Open access • Journal Article • DOI:10.1007/S10456-012-9324-7

Matrigel plug assay: evaluation of the angiogenic response by reverse transcription-quantitative PCR — Source link

Daniela Coltrini, Emanuela Di Salle, Roberto Ronca, Mirella Belleri ...+3 more authors

Institutions: University of Brescia, Uppsala University

Published on: 01 Apr 2013 - Angiogenesis (Springer Netherlands)

Topics: Matrigel and Angiogenesis

Angiogenesis
DOI 10.1007/s10456-012-9324-7

BRIEF COMMUNICATION

Matrigel plug assay: evaluation of the angiogenic response by reverse transcription-quantitative PCR

Daniela Coltrini · Emanuela Di Salle · Roberto Ronca · Mirella Belleri · Chiara Testini · Marco Presta

Received: 30 July 2012 / Accepted: 6 November 2012

Abstract The subcutaneous Matrigel plug assay in mice is a method of choice for the in vivo evaluation of pro- and anti-angiogenic molecules. However, quantification of the angiogenic response in the plug remains a problematic task. Here we report a simple, rapid, unbiased and reverse transcription-quantitative PCR (RT-qPCR) method to investigate the angiogenic process occurring in the Matrigel plug in response to fibroblast growth factor-2 (FGF2). To this purpose, a fixed amount of human cells were added to harvested plugs at the end of the in vivo experimentation as an external cell tracer. Then, mRNA levels of the pan-endothelial cell markers *murine CD31* and *vascular endothelial-cadherin* were measured by species-specific RT-qPCR analysis of the total RNA and data were normalized for *human GAPDH* or *β-actin* mRNA levels. RT-qPCR was used also to measure the levels of expression in the plug of various angiogenesis/inflammation-related genes. The procedure allows the simultaneous, quantitative evaluation of the newly-formed endothelium and of non-endothelial/inflammatory components of the cellular infiltrate in the Matrigel implant, as well as the expression of genes involved in the modulation of the angiogenesis process. Also, the method consents the quantitative assessment of the effect of local or systemic administration of anti-angiogenic compounds on the neovascular response triggered by FGF2.

Keywords Angiogenesis · Anti-angiogenic drugs · FGF2 · Matrigel · PCR · Quantification

Introduction

Angiogenesis plays a key role in various physiological and pathological conditions, including embryonic development, wound repair, inflammation, and tumor growth [1]. The uncontrolled release of angiogenic growth factors, including fibroblast growth factor-2 (FGF2), vascular endothelial growth factor-A (VEGF-A) and other members of the FGF and VEGF families [2, 3], is responsible for the endothelial cell proliferation that takes place during tumor neovascularization and angiogenesis-dependent diseases [4]. 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 angiogenesis-dependent neoplastic and non-neoplastic diseases with anti-VEGF drugs [5].

Various in vivo models have been developed in different animal species, ranging from zebrafish to rodents, to investigate the angiogenesis process and for the screening of pro- and anti-angiogenic compounds, each with its own unique characteristics, advantages and disadvantages [6–

Electronic supplementary material The online version of this article (doi:10.1007/s10456-012-9324-7) contains supplementary material, which is available to authorized users.

D. Coltrini
Unit of Histology, Department of Biomedical Sciences and Biotechnology, University of Brescia, Brescia, Italy

E. Di Salle · R. Ronca · M. Belleri · C. Testini · M. Presta (✉)
Unit of General Pathology and Immunology, Department of Biomedical Sciences and Biotechnology, University of Brescia, Viale Europa 11, 25123 Brescia, Italy
e-mail: presta@med.unibs.it

Present Address:
C. Testini
Department of Immunology, Genetics and Pathology-IGP, Uppsala University, Uppsala, Sweden

Published online: 11 November 2012

10]. Among them, the Matrigel plug assay has become a method of choice [10, 11]. Matrigel, a laminin-rich reconstituted matrix is an extract of the Engelbreth-Holm-Swarm tumor composed of basement membrane components [12]. In the Matrigel plug assay, an angiogenic stimulus (usually represented by recombinant growth factors or tumor cells) is introduced into cold liquid Matrigel which, following subcutaneous (s.c.) injection in mice, gelifies and allows the recruitment of a new microvascular network [11].

Assessment of the angiogenic response in the Matrigel plug assay is routinely achieved by determination of the hemoglobin content of the plug or by histological/immunohistochemical analysis of vascular density in selected regions of plug slides [11]. However, both procedures present significant drawbacks that may affect the accurate quantification of the new microvascular network [7, 10]. Indeed, evaluation of the hemoglobin content is affected by the patency and size of the infiltrating vessels and by the presence of stagnant pools of blood, especially when heparin is added to the gel with possible hemorrhagic consequences. On the other hand, histological/immunohistochemical assessment of vascular density is strongly affected by its high intra-sample heterogeneity (see below) that may be overcome only by averaging the data from numerous tissue slides for each plug, thus resulting in a tedious, operator's subjective procedure [7].

Here we report a simple, rapid and unbiased method to quantify the new microvascular network elicited by FGF2 in the Matrigel plug assay by measuring the steady-state transcript levels of endothelial cell markers present in the plug by reverse transcription-quantitative PCR (RT-qPCR). The procedure also allows the simultaneous, quantitative evaluation of non-endothelial components of the cellular infiltrate and of the expression of genes involved in the modulation of the angiogenesis process in the Matrigel plug. RT-qPCR analysis was used also to assess the anti-angiogenic effect exerted in vivo by the local or systemic administration of FGF2 antagonists. This procedure will facilitate the quantification of in vivo angiogenesis experiments, essential for testing novel angiogenic and anti-angiogenic agents.

Materials and methods

Reagents and cells

Human recombinant FGF2 was from Tecnogen (Piana di Monteverna, Caserta, Italy). Matrigel (Cultrex® BME Growth Factor Reduced) was from Trevigen (Gaithersburg, MD), SU5402 from Calbiochem (Darmstadt, Germany), PD173074 from Sigma-Aldrich (St. Louis, MO), and FGF-ligand trap (FGFR1-Fc) from Relia Tech GmbH (Wolfenbuttel, Germany). Human endometrial adenocarcinoma HEC-1-B cells [13] were obtained from American Type Culture Collection (Rockville, MD) and grown in MEM supplemented with 1 % nonessential amino acids, 1 % sodium pyruvate, and 10 % fetal bovine serum. Murine lung microvascular endothelial 1G11 cells [14] were obtained from A. Vecchi (Istituto Scientifico Humanitas, Rozzano, Milan, Italy) and cultured in Dulbecco modified Eagle medium (DMEM) containing 20 % inactivated fetal bovine serum (FBS).

Matrigel plug angiogenesis assay

Liquid Matrigel (10–16 mg of protein/ml) was mixed at 4 °C with FGF2 dissolved in PBS at a final concentration equal to 1.0 μg/ml unless specified otherwise and injected subcutaneously (0.5 ml/mouse) into the flank of 6–8 week-old C57BL/6 mice (Charles River, Calco, Italy). Matrigel with PBS alone was used as negative control. One week after injection, mice were sacrificed and plugs were harvested, weighted and processed for RT-qPCR analysis. When specified, harvested plugs were divided in two parts, one half underwent RT-qPCR analysis whereas the other half was embedded in Tissue Tec OCT (Sigma-Aldrich), snap-frozen by immersion in liquid nitrogen-cooled isopentane and analysed by immunofluorescence microscopy.

RNA extraction and RT-qPCR analysis of Matrigel plugs

Total RNA was extracted from Matrigel plugs using TRIzol reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Before RNA extraction, plugs were added with an appropriate volume of TRIzol solution containing human HEC-1-B cells to a final concentration of 1×10^4 cells per mg of Matrigel. Purified total RNA were dissolved in RNase free water (1 μl per mg of Matrigel). Contaminating DNA was digested using DNase as in the DNase 1 Amplification Grade kit (Sigma-Aldrich). Four μl of total RNA were retro-transcribed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) using random hexaprimers in a final 20 μl volume. Quantitative PCR was performed with a Biorad iCycler iQ™ Real-Time PCR Detection System using a iQ™ SYBR Green Supermix (Biorad) according to manufacturer's instructions. Each PCR reaction was performed in triplicate on one plate and fluorescence data were recorded using iCycler software (BioRad). Relative expression ratios were calculated by use of Pfaffl equation and Relative Expression Software Tool (http://www.gene-quantification.info). The mRNA expression levels of target genes were normalized to the levels of *mGAPDH* and/or *hGAPDH* or *hβ-actin* housekeeping genes, as specified in the text. In each experiment, an arbitrary value equal to 1.0 was assigned to the levels of

expression of the gene(s) measured in one PBS plug that was used as reference sample.

In one in vitro preliminary experiment, increasing amounts of murine endothelial 1G11 cells were resuspended in 800 µl of liquid Matrigel, then cell suspensions were divided in two aliquots and incubated at 37 °C to allow gelification. Then, each aliquot was added with 500 µl of TRIzol reagent in the absence or in the presence of human HEC-1-B cells (1×10^4 cells per mg of Matrigel). Finally, total RNA was extracted from Matrigel samples for RT-qPCR analysis. The list of oligonucleotide primer sequences utilized in the present work (final concentration 400 nM) are shown in Online Resource 1, Table S1. Control experiments confirmed the lack of murine/human species cross-reactivity of these primers.

Immunoistochemical analysis of the Matrigel plugs

Seven µm-frozen sections of Matrigel plugs were fixed in ice-cold acetone and blocked with 10 % goat serum in Tris-buffered saline. To evaluate microvessel density, sections were stained with rat IgG2a anti-mCD31 monoclonal antibody (1/200 dilution, BD Pharmingen, San Diego, CA) followed by incubation with biotinylated goat anti-rat IgG antibody (1/200 dilution Santa Cruz, CA). Sections were then exposed to avidin–biotin-peroxidase complex Vectastain ABC Kit (Vector, Burlingame, CA) and peroxidase color reaction was developed with 3-amino 9-ethyl carbazole AEC Peroxidase substrate kit (Vector). Finally, sections were lightly counterstained with Mayer's hematoxylin. Images were acquired using a Zeiss fluorescence microscope (Zeiss Inc., North America) equipped with an Olympus N547 digital camera (Olympus, Hamburg, Germany) at 200× magnification. The regions containing the most intense $mCD31^+$ areas of neovascularization ("hotspots") were chosen for quantification (see Online Resource 1, Fig. S1). Five hotspots per Matrigel section and two sections per Matrigel plug were analysed at 200× magnification. The ImagePro Plus analysis system was used to measure $mCD31^+$ areas in each hotspot that were expressed as μm^2 per microscopic field.

Statistical analysis

Statistical analysis was performed with 2-tailed Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

Results and discussion

FGF2 induces a robust angiogenic response in the murine s.c. Matrigel plug assay characterized by the presence of numerous $CD31^+$ blood vessels and an abundant inflammatory cell infiltrate [15]. In a first set of experiments, in order to quantify the newly formed endothelium recruited by FGF2 in Matrigel plugs, we attempted to measure the relative mRNA levels of the murine pan-endothelial marker *mCD31* in FGF2-embedded and control implants by RT-qPCR. However, the limited cellularity in control plugs resulted in a very low yield of total RNA when compared to FGF2 plugs, making the comparison between the two sets of samples unreliable. Moreover, no significant difference was observed between the two experimental groups when the levels of *mCD31* mRNA in each plug were normalized for the levels of expression of the murine housekeeping gene *mGAPDH* (data not shown). Indeed, as stated above, FGF2 driven neovessel formation in the Matrigel plug is paralleled by the recruitment of numerous inflammatory cells. Thus, endothelial cells represent a roughly constant fraction of the total cells infiltrating the plug both in the absence and in the presence of FGF2 [15]. Thus, no significant differences are detectable between FGF2 and control plugs when the transcript levels of an endothelial marker are normalized for a pan-cellular murine housekeeping gene.

To overcome these technical drawbacks, we decided to add an independent external cellular tracer to each harvested plug at the end of experimentation to improve RNA recovery and to provide an internal control for RNA yield and for the efficiency of RT-qPCR amplification among the different samples. The cellular tracer is represented by a fixed number of human cells added to the plug per mg of pellet. Then, the levels of *mCD31* mRNA and of human *GAPDH* (*hGAPDH*) are measured in the final total cellular extract using species-specific oligonucleotide primers and data are expressed as *mCD31*/*hGAPDH* mRNA ratio. This approach overcomes the problem of the poor cellularity of control versus FGF2-plugs and allows the quantification of murine endothelial markers independently of other components of the host cellular infiltrate.

A preliminary experiment was carried out in vitro to assess whether the addition of human cells to the plugs could interfere with the quantification of the *mCD31* mRNA and to estimate the linearity of the method. To this purpose, murine $CD31^+$ endothelial 1G11 cells [14] were added in vitro to the gel suspension in absence or in the presence of a fixed amount of human cells (1×10^4 cells per mg of Matrigel). In the present work, human endometrial adenocarcinoma HEC-1-B cells [13] were used for normalization even though similar results can be obtained with any other human cell line available. After gelling, total RNA was extracted from each sample and the levels of *mCD31*, *mGAPDH* and *hGAPDH* transcripts were assessed by RT-qPCR using species-specific oligonucleotide primers (Online Resource 1, Table S1). Control experiments showed that addition of the human cells to the

plugs does not interfere with murine mRNA quantification (data not shown). When a standard curve of 1G11 cells (ranging between 2×10^5 and 5×10^6 cells) was prepared in the presence of human cells, normalization of the data demonstrated that the *mCD31*/*mGAPDH* mRNA ratio was constant for all the samples tested whereas the *mCD31*/*hGAPDH* mRNA ratio and the *mGAPDH*/*hGAPDH* mRNA ratio were linearly related to the number of 1G11 cells present in the sample (Fig. 1).

On this basis, control and FGF2-Matrigel plugs were injected subcutaneously into the flank of 6–8 week-old C57BL/6 mice. After 1 week, plugs were harvested, weighed and homogenized in a TRIzol solution. Then, each sample was divided in two aliquots and one aliquot was added with human cells resuspended in TRIzol to a final concentration of 1×10^4 human cells per mg of plug. After extraction, total RNA was dissolved in water (1 µl of H_2O per mg of Matrigel) and a fixed volume was retro-transcribed and used for qPCR analysis of *mCD31*, *mGAPDH* and *hGAPDH* transcript levels in each sample. An arbitrary value equal to 1.0 was assigned to the levels of expression of the different genes measured in one PBS plug that was used as reference sample. Again, addition of human cells did not affect the quantification of *mCD31* and *mGAPDH* transcripts in the different samples both in control and FGF2-plugs (data not shown). Also, only a limited 2 fold increase of the *mCD31*/*mGAPDH* mRNA ratio was observed in FGF2 plugs when compared to controls,

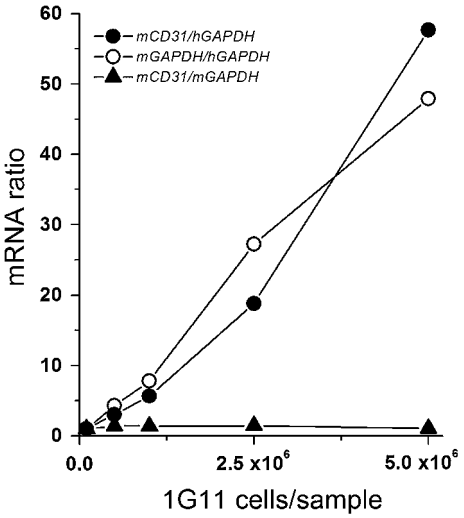

Fig. 1 Quantification of endothelial cell-derived *mCD31* mRNA levels in Matrigel. Increasing concentrations of murine endothelial 1G11 cells (ranging between 1×10^5 and 5×10^6 cells) were added in vitro to liquid Matrigel in the presence of human HEC-1-B cells (1×10^4 cells per mg of Matrigel). Then, RT-qPCR analysis was performed on the total RNA extracted from gelled Matrigel samples. RT-qPCR data are expressed as *mCD31*/*hGAPDH* (*filled circle*), *mGAPDH*/*hGAPDH* (*open circle*) and *mCD31*/*mGAPDH* (*filled triangle*) mRNA ratio. Each point is the mean of 3 determinations. Sample variability was <5 % of the mean values

further confirming that data normalization for *mGAPDH* expression levels masks the anticipated differences in plug vascularity between the two experimental groups (Fig. 2a). At variance, when the expression of *mCD31* mRNA was normalized for the *hGAPDH* transcript levels, a dramatic increase of the *mCD31*/*hGAPDH* mRNA ratio was observed in FGF2 versus control plugs (Fig. 2a). Accordingly, in a second set of experiments we observed a 20–22 fold increase of *mCD31* mRNA expression in FGF2 ($n = 6$) versus control ($n = 4$) plugs both when data were normalized for *hGAPDH* mRNA levels or when normalized for a different human housekeeping gene like *human β-actin*; again, no significant difference between the two experimental groups was observed when data were normalized for *murine β-actin* expression (data not shown).

To further validate this experimental procedure, animals were injected with Matrigel containing increasing amounts of FGF2 ranging between 0.1 and 1.0 µg/ml. After 1 week, plugs were harvested and processed for RT-qPCR analysis after addition of human cells as external tracer as described above. As shown in Fig. 2b, the results demonstrate a dose-dependent increase of the *mCD31*/*hGAPDH* mRNA ratio, thus confirming the quantitative capability of this in vivo assay.

Even though CD31 is commonly used as an endothelial cell marker, its expression is not restricted to endothelium, being expressed to different degrees also on leukocyte subtypes and platelets [16]. Moreover, cytokine combinations may affect *CD31* expression in endothelial cells (see [16] and references therein). On this basis, we evaluated the transcription levels of the murine endothelial marker *vascular endothelial-cadherin* (*mVE-cad*) in the same plugs used for the dose–response experiment described above and originally analyzed for *mCD31* expression. As shown in Fig. 2b, the results demonstrate that similar dose-dependent responses are obtained with FGF2 when quantification of the recruited endothelium is expressed as *mVE-cad*/*hGAPDH* or *mCD31*/*hGAPDH* mRNA ratio. Thus, the simultaneous, quantitative assessment of various endothelial markers in the same sample represents a further advantage of this procedure and may provide an useful control for those experimental conditions in which the modulation of the expression of an endothelial marker may occur.

The Matrigel plug assay is frequently used for the study of anti-angiogenic compounds [10, 11]. On this basis, we assessed the anti-angiogenic effect exerted in vivo by the local or systemic administration of various FGF2 antagonists. Addition to FGF2 plugs of a FGF-ligand trap (a fusion of mouse immunoglobulin Fc with a soluble FGFR1 construct [17]) or of the tyrosine kinase FGFR inhibitor SU5402 [18] causes a significant decrease of newly-formed endothelium as assessed by the reduction of *mCD31*/

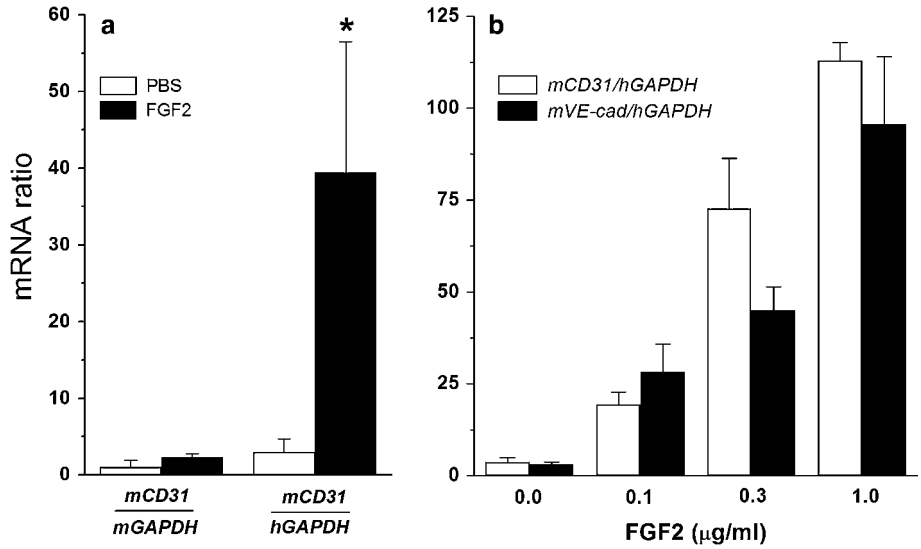

Fig. 2 Quantification of neovessel formation in Matrigel plugs by RT-qPCR analysis. **a** Matrigel plugs containing PBS (*open bars*) or 0.3 μg/ml of FGF2 (*black bars*) were implanted s.c. in mice. After 1 week, plugs were harvested, weighted, homogenized in a TRIzol solution, added with 1×10^4 human cells per mg of plug and assessed for *mCD31*, *mGAPDH* and *hGAPDH* expression levels by RT-qPCR. Data are the mean ± SEM of 7 plugs and are expressed both as *mCD31/mGAPDH* and *mCD31/hGAPDH* mRNA ratios. $^*P < 0.05$. **b** Matrigel pellets containing increasing concentrations of FGF2 were implanted s.c. in mice. After 1 week, plugs were processed as described in panel (**a**) and assessed for *mCD31*, *mVE-cad* and *hGAPDH* expression levels by RT-qPCR. Data are the mean ± SEM of 6 plugs per experimental point and are expressed as *mCD31/hGAPDH* (*open bars*) and *mVE-cad/hGAPDH* (*black bars*) mRNA ratios

hGAPDH and *mVE-cad/hGAPDH* mRNA ratios (Fig. 3, open bars). A similar inhibitory effect was exerted by the systemic administration of the tyrosine kinase FGFR inhibitor PD173074 [19]. Of note, very similar results were obtained when *mCD31* and *mVE-cad* expression data were normalized for the human housekeeping gene *hβ-actin* (Fig. 3, black bars). These data demonstrate that RT-qPCR analysis can be used to assess the effect of anti-angiogenic compounds in the Matrigel plug assay.

Analysis of the vascularization of tumor biopsies is usually performed by immunohistochemical staining of tissue slides for a given endothelial antigen (e.g. CD31 or Factor VIII-related antigen) followed by identification of vascular "hotspots" and their quantification by computerized image analysis [20]. Immunohistochemical assessment of vascularity can be applied also for the analysis of Matrigel plugs [10, 11]. On this basis, we compared the results obtained when Matrigel plug vascularization was quantified by RT-qPCR as described above or by mCD31 immunostaining. To this purpose, 12 mice were inoculated s.c. with FGF2 plugs and 4 animals with control PBS plugs. After 1 week, harvested plugs were divided in two parts: one half was processed for RT-qPCR analysis whereas the other half of the plug was included in OCT for mCD31 immunostaining and computerized image analysis of the $mCD31^+$ hotspots. Various counting procedures have been used for the immunohistochemical assessment of vascularity in tumors, including differences in the number of tissue blocks and of hotspots analyzed per sample (see Ref. [21] for a thoughtful discussion of this point). Because of the high heterogeneity of vascularization in different areas of the same Matrigel plug (see below), the value of $mCD31^+$ vascular density for each PBS or FGF2 plug was calculated as the mean of the values measured in two tissue sections on a total of 10 hotspots per plug.

As shown in Fig. 4a, immunohistochemical analysis demonstrates that the observed increase of the *mCD31/hGAPDH* mRNA ratio in FGF2 versus control plugs is paralleled by a significant increase of $mCD31^+$ vessel density within the implant. As frequently observed in animal experimentation, a remarkable inter-sample variability occurs in PBS and FGF2 experimental groups (Fig. 4b and Online Resource 1, Table S2). Indeed, the mean $mCD31^+$ microvascular density ranged between 0.47×10^3 and 2.83×10^3 μm^2/field and between 3.03×10^3 and 12.05×10^3 μm^2/field for PBS and FGF2 implants, respectively. A similar variability was observed when data were calculated as median values (Online Resource 1, Table S2). Accordingly, the range of values measured for the *mCD31/hGAPDH* mRNA ratio in the different plugs was equal to 0.5–11.8 and 70.7–188.4 in PBS and FGF2 groups, respectively (Fig. 4b). Next, *mCD31* expression levels and $mCD31^+$ vascular density measured in the same Matrigel implants were compared by linear regression analysis (Fig. 4b). Even though statistically significant ($P = 0.013$), the correlation between the

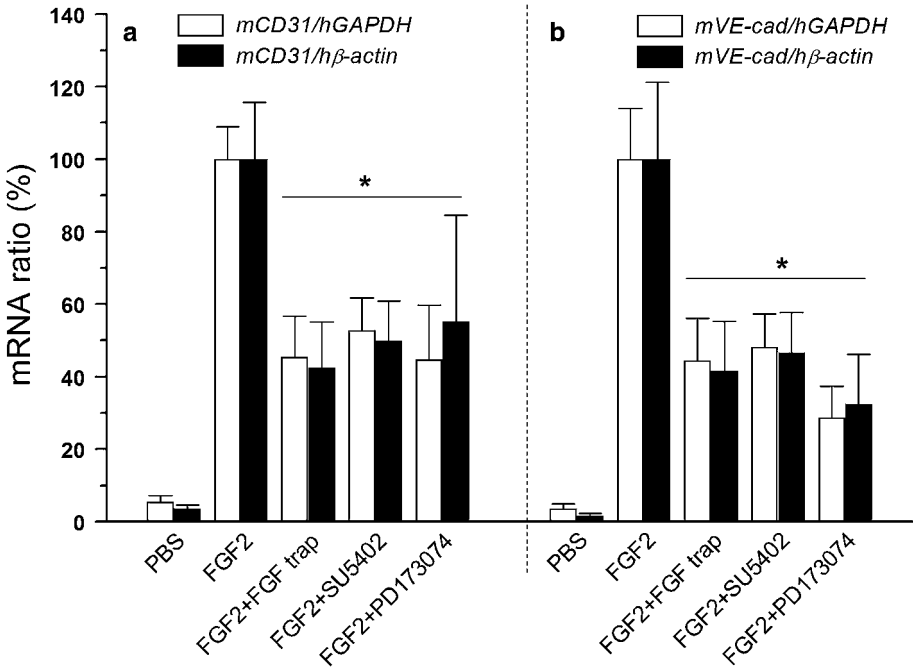

Fig. 3 Effect of local and systemic administration of FGF antagonists on FGF2-induced angiogenesis. Matrigel plugs containing 250 ng/plug of FGF2 were added with vehicle, 5 μg/plug of FGF-ligand trap, or 12 μg/plug of SU5402 and implanted s.c. in mice. In the same experiment, one group of animals implanted with FGF2 plugs was treated i.p. three times per week with 20 mg/kg of PD173074 dissolved in DMSO. After 1 week, plugs were harvested, weighted, homogenized in a TRIzol solution, added with 1×10^4 human cells per mg of plug, assessed for *mCD31* (**a**) and *mVE-cad* (**b**) expression levels by RT-qPCR and data were normalized for *hGAPDH* (*open bars*) and *hβ-actin* (*black bars*) expression. Data are the mean ± SEM of 6 plugs per group. All treatments resulted in a significant inhibition of FGF2 activity: $^*P < 0.05$ or better

two assays was characterized by an elevated dispersion of the data around the fitted line ($R^2 = 0.36$). This is likely due to the very high intra-sample variability of $mCD31^+$ hotspot quantification analysis with plugs showing highly vascularized zones together with poorly vascularized areas (Online Resource 1, Fig. S1). Indeed, the value of a single $mCD31^+$ hotspot ranged between 0.00 and 4.93×10^3 μm^2/field for PBS implants and between 1.38×10^3 and 24.66×10^3 μm^2/field for FGF2 implants (Online Resource 1, Table S2). Possibly, this variability may be overcome by averaging data from a much higher number of tissue slides for each plug, resulting however in a tedious, time-consuming and in any case operator's subjective procedure. At variance, RT-qPCR analysis allowed the rapid, unbiased quantification of the levels of endothelial cell transcripts present in the whole sample, thus minimizing the intra-sample variability.

As stated above, the angiogenic response triggered by FGF2 in the Matrigel plug assay is paralleled by an abundant $CD45^+$ cellular infiltrate with numerous $CD11b^+$ monocytes [15, 22] and $NG2^+$ pericytes [23], whereas only scattered $Gr\text{-}1^+$ neutrophils, $CD8^+$ or $CD4^+$ T-lymphocytes and no $CD19^+$ B-lymphocytes and $NK1.1^+$ natural killer cells are detectable in FGF2 plugs [15, 22]. RT-qPCR analysis confirmed that the significant increase of total cellular infiltrate in FGF2 plugs (indicated by the increase of the housekeeping *mGAPDH/hGAPDH* mRNA ratio) was paralleled by a significant increase of the *mCD45/hGAPDH*, *mCD11b/hGAPDH* and *mNG2/hGAPDH* mRNA values (Fig. 5). At variance, in keeping with the immunohistochemical data [15, 22], *mGr-1*, *mCD8*, *mCD4*, *mCD19*, and *mCD161* were expressed at very low levels in the plugs and their transcripts were detected by RT-qPCR analysis only after a very high number of amplification cycles, making the data nonquantitative and unreliable (data not shown).

The induction of a pro-angiogenic/inflammatory infiltrate by FGF2 goes along with the capacity of this growth factor to up-regulate the expression of various pro-angiogenic/pro-inflammatory genes as assessed on endothelial cells in vitro [15, 22]. On this basis, to get a further insight on the microenvironmental response elicited in vivo by FGF2, we measured the levels of expression of the FGF2-inducible pro-angiogenic/pro-inflammatory genes *mVEGF-A*, *cyclooxygenase-2* (*mCox-2*), *chemokine (C–C motif) ligand 2/MCP-1* (*mCcl2*) and *chemokine* (*C–C motif*) *ligand 7/MCP-3* (*mCcl7*) [15, 22] in PBS and FGF2 plugs. As shown in Fig. 6a, the values of *murine gene/hGAPDH* mRNA ratios are significantly increased in FGF2 versus PBS implants for all the genes investigated, thus indicating an absolute increase of their steady-state mRNA levels in

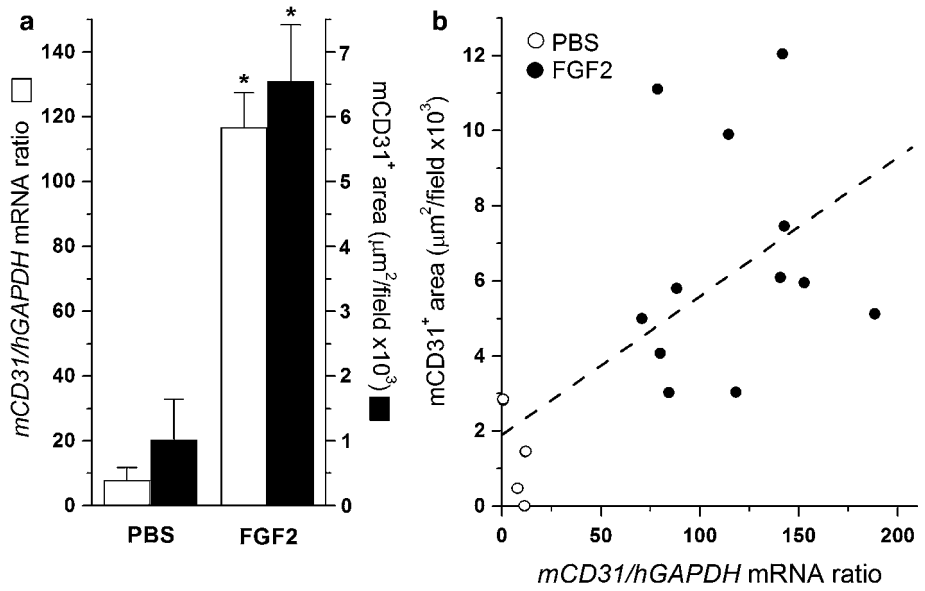

Fig. 4 Quantification of neovessel formation in Matrigel plugs: RT-qPCR versus immunohistochemical analysis. **a** Matrigel plugs containing PBS (n = 4) or 1.0 μg/ml of FGF2 (n = 12) were implanted s.c. in mice. After 1 week, plugs were harvested and divided in two aliquots. One aliquot was processed for determination of the *mCD31/hGAPDH* mRNA ratio by RT-qPCR analysis (*open bars*) whereas the second aliquot was processed for mCD31 immunostaining and evaluation of the $CD31^+$ hotspot areas by computerized image analysis (*black bars*). Data are expressed as mean ± SEM. $^*P < 0.05$. **b** For each plug, the individual value of *mCD31/hGAPDH* mRNA ratio was plotted against the mean value of its $CD31^+$ hotspot area. A statistically significant correlation ($P = 0.013$) characterized by an elevated dispersion of the data around the fitted linear regression line ($R^2 = 0.36$) was observed between the two parameters. *open circle*, PBS plugs; *filled circle*, FGF2 plugs

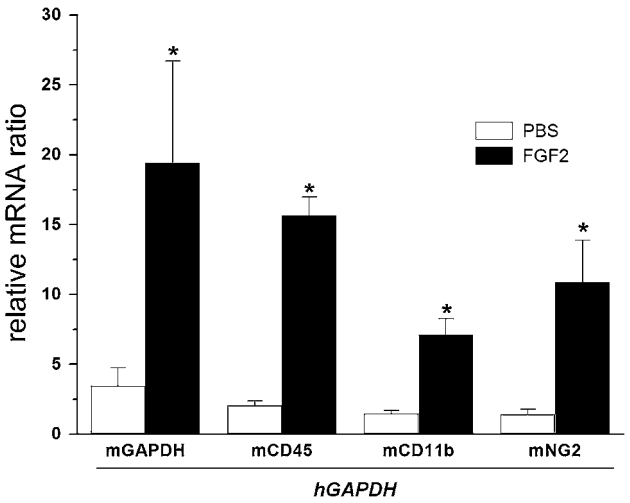

Fig. 5 Evaluation of the cellular infiltrate in Matrigel plugs by RT-qPCR analysis. Matrigel plugs containing PBS (*open bars*) or 1.0 μg/ml of FGF2 (*black bars*) were implanted s.c. in mice. After 1 week, plugs were processed for the evaluation of *mGAPDH*, *mCD45*, *mCD11b* and *mNG2* transcription levels by RT-qPCR and data were expressed as *murine gene/hGAPDH* mRNA ratios. Data were normalized to those measured in one PBS plug used as baseline reference sample in which an arbitrary value equal to 1.0 was assigned to the levels of expression of each investigated gene. The procedure allows the comparison of mRNA levels between control and FGF2 plugs for each single gene but not among the different genes. The data are the mean ± SEM of 5–6 plugs per experimental point; $^*P < 0.05$

FGF2 plugs. At variance, when the data were calculated as *murine gene/mGAPDH* mRNA ratios, only *mCcl2* expression appeared to be significantly upregulated (Fig. 6b). Together, the data strongly suggest that the observed increase of *mVEGF-A*, *mCox-2* and *mCcl7* transcript levels may represent the consequence of the increase of the total cellular infiltrate in FGF2 plugs whereas the increase of *mCcl2* transcripts is due, at least in part, to a FGF2-mediated upregulation of gene expression in infiltrating cells [15, 22]. Even though in situ hybridization analysis will be required to fully elucidate this point, our findings demonstrate that the RT-qPCR approach may represent an useful tool to investigate the molecular events that go along with the angiogenic process in Matrigel plugs.

The method herewith described is rapid, reproducible and shows several advantages when compared to other experimental procedures commonly used to quantify the angiogenic response in the Matrigel plug assay, including evaluation of the haemoglobin content of the plug or immunohistochemical analysis of the newly formed capillary network. Overall, the results validate the use of RT-qPCR analysis for the simultaneous, quantitative evaluation of newly-formed endothelium, non-endothelial components of the cell infiltrate, and expression of genes involved in the modulation of the angiogenesis process in the Matrigel plug. Moreover, the same procedure was used to assess the effect

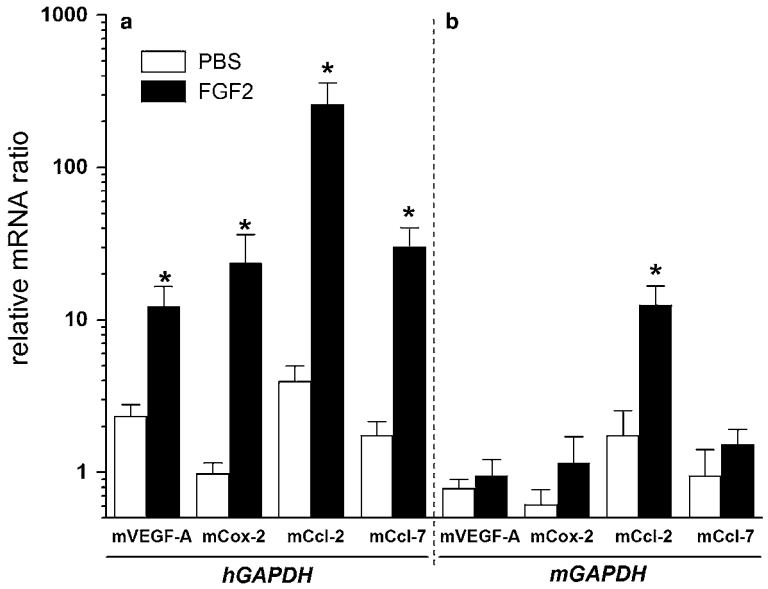

Fig. 6 Expression of pro-angiogenic/pro-inflammatory genes in Matrigel plugs. Matrigel plugs containing PBS (*open bars*) or 1.0 μg/ml of FGF2 (*black bars*) were implanted s.c. in mice. After 1 week, plugs were processed for the evaluation of *mVEGF-A*, *mCox-2*, *mCcl2* and *mCcl7* transcription levels by RT-qPCR and data were expressed both as *murine gene/hGAPDH* (**a**) and *murine gene/mGAPDH* (**b**) mRNA ratios. Data were normalized to those measured in one PBS plug used as baseline reference sample in which an arbitrary value equal to 1.0 was assigned to the levels of expression of all the genes investigated. The procedure allows the comparison of mRNA levels between control and FGF2 plugs for each single gene but not among the different genes. The data are the mean ± SEM of 5–6 plugs per experimental point; $*P < 0.05$

of local or systemic administration of putative anti-angiogenic compounds. A similar quantitative approach can be applied after addition to the gel of a suspension of pro-angiogenic cells (e.g. syngeneic tumor cell lines) or of their conditioned media (data not shown), further supporting the handiness of this RT-qPCR procedure. Clearly, types and amount of recruited cells in the Matrigel plug may vary depending on experimental conditions. The use of primers for cell type-specific markers (including endothelial, stromal and inflammatory cells) rather than of nonspecific murine housekeeping gene(s) may allow an accurate analysis of the neovascularization process and cell infiltrate occurring in the Matrigel plug under the different experimental conditions. This method will facilitate the analysis and quantification of in vivo angiogenesis experiments, essential for testing novel angiogenic and anti-angiogenic agents.

Acknowledgments This work was supported in part by grants from Ministero dell'Istruzione, Università e Ricerca (MIUR, Centro IDET, FIRB project RBAP11H2R9 2011) and Associazione Italiana per la Ricerca sul Cancro (AIRC grant no 10396) to MP.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407:249–257
2. Ferrara N (2010) Binding to the extracellular matrix and proteolytic processing: two key mechanisms regulating vascular endothelial growth factor action. Mol Biol Cell 21:687–690
3. Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R, Rusnati M (2005) Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. Cytokine Growth Factor Rev 16:159–178
4. Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1:27–31
5. Wu JM, Staton CA (2012) Anti-angiogenic drug discovery: lessons from the past and thoughts for the future. Expert Opin Drug Discov 8:723–743
6. Hasan J, Shnyder SD, Bibby M, Double JA, Bicknel R, Jayson GC (2004) Quantitative angiogenesis assays in vivo–a review. Angiogenesis 7:1–16
7. Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N (2003) Angiogenesis assays: a critical overview. Clin Chem 49:32–40
8. Nicoli S, Presta M (2007) The zebrafish/tumor xenograft angiogenesis assay. Nat Protoc 2:2918–2923
9. Ribatti D, Nico B, Vacca A, Presta M (2006) The gelatin sponge-chorioallantoic membrane assay. Nat Protoc 1:85–91
10. Norrby K (2006) In vivo models of angiogenesis. J Cell Mol Med 10:588–612
11. Passaniti A, Taylor RM, Pili R, Guo Y, Long PV, Haney JA, Pauly RR, Grant DS, Martin GR (1992) A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. Lab Invest 67:519–528
12. Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW, Martin GR (1986) Basement membrane complexes with biological activity. Biochemistry 25:312–318
13. Coltrini D, Gualandris A, Nelli EE, Parolini S, Molinari-Tosatti MP, Quarto N, Ziche M, Giavazzi R, Presta M (1995) Growth advantage and vascularization induced by basic fibroblast growth factor overexpression in endometrial HEC-1-B cells: an export-dependent mechanism of action. Cancer Res 55:4729–4738

14. Dong QG, Bernasconi S, Lostaglio S, De Calmanovici RW, Martin-Padura I, Breviario F, Garlanda C, Ramponi S, Mantovani A, Vecchi A (1997) A general strategy for isolation of endothelial cells from murine tissues. Characterization of two endothelial cell lines from the murine lung and subcutaneous sponge implants. Arterioscler Thromb Vasc Biol 17:1599–1604
15. Andres G, Leali D, Mitola S, Coltrini D, Camozzi M, Corsini M, Belleri M, Hirsch E, Schwendener RA, Christofori G, Alcami A, Presta M (2009) A pro-inflammatory signature mediates FGF2-induced angiogenesis. J Cell Mol Med 13:2083–2108
16. Woodfin A, Voisin MB, Nourshargh S (2007) PECAM-1: a multi-functional molecule in inflammation and vascular biology. Arterioscler Thromb Vasc Biol 27:2514–2523
17. Compagni A, Wilgenbus P, Impagnatiello MA, Cotten M, Christofori G (2000) Fibroblast growth factors are required for efficient tumor angiogenesis. Cancer Res 60:7163–7169
18. Mohammadi M, McMahon G, Sun L, Tang C, Hirth P, Yeh BK, Hubbard SR, Schlessinger J (1997) Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science 276:955–960
19. Mohammadi M, Froum S, Hamby JM, Schroeder MC, Panek RL, Lu GH, Eliseenkova AV, Green D, Schlessinger J, Hubbard SR (1998) Crystal structure of an angiogenesis inhibitor bound to the FGF receptor tyrosine kinase domain. EMBO J 17:5896–5904
20. Hasan J, Byers R, Jayson GC (2002) Intra-tumoural microvessel density in human solid tumours. Br J Cancer 86:1566–1577
21. Nico B, Benagiano V, Mangieri D, Maruotti N, Vacca A, Ribatti D (2008) Evaluation of microvascular density in tumors: pro and contra. Histol Histopathol 23:601–607
22. Presta M, Andres G, Leali D, Dell'Era P, Ronca R (2009) Inflammatory cells and chemokines sustain FGF2-induced angiogenesis. Eur Cytokine Netw 20:39–50
23. Tigges U, Hyer EG, Scharf J, Stallcup WB (2008) FGF2-dependent neovascularization of subcutaneous Matrigel plugs is initiated by bone marrow-derived pericytes and macrophages. Development 135:523–532