Fibroblast growth factor 2-induced angiogenesis in zebrafish: the zebrafish yolk membrane (ZFYM) angiogenesis assay

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Abstract

Angiogenesis plays a key role in tumour growth and metastasis. The teleost zebrafish (*Danio rerio*) represents a promising alternative model in cancer research. Here, we describe a zebrafish yolk membrane (ZFYM) angiogenesis assays based on the injection of 1–30 ng of human recombinant FGF2 (rFGF2) in the perivitelline space of zebrafish embryos in the proximity of developing subintestinal vein vessels (SIVs) at 48 hrs after fertilization. The rFGF2 induces a rapid and dose-dependent angiogenic response from the SIV basket, characterized by the ectopic growth of newly formed, alkaline phosphatase-positive blood vessels. These vessels are formed by proliferating cells that incorporate bromodeoxyuridine and express the endothelial cell markers *vegfr2/kdr* and *fli1*. Microangiography shows that rFGF2-induced vessels are patent and connected to the systemic circulation of the embryo. In keeping with these observations, *fli1*:EGFP⁺ cells isolated from transgenic tg(*fli1*:EGFP)^{Y1} zebrafish embryos express the tyrosine kinase (TK) FGF receptor-1 (FGFR1) and activate extracellular signal-regulated kinase signalling when stimulated *in vitro* by rFGF2. The low molecular weight TK-FGFR1 inhibitor SU5402 and the high molecular weight FGF2 antagonist long-pentraxin 3 inhibit the angiogenic activity of rFGF2 when added to fish water or when co-injected with the growth factor, respectively. Moreover, similar to rFGF2, injection of the zebrafish form of vascular endothelial growth factor-A (VEGF-A) induces a significant angiogenic response in the ZFYM assay that is suppressed by the VEGF receptor-2/KDR TK inhibitor SU5416. The ZFYM assay represents a novel tool for testing the activity of low and high molecular weight inhibitors targeting a defined angiogenic growth factor in zebrafish. The assay may offer significant advantages when compared to other animal models.

Keywords: zebrafish • angiogenesis • FGF2 • inhibitors • embryo

Introduction

Angiogenesis plays a key role in tumour growth and metastasis [1]. Thus, the identification of anti-angiogenic drugs and of angiogenesis-related targets may have significant implications for the development of anti-neoplastic therapies, as shown by the positive outcomes in the treatment of cancer patients with the monoclonal anti-vascular endothelial growth factor-A (VEGF-A) antibody bevacizumab [2].

Various animal models have been developed in rodents and in the chick embryo to investigate the angiogenesis process and for the screening of pro- and anti-angiogenic compounds, each with its own unique characteristics and disadvantages [3].

The teleost zebrafish (*Danio rerio*) represents a promising alternative model in cancer research [4]. When compared to other vertebrate model systems, zebrafish offers many advantages, including ease of experimentation, drug administration, amenability to *in vivo* manipulation, feasibility of reverse and forward genetic approaches [5]. Also, zebrafish possesses a complex circulatory system similar to that of mammals and the optical transparency and ability to survive for 3–4 days without functioning circulation make the zebrafish embryo amenable for vascular biology studies [6]. Interestingly, recent studies have shown the possibility to investigate tumour-induced angiogenesis in zebrafish [7–10].

Fibroblast growth factor-2 (FGF2) is a prototypic angiogenic growth factor that induces cell proliferation, chemotaxis and

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protease production in cultured endothelial cells [11] by interacting with high-affinity tyrosine kinase (TK) receptors (FGFRs) and low-affinity heparan sulphate proteoglycans [12]. FGF2 induces angiogenesis *in vivo* in different animal models and modulates neovascularization during wound healing, inflammation, atherosclerosis and tumour growth [13, 14]. Accordingly, pre-clinical studies demonstrate that FGF2 antagonists inhibit tumour growth and vascularization [13, 15, 16]. On this basis, it appears of primary importance to establish a zebrafish-based assay suitable for the characterization of the angiogenic activity of FGF2 and for the identification of novel inhibitors of the FGF/FGFR system [17].

The basic vascular plan of the developing zebrafish embryo shows strong similarity to that of other vertebrates [18]. At the 13 somite stage, endothelial cell precursors migrating from the lateral mesoderm originate the zebrafish vasculature and a single blood circulatory loop is present at 24 hrs after fertilization (hpf). Blood vessel development continues during the subsequent days by angiogenic processes. In particular, subintestinal vein vessels (SIVs) originate from the duct of Cuvier at 48 hpf. During the next 24 hrs, SIVs will form a vascular plexus across most of the dorsal-lateral aspect of the yolk ball. Here, we developed a zebrafish yolk membrane (ZYFM) angiogenesis assay based on the injection at 48 hpf of nanogram amounts of human recombinant FGF2 (rFGF2) in the perivitelline space in the proximity of developing SIVs. Injected rFGF2 induces a rapid and potent angiogenic response, characterized by the ectopic growth of newly formed blood vessels. We demonstrate that the assay is suitable for testing the activity of low and high molecular weight anti-angiogenic inhibitors of the FGF/FGFR system and that its use can be extended to other angiogenic growth factors, including VEGF-A.

Methods

Chemicals

Human rFGF2 and PTX3 were expressed in *E. coli* and Chinese hamster ovary cells, respectively, and purified as described [19, 20]. Recombinant zebrafish VEGF-A (zVEGF) and human VEGF-A were from R&D Systems and Calbiochem Biochemicals (San Diego, CA, USA), respectively. SU5402 and SU5416 were from Calbiochem Biochemicals.

Fish care

A zebrafish (*Danio rerio*) breeding colony (wild-type AB strain), transgenic tg(*flil*:EGFP)^{y1} [21] and *VEGFR2:G-RCFP* [22] zebrafish lines (the latter one provided by A. Rubinstein, Zygogen, Atlanta, GA, USA) were maintained at 28°C as described [23] at the Zebrafish Facilities of the University of Brescia.

The ZFYM assay

Dechorionated embryos at 48 hpf were anaesthetized with 0.04 mg/ml Tricaine® (Sigma, Milan, Italy) and injected into the perivitelline space

between the yolk and the periderm in the proximity of developing SIVs with different concentrations of rFGF2 dissolved in 4 nl of PBS. Injection was performed using borosilicate needles and a Picospritzer microinjector (Eppendorf, Hamburg, Germany). Then, injected embryos were incubated for 24–48 hrs at 28°C in fresh E3 embryo medium added or not with anti-angiogenic TK inhibitors. At the end of the incubation, zebrafish embryos were fixed in 4% paraformaldehyde for 2 hrs at room temperature and stained for endogenous alkaline phosphatase (AP) activity [24]. Then, embryos were mounted in agarose-coated Petri dishes and photographed under an epifluorescence Leica MZ16 F stereomicroscope equipped with DFC480 digital camera and ICM50 software (Leica, Wetzlar, Germany). For transgenic zebrafish embryos, epifluorescence images were acquired before fixation and AP staining. Confocal images were acquired with Leica TCS SP2 confocal laser microscope and confocal stacks were assembled using Imaris software version 6.0 (Bitplane).

Macroscopic evaluation of the angiogenic response was performed by whole-mount analysis of the modifications of SIV development as demonstrated by AP staining in wild-type AB strain embryos and/or by epifluorescence microscopy in transgenic embryos. A positive angiogenic score was assigned to those embryos characterized by the appearance of ectopic vessels sprouting from the SIV basket. Data are expressed as the number of positive embryos/total injected embryos. Also, computerized image analysis was performed on lateral view images of AP-stained embryos. Briefly, five embryos per group from two independent experiments were mounted in agarose-coated Petri dishes and photographed at 11.5× magnification under a stereomicroscope equipped with digital camera. Then, the total cumulative length of all the AP⁺ vessels forming the SIV plexus, including vessel sprouts, and the number of intersection points of the SIV network were quantified for each embryo using the Image-Pro Plus software (version 4.5.1. Media Cybernetics, Inc., Baltimore, MD, USA).

Whole-mount *in situ* RNA hybridization

Whole-mount *in situ* RNA hybridization was performed on 72 hpf zebrafish embryos using digoxigenin-labelled antisense RNA probes for the zebrafish orthologs of the endothelial cell markers *vegfr2/kdr* [25] and *flil* [26]. Probes were prepared with the DIG System nucleic acid labelling kit (Roche Applied Science, Mannheim, Germany) and visualized using the NBT/BCIP substrate (Boehringer Mannheim, Mannheim, Germany).

5-bromo-2-deoxyuridine (BrdU) incorporation

Dechorionated embryos were anaesthetized with 0.04 mg/ml Tricaine® (Sigma) at 48 hpf and injected into the perivitelline space with a solution containing 10 mM BrdU. After 24 hrs at 28°C, embryos were fixed overnight in 4% paraformaldehyde at 4°C and immunostained with a mouse anti-BrdU antibody followed by a secondary horseradish peroxidase-conjugated antibody (Sigma).

Microangiography

Tetramethylrhodamine isothiocyanate (TRITC)-dextran (molecular weight 2.0×10^6 , Invitrogen) was dissolved in double distilled water at 20 mg/ml and microinjected into the sinus venosus/cardinal vein of zebrafish embryos at 72 hpf as described [18].

Zebrafish cell cultures and immunostaining

Thirty *tg(fli1:EGFP)^{y1}* embryos were dissociated at 5 days after fertilization as described [27]. Then, dissociated *fli1:EGFP* cells were cultured on 1.5% gelatin-coated plates in Leibovitz medium (L-15) added with 20% foetal calf serum (FCS) for 12 hrs at 28°C. Cells were then plated on Lab-Tek™ Chamber Slides (Nunc, Thermo Fisher Scientific, Rochester, NY, USA) coated with 1.5% gelatin for further 24 hrs. Following a 6-hr starvation period in L15 medium *plus* 1.0% FCS, cells were stimulated for 30 min. with 10 ng/ml rFGF2 and fixed for 10 min. with 4% paraformaldehyde at room temperature. Immunostaining was performed with a mouse anti-phospho-ERK antibody (1:100 dilution, Sigma) or with a mouse anti-FGFR1 antibody (1:100 dilution, Santa Cruz Biotechnology) followed by secondary anti-mouse IgG-Alexa Fluor® 594 (1:200 dilution, Invitrogen). Finally, cells were counterstained with 4',6-diamidino-2-phenylindole (1:5000 dilution, DAPI, Sigma).

Results

Grafting of mammalian tumour cells in the proximity of the developing SIV plexus affects the normal developmental pattern of the SIVs by stimulating the formation of ectopic sprouting vessels towards the implant [7, 8]. On this basis, to assess the angiogenic potential of FGF2 in zebrafish, we established a ZFYM assay in which a 4- μ l drop of PBS containing rFGF2 is injected into the perivitelline space between the yolk and the periderm in the proximity of developing SIVs at 48 hpf. PBS alone was used for control embryos. Organization of the SIV plexus was observed after 24 hrs by whole-mount AP staining of blood vessels. As shown in Fig. 1A, rFGF2 injection affects the normal developmental pattern of the SIVs causing the appearance of newly formed AP⁺ blood vessels projecting from the SIV plexus. The effect of rFGF2 on blood vessel sprouting is dose dependent (Fig. 1A) and an angiogenic response is observed in a significant percentage of injected embryos at doses as low as 1.0 ng of rFGF2 per embryo (19/35 *versus* 2/18 positive embryos in rFGF2-treated *versus* PBS-treated embryos; Fisher's exact test: $P < 0.01$) (Fig. 1B). Computerized image analysis of AP-stained embryos confirms the increase of SIV vascularity following rFGF2 administration when quantified as the total cumulative length of the AP⁺ SIV plexus and sprouts, or when expressed as the number of intersection points of the SIV network (Fig. 2).

To confirm the endothelial cell origin of the newly-formed AP⁺ projections, rFGF2 was injected in transgenic *tg(fli1:EGFP)^{y1}* [21] and *VEGFR2:G-RFP* [22] zebrafish embryos. In these animals, endothelial expression of the green fluorescent protein (GFP) is driven by the *fli1* and *vegfr2* promoters, respectively. As shown in Fig. 3, rFGF2 induces the formation of GFP⁺ sprouts from the SIV plexus in both transgenic embryos. Accordingly, rFGF2-induced projections express *fli1* and *vegfr2* transcripts as shown by whole-mount *in situ* RNA hybridization (data not shown). Also, AP⁺ neovessels incorporate BrdU, indicating that endothelial cell proliferation concurs to the formation of these structures (Fig. 4A and B). Finally, microangiography performed 24 hrs after rFGF2

Fig. 1 Angiogenic activity of rFGF2 in the ZFYM assay. Zebrafish embryos were injected with the indicated doses of rFGF2 at 48 hpf. After 24 hrs, whole-mount AP staining was performed and embryos were scored for a positive angiogenic response. **(A)** Representative images (top view) of embryos injected with PBS (a), 1.0 ng of rFGF2 (b) or 7.0 ng of rFGF2 (c) and maintained in fish water in the absence or in the presence (d) of 1.0 μ M SU5402. Arrows point to newly formed AP⁺ blood vessels sprouting out from the SIV basket. **(B)** rFGF2 exerts a dose-dependent angiogenic response. Each point is the mean $\pm$ S.D. of two to three experiments with a total of 20–140 embryos per group.

injection demonstrates that the newly formed vascular network is patent and connected to the systemic circulation of the embryo (Fig. 4C). Taken together, the results demonstrate that injection of rFGF2 induces the ectopic growth of newly formed blood vessels in zebrafish embryos.

To assess the role of TK-FGFR activation in FGF2-triggered angiogenesis, zebrafish embryos were injected with rFGF2 and maintained in fish water containing the TK-FGFR inhibitor SU5402 [28]. As shown in Fig. 5 and Table 1, 1.0 μ M SU5402 significantly inhibits the angiogenic activity of rFGF2 whereas an equimolar concentration of the VEGF receptor-2 (VEGFR2/KDR) TK inhibitor SU5416 [2] was ineffective. To further confirm the role of FGFR signalling in rFGF2-driven angiogenesis in zebrafish, *fli1:EGFP⁺* cells were isolated from *tg(fli1:EGFP)^{y1}* embryos. Immunostaining of cultured cells demonstrate that *fli1:EGFP⁺* cells express FGFR1 (Fig. 6) and activate extracellular signal-regulated kinase (ERK) when stimulated *in vitro* by rFGF2 (Fig. 7). This activation was fully inhibited by the TK-FGFR inhibitor SU5402 (Fig. 7). These proofs of concept experiments indicate that the ZFYM angiogenesis assay may represent a

Fig. 2 Computerized image analysis of the angiogenic response. Representative images of AP-stained SIV basket of embryos injected with PBS (**A**) or 7.0 ng of rFGF2 (**B**) (lateral view, head on the left). All vessels were tracked (in yellow) and the total cumulative length of the vascular plexus and vessels sprouts (●) as well as the number of intersection points of the SIV network (○) were calculated using the Image-Pro Plus software (**C**). Data are the mean $\pm$ S.D. of five embryos from two independent experiments.

short-term *in vivo* assay suitable for the identification of novel inhibitors of the FGF/FGFR system.

Zebrafish embryos are permeable to small molecules that can be assessed for their activity after addition to fish water (see above). This is not true for high molecular weight compounds. The pattern recognition receptor long-pentaxin 3 (PTX3) is a multimeric protein with an apparent molecular weight equal to 370,000 [29]. Recent observations have shown that PTX3 is a potent FGF2 antagonist endowed with a significant anti-angiogenic activity *in vitro* and *in vivo* [30]. On this basis, to evaluate whether the ZFYM assay could be used also to assess the effect of angiostatic macromolecules, the rFGF2 solution was added or not with human PTX3 (0.22 μ M) and injected in zebrafish embryos at 48 hpf. After 24 hrs, whole-mount AP staining was performed and embryos were scored for a positive angiogenic response. The data demonstrate that PTX3 is able to exert a significant inhibitory activity (approximately 50% inhibition) when co-injected with rFGF2 (11/14 *versus* 7/18 positive embryos in rFGF2-treated *versus* rFGF2 + PTX3-treated embryos; Fisher's exact test: $P < 0.01$).

To assess whether the ZFYM assay is also suitable to investigate the angiogenic activity of stimuli different from FGF2, zebrafish embryos were injected with the zebrafish form of the angiogenic growth factor VEGF-A (zVEGF-A) [31] and embryos were maintained in the absence or in the presence of the TK

inhibitors SU5402 or SU5416 in fish water. As shown in Table 1, zVEGF-A exerts a significant angiogenic response in the ZFYM assay that was potently inhibited by the VEGFR2/KDR TK inhibitor SU5416 but not by the TK-FGFR inhibitor SU5402.

Discussion

Here, we developed a novel *in vivo* angiogenesis assay in zebrafish embryo based on the injection at 48 hpf of nanogram amounts of rFGF2 in the perivitelline space in the proximity of developing SIVs. The injected growth factor induces a rapid and potent angiogenic response, characterized by the sprouting of newly formed blood vessels from the SIV plexus. The response is dose dependent and can be expressed as percentage of rFGF2-injected embryos showing a positive response. Also, it can be quantified by computerized image analysis of the AP⁺ SIV plexus.

Zebrafish embryo allows disease-driven drug target identification and *in vivo* validation, thus representing an interesting bioassay tool for small molecule testing and dissection of biological pathways alternative to other vertebrate models [32]. Previous studies had shown that developmental angiogenesis in the

Fig. 3 rFGF2-induced zebrafish neovessels express endothelial cell markers. (A) Confocal laser microscopy showing the SIV plexus in PBS-injected (a) and rFGF2-injected (b) transgenic *VEGFR2:G-RCFP* zebrafish embryos 24 hrs after injection. *, GFP⁺ endothelial cell sprouts (lateral view, head on the right). (B) Transgenic *VEGFR2:G-RCFP* (a) and *tg(fli1:EGFP)^{Y1}* (b) zebrafish embryos were injected with rFGF2 at 48 hpf and photographed under an epifluorescence microscope after 24 hrs (top view). Arrows point to newly formed blood vessels sprouting out from the SIV basket.

zebrafish embryo, leading to the formation of the intersomitic vessels of the trunk [22] and of the SIV plexus [33], represents a target for the screening of anti-angiogenic compounds. In these assays, low molecular weight compounds dissolved in fish water are investigated for their impact on the growth of new blood vessels driven by the complex network of endogenous, developmentally regulated signals. The ZFYM assay herewith proposed differs from these assays because the angiogenic stimulus is represented by a well defined topically delivered exogenous agent that leads to the growth of ectopic blood vessels. This allows the screening of low and high molecular weight antagonists targeting a specific angiogenic growth factor and/or its receptor(s). In this respect, the capacity of a low dose (1.0 μ M) of the TK-FGFR inhibitor SU5402 or of the VEGFR2/KDR TK inhibitor SU5416 to affect selectively the angiogenic activity of rFGF2 and zVEGF-A confirms the sensitivity and specificity of the assay.

Fig. 4 Cell proliferation and patency of rFGF2-induced zebrafish neovessels. (A), (B) Zebrafish embryos were injected with rFGF2 plus 10 mM BrdU at 48 hpf. At 72 hpf, embryos were stained for AP activity (in blue) and immunostained with anti-BrdU antibodies (in red). A) Whole-mount lateral view of a representative embryo (head to the right) where newly formed AP⁺ blood vessels highlighted by the white box are shown at higher magnification in the inset (a). B) Cross-section of an AP⁺ vessel with two BrdU⁺ endothelial cell nuclei (arrows). C) rFGF2-treated *tg(fli1:EGFP)^{Y1}* zebrafish embryos were microinjected into the sinus venosus/cardinal vein with TRITC-dextran at 72 hpf. Arrows point to newly formed *fli1:EGFP⁺* vessels (in green) filled by TRITC-dextran (in red) (lateral view, head to the right).

Interestingly, previous observations had shown that SU5402 is able to inhibit intersomitic vessel outgrowth in zebrafish embryos when delivered in the fish water at 1.0- μ M concentration, similar to SU5416 [34]. This effect was interpreted as the result of a non-specific blockade of VEGFR activity by the TK-FGFR inhibitor or, alternatively, as an indication of the involvement of the FGF/FGFR system in developmental angiogenesis in zebrafish. We have confirmed the ability of both TK inhibitors to prevent inter-segmental vessel outgrowth in *VEGFR2:G-RCFP* transgenic embryos (data not shown). The specific anti-FGF2 effect exerted by SU5402 in the ZFYM assay reinforces the hypothesis that the TK activity of FGFRs is indeed required for the angiogenic process in zebrafish. Further studies are required to unequivocally demonstrate this point.

As stated above, a positive angiogenic response is elicited in the ZFYM assay by zVEGF-A. Previous observations had shown that injection of high doses of human VEGF-A into the yolk of

Fig. 5 Effect of TK receptor inhibitors on the angiogenic activity of rFGF2 in zebrafish embryos. Zebrafish embryos were injected with vehicle (PBS) or with rFGF2 (7.0 ng/embryo) at 48 hpf and incubated in fish water containing the TK-FGFR inhibitor SU5402 or the TK-VEGFR2/KDR inhibitor SU5416 (both at 1.0 μ M). At 72 hpf, whole-mount AP staining was performed and embryos were photographed (lateral view, head on the top). Note the capacity of SU5402, but not of SU5416, to suppress the sprouting of AP⁺ blood vessels from the SIV plexus induced by rFGF2 injection.

Table 1 Effect of TK receptor inhibitors on the angiogenic activity of FGF2 and VEGF-A in zebrafish embryos

TK inhibitor	None	SU5402	SU5416
rFGF2	30/42	18/43**	38/56
zVEGF-A	20/35	7/19	7/35**

Zebrafish embryos were injected with rFGF2 (7.0 ng/embryo) or zVEGF-A (10 ng/embryo) at 48 hpf and incubated in fish water containing the TK-FGFR inhibitor SU5402 or the TK-VEGFR2/KDR inhibitor SU5416 (both at 1.0 μ M). At 72 hpf, whole-mount AP staining was performed and embryos were scored for a positive angiogenic response. Data are expressed as the number of positive embryos/total injected embryos. **, Statistically different from embryos injected with the growth factor and incubated in the absence of any inhibitor, Fisher's exact test: $P < 0.01$.

48 hpf zebrafish embryos (60–80 ng/embryo) induces projections from the SIV basket and enlargement of the SIVs; injection of the same dose of human VEGF-A in the perivitelline space results instead in the disruption of vessel formation and of heart development [33]. Here, we extended these observations by showing that injection in the perivitelline space of a low dose of zVEGF-A (10 ng/embryo) causes a significant angiogenic response (Table 1) in the absence of any effect on heart development (data not shown). When tested at the same low dose, human VEGF-A was instead ineffective (data not shown). The observed difference in potency between zVEGF-A and human VEGF-A is of interest. Indeed, even though *in vitro* and *in vivo* studies have shown little, if any, species specificity in the effects of VEGFs (reviewed in [2]), scattered observations have pointed out that the genetic background may indeed affect endothelial cell responsiveness to VEGF-A [35, 36]. The systematic screening of pro-angiogenic compounds will allow assessing how the ZFYM assay compares in terms of sensitivity and specificity to other well-characterized

Fig. 6 Zebrafish *fli1:EGFP*⁺ cells express FGFR1. *fli1:EGFP*⁺ cells were isolated from 5-day-old tg(*fli1:EGFP*)^{Y1} embryos. Immunostaining of cultured cells demonstrate that *fli1:EGFP*⁺ cells (in green) express FGFR1 (in red).

angiogenesis assays in different animal species (*e.g.* the chick embryo chorioallantoic membrane assay [37] and the murine Matrigel plug assay [38]). In this respect, we have observed that the rat form of the recently characterized pro-angiogenic bone morphogenic binding protein Drm/gremlin [39] is able to elicit a positive response in the ZFYM assay (data not shown).

Our data demonstrate that the ZFYM assay may represent a short-term assay suitable for the identification of novel angiogenesis inhibitors. In this context, it is interesting to note the rapid response of the ZFYM assay to angiogenesis inhibitors (24 hrs) when compared to other angiogenesis assays, including the chick embryo chorioallantoic membrane assay (3–4 days), the murine Matrigel plug assay (5–7 days) and the murine (1 week) and rabbit (2–3 weeks) cornea assays [3]. Also, a large number of zebrafish embryos can be injected with the growth factor under test and maintained in 96-well plates, thus allowing systemic

Fig. 7 rFGF2 causes ERK phosphorylation in isolated zebrafish *fli1:EGFP*⁺ cells. *fli1:EGFP*⁺ cells isolated from *tg(fli1:EGFP)^{y1}* embryos (in green) were left untreated (**A–D**) or were treated *in vitro* with rFGF2 (10 ng/ml) in the absence (**E–H**) or in the presence (**I–L**) of 5.0 μ M SU5402. After 30 min., cells were immunostained with anti-phospho-ERK antibodies (in red) and counterstained with DAPI (in blue). Merged images show the ability of rFGF2 to induce ERK activation (arrows in **H**) that is fully inhibited by the TK-FGFR inhibitor SU5402 (**L**).

in vivo treatment of the animals with minimal amounts of compound. Therefore, dose-response experiments can be performed and numerous compounds can be tested in an effective manner. Also, the use of transgenic *tg(fli1:EGFP)^{y1}* and *VEGFR2:G-RCP* zebrafish embryos, in which endothelial cells express GFP under the control of endothelial gene promoters, may represent an improvement of the method (see Fig. 2), allowing the observation and time-lapse recording of newly formed blood vessels in live embryos by epifluorescence microscopy as well as by *in vivo* confocal microscopy.

The ZFYM assay may also present some drawbacks when compared to other *in vivo* angiogenesis assays. Even though a zebrafish facility is much cheaper and its logistic is much simpler than a mammalian facility, angiogenesis assays in zebrafish require dedicated expertise and technical skills, including microinjection. Also, because of the expected variability frequently observed in any *in vivo* assay, numerous embryos should be injected per experimental point. Moreover, the availability of inbred, transgenic, gene knock-out/knock-in animals, of a wide array of antibodies, as well as of bioinformatic genomic, transcriptomic

and proteomic information represent important tools for angiogenesis studies performed in murine models. These tools are only partially available for zebrafish, even though it can be anticipated that the attractive attributes of this animal model will favour its rapid evolution.

In conclusion, the ZFYM assay represents a novel tool for studying the mechanisms of neovascularization and its use for chemical discovery in angiogenesis may offer significant advantages when compared to other animal models.

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