

# Mammalian Tumor Xenografts Induce Neovascularization in Zebrafish Embryos

Stefania Nicoli,<sup>1</sup> Domenico Ribatti,<sup>2</sup> Franco Cotelli,<sup>3</sup> and Marco Presta<sup>1</sup>

<sup>1</sup>Unit of General Pathology and Immunology, Department of Biomedical Sciences and Biotechnology, University of Brescia, Brescia, Italy; <sup>2</sup>Department of Human Anatomy and Histology, University of Bari, Bari, Italy; and <sup>3</sup>Department of Biology, University of Milano, Milan, Italy

## Abstract

The zebrafish (*Danio rerio*)/tumor xenograft model represents a powerful new model system in cancer. Here, we describe a novel exploitation of the zebrafish model to investigate tumor angiogenesis, a pivotal step in cancer progression and target for antitumor therapies. Human and murine tumor cell lines that express the angiogenic fibroblast growth factor (FGF) 2 and/or vascular endothelial growth factor (VEGF) induce the rapid formation of a new microvasculature when grafted close to the developing subintestinal vessels of zebrafish embryos at 48 h postfertilization. Instead, no angiogenic response was exerted by related cell clones defective in the production of these angiogenic growth factors. The newly formed blood vessels sprout from the subintestinal plexus of the zebrafish embryo, penetrate the tumor graft, and express the transcripts for the zebrafish orthologues of the early endothelial markers Fli-1, VEGF receptor-2 (VEGFR2/KDR), and VE-cadherin. Accordingly, green fluorescent protein–positive neovessels infiltrate the graft when tumor cells are injected in transgenic *VEGFR2:G-RFP* zebrafish embryos that express green fluorescent protein under the control of the VEGFR2/KDR promoter. Systemic exposure of zebrafish embryos immediately after tumor cell injection to prototypic antiangiogenic inhibitors, including the FGF receptor tyrosine kinase inhibitor SU5402 and the VEGFR2/KDR tyrosine kinase inhibitor SU5416, suppresses tumor-induced angiogenesis without affecting normal blood vessel development. Accordingly, *VE-cadherin* gene inactivation by antisense morpholino oligonucleotide injection inhibits tumor neovascularization without affecting the development of intersegmental and subintestinal vessels. These data show that the zebrafish/tumor xenograft model represents a novel tool for investigating the neovascularization process exploitable for drug discovery and gene targeting in tumor angiogenesis. [Cancer Res 2007;67(7):2927–31]

## Introduction

Angiogenesis plays a key role in tumor progression and metastatic spread (1). The angiogenic growth factors released by neoplastic cells, including members of the vascular endothelial growth factor (VEGF; ref. 2) and fibroblast growth factor (FGF;

ref. 3) families, are responsible for tumor neovascularization. In its absence, the tumor remains in a state of dormancy (1). Thus, the identification of selective targets in tumor-induced endothelium may have significant implications for the development of antineoplastic therapies, as shown by the positive outcomes in the treatment of cancer patients with the monoclonal anti-VEGF antibody bevacizumab (2).

The use of tumor cell syngrafts or xenografts in animal models may allow the continuous delivery of angiogenic factors produced by a limited number of tumor cells, thus mimicking the initial stages of tumor angiogenesis and metastasis. Various animal models have been developed in rodents and in the chick embryo to investigate the angiogenic process and for the screening of proangiogenic and antiangiogenic compounds, each with its own unique characteristics and disadvantages (4).

The teleost zebrafish (*Danio rerio*)/tumor xenograft model represents a promising alternative model in cancer research (5). When compared with other vertebrate model systems, the zebrafish model offers many advantages, including ease of experimentation, drug administration, amenability to *in vivo* manipulation, and feasibility of reverse and forward genetic approaches (6). Also, the zebrafish possesses a complex circulatory system similar to that of mammals and the optical transparency and ability to survive for 3 to 4 days without a functioning circulation make the zebrafish embryo especially amenable for vascular biology studies (7). Here, we investigated the possibility that the implantation of murine and human tumor cells could elicit an angiogenic response in zebrafish embryos suitable for studying the effect of antiangiogenic chemicals and for the identification of genes involved in tumor angiogenesis.

## Materials and Methods

**Cell cultures.** Murine aortic endothelial (MAE) cells (obtained from R. Auerbach, University of Wisconsin, Madison, WI) and tumorigenic FGF2-overexpressing FGF2-T-MAE cells (8) were grown in DMEM plus 10% FCS. Human adenocarcinoma Tet-FGF2 cells and VEGF antisense–transfected AS-VEGF/Tet-FGF2 cells were grown as described (9). A2780, MDA-MB-435, and B16-BL16 cells were provided by R. Giavazzi (Mario Negri Institute, Bergamo, Italy).

**Fish care, cell and morpholino injection.** A zebrafish (*D. rerio*) breeding colony (wild-type AB strain) and the transgenic *VEGFR2:G-RFP* (10) zebrafish line (provided by A. Rubinstein, Zygogen, Atlanta, GA) were maintained at 28°C, as previously described (11), at the Zebrafish Facilities of the University of Brescia and University of Milan. Dechorionated embryos at 48 h postfertilization (hpf) were anesthetized with 0.04 mg/mL of tricaine (Sigma, St. Louis, MO) and injected with 1,000 to 2,000 cells per embryo resuspended in 3 to 4 nL of Matrigel (Becton Dickinson, Milan, Italy) using a Picospritzer microinjector (Eppendorf, Hamburg, Germany). SU5402 and SU5416 (Calbiochem Biochemicals, La Jolla, CA) were diluted in system water and added to 48 hpf embryos immediately after cell injection. Zebrafish *VE-cadherin* splice site (5'-TTTACAAGACCGTCTACCTTTCCAA-3')

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

**Requests for reprints:** Marco Presta, Unit of General Pathology, Department of Biomedical Sciences and Biotechnology, Viale Europa 11, 25123 Brescia, Italy. Phone: 39-030371-7311; Fax: 39-030370-1157; E-mail: [presta@med.unibs.it](mailto:presta@med.unibs.it).

©2007 American Association for Cancer Research.  
doi:10.1158/0008-5472.CAN-06-4268

and control (5'-CCTCTTACCTCAGTTACAATTATA-3') morpholinos (Gene Tools, Philomath, OR) were microinjected into one-cell stage embryos (0.4 pmol/embryo).

**Whole-mount alkaline phosphatase staining.** Zebrafish embryos were fixed in 4% paraformaldehyde for 2 h at room temperature and stained for endogenous alkaline phosphatase activity (12). Then, embryos were mounted in agarose-coated Petri dishes and photographed under an epifluorescence Leica MZ16 F stereomicroscope equipped with a DFC480 digital camera and ICM50 software (Leica, Solms, Germany). For transgenic *VEGFR2:G-RCFP* zebrafish embryos, epifluorescence images were acquired before fixation and alkaline phosphatase staining. When indicated, 5- $\mu$ m transversal sections of paraffin-embedded stained embryos were mounted and photographed.

**4',6-Diamidino-2-phenylindole staining and visualization of green fluorescent protein-positive vessels.** Transgenic *VEGFR2:G-RCFP* embryos at 72 hpf were incubated overnight at 4°C in 4% paraformaldehyde/PBS and sequentially incubated with 5% sucrose/PBS for 1 h and with 15% sucrose/PBS for 3 h. Finally, embryos were embedded in gelatin solution (7.5% gelatin plus 15% sucrose in PBS) for 4 h and snap-frozen. Cryosections (15  $\mu$ m) were processed in PBT [3% bovine serum albumin, 0.1% Triton X-100 in PBS (pH 7.3)] and sequentially stained with anti- $\alpha$ -tubulin antibody (1:200) and 4',6-diamidino-2-phenylindole (DAPI) solution (1:2,000; Sigma). Sections were then mounted in DAKO (Glostrup, Denmark) fluorescent mounting medium and double-fluorescence images were acquired using an epifluorescence Zeiss Axiovert 200 M microscope equipped with Hal 100 digital camera and AxioVisionLE software. The percentage of tumor area occupied by green fluorescent protein (GFP)-KDR<sup>+</sup> vessels was calculated by computerized image analysis (Image-Pro Plus; MediaCybernetics, Silver Spring, MD) on four sections per experimental point.

**In situ RNA hybridization and reverse transcription-PCR analysis.** Whole-mount *in situ* RNA hybridization was done on 72 hpf zebrafish embryos using digoxigenin-labeled antisense RNA probes for the zebrafish orthologues of the endothelial cell markers *VEGFR2/KDR* (13), *Fli-1* (14), and *VE-cadherin* (15). Probes were prepared with the DIG System nucleic acid labeling kit (Roche Applied Science, Basel, Switzerland) and visualized using an alkaline phosphatase nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Boehringer Mannheim, Mannheim, Germany). Total RNA was extracted from tumor cell-injected embryos using Trizol reagent (Invitrogen, San Diego, CA). PCRs were done on the retrotranscribed total RNA using Pre-Aliquoted ReadyMix PCR Master Mix (Abgene, Epsom, United Kingdom) with annealing temperature equal to 60°C or 55°C for mammalian and zebrafish transcripts, respectively. The species-specific primers used were:

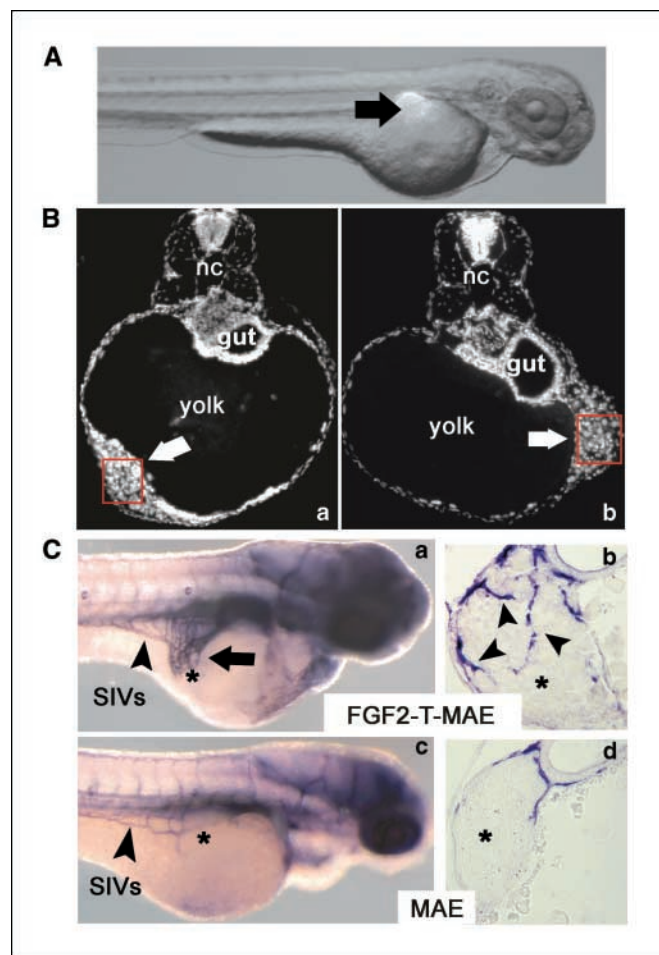
human *FGF2* forward, 5'-AAGCGGCTGACTGCAAAAACG-3';  
human *FGF2* reverse, 5'-AACTGGTGTATTTCTTGACCGGTA-3';  
murine *tubulin* forward, 5'-TCACTGTGCCTGAACCTACC-3';  
murine *tubulin* reverse, 5'-GGAACATAGCCGTAACCTGC-3';  
zebrafish *VE-cadherin* forward, 5'-GATGACTGAGCCTGTCTTCAGGGTT-3';  
zebrafish *VE-cadherin* reverse, 5'-TCTTTCTGATAGCGCCGTCTC-3';  
zebrafish *EF-1* forward, 5'-GGTACTTCTCAGGCTGACTGT-3';  
zebrafish *EF-1* reverse, 5'-CAGACTTGACCTCAGTGGTTA-3'.

## Results and Discussion

Tumorigenic FGF2-overexpressing mouse aortic endothelial cells (FGF2-T-MAE) trigger a potent angiogenic response when compared with the poorly angiogenic, parental MAE cells in a rabbit cornea or in a chick embryo chorioallantoic membrane angiogenesis assay (ref. 8 and references therein). On this basis, the two cell lines were compared for their capacity to induce neovascularization when injected in zebrafish embryos. To this purpose, cells were resuspended in Matrigel and microinjected in zebrafish embryos at 48 hpf (1,000–2,000 cells/embryo) through the perivitelline space between the yolk and the periderm (duct of Cuvier area), close to the developing subintestinal vessels (SIV; Fig. 1A and B). Both cell

types remain viable *in situ*, as shown by the presence of mitotic figures within the grafts and by the expression of murine *tubulin* mRNA (in both cell types) and human *FGF2* mRNA (in FGF2 transfectants only) throughout the whole experimental period (Supplementary Fig. S1). Because of the immaturity of the immune system in zebrafish embryos at 48 to 72 hpf, no graft rejection occurs at this stage.

From 48 to 72 hpf, SIVs form a vascular plexus. Injection of FGF2-T-MAE cells caused the appearance of newly formed alkaline phosphatase-positive blood vessels projecting from the SIV plexus and infiltrating the xenograft. Only limited, if any, change in SIV architecture was instead induced by MAE cell grafts (Fig. 1C). As observed in mammalian models (8), neovascularization occurred in 100% of FGF2-T-MAE cell implants ( $n = 60$ ) when compared with



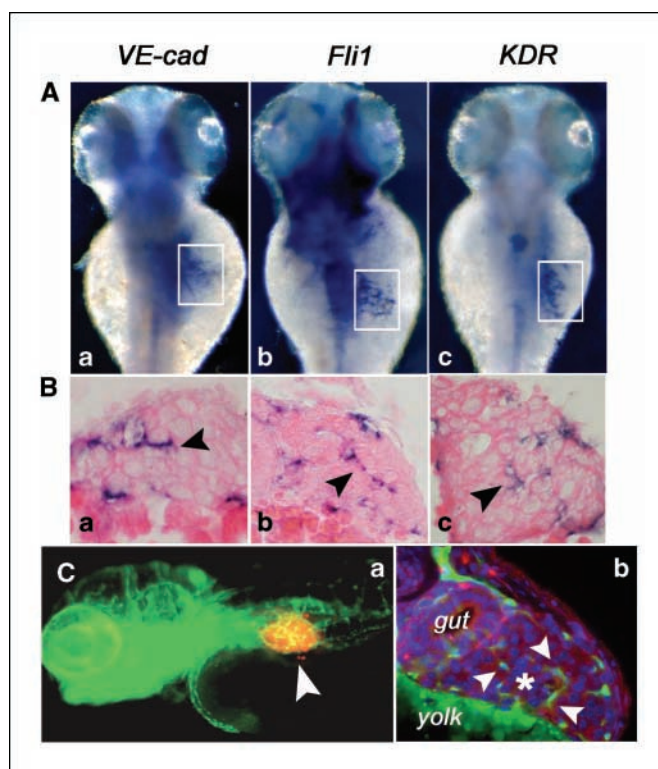
**Figure 1.** Tumor cell xenografts induce angiogenesis in zebrafish embryo. A, zebrafish embryo grafted with GFP-transduced cells to highlight the site of injection (arrow). B, tumorigenic murine FGF2-T-MAE cells (a) and parental MAE cells (b) were injected in the perivitelline space of zebrafish embryos at 48 hpf. After 24 h, transverse sections of the embryos were stained with DAPI to visualize the cell graft (arrow); areas within the boxes are shown at higher magnification in Supplementary Fig. S1 (nc, notochord; original magnification,  $\times 200$ ). C, alkaline phosphatase staining of FGF2-T-MAE cell-injected (a and b) and MAE cell-injected (c and d) embryos at 72 hpf. a and c, whole-mount lateral view showing numerous neovessels originating from the SIV basket that migrate and infiltrate the FGF2-T-MAE cell graft (arrow in a), whereas no neovessel formation is observed in embryos injected with MAE cells (c). b and d, transverse sections of the cell grafts; alkaline phosphatase-positive intratumor blood vessels in the FGF2-T-MAE cell graft (arrowheads; original magnification,  $\times 400$ ). a to d, cell graft (\*).



5% of MAE cell grafts ( $n = 40$ ,  $P < 0.01$ ), no effect being exerted by the injection of Matrigel alone ( $n = 15$ ). *In situ* RNA hybridization confirmed that the novel, tumor-infiltrating alkaline phosphatase-positive SIV structures were formed by zebrafish endothelial cells. Indeed, FGF2-T-MAE cell-induced neovessels highly express the transcripts for the zebrafish orthologues of the early endothelial markers *Fli-1* (14), *VEGFR2/KDR* (13), and *VE-cadherin* (ref. 15; Fig. 2A and B). Accordingly, neovessels express GFP under the control of the VEGFR2/KDR promoter when FGF2-T-MAE cells are injected in transgenic *VEGFR2:G-RCFP* zebrafish embryos (ref. 10; Fig. 2C).

To further confirm the ability of the zebrafish/tumor xenograft model to discriminate between highly angiogenic and poorly angiogenic tumor cell variants, we took advantage of two closely related human endometrial adenocarcinoma cell lines (Tet-FGF2 and AS-VEGF/Tet-FGF2 cells) that differ for VEGF and/or FGF2 expression (9). Indeed, Tet-FGF2 cells constitutively express VEGF and overexpress FGF2 under the control of the tetracycline-responsive promoter (tet-off system); when grafted s.c. in nude mice, Tet-FGF2 cells cause the appearance of fast-growing tumors characterized by high blood vessel density (9). AS-VEGF/Tet-FGF2 cells were generated from Tet-FGF2 cells by stable VEGF antisense transfection. The consequent down-regulation of VEGF production resulted in a significant reduction in the tumorigenic and angiogenic potential of these cells that was further suppressed by the simultaneous inhibition of FGF2 expression following tetracycline administration (9). In keeping with the data obtained in nude mice, angiogenesis occurred in 75% of zebrafish embryos injected with highly angiogenic Tet-FGF2 cells, whereas no response was observed in any of the embryos injected with tetracycline-treated, VEGF antisense-transfected AS-VEGF/Tet-FGF2 cells ( $n = 16$ ,  $P < 0.05$ ). Moreover, neovascularization was observed in 64% of zebrafish embryos ( $n = 36$ ) injected with the proangiogenic human ovarian carcinoma A2780 cells, human breast carcinoma MDA-MB-435 cells, or murine melanoma B16-BL16 cells, showing that this effect was not limited to FGF2 transfectants. Even though only the parallel screening of a variety of cell lines will establish whether the zebrafish-based assay could provide results fully overimposable with those obtained with the classic rodent and chick embryo angiogenesis assays, the data shows the capacity of the zebrafish/tumor xenograft model to discriminate between highly angiogenic and poorly angiogenic tumor cell lines.

Because of the permeability of its embryos to small molecules, the zebrafish model 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 (16). On this basis, to assess the susceptibility of tumor-induced neovessels to systemic exposure of zebrafish embryos to antiangiogenic compounds, live fishes (five embryos per group) were incubated with the FGF receptor-1 tyrosine kinase inhibitor SU5402 (1.0  $\mu\text{mol/L}$ ) or the VEGF receptor-2 (VEGFR2/KDR) tyrosine kinase inhibitor SU5416 (2.5  $\mu\text{mol/L}$ ; refs. 2, 3) immediately after injection of FGF2-T-MAE cells. In keeping with the role of FGF and VEGF signaling in tumor angiogenesis (2, 3), both prototypic antiangiogenic compounds abrogated tumor neovascularization in all the embryos, even though the two molecules did not affect physiologic SIV development at the dose and time point tested (Supplementary Fig. S2). These proof-of-concept experiments indicate that the zebrafish/tumor xenograft



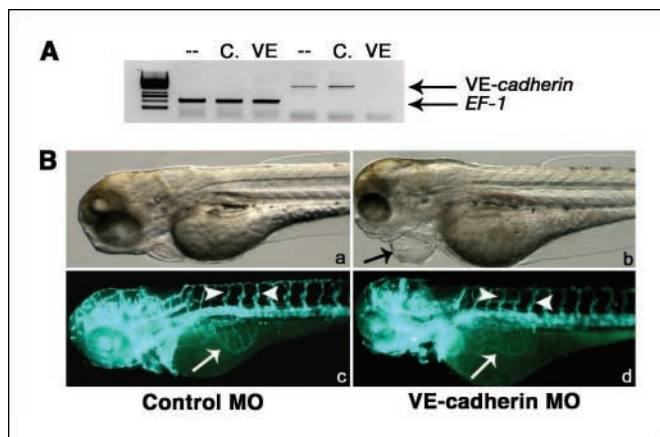
**Figure 2.** Tumor-induced zebrafish neovessels express endothelial cell markers. **A**, whole-mount *in situ* RNA hybridization of 72 hpf zebrafish embryos injected on the right flank with FGF2-T-MAE cells (top view). Embryos were hybridized with specific probes for the zebrafish *VE-cadherin* (a), *Fli-1* (b), or *VEGFR2/KDR* (c) orthologues; intratumor newly formed blood structures are highlighted in the corresponding insets. **B**, transversal sections of the tumor xenografts shown in (A) allow the visualization of intratumor neovessels positive for the corresponding gene transcripts (arrowheads; original magnification,  $\times 1,000$ ). **C**, tumor angiogenesis in transgenic *VEGFR2:G-RCFP* zebrafish embryos. Embryos were injected at 48 hpf with FGF2-T-MAE cells and processed 24 h thereafter. **a**, note the GFP-KDR<sup>+</sup> neovessels (green) penetrating the graft of FGF2-T-MAE cells (arrowhead) preloaded with the fluorescent dye Dil (red; original magnification,  $\times 200$ ). **b**, cryosection of a FGF2-T-MAE tumor xenograft (\*) stained with an anti-murine  $\alpha$ -tubulin antibody (red); nuclear counterstaining with DAPI (blue). Note the GFP-KDR<sup>+</sup> neovessels (green) within the tumor graft (arrowheads; original magnification,  $\times 600$ ).

model may represent a short-term assay suitable for the identification of novel tumor angiogenesis inhibitors. In this context, it is interesting to note the rapid response of this model to angiogenesis inhibitors (24 h) when compared with other tumor graft/angiogenesis assays, including the chick embryo chorioallantoic membrane assay (3–4 days), the s.c. murine Matrigel plug assay (5–7 days), the murine (1 week) and rabbit (2–3 weeks) cornea assays, and the s.c. mouse syngraft and xenograft assays (several weeks; ref. 4). Also, a large number of zebrafish embryos can be injected and maintained in 96-well plates, thus allowing systemic *in vivo* treatment of the animals with minimal amounts of compound. Therefore, dose-response experiments could be done and numerous compounds can be tested in an effective manner.

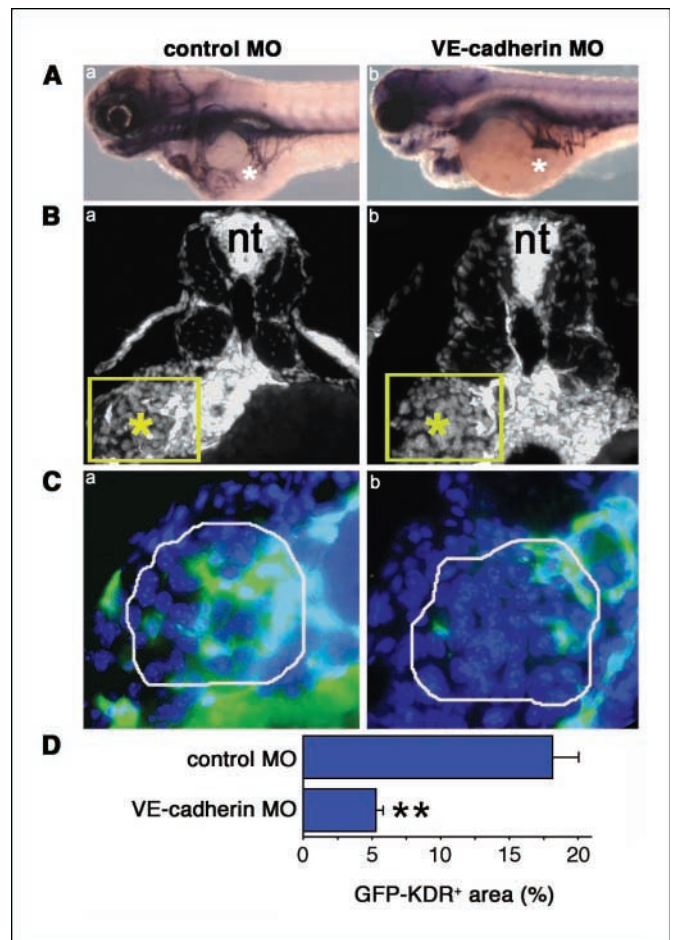
The identification of genes essential for blood vessel formation is of pivotal importance for the understanding of the angiogenic process and for the discovery of novel therapeutic targets. In zebrafish, antisense morpholino oligonucleotides (MO) induce a translational block in gene function, a very rapid experimental procedure compared with gene knockdown in mice (17). The

adherens junction component VE-cadherin plays an essential role in tumor angiogenesis in mice (18). On this basis, transgenic *VEGFR2:G-RCFP* zebrafish embryos were injected at the one-cell stage with a splice site MO targeting the zebrafish *VE-cadherin*. VE-cadherin MO (0.4 pmol/embryo) fully suppresses the maturation of the *VE-cadherin* transcript (Fig. 3A), causing the arrest of blood flow and enlargement of the pericardial cavity without affecting the development of normal intersegmental vessels and SIVs (Fig. 3B). However, the penetrance of the VE-cadherin MO was sufficient to cause a significant decrease in tumor vascularity triggered by FGF2-T-MAE cell implants as shown by alkaline phosphatase staining and quantification of the GFP-KDR<sup>+</sup> vascular area within the tumor graft (Fig. 4). Instead, no effect on tumor vascularization was exerted by control MO. It must be pointed out that the arrest of blood flow following systemic exposure of zebrafish embryos from 24 to 72 hpf with the myofibril ATPase inhibitor 2,3-butanedione monoxime (7.0 mmol/L; ref. 19) had no effect on tumor vascularization, ruling out the possibility that inhibition of tumor-induced neovascularization by VE-cadherin MO may represent a mere consequence of the arrest of blood circulation (data not shown). The capacity of VE-cadherin MO to hamper tumor vascularization without affecting the development of normal embryo vasculature is in keeping with the well-known morphofunctional differences between normal and tumor blood vessels (1), and represents the proof-of-concept that MOs could be used in zebrafish embryos for the rapid *in vivo* identification of genes involved in tumor angiogenesis.

The zebrafish model has exceptional utility as a human disease model system and is suitable for forward genetic screens and transient or permanent gene inactivation via MOs or "targeting-induced local lesions in genes," respectively. Recent studies have shown the feasibility of injecting human melanoma cells in zebrafish embryo to follow their fate and to study their effect on zebrafish development (20). Here, we extend these observations by



**Figure 3.** *VE-cadherin* zebrafish morphants. Transgenic *VEGFR2:G-RCFP* zebrafish embryos were injected at the one-cell stage with control MO or with VE-cadherin MO (0.4 pmol/embryo). A, reverse transcription-PCR analysis done at 24 hpf on total RNA extracted from untreated (-), control MO-injected (C), or VE-cadherin MO-injected (VE) embryos using specific primers for zebrafish *EF-1* and *VE-cadherin*. B, VE-cadherin MO causes arrest of blood flow and enlargement of pericardial cavity in 72 hpf embryos (arrow; b) when compared with control MO embryos (a). However, no gross alterations in GFP-KDR<sup>+</sup> blood vessel formation were observed both in control (c) and VE-cadherin (d) morphants (lateral view). No significant differences in vascular development were observed between control and VE-cadherin morphants also at 24 hpf (data not shown). Arrows, SIVs; arrowheads, intersegmental vessels.



**Figure 4.** VE-cadherin MO inhibits tumor angiogenesis in zebrafish embryo. A, whole-mount alkaline-phosphatase staining of FGF2-T-MAE cell-grafted *VEGFR2:G-RCFP* control (a) and VE-cadherin (b) morphants at 72 hpf (lateral view). B, DAPI-stained transverse sections of tumor-injected control (a) and VE-cadherin (b) morphants [nt, neuronal tube; \*, cell graft; green boxes, areas enlarged in (C); original magnification,  $\times 200$ ]. C, GFP-KDR<sup>+</sup> neovessels (green) within the tumor graft (white line) in *VEGFR2:G-RCFP* control (a) and VE-cadherin (b) morphants (original magnification,  $\times 600$ ). D, quantification by computerized image analysis of the GFP-KDR<sup>+</sup> area in tumors of control and VE-cadherin morphants ( $n = 8$ ;  $P < 0.01$ ).

showing that human and murine tumor xenografts induce angiogenesis in the zebrafish embryo. This process is hampered by the systemic exposure of live embryos to antiangiogenic chemicals or by MO-targeted gene inactivation. The zebrafish/tumor xenograft model represents a novel tool for studying the mechanisms of tumor-induced neovascularization. Its use for chemical discovery and gene targeting in tumor angiogenesis may offer significant advantages when compared with other animal models.

## Acknowledgments

Received 11/24/2006; accepted 2/12/2007.

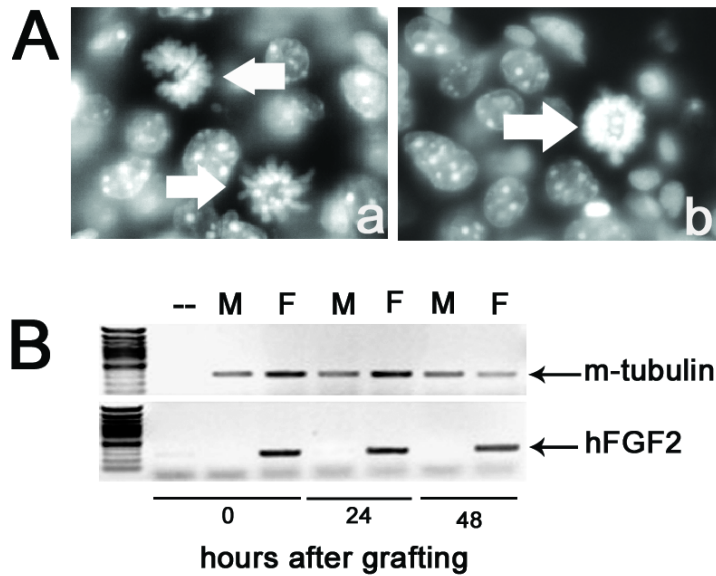
**Grant support:** Associazione Italiana per la Ricerca sul Cancro, MIUR (Centro d'Eccellenza IDET and Cofin projects), Fondazione Berlucci, Istituto Superiore di Sanità (Progetto Oncotecnologico), and EU-FP6 (LSHC-CT-2003-503233, subcontractor) to M. Presta and by MIUR (FIRB) and NOBEL Cariplo to F. Cotelli.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank M. Beltrame (University of Milan) for helpful discussion.

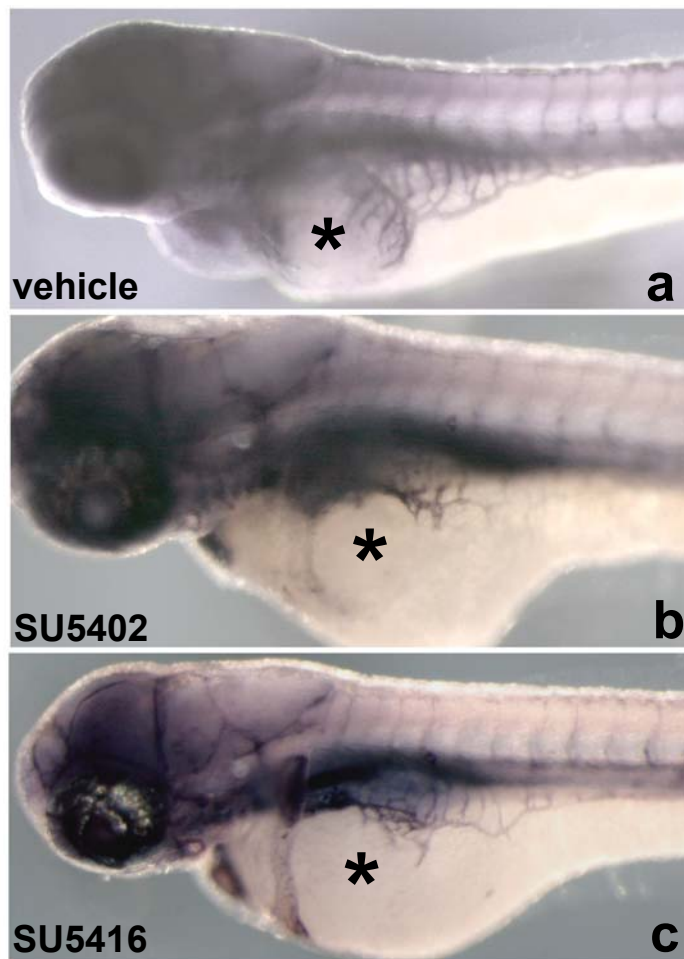
## References

1. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000;407:249–57.
2. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004;25:581–611.
3. Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev* 2005;16:159–78.
4. Hasan J, Shnyder SD, Bibby M, Double JA, Bicknel R, Jayson GC. Quantitative angiogenesis assays *in vivo*—a review. *Angiogenesis* 2004;7:1–16.
5. Lam SH, Wu YL, Vega VB, et al. Conservation of gene expression signatures between zebrafish and human liver tumors and tumor progression. *Nat Biotechnol* 2006;24:73–5.
6. Thisse C, Zon LI. Organogenesis—heart and blood formation from the zebrafish point of view. *Science* 2002;295:457–62.
7. Weinstein B. Vascular cell biology *in vivo*: a new piscine paradigm? *Trends Cell Biol* 2002;12:439–45.
8. Ribatti D, Gualandris A, Belleri M, et al. Alterations of blood vessel development by endothelial cells over-expressing fibroblast growth factor-2. *J Pathol* 1999;189:590–9.
9. Giavazzi R, Sennino B, Coltrini D, et al. Distinct role of fibroblast growth factor-2 and vascular endothelial growth factor on tumor growth and angiogenesis. *Am J Pathol* 2003;162:1913–26.
10. Cross LM, Cook MA, Lin S, Chen JN, Rubinstein AL. Rapid analysis of angiogenesis drugs in a live fluorescent zebrafish assay. *Arterioscler Thromb Vasc Biol* 2003;23:911–2.
11. Westerfield M. *The Zebrafish book*. Eugene (OR): University of Oregon Press; 1995.
12. Serbedzija GN, Flynn E, Willett CE. Zebrafish angiogenesis: a new model for drug screening. *Angiogenesis* 1999;3:353–9.
13. Habeck H, Odenthal J, Walderich B, Maischein H, Schulte-Merker S. Analysis of a zebrafish VEGF receptor mutant reveals specific disruption of angiogenesis. *Curr Biol* 2002;12:1405–12.
14. Brown LA, Rodaway AR, Schilling TF, et al. Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. *Mech Dev* 2000;90:237–52.
15. Larson JD, Wadman SA, Chen E, et al. Expression of VE-cadherin in zebrafish embryos: a new tool to evaluate vascular development. *Dev Dyn* 2004;231:204–13.
16. Pichler FB, Laurenson S, Williams LC, Dodd A, Copp BR, Love DR. Chemical discovery and global gene expression analysis in zebrafish. *Nat Biotechnol* 2003;21:879–83.
17. Nasevicius A, Ekker SC. Effective targeted gene 'knockdown' in zebrafish. *Nat Genet* 2000;26:216–20.
18. Liao F, Li Y, O'Connor W, et al. Monoclonal antibody to vascular endothelial-cadherin is a potent inhibitor of angiogenesis, tumor growth, and metastasis. *Cancer Res* 2000;60:6805–10.
19. Bartman T, Walsh EC, Wen KK, et al. Early myocardial function affects endocardial cushion development in zebrafish. *PLoS Biol* 2004;2:673–81.
20. Topczewska JM, Postovit LM, Margaryan NV, et al. Embryonic and tumorigenic pathways converge via nodal signaling: role in melanoma aggressiveness. *Nat Med* 2006;12:925–32.



**Supplementary Fig. S1.** A, Viability of mammalian cells grafted in zebrafish embryos. FGF2-T-MAE cells (a) and MAE cells (b) were injected in the perivitelline space of zebrafish embryos at 48 hpf. At 72 hpf, transverse sections of the cell grafts were stained with DAPI. Arrows point to mitotic figures within the grafts (original magnification, x 600). B, Murine FGF2-transfected xenografts express FGF2 mRNA in zebrafish embryos. Embryos were injected with vehicle (--), MAE cells (M), or FGF2-T-MAE cells (F) at 48 hpf. At the indicated time points after grafting, total RNA was extracted and steady-state levels of human FGF2 and murine tubulin mRNA were assessed by semiquantitative RT-PCR analysis.





**Supplementary Fig. S2.** Antiangiogenic compounds inhibit tumor-induced neovascularization in zebrafish embryos. Embryos were injected with FGF2-T-MAE cells (\*) at 48 hpf (5 embryos per group). Then, live fishes were incubated with vehicle (a), 1.0  $\mu\text{M}$  of the FGF receptor-1 TK inhibitor SU5402 (b), or 2.5  $\mu\text{M}$  of the VEGFR2/KDR TK inhibitor SU5416 (c). After 24 h, alkaline phosphatase-positive blood vessels were visualized (lateral view). Both compounds cause inhibition of tumor-induced neovascularization in all the embryos tested. No inhibition was observed in vehicle-treated embryos.