Fluorescent Resonance Energy Transfer Imaging of VEGFR Dimerization

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Abstract. *Activation of vascular endothelial growth factor receptor 1 (VEGFR1/FLT1) and 2 (VEGFR2/KDR) involves receptor dimerization. Formation of VEGFR dimer has so far not been visualized in single intact cells. In the present study we describe different optical assays which can be used to observe dimerization of VEGFR1 and VEGFR2. Bimolecular fluorescence complementation (BIFC) assays confirmed homo,- and heterodimerization of transfected receptors. Fluorescence resonance energy transfer (FRET) techniques in living and fixed CHO-K1 cells allowed observation of VEGFR1 homodimer,- and VEGFR1 and VEGFR2 heterodimer formation after ligand stimulation. After inhibition of ligand binding by the VEGFA JH121 antibody VEGFR1 homodimerization was completely abolished. Under the same conditions, cells transfected by VEGFR1 and VEGFR2 maintained relevant receptor heterodimerization. These techniques to monitor VEGFR1 and VEGFR2 homo- and heterodimerization in living and fixed cells may help in the search for new angiogenesis-directed inhibitors of VEGFR dimerization.*

After reaching a critical size, tumors are dependent on angiogenesis, as supply with oxygen and nutrients by diffusion alone are no longer sufficient. Tumors that have undergone neovascularization may then enter a phase of rapid growth and also show increased metastatic potential (1). Among the multitude of endogenous and exogenous factors regulating and supporting tumor growth and progression, angiogenic factors can be considered as some of the most crucial ones (2, 3). The vascular endothelial growth factor (VEGF) family ligands and their receptors are central in angiogenic signaling. VEGF ligands such as VEGFA, VEGFB, VEGFC, VEGFD and placenta growth factor (4) may activate three receptor tyrosine kinases, VEGFR1, VEGFR2, and VEGFR3, thereby leading to formation of VEGFR homodimers and heterodimers (5) which in turn promotes angiogenesis and lymphangiogenesis (6). Some VEGFs interact with multiple receptors while others show very specific receptor binding properties (7). The ligand VEGFA binds to both VEGFR1 and VEGFR2, while VEGFB binds specifically only to VEGFR1. VEGFC and VEGFD preferentially bind to VEGFR3, but both can also bind and activate VEGFR2 (8, 9).

The downstream signaling cascade of VEGFR2 is induced by receptor dimerization and the subsequent phosphorylation of several tyrosine residues at the intracellular domain (10). This, in turn, leads to signal transduction of a series of kinases, which subsequently propagate intracellular signaling (11). It has been reported that endothelial cells in the tumor vasculature overexpress VEGFR2 at about 3- to 5-fold compared to the normal vessels (12). Therefore, VEGFR2 has become an attractive target for antitumor therapy. Several strategies, such as inhibition of VEGF ligand (13) or receptor (14-16), or even of multiple targets in the downstream signaling pathways have been developed as experimental anti-tumor therapeutic strategies (17), and there are numerous compounds which are under various stages of clinical development (18). Since receptor homo- and heterodimerization is crucial for VEGF signal transduction, this may represent another interesting target in the search for novel and effective anti-angiogenic agents. Therefore, methods to visualize dimer formation may be important. We established imaging assays which allow for visualization of receptor dimerization in single intact cells and report their use here.

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Materials and Methods

Chemicals. All chemicals were obtained from Sigma Aldrich (Seelze, Germany). The restriction enzymes were obtained from Thermo Scientific (Schwerte, Germany).

Expression constructs. All plasmid constructs were isolated using the EndoFree™ Plasmid Maxi Kit (Qiagen GmbH, Hilden, Germany). The concentration of the plasmids was determined by spectrophotometric absorbance at 260 nm.

Sequences and reading frames of all constructs derived from Polymerase Chain Reaction (PCR) products were verified by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Constructs designed for Bimolecular Fluorescent Complementation (BIFC) assay: The fragments of linear yellow fluorescent protein (YFP) truncated at the amino acid residues 173 and 155 (YN173 *N*-terminal residues 1-172; YC155 C-terminal residues 155-238 and YC173 C-terminal residues 172-238) were subcloned into the pEYFP-N1 backbone harboring *VEGFR1* or *VEGFR2* cDNA (see below) by replacing the full length YFP within the open reading frame (ORF) 1 between the *Age*I and *Not*I restriction sides. This resulted in three different constructs for each of the VEGFRs used. *VEGFR1*-YN173/YC155/YC173 and *VEGFR2*-YN173/YC155/YC173 were used as BIFC constructs as described (19). Non-complementing YFP fragments of interacting partners in the BIFC assay were included as negative controls.

Constructs designed for Fluorescent Resonance Energy Transfer (FRET) assay: cDNAs containing the coding sequences of human *VEGFR1* (amino acids 286-4302) and human *VEGFR2* (amino acids 303-4373) proteins were kindly provided by Professor L. Claesson-Welsh (20). The *VEGFR1* and *VEGFR2* cDNA fragments were PCR-amplified using Phusion Hot Start II High Fidelity DNA Polymerase (Thermo Scientific) according to the manufacturer instructions and cloned in frame into the pEYFP-N1 and pECFP-N1 vectors, containing yellow and cyan fluorescent proteins respectively (Clontech, Saint-German-en-Laye, France) resulting in *VEGFR1*-CFP, *VEGFR1*-YFP and *VEGFR2*-YFP fluorescent constructs. Primers used were optimized for the expression vectors by adding a start ATG codon, by removing the stop codon TAA and by incorporation of a XhoI site into the sense primers and a SacII site into the antisense primers (shown in bold italics): *VEGFR1*: 5'-gat***ctcgag***ATGGTCAGC TACTGGGACACCGGG-3' (sense), 3'-tata***ccgcgg***GATGGGTGGG GTGGAGTACAGGAC-5' (antisense); *VEGFR2*: 5'-gat***ctcgag***ATG AGCAAGGTGCTGCTGGCCGTCG-3' (sense), 3'-tata***ccgcgg*** AACAGGAGGAGAGCTCAGTGTGGTCCC-5' (antisense).

PCR was performed under the following reaction conditions: Initial denaturation at 98˚C for 30 s, 25 cycles of 98˚C for 5 s, 69˚C for 15 s (for *VEGFR1*) or 74˚C for 15 s (for *VEGFR2*) and 72˚C for 75 s, with a final 5 minutes extension at 72˚C.

The DNA constructs used for positive control FRET experiments in fluorescence microscopic studies were carrier molecules with enhanced YFP (eYFP) and eCFP fluorescent proteins linked by 28 amino acids chain and full-length wild-type (wt) human endoglin (amino acids 1-658, Endo^{wt}) tagged to the *N*-terminal eYFP and eCFP sequences of the pEYFP-N1 and pECFP-N1vectors (Clontech) resulting in Endo-YFP and Endo-CFP, respectively (19).

Cell culture/transfection. Cell media and fetal bovine serum were purchased from PAA Laboratories (Cölbe, Germany). CHO-K1 cells were ordered from DSMZ (Braunschweig, Germany). Cells were cultured and maintained in Hams F12 medium with L-glutamine supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator at 37˚C with 5% CO_2. For live-cell fluorescence microscopy, cells were cultured on glass bottom FluoroDishes (World Precision Instruments, Berlin, Germany). For BIFC and other fixed cell imaging experiments, cells were cultured on 18×18 mm coverslips (Menzel-Glaeser, Braunschweig, Germany) plated in 6-well dishes (Greiner Bio-One, Frickenhausen, Germany).

Plasmids were transfected into CHO-K1 cells using the Fugene HD (Promega, Mannheim, Germany) transfection reagent according to the manufacturer's protocol. After transfection, cells were selected using 400 μg/ml G418 (Sigma Aldrich).

Fluorescent immunostaining. Antibodies to human VEGFR1 and human VEGFR2 were obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany). Rhodamine conjugated bovine anti-goat IgG-R was purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Expression of the transfected cDNAs was analyzed by immunocytochemistry using standard protocols 48 h after transfection. In brief, after fixation with methanol and blocking in phosphate buffered saline (PBS)/5% FBS, cells were incubated for one hour with the diluted primary antibodies to human VEGFR1 or human VEGFR2 (1:100 in PBS/1% FBS). For negative control, transfected cells were stained omitting the primary antibody. Nuclei were counterstained for 10 minutes with 4',6-diamidino-2-phenylindole (DAPI). Thereafter, cells were mounted on Mowiol (+Dapco) (Carl Roth, Germany) for imaging. Additionally, empty vector-transfected and non-transfected cells were included as controls using the full staining protocol.

Stimulation and inhibition experiments. Stimulation and inhibition experiments were performed using CHO-K1 cells that were co-transfected with VEGFR1-CFP and VEGFR1-YFP or alternatively with VEGFR1-CFP and VEGFR2-YFP. Transfected cells were stimulated with different doses of recombinant human the short isoform of VEGFA VEGF121 (R&D Systems), hereafter referred to as VEGF, after overnight starvation in standard medium supplemented with 1% FBS. A subset of the stimulation experiments was conducted as time-lapse experiments with image acquisition and fluorescence resonance energy transfer imaging (FRET-imaging) analysis after 5, 10, 15 min of stimulation. For the inhibition assays, VEGF-containing media supplemented with 10, 20 or 100 ng/ml VEGF121 were pre-incubated for one hour with 10 μg/ml neutralizing VEGFA JH121 antibody (Thermo Scientific).

Fluorescence microscopy.

FRET: FRET measurements were performed on transfected living and fixed CHO-K1 cells. To reduce the background signal, cells were incubated at room temperature in a HEPES-buffered saline (140 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$; 2 mM $MgCl_2$; 10 mM HEPES; 10 mM glucose; 0.1% bovine serum albumin; pH 7.50) prior to microscopy.

For microscopy, an inverted wide-field Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) and Axiovision Version 4.8 software were used. Images were acquired with a Zeiss AxioCam-HR 16-bit CCD camera (Carl Zeiss AG Camera Lens Division, Oberkochen, Germany) running in 2×2 binning mode and with appropriate filter sets for the different fluorophores.

BIFC: After co-transfection of two complementary *VEGFR1* or *VEGFR2* BIFC constructs, which resulted in the complementation of the two EYFP fragments upon dimerization of the monomers, dimer formation was visualized by fluorescence microscopy of fixed cells using filters for YFP and DAPI.

FRET measurement. FRET signals were calculated according to Zal and Gascoigne (21) on a pixel-by-pixel basis through image mathematics. The measurement was carried out by sequential acquisition of three channels: CFP channel (donor excitation and donor emission), YFP channel (acceptor excitation and acceptor emission) and FRET channel (donor excitation, acceptor emission). All measurement parameters, such as camera exposure time, gain and excitation intensities, remained constant throughout the entire experiment. Crosstalk or bleedthrough of fluorescence between the donor and acceptor emission spectra were calculated and images were background corrected. Using previously defined crosstalk coefficients, FRET efficiency was calculated according to Zal and Gascoigne (21): $Eapp={}^{R}/_{R+G}$ where R is a ratio of sensitized emission to donor (CFP) fluorescence during donor excitation and G is a ratio of the sensitized emission to the corresponding amount of donor recovery in the donor channel after acceptor (YFP) photobleaching.

Acceptor photo bleaching (APB). Paraformaldehyde-fixed cells were used for analysis. To evaluate photobleaching compensation, images were taken using the donor (CFP) filter. Thereafter, the acceptor (YFP) was photobleached for 1 minute using the YFP excitation filter. This was repeated in rounds, for a total of 8 min photobleaching time. To calculate the effect of bleaching on the donor chromophore (CFP), only pECFP-transfected cells were used as controls.

Data analysis/statistics. Analyses were carried out using Image J Version 4.8 software. All experiments were performed at least in triplicate. Frequency distribution tests were calculated using Prism 6 software (GraphPad Software Inc, California, USA) and expressed as means.

Results

EYFP-tagged VEGFRs exhibit membrane and peri-nuclear localization in transiently-transfected CHO-K1 cells. The functionality of the cloned cDNA sequences coding for the two VEGFRs (*VEGFR1*, *VEGFR2*) were examined as these sequences were used in all DNA expression constructs throughout the different experiments in this study.

Expression and cellular localization of the VEGFR proteins in transfected cells were analyzed using immuno-fluorescence. In order to distinguish transfected from non-transfected cells within the same sample and to evaluate reactivity of the antibodies, we used VEGFR carrying a *C*-terminal EYFP or ECFP tag. All EYFP/ECFP-positive cells exhibited strong VEGFR staining compared to non-transfected cells within the same sample. Transfected CHO-K1 cells demonstrated strong membranous and a punctuated cytoplasmic staining, with an accumulation in the perinuclear region, verifying proper expression of the transfected *VEGFR* cDNAs. Cells transfected with a mock-EYFP vector did not exhibit any VEGFR antibody-specific staining. Other negative controls created by omitting the primary antibodies did not show non-specific reaction of the secondary antibody with the cells (Figure 1).

BIFC assay demonstrated that transfected VEGFRs dimerize in CHO-K1 cells. In order to demonstrate that the VEGFR cDNA constructs used do indeed dimerize after transfection, a BIFC assay was used. In these assays, complementation of the truncated YFP fragments after dimerization results in fluorescence signal that can be detected.

By tagging truncated YFP fragments to the C-terminus of the respective VEGFRs, three compatible combinations were obtained. BIFC signals for both homodimerization between VEGFR1 proteins (Figure 2a), and heterodimerization between VEGFR1 and VEGFR2 proteins (Figure 2b) were confirmed using the YN173 and YC155 constructs, thus demonstrating that transfected VEGFRs indeed dimerized in CHO-K1 cells.

FRET controls. For positive control, eYFP and eCFP connected by a 28-amino-acid length on the same carrier molecule were used (intra-FRET). As control for inter-molecular FRET (inter-FRET), full-length wt endoglin (amino acids 1-658, $Endo^{wt}$) tagged with eYFP and eCFP were used. Here, donor and acceptor were attached to different endoglins which readily interact to create homodimers. Both positive controls demonstrated FRET, thus defining the maximum of the dynamic range of the optical assay. For negative control, cells were co-transfected with pEYFP-N1 and pECFP-N1, *VEGFR1*-YFP and pECFP-N1 or with *VEGFR2*-YFP and pECFP-N1. In these controls, no FRET was observed (Figure 3a).

Using APB, the positive control groups exhibited a 15% and 5% increase of CFP and a 80% or 60% loss of YFP intensity for the intra- and inter-FRET controls, respectively (Figure 3b).

In the frequency distributions of the FRET efficiency, nearly 100% of the negative-control group gave a calculated FRET efficiency of between 0 and 1, with a peak at 0.5. More than 90% of the positive inter-FRET controls were distributed between 1.0-4.0, with a plateau between 2.0 and 3.0. The intra-FRET positive control group exhibited the highest FRET efficiency but also the broadest frequency distribution, with FRET efficiency values of between 12 and 20 (Figure 3c).

VEGF-induced VEGFR dimerization by FRET. In order to visualize dimerization between VEGFR1 and VEGFR2 proteins, we generated fusion proteins with CFP and YFP. After receptor dimerization, the fluorophores are brought in spatial proximity and thus were expected to undergo FRET. For homodimerization, cells were co-transfected with *VEGFR1*-CFP and *VEGFR1*-YFP. To demonstrate receptor

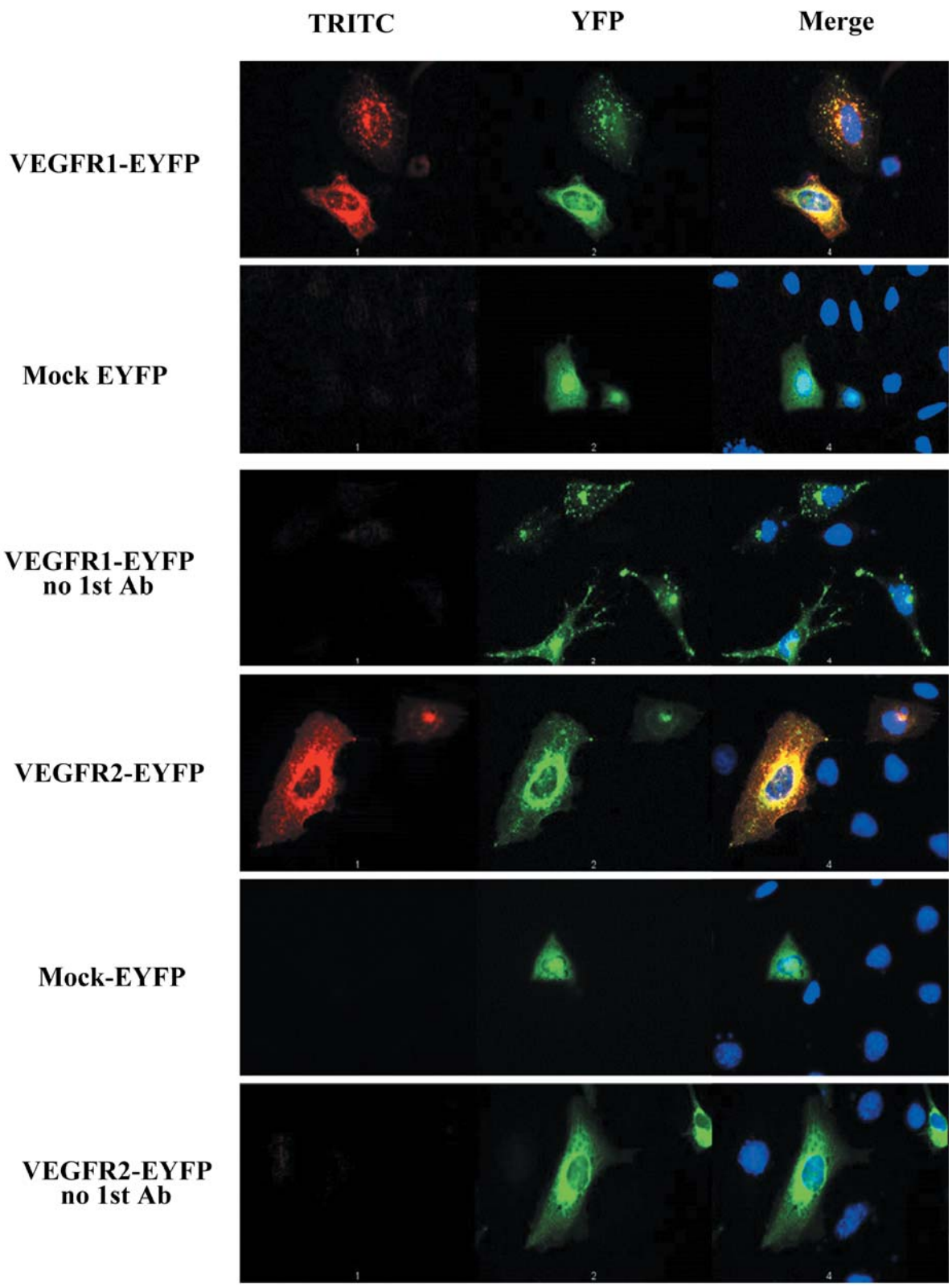

Figure 1. *Antibody staining and localization of vascular endothelial growth factor receptor (VEGFR) in CHO-K1 cells. CHO-K1 cells were transiently transfected by the enhanced yellow fluorescent protein (EYFP)-tagged VEGFR constructs, as indicated and immunostained after 48 h. Only cells transfected by EYFP-VEGFR constructs showed a corresponding TRITC staining. Fluorescent images are presented in pseudocolours.*

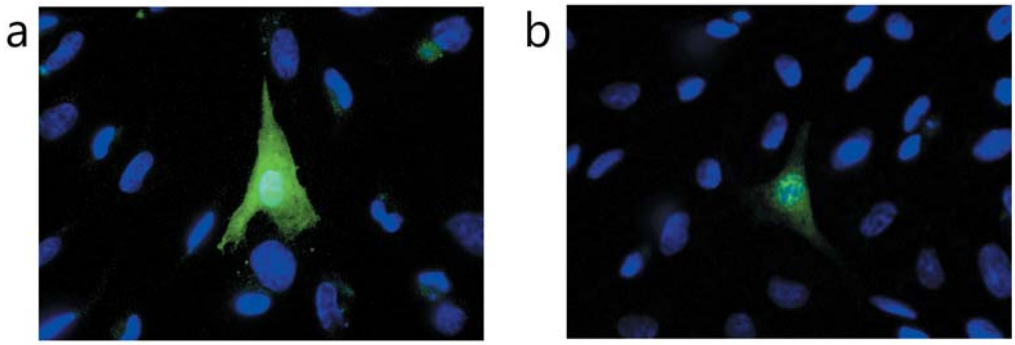

Figure 2. *Bi-molecular fluorescent complementation (BIFC) assay to monitor VEGFR dimerization. CHO-cells were transfected by the complementary BIFC constructs VEGFR1-YN173 and VEGFR1-YC155 to show VEGFR-1 homodimerization (a). To demonstrate heterodimerization of VEGFR1 and VEGFR2 CHO cells were transfected by VEGFR1-YN173 and VEGFR2-YC155 (b). Dimerisation of the corresponding constructs resulted in the complementation of the two enhanced yellow fluorescent protein (EYFP) short fragments upon dimerization of the receptor monomers in co-transfected CHO-K1. Dimer formation was visualized by fluorescence microscopy of fixed cells using filters for YFP and DAPI.*

heterodimerization, cells were co-transfected with *VEGFR1*-CFP and *VEGFR2*-YFP. FRET in all imaging experiments was determined by three-channel sensitized emission imaging and, for confirmation, by APB.

When compared to the positive (inter-FRET with End^{WT}) and negative controls, the FRET efficiency distribution for both VEGFR1 homodimerization, showed a major peak between 0.5 and 1.2 (frequency of 60%) for VEGFR1 homodimerization. The frequency distribution for VEGFR1 and VEGFR2 heterodimerization showed a major peak between 0.5 and 1.2, containing 35% of all cells, with a wide distribution up to 4.0 (including 90% of all cells), which was significantly higher ($p<0.05$) than that of the negative controls (0.5) and lower than that of the positive controls (Figure 4a).

VEGFR1 and VEGFR2 fluoroprotein-expressing CHO-K1 cells were stimulated by VEGF and fixed. The acceptor fluorophore was then photobleached. The intensity of YFP decreased which in turn increased the donor intensity due to lower FRET (Figure 4b).

Dose- and time-dependent VEGF stimulation of homo- and heterodimerization between VEGFRs. To promote dimerization of VEGFR monomers, transfected cells were stimulated by different doses of VEGF, as this ligand is known to bind to both VEGFR1 and VEGFR2. The effect on homodimerization of VEGFR1 and heterodimerization among VEGFR1 and VEGFR2 proteins was imaged. VEGF induced a dose-dependent increase of VEGFR1 homodimers up to a dose of 20 ng/ml, resulting in mean FRET efficiencies of 1.25 and 1.55, respectively. Compared to 20 ng/ml VEGF, stimulation of the cells with 100 ng/ml VEGFA resulted in a markedly lower mean FRET efficiency of 0.95. In contrast, VEGFR1-VEGFR2 pair stimulation showed a marked FRET efficiency of 1.6 even without VEGF, which was not significantly altered by VEGF stimulation, indicating that even without specific stimulation, VEGFR1 and VEGFR2 monomers form heterodimers (Figure 5a).

When cells were stimulated by 10 ng/ml, 20 ng/ml and 100 ng/ml of VEGF for 5, 10, 15 min, respectively, maximum dimer formation was found after 5 min of stimulation.

Neutralizing of VEGF does not suppress VEGFR2 heterodimerization. A (VEGF)-antibody Ab-3 was used which is a competitive inhibitor of VEGFA binding to its receptors. Pre-incubation with the neutralizing antibody reduced FRET efficiency for VEGFR1 homodimerization. No significant reduction of the FRET efficiency for VEGFR1 and VEGFR2 heterodimers was observed (Figure 5b).

Discussion

VEGFs are overexpressed in the vast majority of human tumors so far examined, including those of the brain (22), breast (23, 24), lung adenocarcinoma (25) and gastrointestinal tract (2). In parallel, VEGFRs have been shown to be expressed in different tumor cell types (26, 27) enabling for autocrine stimulation of tumor cell function (28, 29) in addition to the pro-angiogenic function of VEGF on endothelial cells. In mouse models, VEGFA has been shown to be the dominant pro-angiogenic factor involved in the angiogenic switch. Inhibition of its function significantly impaired the formation and growth of solid tumors (30). Since the formation of tumors is dependent on angiogenesis, several strategies have been developed which all target the VEGF signal-transduction pathways. These involve antibodies against VEGF and against VEGFRs, as well as VEGFR tyrosine

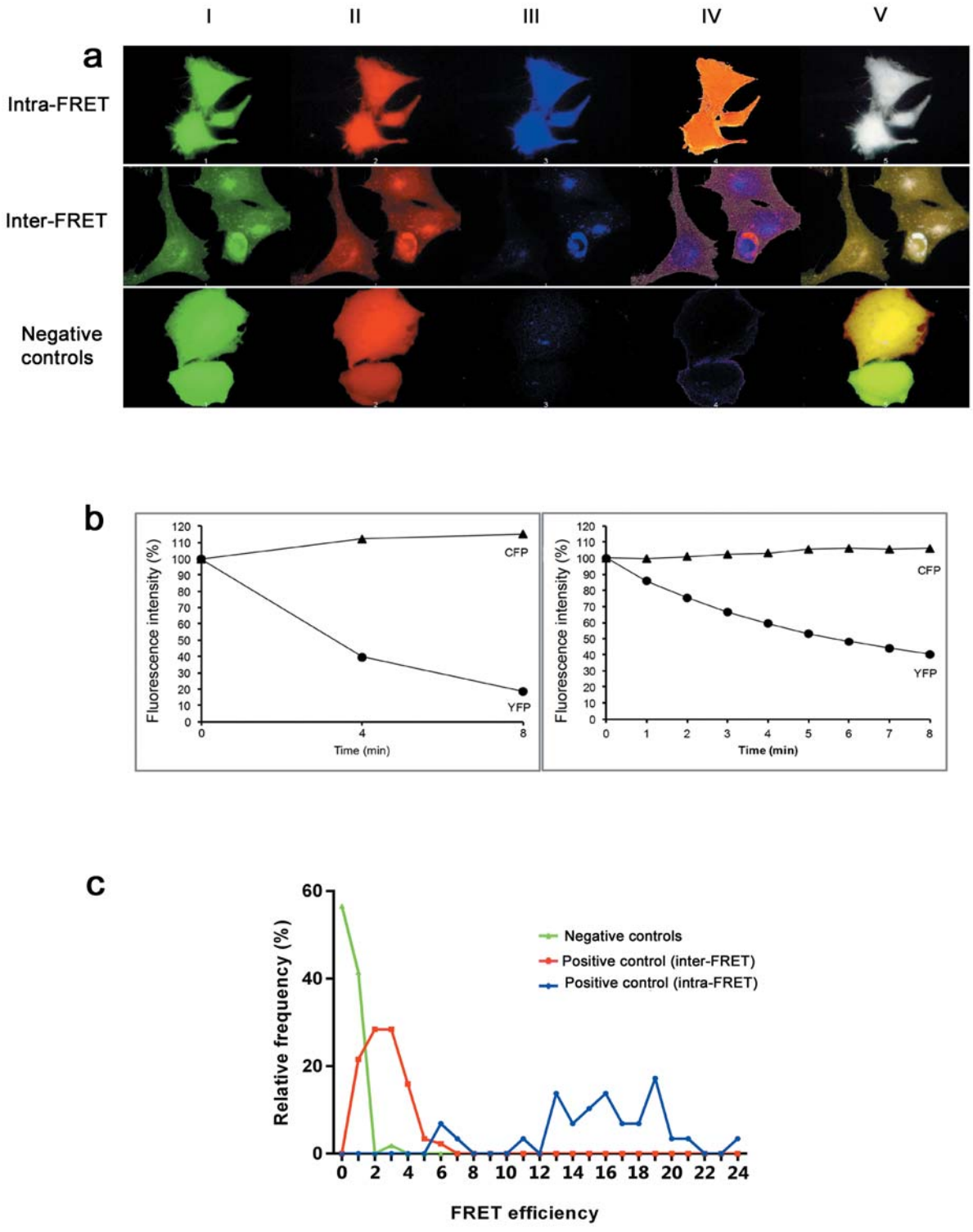

Figure 3. *Fluorescence resonance energy transfer (FRET) imaging controls. a: Pseudo-color images were acquired under the three filter sets: yellow fluorescent protein (YFP) (I), cyan fluorescent protein (II), and filter corrected FRET (III). After subtraction of background and bleed-through signals, net FRET (IV) was localized to the nucleus and cytoplasm in positive intra-FRET (first row), inter-FRET (second row) and weak signals in the negative controls (third row). RGB merged image (V) describes superimposition of YFP, CFP and FRET channels. b: Positive control groups assayed by acceptor photo bleaching showed a loss of YFP and gain of CFP intensity for the intra- (left) and inter-FRET (right) controls, demonstrating FRET. c: FRET efficiency of each control group plotted against the number of events.*

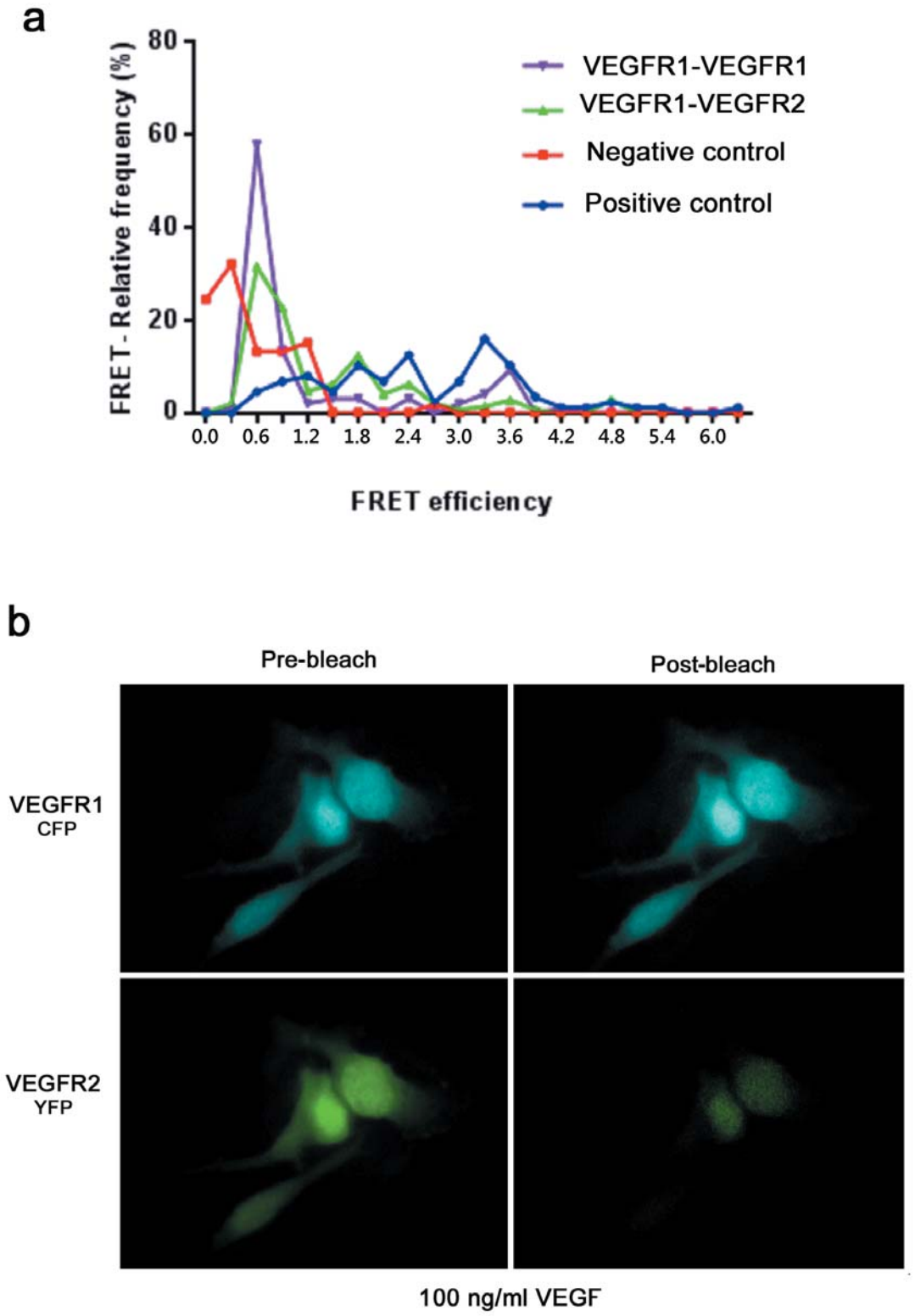

Figure 4. *Fluorescent resonance energy transfer (FRET) efficiency for formation of vascular endothelial growth factor reseptor (VEGFR1) homodimers and VEGFR1–VEGFR2 heterodimers in CHO-K1 cells. a: FRET efficiency of all groups plotted against the number of events. Negative and inter-FRET positive (with End^{WT}) controls are also shown. b: Pre-bleach and post-bleach images from an acceptor photobleaching assay of fixed CHO-K1 cells co-expressing VEGFR1 and VEGFR2.*

kinase inhibitors (31). The humanized monoclonal antibody bevacizumab (Avastin) is the main therapeutic agent currently used in clinical practice. As a selective VEGF inhibitor, bevacizumab is used alone or in combination with standard chemotherapy (32). Despite a proven clinical benefit, recent studies showed that bevacizumab monotherapy brought only limited measurable effect *in vitro* (33). Additionally, chronic inhibition of VEGF signaling in colorectal cancer may lead to an increase in cell migration and invasion *in vitro*, and metastasis *in vivo* (13). Clinically, anti-VEGF treatment seems to be especially effective when begun shortly after tumor initiation, or before the development of advanced malignancy (34). While VEGFA blocking may slow tumor angiogenesis, it does not slow the growth of lymphatic vessels (lymphangiogenesis) which provide an alternative route for cancer cell dissemination (35). Moreover, treatment with bevacizumab was associated with an increased expression of VEGFC and VEGFD in metastatic colon cancer upon disease progression (36), and in glioblastoma cells (37), which may represent a mechanism for acquiring resistance to bevacizumab. Antibodies which specifically target the VEGFR ligand-binding site are another therapeutic approach. Ramucirumab (IMC-1121B) is a fully-humanized therapeutic antibody specifically developed against VEGFR2 which demonstrated survival benefits in second-line monotherapy of patients with gastric cancer (38). Alternatively, receptor kinase inhibitors such as sunitinib and sorafenib are employed which target the intracellular region of VEGFRs and competitively inhibit the ATP-dependent phosphorylation of their tyrosine residues. These have demonstrated clinical benefit in various types of cancer, such as renal cancer, hepatocellular carcinoma and gastrointestinal stromal tumors (39), however, a significant number of treated patients experience disease progression (40-42). In addition, such inhibitors potentiated metastasis and tumor cell extravasation in a syngenic mouse model, an effect not shown by antibodies against VEGF or VEGFRs (43). Therefore the real clinical efficacy of these agents for other tumor entities remains to be shown.

Despite extensive pre-clinical and clinical research on strategies which inhibit VEGF or the VEGF pathway, there are currently no validated agents which selectively target VEGFR2 dimerization, although dimerization is a key regulator of VEGF signal transduction: Guided by the binding properties of the ligands, the VEGFRs are able to form both homodimers and heterodimers (44). As for other growth factor receptors, dimerization of receptor monomers is a critical event in ligand-induced signal transduction. Dimerization of receptors is accompanied by activation of the receptor-kinase activity that leads to the autophosphorylation of the receptors. Phosphorylated receptors recruit interacting proteins and induce the activation of signaling pathways that involve their specific array of second messengers (20). Signaling may either be induced by binding of ligand to one receptor monomer and subsequent coupling of a second receptor monomer to this complex (45), or by binding of ligand to already pre-assembled receptor dimers (46). In our study, stimulation with VEGF induced formation of VEGFR1 homodimers, whereas VEGFR1–VEGFR2 heterodimers were found to be already pre-assembled and their formation was not increased by VEGF stimulation. Ligand-induced formation of VEGFR dimers has been described by a variety of groups (4, 47, 48) but pre-assembly of VEGFR1–VEGFR2 heterodimers has also been reported (49). In the latter report, stimulation with VEGFA also did not elicit increased heterodimer formation, which our findings support. These VEGFR hetero- and homodimers do possess different transduction efficiencies and signaling outcomes. VEGF signaling through VEGFR1–VEGFR2 heterodimers efficiently induced phospholipase Cγ phosphorylation and inositol phosphate accumulation, whereas VEGFR1 homodimers only poorly-induced these responses (48). Using a synthetic ligand that specifically binds to VEGFR1–VEGFR2 heterodimers, it was demonstrated that VEGFR1–VEGFR2 signaling induced migration tube formation of endothelial cells, but did not mediate proliferation and tissue factor production, supporting findings that these functions are dependent on signaling *via* VEGFR2 homodimers. Moreover, signaling through VEGFR1–VEGFR2 heterodimers impaired VEGFA-induced prostacyclin release, extracellular signal-related kinase 1 and 2 phosphorylation and Ca^{2+} mobilization, indicating a role of VEGFR1–VEGFR2 signaling in the regulation of endothelial cell homeostasis (44). In addition heterodimers of VEGFR2 with VEGFR3 have been detected in developing blood vessels and in lymphatic structures in embryoid bodies, and inhibition of receptor dimerization markedly reduced angiogenic sprouting (4); formation of VEGFR2–VEGFR3 heterodimers is essential for VEGFR3 activity induced by VEGFC and -D (47).

Therefore, in theory, inhibition of VEGFR dimer formation would potently impair VEGF signaling. In addition, inhibition of VEGFR dimerization would potentially be more effective even in the presence of high ligand concentrations. Recently, it was shown that inhibition of VEGFR3 dimerization using the monoclonal 2E11 antibody was able to block VEGFC activity up to a concentration of 100 ng/ml, whereas an antibody inhibiting ligand binding failed at this concentration. Combination of the two antibodies exerted synergistic effects (50).

When investigating VEGFR dimerization, different techniques have been employed including *in vitro* and *in vivo* experiments with computational structure studies (46), chemical cross-linking experiments (51), and immuno-precipitation procedures (44). However, no imaging assay to monitor VEGFR dimerization has yet been described.

Since an imaging assay may facilitate the development of inhibitors of VEGFR dimerization, we here applied BIFC constructs and FRET techniques to visualize receptor

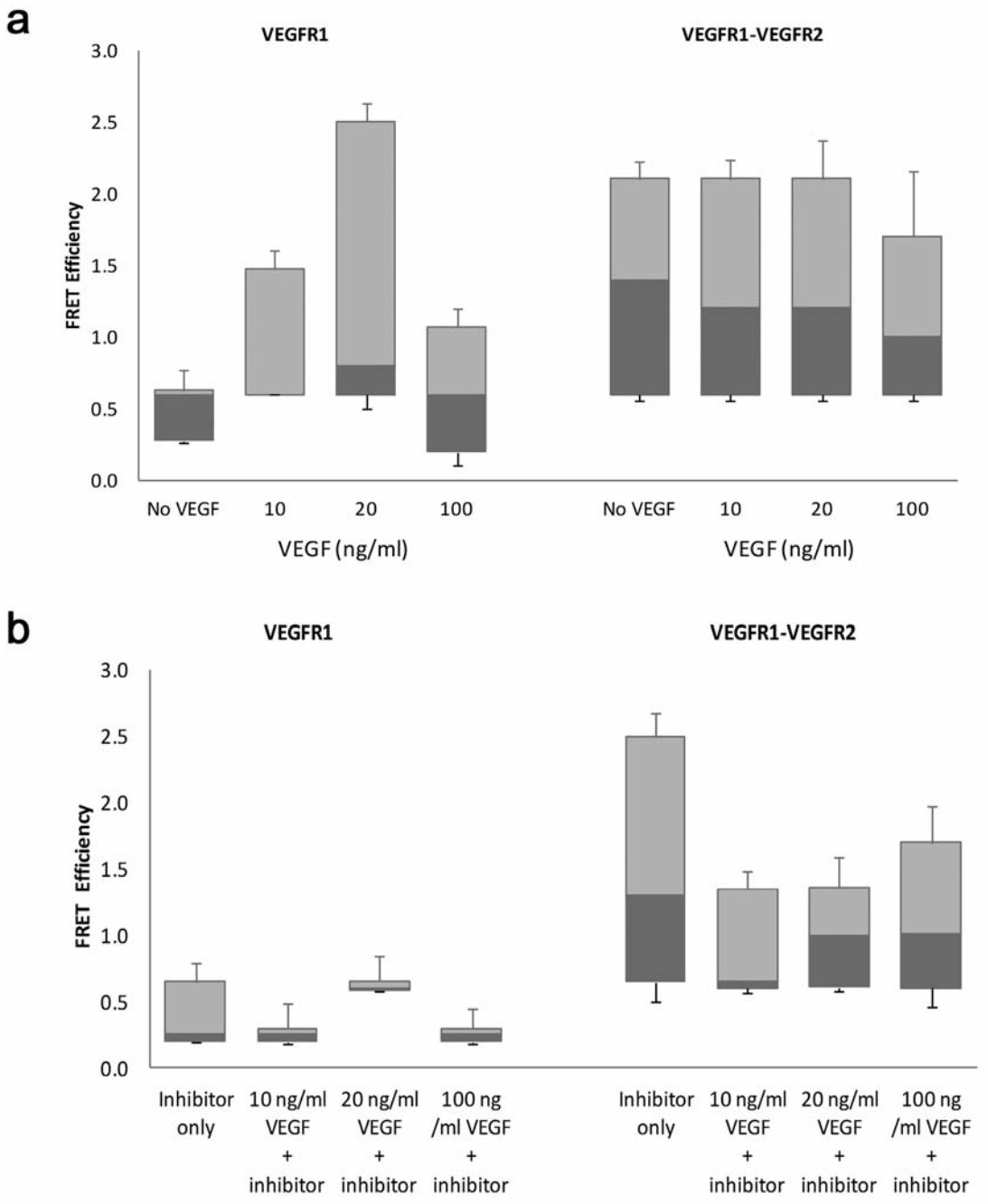

Figure 5. *a: Stimulation of VEGFR homo- and heterodimerization with the short isoform of vascular endothelial growth factor VEGF121. b: After pre-incubation with a neutralizing antibody against VEGF (left), VEGFR-1 homodimer formation was inhibited, whereas no effect was detectable for VEGFR1-VEGFR2 heterodimer formation (right).*

dimerization in single intact cells. In order to avoid possible crosstalk affected by endogenous receptors, we choose CHO-K1 cells, as they do not endogenously express VEGFR under normal conditions. Using both approaches we were able selectively determine VEGFR1 homo- and heterodimerization in single cells. Furthermore, our data support previous studies which indicate that VEGFRs are potently regulated by dimerization (44, 46). Even without specific stimulation, VEGFR1 and VEGFR2 monomers formed dimers. While stimulation with VEGF increased VEGFR1 homodimerization, it did not increase VEGFR1 and VEGFR2 heterodimerization. Moreover, competitive inhibition of VEGF rendered VEGFR2

heterodimerization unchanged, while it potently inhibited VEGFR1 homodimerization. Therefore, either VEGFR1 has a higher affinity for VEGF or the majority of VEGFR2 subunits may already exist as preformed heterodimers.

In summary, our study shows that FRET imaging techniques can be applied successfully to monitor VEGFR homo- and heterodimerization in single cells. In further studies, this may help develop and characterize inhibitors of VEGFR dimerization.

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