

Supplementary Information

Generic Strategy for Pharmacological Caging of Growth Factors for Tissue Engineering

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Supplementary Manuscript

Generic gene assembly design and optimized protein production. For the design of the expression vectors, we constructed a generic vector harboring the Fc-tag in a high-yield optimized mammalian expression vector in which the target gene can easily be integrated by restriction enzyme-free joining PCR or Gibson assembly (Supplementary Fig. 1a, Ref. 1-2). In order to efficiently produce the Fc-tagged proteins³, we devised a robust and generically protein production platform based on human embryonic kidney cells HEK-293T. Optimized production conditions were determined by an analysis of variance (ANOVA) 2^{6-1} fractional factorial experimental design using the following factors: cell density, cell cultivation time, concentration of the histone deacetylase inhibitor valproic acid (VPA), amounts of the plasmids expressing the cell-growth suppressing proteins p18⁴ and p21^{5,6}, as well as the concentration of the plasmid expressing the secreted protein of interest, in this case SEAP_{Fc} as a secreted human model glycoprotein. The first experiment of the optimization process showed a positive effect when the cultivation time, the cell density, VPA concentration, or the amount of p18-expressing plasmid were increased. The amount of the p21- and protein of interest-expressing plasmids did not have a significant effect on the protein yield (Supplementary Fig. 1b). In subsequent experiments, the p21-expressing plasmid was excluded, whereas the protein of interest-expressing plasmid concentration was fixed at the value corresponding to the standard protocol, i.e. 6 µg/Petri dish. Four additional experiment iterations were performed until all parameters were optimized. Maximum yield was reached when seeding $3.5 \cdot 10^6$ cells per Petri dish and transfecting them with 10.5 µg of the p18-expressing plasmid and 6 µg of the constitutive SEAP-expressing plasmid, followed by exposure to 3.5 mM of VPA for a time period of 120 hours.

Supplementary Materials and Methods

Expresion vector construction, mammalian cell culture and protein production

Generic expression vector construction strategy. A restriction enzyme-free cloning strategy was established that can generically be applied to fuse arbitrary genes to the cageable Fc domain via a linker sequence (amino acid sequence: GNYNGPKVASGGGGSGGGGSGGGGS). For this aim the Fc-encoding gene derived from mouse IgG_{2a} was amplified from the plasmid pFUSE-mlgG2A-Fc2 (Invivogen, San Diego, California, cat. no. pfuse-mg2afc2) using the oligos OLMK005 (5'-ggtaactataacggctctaaggtagcgagtggtggaggcgggttcaggcggaggtggctctggcgggtggcggatcgcccagagggcccacaatcaagc-3') and OLMK006 (5'-accataataaccataatagctgtttgccatcaatggtgatggtgatggtgtttaccggagtcggggagaagc-3') (Fc-specific annealing sequence in italics, linker sequence used for annealing in the subsequent fusion PCR underlined). The resulting Fc-encoding PCR product was mixed with PCR products encoding the target genes (see Supplementary Table 1) fused to the common linker motif. Both fragments were fused by joining PCR using the respective target gene-responsive forward oligo (Supplementary Table 1) and the generic reverse oligo OLMK078 (5'-accataataaccataatagctgtttgcc-3', annealing sequence in italics). The resulting fusion gene was cloned in sense orientation into pEF5/FRT/V5-D-TOPO (Invitrogen, Carlsbad, CA, cat. no. K603501) using TOPO cloning under the control of the strong mammalian elongation factor 1 α -derived promoter P_{hEF1 α} . The expression vector pLMK045 encoding for the adaptor protein ZZ-GyrB-His₆ was constructed by amplifying the gene encoding for ZZ from the plasmid pEZZ-18 (GE Healthcare, Piscataway, NJ, cat. no. 27-4810-01) using oligos OLMK250 (5'-caccatggcgcaacacgatgaagc-3') and OLMK251 (5'-ggaggagtcataagaattcgacatgctcgaattcgctgtctac-3'), and the gene encoding for hexahistidine-tagged gyrase B from the plasmid pWW873⁷ using oligos OLMK252 (5'-atgtcgaattcttatgactcctcc-3') and OLMK253 (5'-ccataataaccataatagctgtttgccatcaatggtgatggtgatgatggccttcatag-3') (annealing sequence in italics, linker sequence used for annealing in the subsequent fusion PCR underlined). The both PCR products were fused via joining PCR using oligos OLMK250 and OLMK253. The resulting fusion gene was cloned in sense orientation into pET101/D-TOPO (Invitrogen, cat. no. K101-01) using TOPO cloning under the control of the bacterial T7 promoter. The following plasmids have been described previously (pWW56⁸, pXLG^{HEK}-p18h⁹, pXLG^{HEK}-p21h⁹, pPDGFR-BB¹⁰, pLMK135¹¹) or are commercially available (pFA2-Elk1, Stratagene, La Jolla, CA, cat. no. 219061-51; pFR-Luc, Stratagene, cat. no. 219050-51; pGL4.75, Promega, Madison, WI, cat. no. E6931). The plasmids pXLG^{HEK}-p18h and pXLG^{HEK}-p21h⁹ were kindly provided by Florian Wurm and the plasmid pPDGFR-BB was kindly provided by Andrius Kazlauskas.

Mammalian cell culture. Human embryonic kidney (HEK-293T) cells were cultivated in DMEM medium (Dulbecco's modified Eagle's medium (DMEM, PAN Biotech, Aidenbach, Germany, cat. no.

P03-0710) supplemented with 10 % (v/v) foetal calf serum (FCS, PAN Biotech, cat. no. P281803, lot no. P101003TC) and 1 % (v/v) penicillin/streptomycin solution (PAN Biotech, cat. no. P06-07100)) (DMEM complete). Human umbilical vein endothelial cells (HUVEC; Promocell, Heidelberg, Germany, cat. no. C-12200) were cultivated in endothelial cell medium (Promocell, cat. no. C-22010) supplemented with 10 % (v/v) FCS and 50 µg/ml gentamycin (Alexis, San Diego, CA, cat. no. 380-003-G005). Mouse embryonic fibroblasts (MEFs) were cultivated in DMEM medium with 4.5 g/l glucose (PAN Biotech, cat.no. P04-03600) supplemented with 10 % (v/v) fetal calf bovine serum Gold (PAA, Pasching, Austria, cat. no A15-151, lot no. 30/ A15109-1513), 2 mM L-glutamine (PAN Biotech, cat. no. P04-80100), 10 mM HEPES (PAN Biotech, cat. no. P05-01100) and 2 % (v/v) penicillin/streptomycin solution. MEFs were generated according to a previously described protocol¹². In brief, MEFs were isolated from d13 B-*raf*^{flox/flox} mouse embryos from a mixed C57/Bl6xBalb/C background¹³ (kindly provided by Drs. Manuela Baccarini (Vienna) and Elias Hobeika/Michael Reth, MPI Freiburg). Early passage MEFs were immortalised by retroviral infection with the pQCXIH/Tag retroviral construct (kindly provided by Ruth Lyons and Dr. Roger J. Daly, Garvan Institute, Sydney), which encodes for the large T antigen of Simian Virus 40 (Tag). Following infection, MEF pools were selected with hygromycin B (200 µg/ml, Invivogen, cat.no. ant-hm-1). These MEFs retain their normal morphology and a proper contact inhibition response. In the present study, only B-Raf proficient MEFs with a floxed B-*raf* locus were used. All cell lines were cultured at 37 °C in a humidified atmosphere containing 5 % CO₂. For cell culture experiments involving caged proteins, FCS was replaced by low IgG FCS (PAN Biotech, cat. no. P30-2802, lot. no. P102506UI).

Standard transfection protocol for HEK-293T cells. For transfection of HEK-293T cells, 50'000 cells were seeded in each well of a 24-well plate in 0.5 ml DMEM complete medium and allowed to settle for 18 h prior to transfection. The transfection mix was prepared as follows: 50 µl 0.25 M CaCl₂ solution containing a total amount of 0.75 µg DNA was added drop wise to 50 µl Hepes-buffered saline solution (HBS, 50 mM Hepes/NaOH, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 7.05) while vortexing. After 20 min of incubation at room temperature, the solution was added drop wise to the cells. The cells were centrifuged for 5 min at 1200 x g and incubated for 2 hours prior to fresh medium exchange. Protein production was analyzed 48 h post transfection.

Protocol for the production of cageable proteins. An optimized protocol for the production of cageable proteins was developed using ANOVA fractional factorial experimental designs (see below). Briefly, 24 h prior to transfection 3.5 x 10⁶ HEK-293T cells were seeded in a 10 cm Petri dish in 10 ml DMEM medium containing 10 % (v/v) ultra low IgG FCS and 1 % (v/v) of a penicillin / streptomycin solution. The transfection mix was prepared by dropwise addition of 500 µl of a 250 mM CaCl₂ solution containing 6 µg of the target plasmid and 10.5 µg of the plasmid pXLG^{HEK}-p18h⁹ to 500 µl of a Hepes-

buffered saline solution (HBS, 50 mM Hepes/NaOH, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 7.05) under vortexing. The transfection mix was incubated for 20 min at room temperature and subsequently slowly added to the cells. After 5 hours the medium was exchanged to fresh medium supplemented with 3.5 mM valporic acid (Sigma-Aldrich, cat. no. P4543). Subsequently, the cell culture supernatant was harvested 120 h post-transfection and the secreted Fc-tagged proteins were purified by protein A affinity chromatography (Roche Applied Science, Rotkreuz, Switzerland, cat. no. 11 134 515 001) according to the manufacturer's protocol. The buffer was exchanged to PBS by ultrafiltration (10 kDa MWCO, Corning, Lowell, MA, cat. no. 431483) and the proteins were quantified by an Fc-specific ELISA according to the protocol below.

Production of ZZ-GyrB-His₆. The expression vector pLMK045 encoding for the adaptor protein ZZ-GyrB-His₆ was transformed into *E. coli* BL21 STARTM (DE3) (Invitrogen, cat. no. C601003) and protein production was induced at an OD₆₀₀ = 0.6 by the addition of isopropyl β - D -1-thiogalactopyranoside (IPTG, Carl Roth, Karlsruhe, Germany, cat. no. CN08.3) to a final concentration of 1 mM and carried out for 4 hours at 37 °C. The cells were harvested by centrifugation at 6000 x g for 5 min at 4 °C, resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted using a French press (APV, Albertslund, Denmark, model APV-2000). After cell debris removal by centrifugation at 30000 x g for 20 min at 4 °C, the cell lysate was loaded onto a Ni²⁺-NTA Superflow column (Qiagen, Hilden, Germany, cat. no. 30210). Subsequently, the column was washed with 20 ml each of lysis buffer and wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) and the protein was eluted in 10 ml of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). The buffer was exchanged to phosphate buffered saline (PBS, 50 mM NaH₂PO₄, 150 mM NaCl, pH 8) and the protein was concentrated to 50 mg/ml by ultracentrifugation (10 kDa MWCO, Corning, Lowell, MA, cat. no. 431483). The protein was shock frozen in liquid nitrogen and stored at -80°C.

Cage synthesis and characterization

The cage was synthesized by covalently coupling novobiocin (Carl Roth, Karlsruhe, Germany, cat. no. C247.3) to epoxy-activated agarose resin (Sigma-Aldrich, Steinheim, Germany, cat. no. E6632) through a *nucleophilic addition* reaction¹⁴. Briefly, 100 mg epoxy-activated agarose resin was reswollen for 1 hour at room temperature in 1 ml distilled water and subsequently washed with 50 ml coupling buffer (0.3 M Na-carbonate, pH 9.5). The resin was mixed with 50 mg novobiocin in 15 ml coupling buffer and was gently shaken for 17 h at 37 °C. Excess epoxy-groups were blocked by the addition of ethanolamine (Sigma-Aldrich, cat. no. E-0135) to a final concentration of 1 M and the mixture was shaken for 5 hours at 37 °C, after which the resin was washed in 50 ml each of 0.5 M NaCl in coupling buffer, distilled water, 0.5 M NaCl in 0.1 M Na-acetate pH 4.0 and distilled water. The washing was repeated three times. All

subsequent steps were performed at 4 °C on a rocking platform (Biometra, Goettingen, Germany). Unspecific protein-binding sites of the cage were blocked by incubation in 25 ml of 1 % (w/v) bovine serum albumin (BSA, Fluka, Buchs, Switzerland, cat. no. 05479) in phosphate buffered saline (PBS, 50 mM NaH₂PO₄, 150 mM NaCl, pH 8) overnight. The cage was functionalized with the adaptor protein ZZ-GyrB by incubating 100 mg cage in 25 ml of a 100 µg/ml ZZ-GyrB solution in PBS containing 1 % (w/v) BSA for 4 h. Excess linker was removed by three washing steps (30 min each) in tris-buffered saline (TBS, 50 mM tris, 150 mM NaCl, pH 7.6) supplemented with 0.05 % (v/v) Tween 20 and 1 % (w/v) BSA. The cargo proteins were caged by incubating 100 mg adaptor-functionalized cage directly in 25 ml cell culture supernatant (Dulbecco's modified Eagle medium (DMEM, Pan Biotech, Aidenbach, Germany, cat. no. P03-0710) supplemented with 10 % ultra low *IgG* fetal calf serum (FCS) (PAN Biotech, cat. no. P30-2802, lot. no. P102506UI) and 1 % (v/v) of a penicillin / streptomycin solution (PAN Biotech, cat. no. P06-07100)) containing 200 ng/ml cargo protein for 4 h followed by three washing cycles as indicated above. The caged proteins were stored at 4 °C in PBS containing 0.1 % (w/v) BSA. For characterization of the uncaging kinetics in mammalian cell culture medium, caged SEAP_{Fc} (8 mg cage) was incubated in 5 ml DMEM supplemented with 1% ultra low *IgG* FCS and 1 % (v/v) of a penicillin / streptomycin solution at room temperature on a rocking platform in the presence of increasing novobiocin concentrations. Uncaging kinetics were determined by profiling the increase of SEAP activity in the supernatant for 5 h. The released SEAP_{Fc} fraction was determined by dividing the uncaged protein amount by the total caged protein amount. The total amount of caged SEAP_{Fc} was calculated according to Lambert-Beer's law¹⁵ from the increase in absorbance measured at 405 nm when incubating 8 mg cage in 5 ml DMEM supplemented with 1% ultra low *IgG* FCS and 1 % (v/v) of a penicillin / streptomycin with 6.25 ml 2x SEAP buffer (20 mM homoarginine, 1 mM MgCl₂, 21% (v/v) diethanolamine, pH 9.8) and 1.25 ml pNPP solution (120 mM para-nitrophenyl phosphate). The caging efficiency was determined by incubating the cage with saturating amounts of SEAP_{Fc}, whereas the amount of non-specific adsorbed protein was determined by mixing the proteins with novobiocin-free cage.

Analysis of mesenchymal progenitor cells (MSC) mobilization

For the analysis of MSC mobilization spheroids were embedded into matrix metalloprotease-sensitive polyethylene glycol-based hydrogels together with caged PDGF-BB_{Fc}. MSC mobilization was monitored by time lapse microscopy and quantitatively analyzed.

Formation of MSC spheroids. MSCs from human term placenta were isolated as described previously¹⁶. For spheroid formation 25'000 cells/ml were suspended in serum-free DMEM medium supplemented with 1 % (v/v) penicillin / streptomycin solution and 0.2 % (w/v) methyl cellulose (Sigma-Aldrich, cat. no. M0512). Droplets of 30 µl were placed in bacteriological dishes and cultured upside-down as hanging

drops overnight. The resulting spheroids were harvested in PBS containing 1 % (w/v) BSA, washed once with serum-free DMEM medium and were subsequently embedded into the hydrogels.

Hydrogel formation. PEG-based, matrix metalloprotease-sensitive hydrogels were synthesized as described previously¹⁷. In brief, stoichiometric amounts (final concentration: 1.4 %, w/v) of 8-PEG-MMP_{sensitive}-Lys and 8-PEG-Gln were mixed with 50 μ M Gln-RGD peptide, 400 spheroids / ml and 10 U / ml thrombin-activated factor XIIIa in 50 mM Tris pH 7.6 supplemented with 50 mM CaCl₂. Droplets of 20 μ l were placed between two siliconized glass slides (Sigmacote, Sigma-Aldrich, cat. no. SL2) using spacers of 1 mm thickness. In order to prevent spheroid sedimentation, the glass slides were slowly rotated at room temperature until onset of gelation, and subsequently incubated for 30 min at 37 °C. The hydrogels were thereafter released and transferred into a 48 well plate. Hydrogels containing caged PDGF-BB_{Fc} were prepared as described above, except that the hydrogel precursor solution (80 μ l volume per gel containing 40 mg cage) was cast onto the spheroid-containing hydrogels in the 48-well plates so that both gels were fused together. The final gels were incubated in 100 μ l serum-free DMEM medium supplemented with 0.1 % (w/v) BSA (AppliChem, Darmstadt, Germany, cat. no. A1391, lot. no. 1U002751) at 37 °C and 5 % CO₂ in the presence (6 μ M) or absence of the uncaging stimulus novobiocin.

Image recording and image analysis. Cell mobilization was monitored by time lapse microscopy (Zeiss Axiovert 200M) every 10 min for a total period of 16 h. The samples were fixed by incubation in 4 % (w/v) para-formaldehyde (Fluka, cat. no. 76240) and 0.2 % Triton in PBS for 20 min and subsequently washed once with 0.1 M glycine in PBS and twice with PBS. DAPI staining was performed by incubation with DAPI reagent (Sigma-Aldrich, cat. no. D5942) for 15 min followed by washing with PBS. Fluorescent Z-stack images (140 μ m in total thickness) of DAPI stained MSC microtissues were acquired using a Zeiss Axiovert 200M microscope. Maximum intensity projections and uniform threshold adjustments were performed in ImageJ. The total number of migrated cells was counted manually.

Analytics

Quantification of Fc-tagged proteins. The Fc-tagged proteins were quantified via enzyme-linked immunosorbent assay (ELISA) according to the following protocol. Capture antibody (goat anti-mouse IgG, Fc-specific, Sigma-Aldrich, Steinheim, Germany, cat. no. M4280) was diluted 1:500 in PBS of which 100 μ l was added to each well of a 96-well ELISA plate (Corning, cat. no. 3590) and incubated overnight at room temperature. Subsequently, the plate was washed three times with PBS containing 0.05 % (v/v) Tween-20. The non-specific binding sites in the wells were blocked by adding 300 μ l of blocking buffer (PBS containing 1% (w/v) BSA) to each well and incubating for 1 h. The plate was

thereafter washed as described above. The Fc-tagged proteins and the standard protein (anti his-tag monoclonal antibody, Novagen, Madison, WI, cat. no. 70796-3) were added to the wells and incubated for 2 h. The plate was washed as indicated above and 100 μ l of detection antibody (anti-mouse IgG x HRP, Amersham Life Science, Piscataway, NJ, cat. no. NA931) diluted 1:2500 in blocking buffer was added to each well. After 30 min, the plate was washed and the assay was developed by the addition of 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) reagent (0.03%, w/v, Sigma-Aldrich, cat. no. A1888), and H₂O₂ (0.03%, v/v) in sodium citrate buffer (50 mM, pH 4.0). The absorbance at 405 nm was measured using a SynergyTM 4 multi-mode microplate reader (BioTek[®] Instruments, Inc., Winooski, VM).

SEAP activity assay. SEAP activity was measured using a *p*-nitrophenylphosphate-based light absorbance kinetic assay according to a previously described protocol¹⁵.

Dual luciferase assay. For quantification of the firefly and renilla luciferase activities, the cells were lysed for 15 min with 250 μ l cell lysis buffer (1 % (v/v) Triton X-100, 15 mM MgSO₄, 4 mM EGTA, 1 mM DTT, 25 mM Tris-HCl, pH 7.8) per well of a 24-well plate. After 15 min incubation, the cells were resuspended by pipetting up and down and 100 μ l of the cell lysate were added to each well of a white flat bottom 96-well plate. 20 μ l of either firefly luciferase substrate (20 mM Tricine, pH 7, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 0.52 mM ATP, 0.27 mM Acetyl-CoA, 5 mM NaOH, 50 mM MgCO₃, 0.47 mM luciferin) or renilla luciferase substrate (5 μ M coelenterazine (Carl Roth, cat. no. 4094.3) in PBS) were added to each well and the luciferase activity was monitored with a 10-min overall integration time. All measurements were performed using a SynergyTM 4 multi-mode microplate reader.

VEGF bioactivity assay. VEGF_{Fc} bioactivity was determined by the HUVEC cell proliferation assay: 1'500 HUVECs were seeded in 100 μ l endothelial cell medium supplemented with various VEGF concentrations in each well of a 96-well plate. In order to ensure a uniform oxygen supply, the cells were covered with a gas-permeable membrane (BREATSeal, Greiner Bio-One, Frickenhausen, Germany, cat. no. 676051). After 96 h of incubation the cell number was quantified by the WST-1 assay (Roche Applied Science, cat. no. 11 644 807 001) according to the manufacturer's protocol.

IGF1 bioactivity assay. The bioactivity of IGF1_{Fc} was assessed using the aforementioned MEF system¹². Cells were seeded in six-well plates at a density of 2 x 10⁵ cells/well. Cells were allowed to settle for 24 h before they were starved for 18-24 h in DMEM medium containing 0.5 % (v/v) FCS. IGF1_{Fc} was added to the starvation medium to a final concentration of 50 ng/ml. Following stimulation, the cells were lysed in 200 μ l RIPA buffer (50mM Tris-HCL pH 7.4, 1% Triton-X 100, 137mM sodium chloride, 1%

glycerine, 1mM sodium orthovanadate, 0.5mM EDTA pH 8.0, 0.5% sodium-deoxycholate, 0.1% SDS protease inhibitors: 0.2µg/µl aprotinine, 0.01g/µl leupeptin, 1mM AEBSF composition) and processed as described previously¹⁸. In brief, cell lysates cleared by centrifugation were supplemented with equal volume of 2x Laemmli buffer (20% (v/v) glycerol, 6% (v/v) SDS, 6% (v/v) β-mercaptoethanol, 0.6% (v/v) bromophenol blue) and heated for 5 min at 95°C to denature proteins. Protein lysates were loaded to 10% polyacrylamide gels and analyzed by Western blot using the following antibodies: anti p44/42 MAPK(ERK1/2) (1:2000, Cell Signaling technology, Boston, MA, cat. no. 9101), anti pAKT S473 (1:1000, Cell Signaling technology, cat. no. 9271), anti Egr-1 (C19) (1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, cat.no. sc-189) and anti β-actin (C4) (1:2000, Santa Cruz Biotechnology Inc., cat. no. sc-47778).

PDGF-BB bioactivity assay. The PDGF-BB_{FC} bioactivity assay was performed by transiently co-transfecting HEK-293T cells seeded in a 24-well plate with the plasmids for the firefly luciferase-based PDGF reporter system (pPDGFR-B; PDGF-BB receptor, pFA2-Elk1; GAL4-Elk1 fusion protein, pFR-Luc; GAL4-responsive firefly luciferase reporter) as well as the constitutive renilla firefly expression vector (pGL4.75). The cells were cultivated for 48 h in DMEM complete supplemented with various concentrations of PDGF-BB_{FC} prior to quantification of luciferase activity.

For analyzing the bioactivity of uncaged PDGF-BB_{FC}, HEK-293T cells were seeded and transfected as above and cultivated in DMEM supplemented with 1 % (v/v) ultra low IgG FCS and 1% penicillin/streptomycin solution in the presence (6 µM) or absence of uncaging novobiocin. 8 mg PDGF-BB_{FC}-loaded cage was added in a transwell cell culture insert (Corning, cat. no. 93413) and the activity of uncaged PDGF-BB_{FC} was determined at different time points as indicated above. The amount of uncaged PDGF-BB_{FC} was quantified via a PDGF-BB ELISA (Peprotech, Hamburg, Germany, cat. no. 900-K04) according to the manufacturer's protocol. In order to avoid unspecific binding of the ELISA antibodies to the ZZ-domain, the samples were diluted in FCS (1:100) and subsequently incubated at room temperature for 2 hours prior to quantification, allowing the IgGs in the FCS to saturate the ZZ domain.

Receptor-ligand binding affinity. Receptor-ligand binding affinity experiments were performed via enzyme-linked immunosorbent assay (ELISA) according to the following protocol. 100 µl of IGFI receptor (1.4 ng/ml, R&D Systems, Minneapolis, MN, cat. no. 391-GR-050), KDR (VEGFR2) receptor (0.1 ng/ml, Life Technologies, Karlsruhe, Germany, cat. no. PV3660), or PDGF-BB receptor (0.6 ng/ml, Sino Biological Inc., Beijing, China, cat. no. 10514-H08H) diluted in PBS were added to each well of a 96-well ELISA plate (Corning, cat. no. 3590) and incubated overnight at room temperature. Subsequently, the plate was washed three times with PBS containing 0.05 % (v/v) Tween-20. The non-specific binding

sites in the wells were blocked by incubation for 1 h with 300 μ l blocking buffer (PBS containing 5% (w/v) BSA) and the plate was washed as described above. The wild type proteins (human IGF-I (Peprotech, cat. no. 100-11), human VEGF (Peprotech, cat. no. 900-K10), and human PDGF-BB (Peprotech, cat. no. 900-K04)), the Fc-tagged proteins, and the Fc-tagged proteins bound to the adaptor protein (molar ratio 1:1) were diluted in PBS containing 1 % BSA. In order to avoid unspecific binding of antibodies to the ZZ-domain, the samples were incubated for 2 h at room temperature in the presence of an excess amount of IgGs (purified from FCS by protein A affinity chromatography). The samples were added to the plate and incubated at room temperature for 4 h to allow for receptor-protein binding. The plate was washed as described above and receptor-bound proteins were detected according to the manufacturer's protocol using the IGFI-HRP conjugate antibody from the human IGF-I ELISA (R&D Systems, cat. no. DG100), the VEGF detection antibody and the avidin-HRP conjugate antibody from the VEGF ELISA (Peprotech, cat. no. 900-K10), as well as the PDGF-BB detection antibody and the avidin-HRP conjugate antibody from the PDGF-BB ELISA (Peprotech, cat. no. 900-K04). The maximum specific binding B_{MAX} and dissociation constant K_{D} for the various proteins were determined by fitting the experimental saturation data to the binding curve $y = xB_{\text{MAX}}/(K_{\text{D}} + x)$ using OriginPro. The Scatchard plots were constructed under the assumption that the bound protein fraction is negligible in the total amount, i.e. the added protein concentration closely approximates the free protein concentration.

Experimental design and statistical analysis

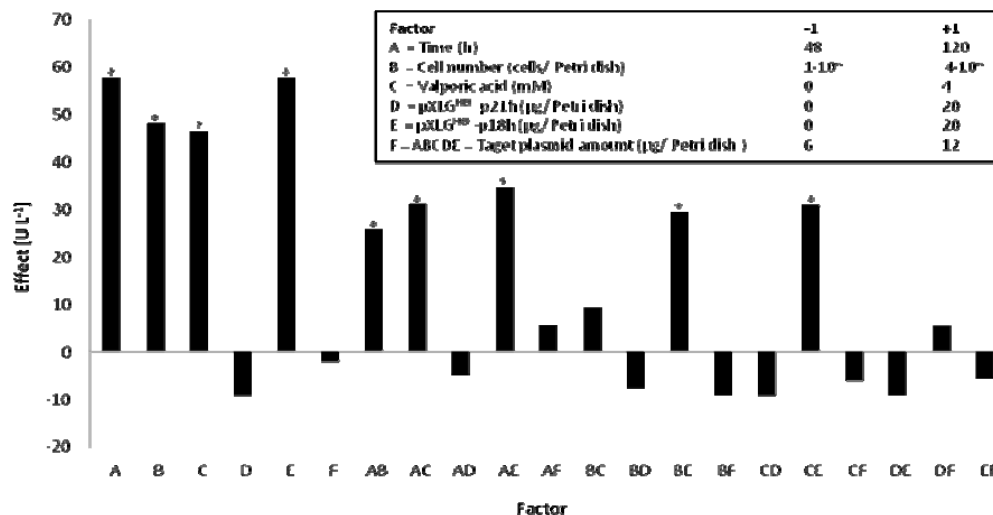
Analysis of variance (ANOVA) fractional factorial experimental design. The yield of secreted protein production in HEK-293T cells was optimized using an Analysis of Variance (ANOVA) 2^{6-1} fractional factorial experimental design¹⁹. The following factors were varied: cell density (A), cell cultivation time (B), Valporic acid (VPA) concentration (C), p18-expressing plasmid (pXLG^{HEK}-p18h)⁹ amount (D), p21-expressing plasmid (pXLG^{HEK}-p21h)⁹ amount (E), as well as the amount of the SEAP-expressing plasmid pWW56⁸ (F=ABCDE). The factors were assigned either a low (-1) or a high (+1) value. The secreted protein yield was quantified by measuring the enzymatic activity of SEAP. Analysis of the main effects and the two-factor interactions was performed using ReliaSoft's *DOE++* software (Tucson, USA), were all factors with a p-value < 0.05 were considered statistically significant. Factors determined not to be significant were fixed at constant values in subsequent experiments. Optimum yield was reached after 5 experimental iterations.

Supplementary Figures

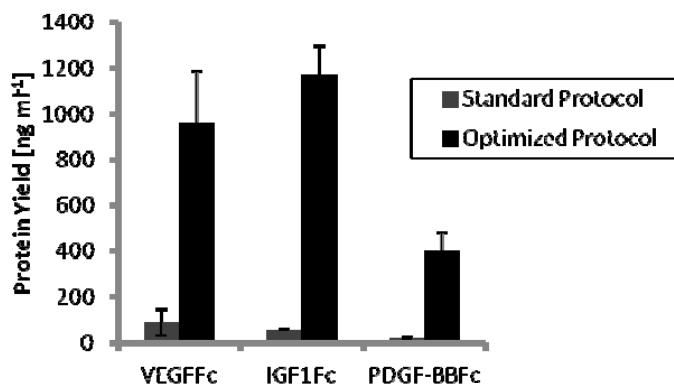
a)



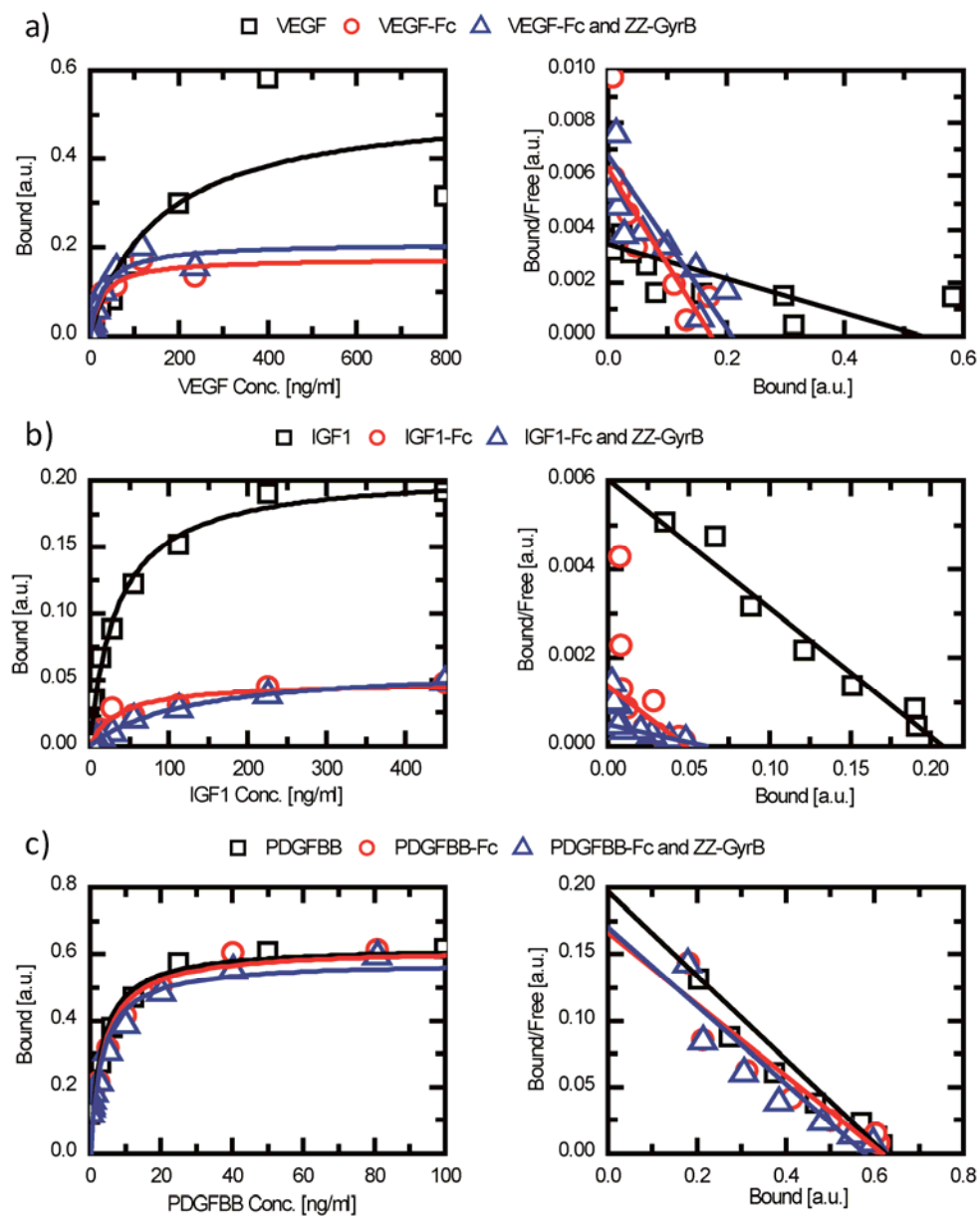
b)



c)



Supplementary Figure 1. Generic strategy for the high-yield production of cageable proteins (a) Generic design of expression vectors for the production of Fc-tagged proteins. Arbitrary genes encoding secreted factors are amplified using primers with compatible linkers, fused to the Fc-encoding gene of mouse IgG_{2a}, and cloned in a restriction enzyme-free manner into the mammalian expression vector. gs-linker, glycine-serine linker; his₆, hexahistidine tag; pA, polyadenylation signal; P_{hEF1α}, human elongation factor-1 alpha promoter. (b) Systematic optimization of the production of cageable proteins. A 2⁶⁻¹ fractional factorial experimental design was implemented to analyze the direct effects and first order effects of the indicated factors (A-F) on the yield of the recombinant model protein SEAP. For each factor the indicated levels were applied. The experiments were performed in triplicates, the asterisks indicate statistically significant effects with $p < 0.05$. (c) Production of cageable proteins using the optimized production protocol. VEGF_{Fc}, IGF1_{Fc} and PDGF-BB_{Fc} were produced according to the standard protocol and the improved protocol. Data indicate the mean and the standard deviation of four experiments.



Supplementary Figure 2. Saturation curve and Scatchard plot for the binding of the different growth factor variants to their recombinant receptor. (a), VEGF; (b), IGF1; (c), PDGF-BB.

Supplementary Tables

Gene	<i>vegfl21</i>	Template	pBM043 ²⁰	Vector name	pLMK006
	Forward oligo OLMK009	5'-caccatgaactttctgctgtcttgggtgcattg-3'			
	Reverse oligo OLMK010	5'-tcgctaccttaggaccgttatagttacccgcctcggcttgcacattttcttg-3'			

Gene	<i>igfl</i>	Template	Clone ID: LIFESEQ780 074 (Thermo Scientific, Waltham, MA)	Vector name	pLMK040
	Forward oligo OLMK055	5'- caccatgggaaaaatcagcagttctccaaccc -3''			
	Reverse oligo OLMK056	5'- tcgctaccttaggaccgttatagttaccatcctgtagttcttgttctctgcactc -3'			

Gene	<i>pdgf-bb</i>	Template	Clone ID:5174750 (Thermo Scientific)	Vector name	pLMK134
	Forward oligo OLMK038	5'- caccatgaatcgctgctgggcgctctt -3'			
	Reverse oligo OLMK0163	5'- tcgctaccttaggaccgttatagttaccgggtcacaggccgtgcagctg -3'			

Supplementary Table 1. Cloning strategy for expression vectors of cageable growth factors. The indicated genes were PCR-amplified (annealing sequence underlined, primer sequences indicated) and fused to the Fc-encoding sequence using the generic cloning strategy described above.

Supplementary Videos

Supplementary video 1. Time-lapse microscopy images showing the stimulation of MSC migration by controlled uncaging of PDGF-BB_{Fc}. Microtissues of MSCs were incorporated into matrices together with the cage containing trapped PDGF-BB_{Fc} and cultivated in the (a) absence or the (b) presence of novobiocin. Images at 10 x magnification were recorded every 10 min over a time period of 16 h.

Supplementary References

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