BMP-2 gene by rabbit chondrocytes (50,000 per clot) embedded in a fibrin-vector-matrix with *COPROGs* carrying the BMP-2 gene. BMP-2 was detected by ELISA in the clots (*black bars*) as well as in the cell culture medium supernatants (*grey bars*). Peak expression levels were found on day 6 among the tested time points. The *vertical dotted line* shows maximum background BMP-2 expression found when cells were embedded in vector-free ("empty") clots. Alkaline phosphatase expression as a marker of BMP-2 function closely correlated with BMP-2 expression (*black line*, *black circles*, *right y-axis*) and was up to three-fold above background levels in control samples ("GFP", *bars to the furthest right*. Measurements taken from cells growing in GFP-*COPROG* clots). Mean value and standard deviations from triplicates. **C**–**E** Positive alcian blue staining as a marker of proteoglycan production was only found when cells were grown in BMP-2-*COPROG* clots **D**, **E**, and not in control clots **C**, but only when the clots were cultivated in supplemented media. The microscopic pictures show alcian blue-PAS stainings of rabbit chondrocytes that have outgrown from clots after 3 weeks in culture. **C** Cells grown in a plain fibrin clot without vector. **D**, **E** Cells grown in a BMP-2-*COPROG* clot. **F**–**H** Similar results were obtained with toluidine blue stainings of histological sections derived from chondrocyte-colonized fibrin clots after 10 days in culture. **H** Control clot without vector, ×10 magnification. **G**, **H** BMP-2-*COPROG* clot, ×10 and ×20 magnification, respectively.

effect of the *COPROG* method regarding cell viability and mineralization. Cells in the sections of every animal in the hBMP2/*COPROG* group were predominantly viable and there was no abnormal mineralization.

Immunohistology

Paraffin sections of regeneration tissue of six animals per group were analysed after 4 and 12 weeks after hBMP2/*COPROG* clot implantation. Collagen I, collagen II, aggrecan and aggrecan-link-protein were immunostained (Table I). Staining intensity was graded semi-quantitatively as described in the materials section. The results for all analysed proteins in the hBMP2/*COPROG* group were comparable to controls and therefore hBMP2/*COPROG*-transfection was without any effect on these proteins (data not shown).

RT-PCR Analysis

The expression of different genes in regeneration tissue of osteochondral defects in the rabbit model was analysed by quantitative RT-PCR after 12 weeks (Fig. 10). This analysis showed a slight but not significant increase in hBMP2 expression for the hBMP2/*COPROG* transfected group in comparison to controls. However, the slight increase in hBMP2 expression was without any effect for the expression of other BMP2-dependent proteins. RNA levels for collagen I, II and X were comparable to controls. Expression of Sox9, a typical transcription factor of the Smad pathway, was not increased showing missing additional activation of the BMP2 signal cascade. Finally, the transcription factor Cbfa1, a parameter for hypertrophic chondrocytes, was not altered. Therefore, the quantitative RT-PCR results are in accordance to the histology and immunohistology analysis showing a missing *in-vivo* long-term effect of hBMP2/*COPROG*-transfection.

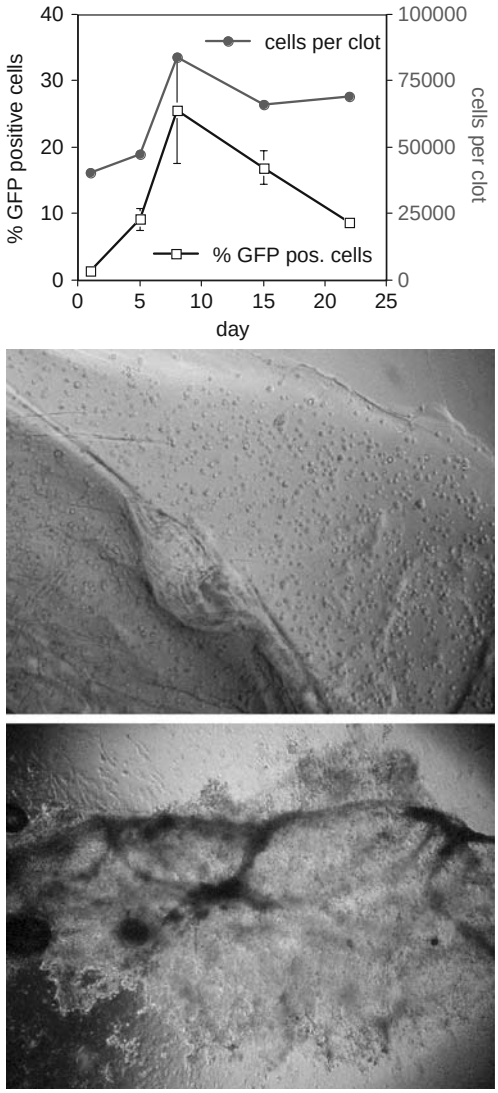

Fig. 6. Cell growth and percentage of transfected cells in vector-loaded fibrin clots. Primary rabbit chondrocytes were grown in GFP-*COPROG* clots. The cell number per clot doubled within the first week and then declined as clot resorbtion and cell outgrowth from the clot proceeded (*filled circles in the graph to the left*, *right y-axis*). In parallel, the percentage of transfected cells increased to above 25% until day 8 and then declined. Clot resorbtion and cell outgrowth can be seen in microscopic examination. *Upper right*: Microscopic aspect of a clot after 2 days in culture (×10 original magnification). The speckled structures are the cells suspended in the clot. *Lower right*: Clot after 4 weeks in culture. Only remnants of the clot can be observed and cells gown out from the clot can be observed in the periphery.

DISCUSSION

The "gene-activated matrix" approach to tissue engineering exploits the *in situ* transfection of cells in an implantable biomaterial matrix to result in the *in situ* generation of growth factors in order to direct tissue (re)generation (3,39). Fibrin glue is an excellent tissue sealant in clinical application and has been used in *ex vivo* cartilage tissue engineering (40,41). Several research teams have reported highly promising pre-clinical results in tissue (re)generation *in vivo* upon localized growth factor gene delivery mediated by nonviral or viral gene vectors immobilized at a target site with fibrin glues (19,20,24–26). These model applications cover a diverse range of medical indications, including neoangiogenesis in ischemic myocardium or in full thickness dermal wounds, epithelialization or skin engraftment in cutaneous wounds and bone healing in dental implant defects. Another successful approach, used for example in articular cartilage repair, is *ex vivo* transduction/transfection of autologous chondrocytes or mesenchymal cells with an appropriate growth factor gene (e.g. BMP-2, IGF-1) and fixation of these cells in cartilage lesions using fibrin glue (42,43). These preclinical studies clearly demonstrate the feasibility and utility of gene therapy in supporting tissue repair. A transition from bench to bedside however will require simple, safe and efficient ways of administering such therapies. This means that handling steps, safety risks and variability must be reduced to a minimum. Safety concerns are in support of using nonviral vectors. These are usually assembled from at least two components directly before use which is an inherent source of variability of the formulation. Therefore, the major aims of this study were generating a pre-formed lyophilized fibrinogen component of a fibrin glue comprising a nonviral gene vector and demonstrating its competence in transfection of relevant primary cells.

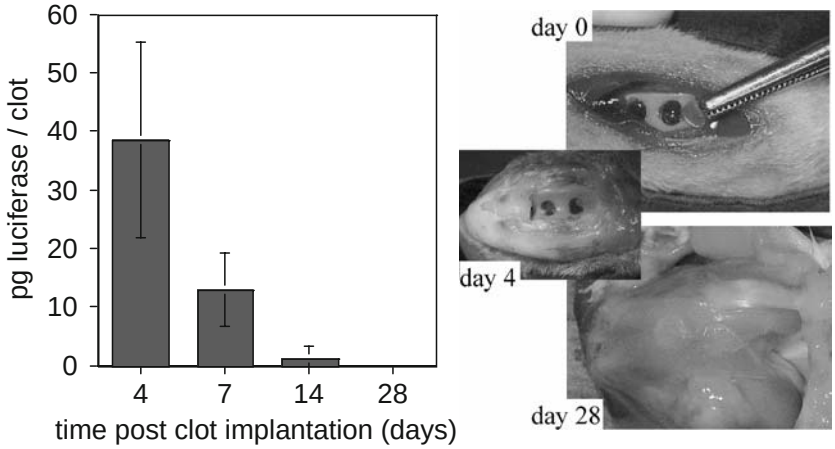

Fig. 7. Luciferase expression *in vivo* from luciferase-*COPROG* clots with suspended autologous chondrocytes implanted in osteochondral defects in rabbits (*left*). Implants were recovered from the implantation sites at the specified time points, the tissue was homogenized, and luciferase expression was determined in tissue lysates. Similarly to the *in vitro* situation, the expression declines rapidly. Mean value and standard deviation ($n=3$ for the day 1 group, $n=4$ for all other groups). *Right*: Aspect of implant sites directly after clot formation in the osteochondral defect, 4 days later and on day 28 after implantation.

We considered using naked DNA, PEI-DNA polyplexes and *COPROGs* (Supplementary Fig. 1), all of which we have previously examined with collagen sponges as gene carriers (14). A first question was whether these entities would impair the clotting properties of fibrinogen, which was in fact the case for PEI polyplexes (Supplementary Fig. 3) which we therefore excluded from further examinations. The known anticoagulant properties of PEI (44) are obviously effectively shielded in *COPROGs*, in analogy to reduced complement activation and interaction with serum components seen with these vectors (15,16). The ROTEG analysis defined a clotting-compatible concentration window of up to 20 μg DNA/mg fibrinogen for *COPROGs*. Clotting was compatible with co-lyophilization of the fibrinogen component with naked DNA and *COPROGs* throughout the dose range (data not shown), which is important from a manufacturing and ease-of-application perspective.

Next we were interested in the release kinetics of naked DNA and *COPROGs* from fibrin clots which is an important parameter with respect to the spatio-temporal control of delivery. The latter is particularly important in growth factor delivery as has been pointed out by Cao and Mooney (10). The GAM concept adds a level of complexity to growth factor delivery, because spatio-temporal control of both gene delivery and the release of the expressed growth factor will need to be considered. Concerning vector release from a GAM, basically three concepts can be pursued: The first is, having the matrix serve as a controlled release depot of the vector, that is, vector release is supposed to govern transfection/transduction at an implantation site. The second is having the matrix immobilize the vector such that matrix colonization by cells governs transfection/transduction. And the third is like the second plus having matrix degradation govern vector release and in this manner influence transfection/transduction. Our approach presented here corresponds to the third concept. *COPROGs* are efficiently immobilized in the fibrin matrix and vector release is slow (0.29% release per day) with only a minor dependence on the density of the fibrin matrix, as opposed to naked DNA the release of which is above 70% within 7 days (in agreement with the results of Trentin *et al.* (45)) with some dependence on fibrin density (Fig. 1). *COPROGs* are evenly dispersed in the fibrin matrix as evidenced by fluorescence microscopy (Fig. 3). When chondrocytes are incorporated in *COPROG*-clots they get rapidly associated with the vector, likely already upon mixing with the rehydrated fibrinogen component before thrombin addition (Fig. 3). The vectors in fibrin clots are large structures (Fig. 2) unlike freshly assembled *COPROGs* which have a size of 20–30 nm (15). Interestingly, electron microscopy reveals two morphologies: Potato-like structures of 300–500 nm size and tubular structures, similar to the ones observed with PEGylated PEI polyplexes by Erbacher *et al.* (46,47). Presently, we do not know what gives rise to the formation to one or the other morphology. We can only speculate that either structure is the result of some association with a component of the fibrinogen preparation which may form during the lyophilization process. In any case, chondrocytes internalize these structures by what appears to be phagocytosis and macropinocytosis (Fig. 2).

The release characteristics alone would predict that naked DNA is rapidly lost for the transfection of cells growing in the matrix. In fact, transfection by naked DNA was essentially undetectable using the luciferase assay while *COPROGs* gave rise to high expression both in primary keratinocytes and primary chondrocytes (Fig. 4 and 5). At the *COPROG* dose per clot which was chosen in most of our experiments, the level of reporter gene expression was dependent on the cell density in the clot, particularly with keratinocytes (Fig. 4B). The sharp decline of reporter gene expression above 100,000 cell per clot may be explained by insufficient nutrient supply to the cells during the initial days but also by the fact that for PEI-based vectors a certain minimum dose of vector per cell is known to be required to yield efficient transfection (48). In any case, the expression of the transfected gene dropped rapidly after having reached peak levels within a few days, nevertheless remained detectable for 21 days. The expression kinetics was

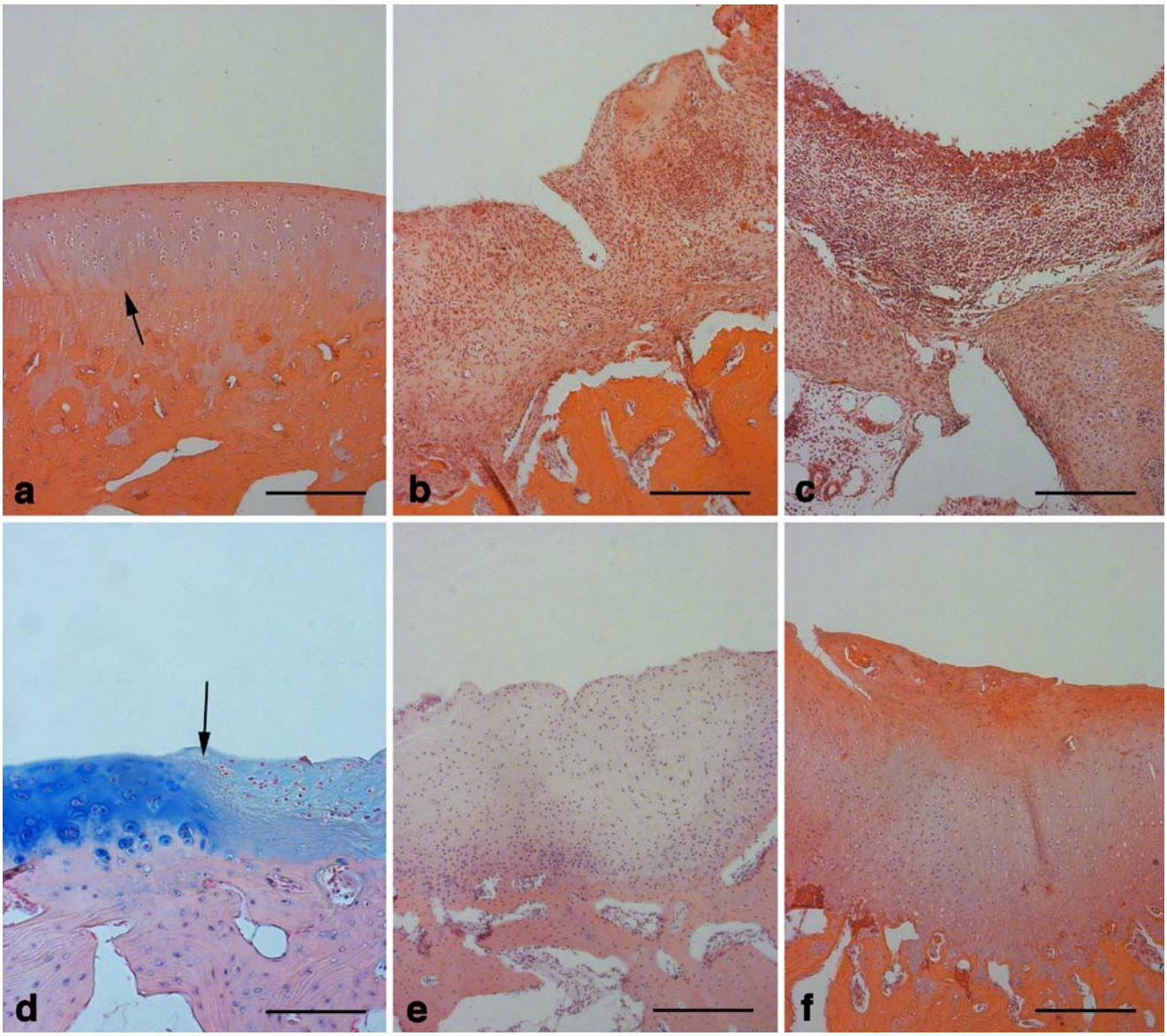

Fig. 8. Histology. **a** Normal hyaline cartilage of untreated rabbit with abundant extracellular matrix and typical cellular morphology. *Arrow marks* intact tide mark. **b** Overview of 4 week control animal, showing mainly unorganized fibrous tissue with discontinuous surface. **c** Plasmid animal after 4 weeks presenting the same quality of the regenerated tissue as the control animals. **d** Alcian Blue staining (12 week plasmid animal) showing boarder between native (*left side*) and regenerated (*right side*) cartilage. Note decreased staining, indicating less proteoglycan content in the newly formed tissue. **e** Twelve week control animal showing increasing organization of tissue with time. Tidemark has not been regenerated and no hyaline cartilage can be seen. **f** Comparable results of the 12 week plasmid group. HE staining: **a**, **b**, **c**, **e**, **f** Alcian Blue staining: **d**. *Bar* size 250 μm, except figure **d** 125 μm.

dependent on the cell type and on the transfected gene which is a commonly observed phenomenon in gene delivery which may have to do with differential rates of vector uptake and cell division and general differences in cell physiology. All plasmid constructs used in this study had the expression of the transfected gene under the control of the CMV promoter. This explains the rapid decline of expression which has been observed by many groups when using this promoter. The percentage of transfected cells increased within the first 8 days suggesting sustained transfection while the cells expand in the matrix (Fig. 6). When primary chondrocytes were transfected in BMP-2-*COPROG* clots, the expressed growth factor was readily released from the clot, induced the expression of alkaline phosphatase (a widely recognized biochemical marker for osteoblast activity and hypertrophic chondrocytes) and led to increased proteoglycan production (Fig. 5) which was detectable in out-grown chondrocytes even after 3 weeks in culture. As BMP-2 expression had returned to baseline levels by this time point, this observation indicates that a short term stimulus by BMP-2 transfection has a sustained effect on the cell phenotype under the culture conditions used here. However, the BMP-2 expression levels were at least 500-fold lower than the ones achieved by retroviral (49) or adenoviral (50) transduction of chondrocytes and about three- to ten-fold

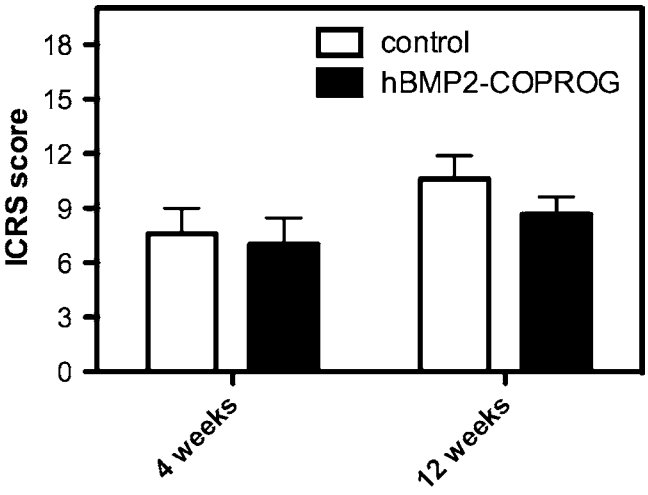

Fig. 9. ICRS score. Paraffin sections were analysed regarding the ICRS score. There was no significant change in cartilage quality by hBMP2-COPROG transfection in comparison to controls (mean± SEM, $n=6$, $p>0.05$).

lower than the minimal concentration of BMP-2 that was required for the stimulation of rat perichondral cells in a study published by Gelse *et al.* (43).

With this in mind we started using the BMP-2-*COPROG* preparations in an osteochondral defect model that we have described previously (51), similar to the one also used by Gelse *et al.* (43). Pilot experiments with the luciferase reporter gene had yielded reasonable expression levels at early time points (Fig. 7). However, the BMP-2-*COPROG* preparations were insufficient in improving any of the parameters used to characterize cartilage repair (Figs. 8, 9, and 10) compared to controls. As the same setup using retrovirally transduced chondrocytes (49) yields highly efficient cartilage repair (S.V., unpublished results), we conclude that both, expression levels and expression kinetics achieved with our setup are insufficient to stimulate cartilage repair. The good news is that the chondrocytes seeded in the *COPROG*-fibrinogen preparation to fill the osteochondral defects were entirely viable and that there are tools to improve expression levels and persistence by choosing appropriate promoters, enhancers, and CpG-free sequences in the plasmid construct (52).

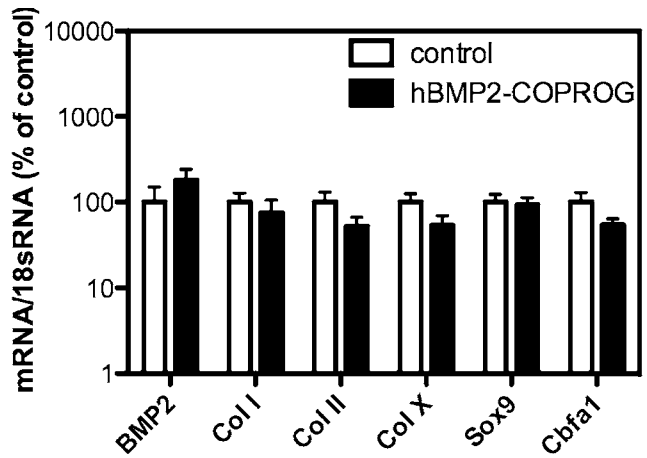

Fig. 10. RT-PCR analysis of regeneration tissue in osteochondral defects. Data showed no influence of hBMP2-COPROG transfection on RNA levels of different proteins after 12 weeks (mean±SEM, $n=6$, $p>0.05$).

In summary, we have demonstrated that fibrinogen can be formulated as carrier matrix for gene vectors such that it can serve as a lyophilized component of a fibrin glue. This makes it easy to use as an injectable implant which mediates gene delivery. Further improvements in vector and plasmid design are needed to yield high and persistent transgene expression which is required in some applications, such as the osteochondral defect model examined in this study.

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