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Pharmacologically Controlled Protein Switch for ON-OFF Regulation of Growth Factor Activity

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Maria Karlsson^{1,3}, Balder Rebmann^{1,2}, Philipp S. Lienemann^{4,5}, Natallia Sprossmann^{1,2}, Martin Ehrbar⁴, Gerald Radziwill^{1,2} & Wilfried Weber^{1,2,3}

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Correspondence and requests for materials should be addressed to W.W. (wilfried.weber@biologie.uni-freiburg.de)

¹Faculty of Biology, University of Freiburg, Schänzlestrasse 1, 79104 Freiburg, Germany, ²BIOSS Centre for Biological Signalling Studies, University of Freiburg, Schänzlestrasse 18, 79104 Freiburg, Germany, ³Department of Biosystems Science and Engineering, ETH Zurich, Mattenstrasse 26, 4058 Basel, Switzerland, ⁴Department of Obstetrics, University Hospital Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland, ⁵Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne, Station 15, Bld AI 1109, 1015 Lausanne, Switzerland.

The precise manipulation of growth factor signaling is central to the progress of tissue engineering. Methods for direct time-resolved activation of signaling pathways through controlled receptor dimerization have been reported; however, these suffer from the risks associated with gene transfer. Here we present an alternative gene transfer-free approach in the form of a protein switch featuring pharmacologically controlled ON-OFF regulation of growth factor activity. The reversible operation of the switch enables stimulation of target processes within a defined period of time. The protein switch provides a means for both studying and manipulating signaling processes, and is thus believed to be a valuable tool for basic research as well as tissue engineering and biomedical applications.

Controlled growth factor signaling plays a key role in the advancement of tissue engineering and can provide an improved insight into growth factor-mediated cellular processes. Existing methods for the direct activation of growth factor signaling pathways are based on controlled dimerization of the receptor^{1–3}. Although these methods provide a means for controlling growth factor-mediated pathways in a highly specific and time-resolved manner, they rely on gene transfer and therefore suffer from the risk of serious side effects^{4,5}. Here we address this drawback by proposing an alternative, gene transfer-free, approach based on the control of the growth factor rather than the receptor. The control of growth factors belonging to the cysteine-knot superfamily is of special interest, since these are pivotal in a wide spectrum of cellular processes, such as cell development, differentiation, and reprogramming^{6,7}. The cysteine-knot growth factors bind their cognate receptors as dimers, which brings the receptor subunits into close association and initiates downstream signaling processes⁸. We have exploited these properties to construct a generic “protein switch” featuring small molecule-induced ON-OFF regulation of the activity of cysteine-knot growth factors.

Results

The switch consists of a chimeric protein with an engineered monomeric variant of the protein of interest (POI) fused to the inducible dimerization domain of the bacterial protein gyrase B (GyrB) (Fig. 1). In its default state the switch is OFF due to its monomeric structure, which prohibits dimerization and thereby activation of the protein receptor. The switch is turned ON upon the addition of coumermycin. Each coumarine ring of the coumermycin bind a GyrB, leading to dimerization of the protein and thus the activation of the receptor and downstream signaling processes. The switch is turned OFF by the administration of novobiocin, upon which the single coumarine ring of the novobiocin competitively inhibits binding of coumermycin to GyrB, returning the protein to its monomeric state.

In this study the vascular endothelial growth factor A isoform 121 (VEGF), essential for angiogenesis and vasculogenesis, was used as an example protein for the characterization of the switch. In order to generate a monomeric version of VEGF the cysteine residues on positions 51 (C51) and 60 (C60) responsible for the formation of two antiparallel intermolecular disulfide bonds were substituted with alanine residues (VEGF (C51-A, C60-A)) using site-specific mutagenesis. The resulting gene was fused to a flexible glycine-serine

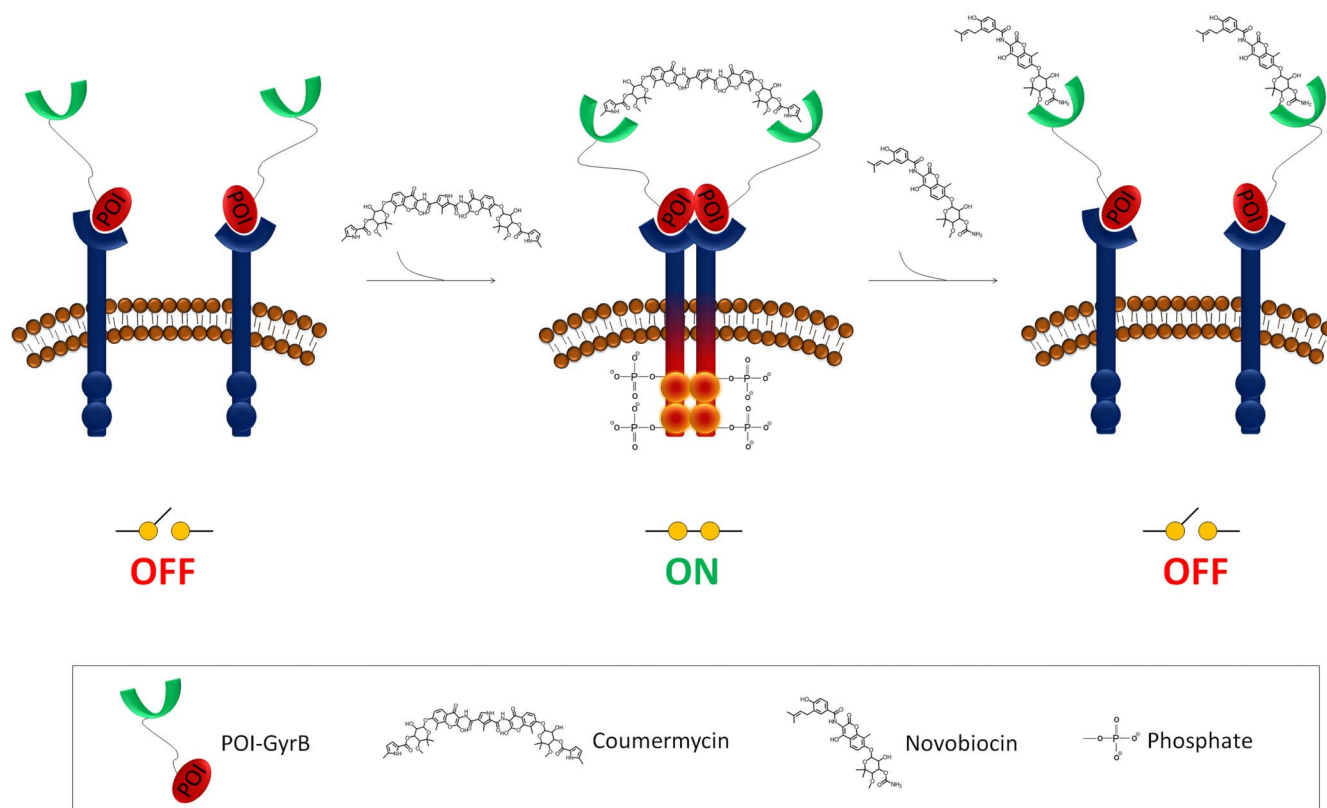


Figure 1 | Schematic representation of the protein switch. The protein switch is based on a chimeric protein consisting of an engineered monomeric variant of the protein of interest (POI) that is fused to the inducible dimerization domain of the bacterial protein gyrase B (GyrB). The switch is OFF in its default state; the monomeric configuration of the switch prohibits dimerization and thereby activation of the growth factor receptor. The switch is turned ON upon the addition of coumermycin. Each coumarin ring of the coumermycin binds one GyrB, leading to the dimerization of the growth factor and subsequently phosphorylation of the receptor and activation of the downstream signaling pathways. The switch is turned OFF by the addition of an excess novobiocin amount. The coumarin ring of the novobiocin competitively inhibits binding between coumermycin and GyrB whereby the switch returns to an inactive monomeric state.

encoding linker (gs-linker) followed by the gene encoding for the N-terminal domain of GyrB and the sequence for a hexahistidine tag (His₆) (VEGF_{SWITCH}: VEGF (C51-A, C60-A)-gs-linker-GyrB-His₆) (Supplementary Information (SI) Fig. 1a). VEGF_{SWITCH} was produced as a soluble cytoplasmic protein in *Escherichia coli* and purified by immobilized-metal affinity chromatography (SI Fig. 1b). The non-essential cysteine residue on position 116 is known to form a third interchain disulfide bridge, but was in this study not mutated since it is suggested to improve protein stability⁹. In order to avoid spontaneous dimerization, the free cysteines were alkylated and the monomeric fraction of the switch was subsequently isolated using size exclusion chromatography (SI Fig. 1c).

Inducible dimer- and monomerization of the switch were characterized by size exclusion chromatography. The molecular weight peak at 50 kDa shown in Fig. 2a confirms the monomeric nature of the switch (VEGF_{SWITCH-OFF}). The addition of coumermycin shifts the molecular weight peak to 100 kDa, indicating an inducible transition to a dimeric state (VEGF_{SWITCH-ON}). By subsequently adding an excess amount of novobiocin the molecular weight peak returns to its initial position at 50 kDa, which demonstrates the fully reversible operation of the switch (Fig. 2a). The binding affinity of the three VEGF_{SWITCH} variants to the VEGF receptor 2 (VEGFR2) was examined by fitting the saturation curve $y = xB_{MAX}/(K_D + x)$ to the experimentally determined receptor-bound protein fraction (y) versus the protein concentration (x). The relative binding parameters B_{MAX} and K_D are similar for the three different VEGF_{SWITCH} variants, as seen in Fig. 2b.

In order to examine the bioactivity of the dimeric protein configuration (VEGF_{SWITCH-ON}), its ability to stimulate VEGFR2-phosphorylation in human umbilical vein endothelial cells (HUVECs) was compared to that of intact VEGF without the GyrB domain (VEGF) and intact VEGF fused to GyrB (VEGF_{GyrB}) using Western blotting (Fig. 3a) with GyrB as a negative control. It can be seen that VEGF_{SWITCH-ON} is bioactive, albeit at a lower level than VEGF and VEGF_{GyrB} at equal concentrations. However, by increasing the VEGF_{SWITCH-ON} concentration similar activity levels as for VEGF and VEGF_{GyrB} were reached (Fig. 3a). In addition, the ability of VEGF_{SWITCH-ON} to dose-dependently activate downstream protein expression in HUVECs was confirmed by examining the endogenous delta like ligand 4 (DLL4) levels in the cells after stimulation with VEGF_{SWITCH-ON} at various concentrations (Fig. 3b).

VEGF-governed endothelial cell migration is a complex process that relies on the coordination between numerous signaling pathways¹⁰. As this process is a key component in angiogenesis, methods enabling the study and regulation of endothelial cell migration are highly valuable for both basic research and tissue engineering. Here we demonstrate the use of VEGF_{SWITCH} for time-resolved stimulation of three-dimensional migration of HUVECs assembled into microtissues under *in vivo*-like conditions. As shown in Fig. 4a, only background migration of the cells from the microtissue is observed when the switch is in its default OFF-state, i.e. without any inducer. The addition of coumermycin turns the switch ON, resulting in stimulated cell migration (Fig. 4b). Fig. 4c and Fig. 4d show the microtissues after turning the switch OFF by administrating

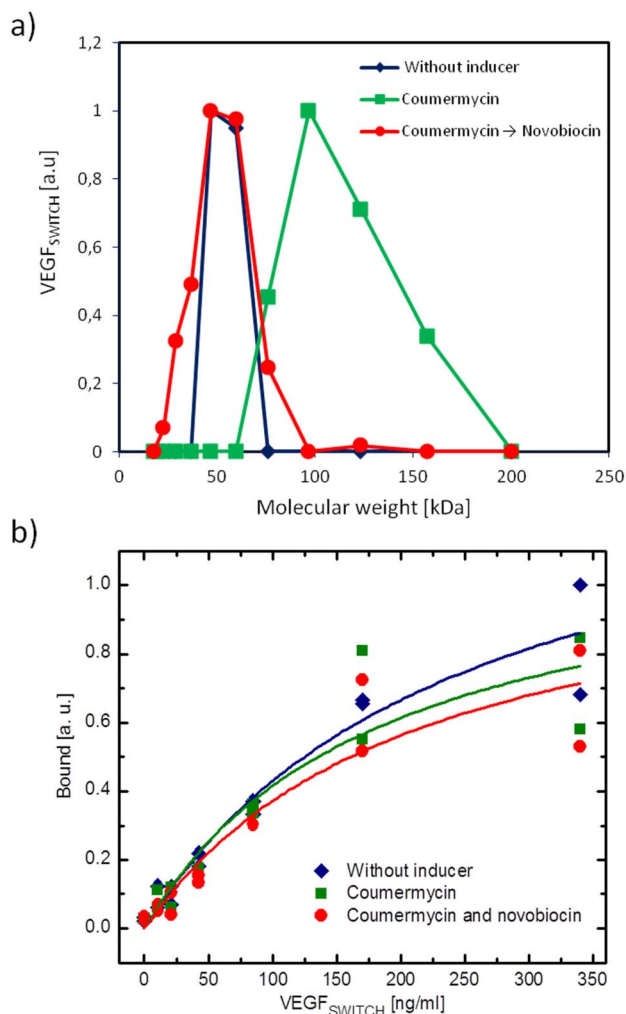


Figure 2 | Characterization of inducible dimer- and monomerization and receptor binding affinity. (a) Inducible dimer- and monomerization of the switch. VEGF_{SWITCH} was incubated at room temperature without or with coumermycin for 60 min, or with coumermycin for 30 min followed by 30 min with novobiocin added. The resulting molecular weights were determined using size exclusion chromatography. (b) Receptor-ligand binding affinity. Saturation curve for the binding of the different VEGF_{SWITCH} variants to VEGFR2.

novobiocin immediately respectively 1 hour after the coumermycin. The immediate addition of novobiocin prevents any migration, whereas limited migration is observed in the case where the cells were stimulated for a time period of 1 hour.

Discussion

We have reported on the construction of a protein switch providing ON-OFF regulation of the protein activity, which enables stimulation of the target process over a precise period of time. The switch was realized by engineering a VEGF version, which is inactive in its default monomeric state and active in its dimeric state. We showed inducible dimerization and subsequent re-monomerization of the switch and confirmed bioactivity in the dimerized ON-state by receptor phosphorylation as well as stimulated downstream protein expression. ON-OFF regulation was demonstrated by using the switch for activating and deactivating the three-dimensional migration of HUVECs in a time-resolved and tunable manner.

Compared to existing methods for time-resolved growth factor signaling based on controlled receptor dimerization^{1–3}, our switch has the advantage of being gene transfer-free and thus avoids the associated side-effects^{4,5}. VEGF variants that bind to their receptor

but fail to activate it are known to function as antagonists¹¹. In potential *in vivo* applications we therefore expect excess amounts of VEGF_{SWITCH} to outcompete endogenous VEGF as it binds to its receptor in both the monomeric OFF-state and in the induced dimeric ON-state. The present switch design may serve as a blueprint for the construction of a wide range of protein switches using other dimeric growth factors, primarily those found in the cysteine-knot superfamily. The precise ON-OFF operation of the switch illustrates its potential as a tool for elucidating the complex temporal orchestration of the signaling pathways underlying endothelial cell migration, as well as for the guidance of angiogenic processes in tissue engineering.

Methods

Construction of expression vectors. The vascular endothelial growth factor isoform 121 (VEGF) was amplified from plasmid pBM043¹² and the cysteine residues on amino acid positions 51 and 60 were replaced with alanine residues using site-specific mutagenesis. The encoding sequence for the N-terminal domain of the bacterial gyrase subunit B (GyrB) protein and the sequence for a hexahistidine tag (His₆) were amplified from plasmid pWW873¹³. The two fragments were fused together via a glycine-serine flexible encoding linker sequence (gs-linker) and cloned into the vector pWW301¹⁴, resulting in the bacterial T7 promoter-based expression vector pLMK550 for the production of VEGF_{SWITCH} (VEGF (C51-A, C60-A)-gs-linker-GyrB-His₆) with the following amino acid sequence:

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MAPMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDFQYEPDEIEYIFKPS
AVPLMRCGGACNDEGLECVPTESITMQIMRIKPHQGQHIGEMSFLOHNKCE
CRPKKDRARQEKCDKPRRDIGGGGSGGGGSGGGGSSARMSNSYDSSSIKVLK
GLDAVRKRPGMYIGDIDDGTGLHHMVFEVVDNAIDEALAGHCKEIVTI
HADNSVSVQDDGRGPTGIHPPEEGVSAAEVIMTVLHAGGKFDNDNSYKV
SGGLHGVGVSVVNALSQKLELVIQREGKIHRQIYEHGVPQAPLAVTGETEK
TGMTVRFWPSLETFTNVTEFEYELAKRRLRELSFLNSGVSIRLRDKRDGKED
HFHYEGHHHHHHH.
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VEGF, bold and italics; Mutations, bold, italics and underlined; GS-linker, underlined; GyrB, bold; his₆, italics.

The expression vector for a non-mutated version of the fusion protein was cloned in the same way as pLMK550 except that the site-specific mutagenesis was excluded, resulting in plasmid pLMK619 for the expression of VEGF_{GyrB} (VEGF-gs-linker-GyrB-His₆). The construction of the VEGF^{15,16} and the GyrB¹³ expression vectors has been described previously. Details regarding protein production and size exclusion chromatography are given in the Supplementary Information.

Inducible dimerization and monomerization of VEGF_{SWITCH}. The monomeric structure, inducible dimerization, and reversible monomerization of the switch were characterized by incubating the protein (40 ng ml⁻¹) at room temperature with, respectively, dimethyl sulfoxide alone (DMSO, Pan Biotech, Aidenbach, Germany, cat. no. P60-36720100) (0.23% v/v) for 60 min, 1 ng ml⁻¹ coumermycin (Sigma-Aldrich, Steinheim, Germany, cat. no. C9270) dissolved in DMSO, or with 1 ng ml⁻¹ coumermycin for 30 min followed by 30 min with 1.8 μg ml⁻¹ novobiocin (Carl Roth, Karlsruhe, Germany, cat. no. C247.3) added. The protein molecular weights resulting from the different incubation conditions were subsequently analyzed by size exclusion chromatography according to the above described protocol. The VEGF concentrations in the fractions eluted between 62.5 ml and 92.5 ml, corresponding to molecular weights ranging from 14 kDa to 200 kDa, were quantified via VEGF ELISA (Peprotech, Hamburg, Germany, cat. no. 900-K10). The coumermycin and novobiocin concentrations given above were used in all subsequent experiments.

Receptor-ligand binding affinity. The receptor-ligand binding affinity experiment was performed by ELISA. VEGFR2 (Life Technologies, Karlsruhe, Germany, cat. no. PV3660) was diluted in Tris-buffered saline (TBS; 50 mM Tris pH 7.8 and 150 mM NaCl) to a final concentration of 4 ng ml⁻¹ and 100 μl were added to each well of a 96-well ELISA plate (Corning, Lowell, MA, cat. no. 3590) followed by overnight incubation at room temperature. The plate was washed three times with TBS containing 0.05% (v/v) Tween-20; the same procedure was used for all subsequent washing steps. Non-specific binding sites were blocked by incubation for 2 h with 300 μl blocking buffer (TBS containing 5% (w/v) bovine serum albumin (BSA, Fluka, Buchs, Switzerland, cat. no. 05479)) after which the plate was washed. The various VEGF_{SWITCH} versions were diluted in TBS containing 5% BSA of which 100 μl were added to each well. The plate was washed after 2 h of incubation at room temperature. The receptor-bound proteins were detected according to the manufacturer's protocol using the VEGF detection antibody and the avidin-HRP conjugate antibody from the VEGF ELISA. After washing, the ELISA was developed by adding 100 μl per well of 2, 2'-azino-bis (3-ethylbenzothiazoline-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 sodium citrate, pH 4.0). The absorbance at 405 nm was determined using a Synergy™ 4 multi-mode microplate reader (BioTek® Instruments, Inc., Winooski, VT). The binding curve $y = xB_{MAX}/(K_D + x)$ was fitted to the saturation data in

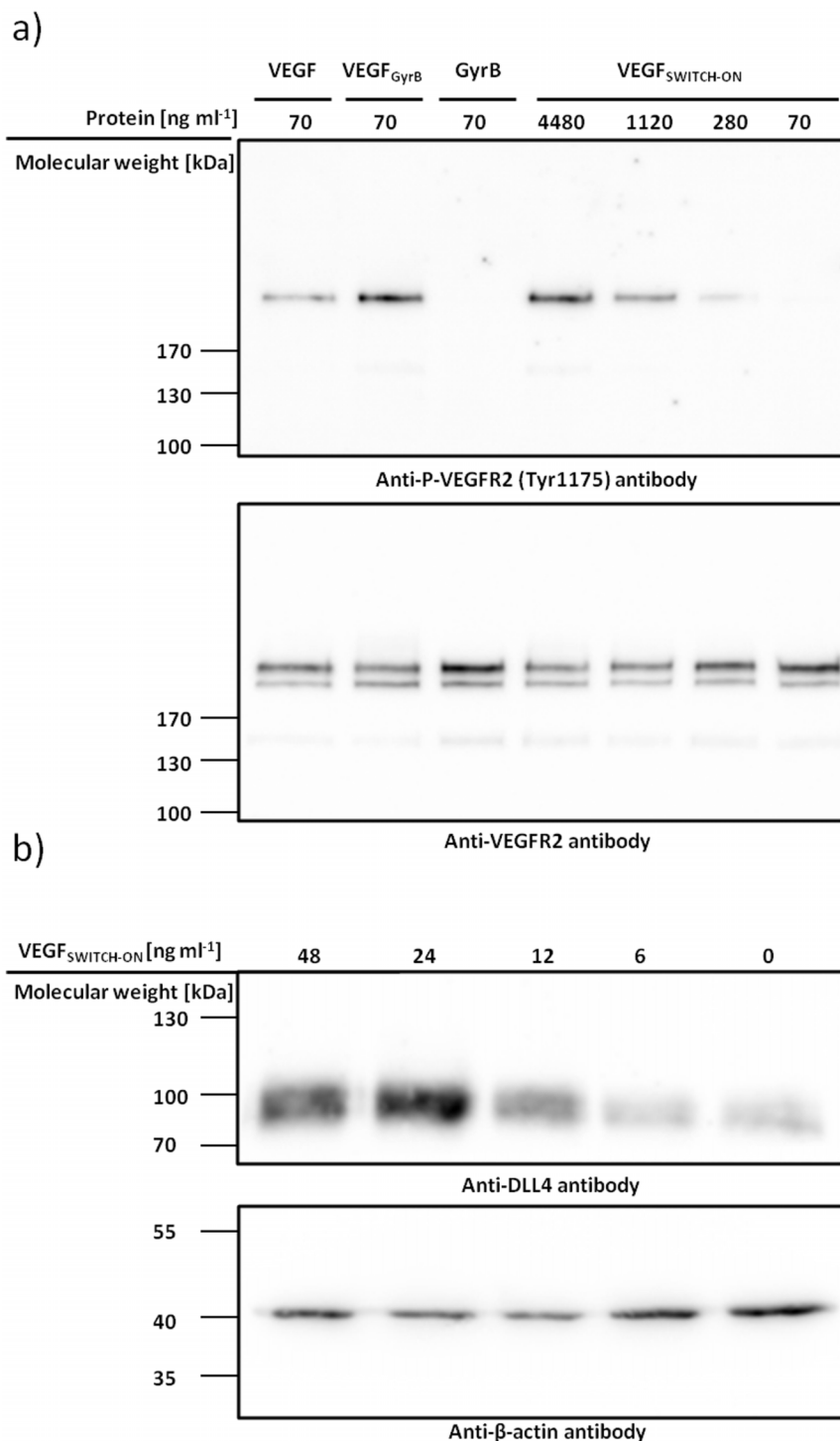


Figure 3 | VEGF_{SWITCH-ON} bioactivity. (a) Comparison of VEGFR2 phosphorylation induced by VEGF_{SWITCH-ON}, VEGF and VEGF_{GyrB} with GyrB as a negative control. HUVECs were stimulated for 15 min with the indicated protein concentrations after which whole-cell extracts were subjected to SDS-PAGE followed by Western blot analysis using an antibody against phosphorylated VEGFR2 (P-VEGFR2 (Tyr 1175))¹⁸. Subsequently, the same membrane was re-probed with an anti-VEGFR2 antibody¹⁸ for detection of the total levels of VEGFR2. (b) Stimulation of DLL4 expression. HUVECs were stimulated for 4 days with the indicated VEGF_{SWITCH-ON} concentrations after which whole-cell extracts were resolved by SDS-PAGE followed by Western blot analysis. After transfer of the proteins to a PVDF-membrane, the membrane was cut into two pieces. The upper part of the membrane (containing proteins with a molecular weight larger than 60 kDa) was probed with an anti-DLL4 antibody¹⁹. As a loading control, the levels of β-actin on the lower part of the membrane (containing proteins with a molecular weight below 60 kDa) were detected using an anti-β-actin antibody²⁰.

order to determine the relative maximum specific binding B_{MAX} and the dissociation constant K_D of the VEGF_{SWITCH} variants.

VEGFR2 phosphorylation and stimulation of DLL4 expression. Human umbilical vein endothelial cells (HUVECs, Promocell, Heidelberg, Germany, cat. no. C-12200)

were cultivated in endothelial growth medium II (Lonza, Basel, Switzerland, cat. no. C-22111). For VEGFR2 phosphorylation experiments, 150,000 cells were seeded in 1 ml of endothelial growth medium (Lonza, Basel, Switzerland, cat. no. C-22010) per well of a 12-well plate and grown to confluence over 2 days. Subsequently, the cells were starved in FCS-free endothelial growth medium containing 0.1% (w/v) BSA for

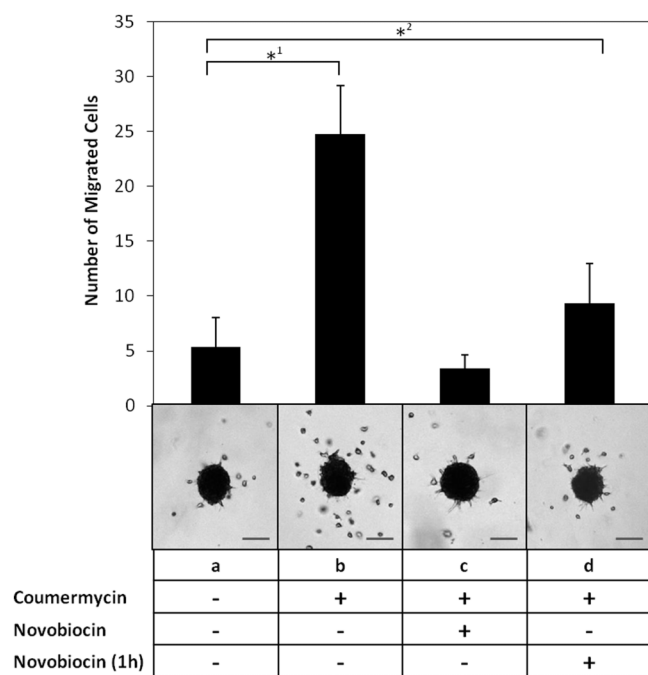


Figure 4 | VEGF_{SWITCH} for ON-OFF stimulation of HUVEC mobilization. HUVECs assembled into microtissues were incorporated into a metalloprotease-sensitive polyethylene glycol-based hydrogel and incubated together with VEGF_{SWITCH} as well as the inducers indicated in the table. The cells were fixed, stained with DAPI, and the total number of migrated cells was determined. Light microscopy images of representative spheroids and quantitative data after 15 h incubation are shown. The data represent the mean and the standard deviation of $n \geq 3$ microtissues and the asterisk indicates a statistically significant difference to the control with $p(1) = 1.0 \cdot 10^{-6}$ and $p(2) = 0.02$. Scale bar, 100 μm .

6 h. The cells were thereafter stimulated for 15 min with either 70 ng ml^{-1} VEGF, 70 ng ml^{-1} VEGF_{GyrB}, 70 ng ml^{-1} GyrB, or with 70 ng ml^{-1} , 280 ng ml^{-1} , 1120 ng ml^{-1} or 4480 ng ml^{-1} VEGF_{SWITCH-ON}. In all experiments the plates were covered with an air-permeable filter (BREATSeal, Greiner Bio-One, Frickenhausen, Germany, cat. no. 676051) to ensure a homogenous oxygen supply. After stimulation, the medium was discarded, the cells were washed with 1 ml ice-cold PBS and lysed in 90 μl ice-cold lysis buffer (Cell Signaling Technology, Danvers, MA, cat. no. 9803) containing 1.0 mM phenyl-methylsulfonyl fluoride (Sigma-Aldrich, cat. no. P-7626) for 5 min on ice. The cells were detached with a cell culture scraper and transferred to an ice-cold eppendorf tube, after which they were boiled in SDS loading buffer (2% (v/v) glycerol, 10 mM Tris/HCl, 4 g l^{-1} SDS, 0.5 g l^{-1} bromophenol blue, 2% (v/v) β -mercaptoethanol, pH 6.8) for 5 min at 95°C. The proteins were separated by SDS-PAGE (10% w/v) and phosphorylated VEGFR2 was detected by Western blotting using an anti-phospho-VEGFR2 (Tyr1175) antibody (1 : 1000, Cell Signaling Technology, cat. no. 2478) followed by incubation with an HRP-conjugated anti-rabbit antibody. As a loading control, the total amount of VEGFR2 was detected by drying the polyvinylidene difluoride (PVDF) membrane, reactivating it in methanol, and incubating it with an anti-VEGFR2 antibody (1 : 1000, Cell Signaling Technology, cat. no. 2479) followed by an HRP-conjugated anti-rabbit antibody. In all experiments the HRP was detected with Enhanced Chemiluminescent (ECL) reagent (GE Healthcare, Chalfont UK, cat. no. RPN2232) and the images were acquired using a LAS-4000 imager (Fujifilm, Düsseldorf, Germany).

For stimulation of DLL4 expression, 150,000 HUVECs were seeded in 1 ml of endothelial growth medium per well of a 12-well plate and incubated for 4 days in the absence, or in the presence of either 6 ng ml^{-1} , 12 ng ml^{-1} , 24 ng ml^{-1} or 48 ng ml^{-1} VEGF_{SWITCH-ON}. After stimulation, the cells were lysed as described above and boiled in SDS loading buffer for 5 min at 95°C. DLL4 expression was resolved by SDS-PAGE (10%, w/v) followed by Western blot analysis. After transfer, the membrane was cut into two pieces. The upper part of the membrane (containing proteins with a molecular weight larger than 60 kDa) was probed with an anti-DLL4 antibody (1 : 1000, Cell Signaling Technology, cat. no. 2589) followed by an HRP-conjugated anti-rabbit antibody; whereas the lower part of the membrane (containing proteins with a molecular weight below 60 kDa) was probed with an anti- β -actin antibody (1 : 1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, cat. no. sc-47778) followed by an HRP-conjugated anti-mouse antibody (1 : 2500, Amersham Life Science, Piscataway, NJ, cat. no. NA931).

Formation of endothelial microtissues. HUVECs were cultivated in endothelial growth medium (Lonza, Basel, Switzerland, cat. no. CC-3162) supplemented with 10% (v/v) foetal calf serum (FCS, Gibco Life Technologies, Karlsruhe, Germany, cat. no. 10500). For microtissue formation, 25'000 cells ml^{-1} were suspended in 0.2% (w/v) methyl cellulose (Sigma-Aldrich, cat. no. M0512) in DMEM:EBM (4 parts DMEM/F-12 + GlutaMAX™ (Gibco Life Technologies, cat. no. 31331-028) and 1 part endothelial basal medium 2 (EBM-2, Lonza, cat. no. CC-3156) supplemented with 1% (v/v) penicillin/streptomycin solution (Gibco Life Technologies, cat. no. 15140-122) and 10% (v/v) FCS). Droplets of 30 μl were placed in non-adhesive cell culture dishes (Greiner Bio-One, Frickenhausen, Germany, cat. no. 633180) and cultured overnight as hanging drops. The resulting spheroids were harvested in DMEM:EBM, washed once with DMEM:EBM, and 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 μM Gln-RGD peptide, 400 spheroids ml^{-1} , and 10 U ml^{-1} thrombin-activated factor XIIIa in 50 mM Tris-HCl (pH 7.6) supplemented with 50 mM CaCl₂. Droplets of 20 μl were placed between two siliconized glass slides (Sigma, Sigma-Aldrich, cat. no. SL2) using 1-mm-thick spacers. In order to prevent spheroid sedimentation, the glass slides were slowly rotated at room temperature until the onset of gelation, and subsequently incubated for 30 min at 37°C. The hydrogels were thereafter released and transferred into a 24-well plate. The final gels were incubated for 15 h in 500 μl DMEM:EBM medium at 37°C and 5% CO₂ in the presence of 1 μg VEGF_{SWITCH} ml^{-1} and either only DMSO, 25 ng ml^{-1} coumermycin dissolved in DMSO, or 25 ng ml^{-1} coumermycin and 45 $\mu\text{g ml}^{-1}$ novobiocin.

Analytics. Z-stack phase contrast images (200 μm depth) were acquired at 10 \times magnification using a Zeiss Axiovert 200 M. HUVECs mobilization was quantified by measuring the migration distance from the microtissue center. Cells with a migration distance larger than 75 μm were considered in the statistical analysis. The results were evaluated by a t-test, where p-values < 0.05 were considered to be statistically significant.

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Author contributions

The experiments were designed by M.K., B.R., P.S.L., M.E., G.R. and W.W. and the experimental work was performed by M.K., B.R., P.S.L. and N.S. The manuscript was written by M.K. and W.W.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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