



Paracrine and autocrine effects of fibroblast growth factor-4 in endothelial cells

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Recombinant Fibroblast Growth Factor-4 (FGF4) and FGF2 induce extracellular signal-regulated kinase-1/2 activation and DNA synthesis in murine aortic endothelial (MAE) cells. These cells co-express the IIIc/Ig-3 loops and the novel glycosaminoglycan-modified IIIc/Ig-2 loops isoforms of FGF receptor-2 (FGFR2). The affinity of FGF4/FGFR2 interaction is 20–30 times lower than that of FGF2 and is enhanced by heparin. Overexpression of FGF2 or FGF4 cDNA in MAE cells results in a transformed phenotype and increased proliferative capacity, more evident for FGF2 than FGF4 transfectants. Both transfectants induce angiogenesis when applied on the top of the chick embryo chorioallantoic membrane. However, in contrast with FGF2-transfected cells, FGF4 transfectants show a limited capacity to growth under anchorage-independent conditions and lack the ability to invade 3D fibrin gel and to undergo morphogenesis *in vitro*. Also, they fail to induce hemangiomas when injected into the allantoic sac of the chick embryo. In conclusion, although exogenous FGF2 and FGF4 exert a similar response in MAE cells, significant differences are observed in the biological behavior of FGF4 versus FGF2 transfectants, indicating that the expression of the various members of the FGF family can differently affect the behavior of endothelial cells and, possibly, of other cell types, including tumor cells. *Oncogene* (2001) 20, 2655–2663.

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Introduction

The *hst* oncogene encodes for Fibroblast Growth Factor-4 (FGF4), a member of the FGF family that transforms 3T3 cells with high efficiency (Delli Bovi *et al.*, 1987; Moscatelli and Quarto, 1989). *Hst* activation results in the overexpression of the growth factor that is efficiently secreted and binds to cell surface tyrosine kinase FGF receptors (FGFRs), thus creating an

autocrine loop of stimulation (Moscatelli and Quarto, 1989). Also, FGF4 stimulates endothelial cell proliferation, migration, and protease production *in vitro* and neovascularization *in vivo* (Delli Bovi *et al.*, 1988; Yoshida *et al.*, 1994). FGF2 shares with FGF4 a potent angiogenic activity (Basilio and Moscatelli, 1992). The single-copy human *fgf-2* gene encodes multiple FGF2 isoforms with molecular weight (MW) ranging from 18 000–24 000 (Florkiewicz and Sommer, 1989). At variance with FGF4, FGF2 isoforms lack a leader sequence for secretion and have different intracellular fates (Renko *et al.*, 1990). Nevertheless, FGF2 isoform(s) can be secreted in limited amounts by an alternative secretion pathway (Mignatti *et al.*, 1992) and accumulate in the extracellular matrix, from where they are released by degrading enzymes (Ribatti *et al.*, 1999a and references therein). Overexpression of the different FGF2 isoforms may have different biological consequences in transfected cells, suggesting that they may play different intracellular/extracellular functions (Couderc *et al.*, 1991; Bikfalvi *et al.*, 1995; Escaffit *et al.*, 2000; Gaubert *et al.*, 2001). However, both M_r 18 000 and M_r 24 000 FGF2 isoforms show angiogenic activity *in vitro* and *in vivo* (Gualandris *et al.*, 1994).

A dual receptor model has been proposed for FGFs in which interaction with low affinity heparan sulfate (HS) proteoglycans (HSPGs) is required for the high affinity interaction with FGFRs (Klagsbrun and Baird, 1991). HSPGs may induce FGF oligomerization required for receptor dimerization, autophosphorylation and activation of intracellular signaling (Spivak-Kroizman *et al.*, 1994). Four FGFR genes have been cloned (*FGFR1/fg*: Lee *et al.*, 1989; *FGFR2/bek*: Dionne *et al.*, 1990; *FGFR3*: Keegan *et al.*, 1991; and *FGFR4*: Partanen *et al.*, 1991). They are characterized by three extracellular immunoglobulin (Ig)-like domains, an acidic box located between the first and second Ig-like loop, a transmembrane domain, and a splitted intracellular catalytic tyrosine-kinase domain. Several alternative spliced RNA variants that differ in the number of Ig-like loops and/or in the absence of the intracellular domain were also described (Johnson and Williams, 1993). The growth factor binding site of FGFR includes the second half of the third Ig-like loop (Plotnikov *et al.*, 2000); three variants of this region, encoded by different exons, have been described

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(Johnson and Williams, 1993; Chellaiah *et al.*, 1994). IIIc isoforms show a broader spectrum of ligands when compared to IIIb variants (Ornitz *et al.*, 1996). In particular, FGF2 and FGF4 bind to murine FGFR2/IIIc/Ig-3 with similar affinity in CHO transfected cells (Mansukhani *et al.*, 1992).

FGFs may exert their effects on endothelial cells via a paracrine mode consequent to their release from producing cells and/or by mobilization from the extracellular matrix. FGF2 may also play an autocrine role in endothelial cells (Gualandris *et al.*, 1996, and references therein). Accordingly, FGF2 has been implicated in the pathogenesis of lesions of endothelial cell origin, including hemangiomas (Takahashi *et al.*, 1994) and Kaposi's sarcoma (KS) (Ensoli *et al.*, 1994a, b). Interestingly, recent observations have also shown that the *FGF4* gene is amplified and expressed in HHV8-positive KS biopsies (Kiuru-Kuhlefeld *et al.*, 2000). These data suggest that FGFs produced by cells of the endothelial lineage, including KS-derived spindle cells, may play important autocrine and paracrine roles in angiogenesis and in the pathogenesis of vascular lesions.

To assess the biological consequences of endothelial cell activation by endogenous FGFs, we originated an FGF2-overexpressing mouse aortic endothelial cell line (pZipFGF2 MAE cells: Gualandris *et al.*, 1996). FGF2 transfectants show an invasive and morphogenic behavior *in vitro*. *In vivo*, they are angiogenic, cause the formation of opportunistic vascular tumors in nude mice, and induce hemangiomas when injected in the allantoic sac of the chick embryo (Gualandris *et al.*, 1996; Ribatti *et al.*, 1999b). This latter effect appears to be due to an as yet unidentified heparin-binding FGF2-inducible protein released by pZipFGF2 MAE cells and able to confer morphogenic and invasive capacity when added to parental MAE cells (Ribatti *et al.*, 1999b).

In the present work, we decided to investigate the interaction of exogenous FGF4 with MAE cells and to assess the impact of FGF4 overexpression on the biological behavior of MAE transfectants *in vitro* and *in vivo* when compared to FGF2 transfectants. The results show that exogenous FGF2 and FGF4 exert a similar response in MAE cells. In contrast, significant qualitative differences are observed in the biological behavior of these cells following the endogenous expression of the two growth factors. This may reflect differences in the intracellular/extracellular fate of FGF4 in respect to FGF2 isoforms.

Results

Interaction of exogenous FGF4 with MAE cells

MAE cells express FGFR2 (Bastaki *et al.*, 1997). Accordingly, recombinant FGF2 and FGF4 stimulate DNA synthesis when administered to these cells. The mitogenic activity of FGF4 appears to be approximately 10 times less potent than that of FGF2 and is

enhanced by heparin (Figure 1a). Also, both growth factors trigger ERK_{1/2} phosphorylation at a saturating concentration equal to 10 ng/ml (Figure 1a, insert). In agreement with its mitogenic capacity, FGF4 was 20–30 times less potent than FGF2 in competing for the binding of ¹²⁵I-FGF2 to FGFRs in MAE cells (Figure 1b). Unmodified heparin, but not 2-*O*-desulfated heparin, was able to increase significantly the affinity of FGF4 for the receptor (Figure 1b), suggesting that cell surface HS may play a role in FGF4/FGFR2 interaction in these cells. No significant effect was instead exerted by 100 nM unmodified heparin or 2-*O*-desulfated heparin on FGF2/FGFR2 interaction (data not shown).

FGFR2/IIIc/Ig-3 loops isoform binds FGF2 and FGF4 with similar affinity (Mansukhani *et al.*, 1992). In order to identify the FGFR2 isoform(s) expressed by MAE cells, we designed different oligonucleotide primers spanning the extracellular domain of the murine receptor (Figure 2a). When MAE cells were compared by RT-PCR analysis to NIH3T3 cells transfected with the FGFR2/IIIc/Ig-3 loops isoform (see Materials and methods), an identical pattern of amplification was observed using primers spanning the second and third Ig-like loops. In contrast, one extra band of approximately 100 bp was detected in MAE cells using primers that span the first Ig-like loop (Figure 2b). On this basis, a more expanded fragment spanning the region anticipated to contain the signal peptide sequence, the first Ig-like loop, and the acidic box was amplified and cloned. Nucleotide sequence (not shown) indicates that this fragment actually encodes for the N-terminus of the FGFR2/IIIc/Ig-2 loops variant (Twigg *et al.*, 1998) and contains the HS-modified Ser-Ser-Gly motif in its acidic domain (Sakaguchi *et al.*, 1999) (Figure 2c). Cross-linking of MAE cell surface receptors with ¹²⁵I-FGF2, followed by SDS-PAGE and 1 week exposure of the gel to a FLA2000 PhosphoImager screen (Fuji), showed the presence of a faint broad band with M_r 140 000–210 000 (data not shown), as already observed for NIH3T3 cells overexpressing the HS-modified FGFR2/IIIc/Ig-2 loops isoform (Sakaguchi *et al.*, 1999), suggesting that both the IIIc/Ig-3 loops and the IIIc/Ig-2 loops isoforms of FGFR2 are exposed on the cell surface. However, the limited number of FGFRs and the coexpression of two isoforms made unfeasible the assessment of the contribution of each individual FGFR2 isoform to FGF2 and FGF4 binding.

FGF4 overexpression in MAE cells: effects in vitro

FGF2 overexpression confers a transformed, angiogenic phenotype to transfected MAE cells (pZipFGF2-MAE cells) associated with an invasive and morphogenic behavior *in vitro* (Gualandris *et al.*, 1996). To assess the impact of FGF4 overexpression in MAE cells, human FGF4 cDNA was cloned in the same retroviral expression vector utilized to generate the pZipFGF2-MAE cells, and the construct was transfected in the parental cell line. After G418

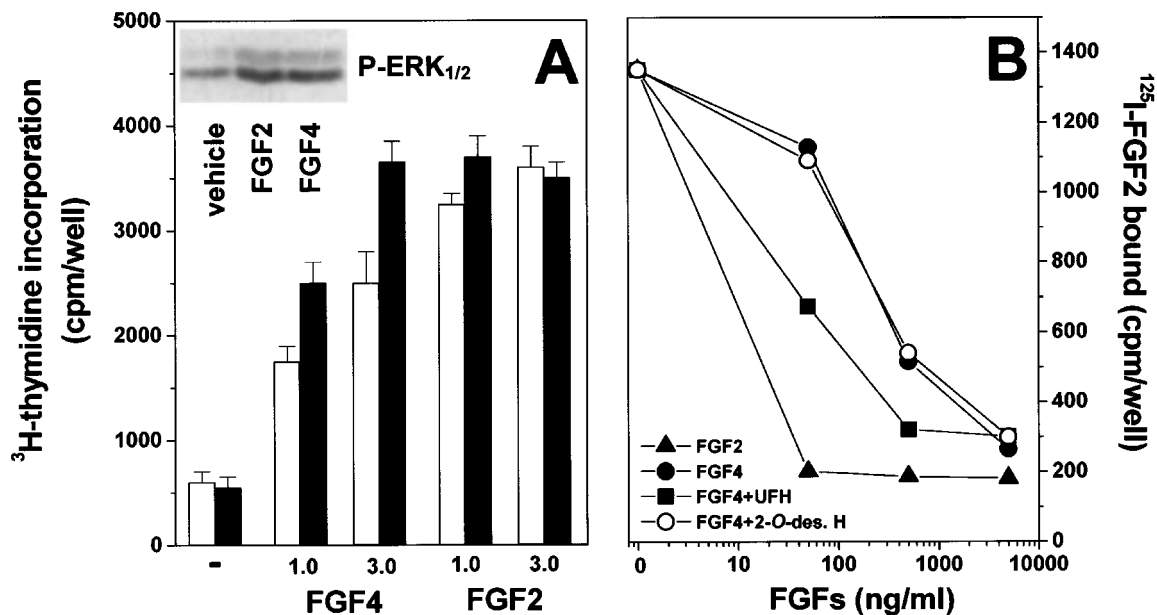


Figure 1 Biological activity of FGF2 and FGF4 in MAE cells. (a) Recombinant FGF4 and FGF2 were added to quiescent MAE cells at 1.0 and 3.0 ng/ml in the absence (open bars) or in the presence (black bars) of 100 nM heparin. Then, [³H]thymidine incorporation was measured as described in Materials and methods. The results are the mean of two experiments in triplicate (*Inset:*) MAE cells were treated with 10 ng/ml FGF2 or FGF4. After 20 min, ERK_{1/2} phosphorylation (P-ERK_{1/2}) was evaluated by Western blot analysis of the cell extracts. (b) MAE cells were incubated for 2 h at 4°C with ¹²⁵I-FGF2 (3 ng/ml) in the presence of increasing concentrations of unlabeled FGF2 (▲) or FGF4 added alone (●) or with 100 nM unmodified heparin (UFH, ■) or 100 nM 2-*O*-desulfated heparin (2-*O*-des.H, ○). Then, radioactivity bound to high affinity FGFRs was measured

selection, conditioned media from different clones were loaded on heparin-Sepharose columns and 2.0 M NaCl eluates were assessed for FGF4 protein expression. Among them, one clone, named pZipFGF4-MAE cells, was used for further experiments. As shown in Figure 3a, FGF4 is released by pZipFGF4-MAE cells at approximately 3 ng/24 h/10⁶ cells and accumulates in the conditioned medium. In contrast, both the M_r 18 000 and the HMW FGF2 isoforms accumulates intracellularly in pZipFGF2-MAE cells (Figure 3a). These cells actually release limited amounts of FGF2 in their conditioned medium, corresponding approximately to 30 pg/24 h/10⁶ cells (Ribatti *et al.*, 1999b).

In agreement with previous observations (Gualandris *et al.*, 1996), pZipFGF2-MAE cells showed a transformed phenotype (Figure 4) and a marked increase in cell proliferation and saturation density when compared to parental cells (Figure 3b). Moreover, they underwent morphogenesis when seeded on Matrigel and showed an invasive behavior and sprouting activity in 3D fibrin gel (Figure 4). FGF4 transfectants acquired a transformed morphology and showed a 2.5-fold increase of their proliferation rate and saturation density. However, they failed to invade fibrin gel and to form tube-like structures on Matrigel (Figures 3b and 4). Also, pZipFGF4-MAE cells showed a limited capacity to grow under anchorage-independent conditions when compared to FGF2 transfectants (104 ± 17, 15 ± 3, and 2 ± 1 colonies/field for pZipFGF2, pZipFGF4, and parental cells, respectively). Moreover, the size of pZipFGF4-MAE colonies

was significantly smaller than that of pZipFGF2-MAE colonies (Figure 4).

FGF4 overexpression in MAE cells: effects *in vivo*

pZipFGF2-MAE cells induce angiogenesis when implanted on the top of the chorioallantoic membrane (CAM) and cause hemangioma formation when injected into the allantoic sac of the chick embryo (Table 1 and Ribatti *et al.*, 1999b). The former but not the latter activity is inhibited by neutralizing anti-FGF2 antibody (Ribatti *et al.*, 1999b). When tested under the same experimental conditions, pZipFGF4-MAE cells induce angiogenesis on the CAM but are unable to cause hemangiomas when injected into the allantoic sac (Table 1).

An as yet unidentified heparin-binding FGF2-inducible protein appears to be responsible for the hemangioma-inducing capacity of FGF2 transfectants (Ribatti *et al.*, 1999b). This molecule stimulates parental MAE cells to originate endothelial sprouts in 3D fibrin gel (Ribatti *et al.*, 1999b). In agreement with the *in vivo* observations, only the pZipFGF2-MAE cell-conditioned medium stimulates endothelial sprouting whereas the conditioned media of both transfectants exerted a significant mitogenic activity on MAE cells (Figure 5). Accordingly, the conditioned medium of FGF2 transfectants stimulates the sprouting of endothelial 1G11 cells (Dong *et al.*, 1997) whereas the conditioned medium of pZipFGF4-MAE cells is ineffective (Figure 5), despite the fact that both

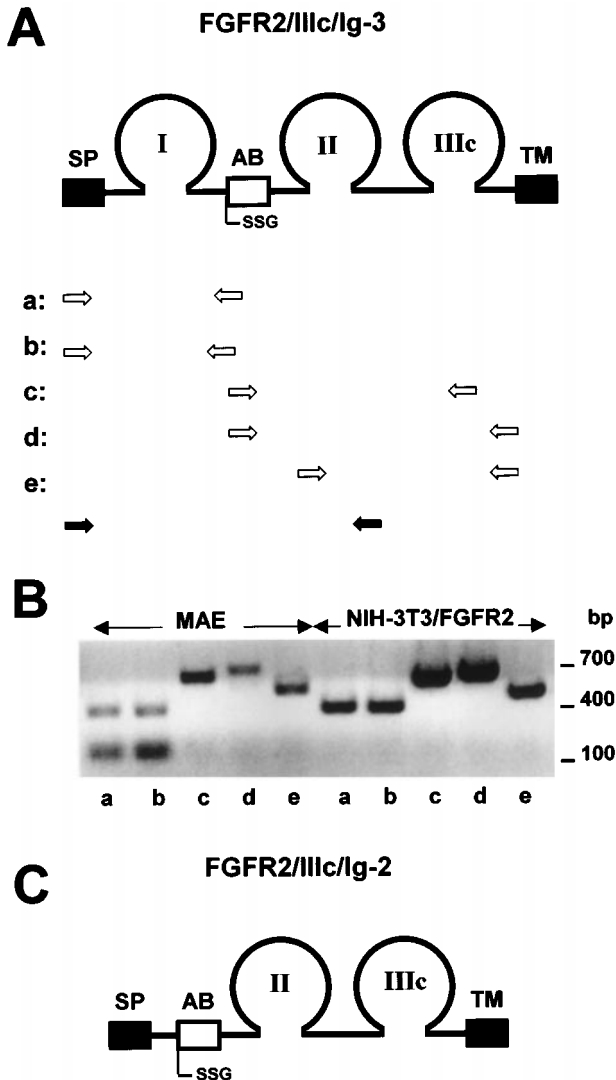


Figure 2 Identification of FGFR2 isoforms expressed by MAE cells. (a) Schematic representation of the FGFR2/IIIc/Ig-3 isoform showing the oligonucleotide primers used for RT-PCR analysis of cellular transcripts. (a) forward 5'-CCTCCTTCAGTT-TAGTTGAGGATACCA-3', reverse 5'-CTCCTGTTCTCACT-GACAACGTCTT-3', (b) forward 5'-CCTCCTTCAGTT-TAGTTGAGGATACCA-3', reverse 5'-GAAGATCCAAGTTT-CACTGTCTACCG-3', (c) forward 5'-GACACAGATAGTCC-GAAGACGTT-3', reverse 5'-TGCAGAGTGAAAGGATAT-CCCG-3', (d) forward 5'-GACACAGATAGTCCGAAGAC-GTT-3', reverse 5'-GATAATCTGGGGAAGCCGTGAT-3', (e) forward 5'-CGCATGGAGGCTATAAGGTACG-3', reverse 5'-GATAATCTGGGGAAGCCGTGAT-3', black arrows indicate the primers used to clone the FGFR2/IIIc/Ig-2 isoform: forward 5'-CCTCCTTCAGTTT-TAGTTGAGGATACCA-3', reverse 5'-TGGTTGATGGACCCGTAGGCA-3'. (b) RT-PCR analysis of MAE and NIH3T3/FGFR2 cellular transcripts. In each lane, the letter identifies the oligonucleotide primers used for the analysis. The arrowhead points to the extra band in MAE cells belonging to FGFR2/IIIc/Ig-2 isoform schematized in panel c. SP, signal peptide; AB, acidic box; TM, transmembrane domain; SSG, Ser-Ser-Gly-motif

FGF2 and FGF4 are able to exert a significant mitogenic response also in this endothelial cell type (data not shown). Similar results were obtained when

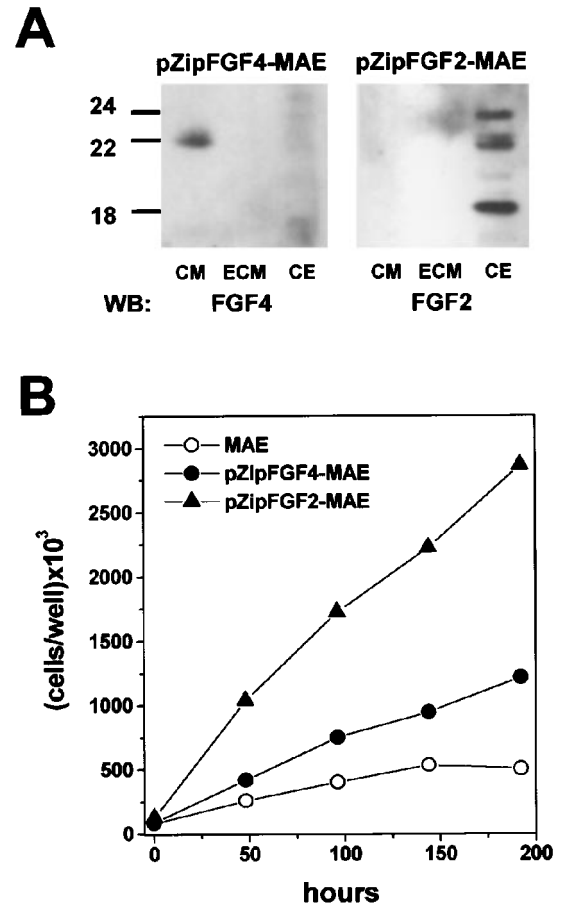


Figure 3 FGF2 and FGF4-transfected MAE cells. (a) Western blot analysis of the conditioned medium (CM), extracellular matrix (ECM), and cell extract (CE) of pZipFGF4-MAE and pZipFGF2-MAE cells with anti-FGF4 and anti-FGF2 antibodies, respectively (see Material and methods) (b) Parental (○), pZipFGF2 (▲), and pZipFGF4 (●) MAE cells were seeded at 50 000 cells/cm² in 24-well plates in complete medium and counted at the indicated periods of time. Data represent the mean of two independent experiments in triplicate

conditioned media and recombinant proteins were tested in the fetal bovine aortic endothelial GM7373 cell line (Grinspan *et al.*, 1983) (data not shown).

Discussion

In the present paper we investigated the effects of exogenous and endogenous FGF4 on MAE cell behavior *in vitro* and *in vivo*. Northern blot analysis demonstrated that MAE cells express FGFR2 (Bastaki *et al.*, 1997). The IIIc/Ig-3 loops isoform of this receptor had been shown to bind FGF2 and FGF4 with similar affinity (Mansukhani *et al.*, 1992). Our data demonstrate that human recombinant FGF2 and FGF4 elicit similar biological responses in MAE cells by triggering receptor downstream signaling, exemplified by ERK_{1/2} phosphorylation (Besser *et al.*, 1995), and stimulating DNA synthesis, although the potency of the mitogenic activity of FGF4 appears to be lower

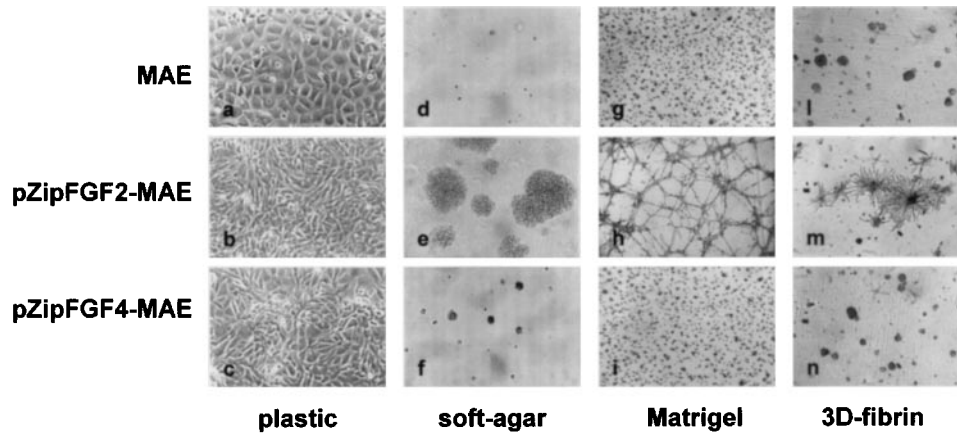


Figure 4 Morphological features of FGF2 and FGF4 transfectants. Parental (a, d, g, l), pZipFGF2 (b, e, h, m) and pZipFGF4 (c, f, i, n) MAE cells were cultured on plastic (a, b, c), in soft-agar (d, e, f), on Matrigel (g, h, i), or in 3D fibrin gel (l, m, n) and photographed under an inverted microscope. Original magnification: 100× (a–f), 40× (g–n)

Table 1 Effect of FGF4 and FGF2 transfected MAE cells on the developing microvasculature of the chick embryo CAM

	Hemangioma formation ^a (positive eggs/ total eggs)	Angiogenic response ^b (positive eggs/ total eggs) (microvessel density)	
Parental MAE	0/20	0/20	0
pZipFGF2-MAE	14/20	18/20	30 ± 3
pZipFGF4-MAE	0/20	17/20	27 ± 4

^aCells were injected into the allantoic sac; ^bCells were implanted on the top of the CAM. See Materials and methods for details

than that of FGF2 and is enhanced by heparin. Accordingly, competition experiments with ¹²⁵I-FGF2 have shown that the affinity of FGF4 for FGFRs in MAE cells is lower than that of FGF2. Heparin causes a significant increase of the affinity of FGF4 for FGFR interaction. The effect appears to be specific since 2-*O*-desulfated heparin, that does not interact with FGF4 (Guimond *et al.*, 1993), is ineffective and unmodified heparin does not affect the receptor-binding capacity of FGF2. Recent data have shown that the acidic box of the FGFR2/IIIc/Ig-2 loops isoform can be covalently modified by HS chains in its Ser-Ser-Gly-motif (Sakaguchi *et al.*, 1999) and that both the receptor core protein and the glycosaminoglycan modification are required for binding. By RT-PCR analysis of the cellular transcripts, we demonstrate that MAE cells express both the FGFR2/IIIc/Ig-3 loops and the FGFR2/IIIc/Ig-2 loops isoforms and that the latter one contains the Ser-Ser-Gly-motif. These observations, together with the data obtained from cross-linking experiments with radiolabeled FGF2, strongly suggest that both isoforms are expressed on MAE cell surface and that the FGFR2/IIIc/Ig-2 loops isoform is indeed HS modified. To our knowledge this is the first demonstration of the expression of different FGFR2 isoforms in endothelial cells. Further experiments on appropriate FGFR2 transfectants will be required to

assess the contribution of each isoform and of HS modification to FGF2 and FGF4 binding and biological responses.

Taken together, our data indicate that exogenous FGF2 and FGF4 act on endothelial cells *in vitro* in a similar manner even though significant differences may exist in their affinity for FGFRs and in their susceptibility to the extracellular glycosaminoglycan milieu. This appears to be in keeping with recent observations concerning the role of cell surface HSPGs in modulating the specificity of FGFR2/IIIc (Uematsu *et al.*, 2000).

FGF2 overexpression deeply affects the biological behavior of endothelial cells (present paper and Gualandris *et al.*, 1996). Indeed, pZipFGF2-MAE cells show a transformed morphology, an increased saturation density, and the capacity to grow under anchorage-independent conditions. Also, they show an invasive behavior and sprouting activity in three-dimensional fibrin gels, and the ability to form a complex network of branching cord-like structures connecting foci of infiltrating cells when seeded on Matrigel. Furthermore, they are able to induce an angiogenic response in the CAM and cause the formation of hemangiomas when injected into the allantoic sac of the chick embryo (present paper and Ribatti *et al.*, 1999b). To investigate the impact of the expression of endogenous FGF4 on endothelial cell behavior, MAE cells were transfected with a human FGF4 cDNA. pZipFGF4-MAE cells were characterized by an elevated production and release of FGF4 protein. FGF4-overexpression induces a transformed phenotype in NIH3T3 cells as the consequence of an extracellular autocrine loop of stimulation (Talarico and Basilico, 1991). Accordingly, pZipFGF2-MAE cells are characterized by a transformed morphology, an increased saturation density, but a limited capacity to grow under anchorage-independent conditions. As observed for NIH3T3 cell transfectants (Moscatelli and Quarto, 1989), suramin, a polysulfonate compound able to prevent FGF/FGFR interaction (Sato and

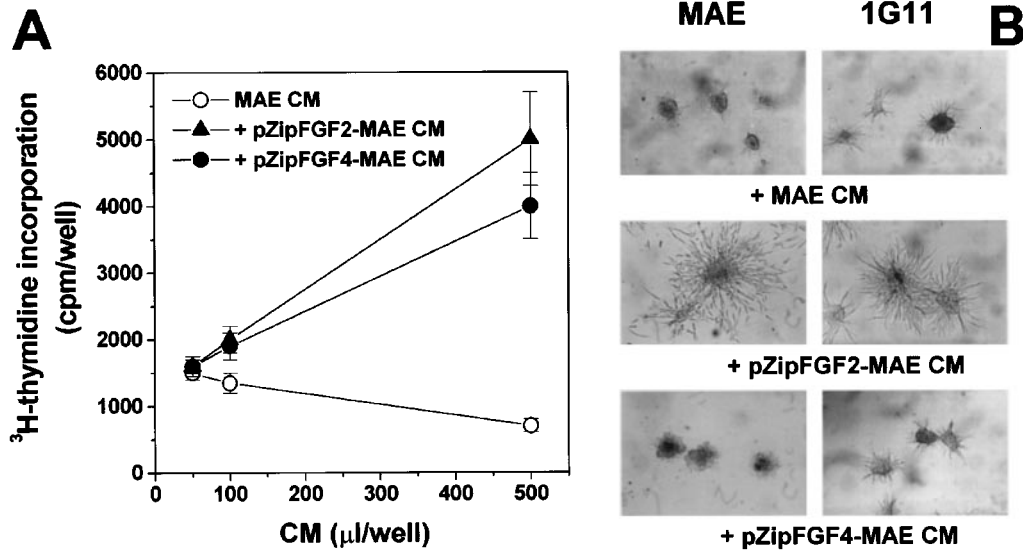


Figure 5 Biological activity of the conditioned medium of FGF2 and FGF4 transfectants. (a) Increasing concentrations of the conditioned medium (CM) of parental (○), pZipFGF2 (▲), and pZipFGF4 (●) MAE cells were added to quiescent MAE cells. Then, [³H]thymidine incorporation was measured as described in Materials and methods. (b) Conditioned media were added to aggregates of parental MAE cells or of 1G11 cells grown in 3D fibrin gels. Cell cultures were photographed after 48 h. Original magnification: 100 ×

Rifkin, 1988), reverts the pZipFGF4-MAE cell phenotype when added to their culture medium, thus confirming the extracellular mode of action of endogenous FGF4 (data not shown). Released FGF4 is indeed biologically active, as demonstrated by the capacity of pZipFGF4-MAE cell conditioned medium to induce a significant increase of [³H]thymidine incorporation in NIH3T3 cells (data not shown) and in parental MAE cells. Also, in keeping with the angiogenic activity of the recombinant protein (Yoshida *et al.*, 1994), pZipFGF4-MAE cells trigger an angiogenic response when implanted on the top of the chick embryo CAM.

Nevertheless, significant differences exist between FGF2- and FGF4-transfected MAE cells both *in vitro* and *in vivo*. pZipFGF4-MAE cells showed a proliferation rate lower than that of FGF2 transfectants, were unable to sprout in 3D fibrin gel, and did not undergo morphogenesis on Matrigel. Also, they lack the ability to induce hemangiomas in the CAM. Furthermore, their conditioned medium does not show any endothelial sprouting-inducing activity *in vitro*. All these biological features were instead present in FGF2 transfectants and appear to be the consequence of the release of an as yet unidentified heparin-binding factor that can be isolated from pZipFGF2-MAE conditioned medium (Ribatti *et al.*, 1999b). Interestingly, experimental evidences suggest that the production of this protein may be due to an intracellular mode of action of the overexpressed, endogenous FGF2 (Ribatti *et al.*, 1999b).

FGF2 cDNA overexpression leads to the production of both low and HMW FGF2 isoforms that are poorly released by transfected cells. Experimental evidences point to different functions of FGF2 isoforms

(Couderc *et al.*, 1991; Bikfalvi *et al.*, 1995; Escaffit *et al.*, 2000; Gaubert *et al.*, 2001), possibly related to differences in their subcellular localization and release. Indeed, HMW FGF2 isoforms are mostly recovered in the nucleus whereas M_r 18 000 FGF2 is mostly cytosolic (Renko *et al.*, 1990). In adult bovine aortic endothelial cells and NIH3T3 cells, the constitutive overexpression of HMW FGF2 isoforms leads to cell immortalization whereas M_r 18 000 FGF2 overexpression induces a transformed phenotype (Couderc *et al.*, 1991; Quarto *et al.*, 1991). In contrast, FGF4 is efficiently released by producing cells and does not appear to play an intracellular role (Talarico and Basilico, 1991). On this basis, it is tempting to hypothesize that the biological differences observed between pZipFGF4-MAE and pZipFGF2-MAE cells are due to intracrine function(s) exerted by the HMW FGF2 isoforms in the latter transfectants. Further experiments aimed to investigate the pattern of gene expression in pZipFGF4-MAE cells, pZipFGF2-MAE cells, and in MAE cells transfected with the individual FGF2 isoforms are in progress in our laboratory to assess this hypothesis.

KS-derived spindle cells of endothelial origin express both FGF2 and FGF4 at high levels (Ensoli *et al.*, 1994a,b; Kiuru-Kuhlefelt *et al.*, 2000). Our data shed a new light on the biological significance of the overexpression of the two angiogenic factors in KS. Also, *FGF4/hst* gene amplification has been observed in human tumors of different origin (Theillet *et al.*, 1989; Shimon *et al.*, 1998; Oliver, 1999). In this context, our data indicate that FGF2 and FGF4 expression may affect angiogenesis by a paracrine mechanism of action and exert different autocrine effects on the producing cells.

In conclusion, our data demonstrate that exogenous FGF2 and FGF4 exert a similar response in MAE cells. In contrast, significant differences are observed in the biological behavior of these cells following the endogenous expression of the growth factors. Overexpression of different members of the FGF family in endothelial cells may represent a novel approach to investigate their impact on endothelial cell behavior *in vitro* and *in vivo*.

Materials and methods

Cell cultures and transfection

Balb/c mouse aortic endothelial 22106 cells (MAE cells; Bastaki *et al.*, 1997) and pZipFGF2-MAE cells (Gualandris *et al.*, 1996) were grown in DMEM plus 10% fetal calf serum (FCS) in the absence or in the presence of 500 $\mu\text{g/ml}$ of G418 sulfate (Calbiochem), respectively.

The expression vector pZipFGF4 was obtained by inserting the human FGF4 cDNA (Delli Bovi *et al.*, 1987) under the control of the Mo-MuLV LTR elements into pZipNeoSV(X), the expression vector used to generate pZipFGF2-MAE cells (Gualandris *et al.*, 1996). MAE cells were transfected with a calcium phosphate precipitate containing 20 μg of plasmid DNA [either pZipFGF4 or pZipNeoSV(X)]. After G418 selection, conditioned media from different clones were loaded on heparin-Sepharose columns and 2.0 M NaCl eluates were screened for FGF4 presence by Western blot analysis and for the capacity to induce [^3H]thymidine incorporation in NIH3T3 cells.

NIH3T3 cells were transfected with the expression vector pZipNeoSV(X) harboring the murine FGFR2/IIIc/Ig-3 isoform cDNA (Mansukhani *et al.*, 1992). Stable transfectants were characterized for [^{125}I]FGF-2 binding as described below.

1G11 cells (Dong *et al.*, 1997) were grown on gelatin-coated dishes in DMEM supplemented with 1 mM glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate and 20% FCS.

Immunoblot analysis

Subconfluent cultures were washed with serum-free medium and incubated for 48 h with fresh medium. At the end of the incubation, conditioned medium was collected. Cell monolayers were washed twice with PBS and once with 2 M NaCl in PBS. The 2 M NaCl wash was collected and diluted with PBS to 0.5 M NaCl. Conditioned medium, containing the free growth factor(s), and diluted 2 M NaCl wash, containing the growth factor(s) released from the extracellular matrix, were loaded onto 100 μl heparin-Sepharose columns. The resin was transferred to an Eppendorf tube, washed twice with PBS and added with SDS-PAGE reducing sample buffer. In the meantime, cell monolayers were scraped from the dish and sonicated in SDS-PAGE reducing sample buffer with three bursts of 10 s each at 50 W. Cells extracts and resin beads were boiled, proteins were run on SDS-15% PAGE, transferred to nitrocellulose membranes, and probed with anti-FGF2 or anti-FGF4 polyclonal antibodies (provided by DB Rifkin and C Basilico, New York University Medical Center, NY, USA, respectively).

To evaluate extracellular signal-regulated kinase-1/2 (ERK $_{1/2}$) phosphorylation, MAE cells were treated for

20 min with 10 ng/ml of FGF2 or FGF4. Western blot analysis of the cell extracts was performed by using anti-phospho-ERK $_{1/2}$ antibody (Santa Cruz Biotechnology). Immunocomplexes were visualized by chemiluminescence utilizing the Supersignal[®] West Pico chemiluminescent substrate (Pierce) according to manufacturer's instructions.

RT-PCR analysis and characterization of FGFR2 isoforms

RNA was extracted from exponentially growing cells as described (Chomczynski and Sacchi, 1987). Two μg of total RNA were retrotranscribed with Ready-To-Go[™] You-Prime First Strand Beads (Amersham Pharmacia Biotech), using a 18 mer oligo-dT primer. One tenth of the reaction was subjected to 35 cycles of polymerase chain reaction (PCR) using different oligonucleotide primers spanning the extracellular domain of the murine receptor (Figure 2a). The fragment obtained with the primers indicated by the black arrows in Figure 2a was cloned in pSTBlue-1 using a Perfectly Blunt[™] cloning kit (Novagen), and automatically sequenced with a 373 Applied Biosystem Sequence Analyzer.

Cell proliferation assay

Human recombinant FGF2 (Isacchi *et al.*, 1991) and FGF4 (provided by C. Basilico) and serum-free conditioned media from parental MAE, pZipFGF2-MAE, and pZipFGF4-MAE cells were tested for the ability to stimulate [^3H]thymidine incorporation into the DNA of MAE cells. To this purpose, cell cultures were incubated for 2 days with 0.4% FCS. Quiescent cells were then loaded with the indicated stimulus. After 16 h, cells were pulse-labeled with ^3H -thymidine (1 $\mu\text{Ci/ml}$) for 6 h. Then, the amount of radioactivity incorporated into the trichloroacetic acid-precipitable material was measured.

[^{125}I]FGF-2 binding assay

FGF2 was labeled at 1800 c.p.m./fmol with ^{125}I (37 GBq/ml; NEN Life Science) using Iodogen (Pierce) (Moscatelli, 1987). Cells were seeded in 24-well dishes at the density of 80 000 per cm^2 . After 24 h, cells were washed with ice-cold PBS and incubated for 2 h at 4°C in serum-free medium containing 0.15% gelatin, 20 mM HEPES buffer, pH 7.5, added with 3 ng/ml ^{125}I -FGF2 in the absence or in the presence of increasing concentrations of unlabeled FGF2 or FGF4. Then, cells were washed with 2 M NaCl in 20 mM HEPES buffer (pH 7.5) to remove [^{125}I]FGF-2 bound to low affinity HSPGs and with 2 M NaCl in 20 mM sodium acetate (pH 4.0) to remove [^{125}I]FGF2 bound to high affinity FGFRs (Moscatelli, 1987). When indicated, 100 nM unmodified or 2-O-desulfated heparin (Coltrini *et al.*, 1994) were added to the binding medium.

Soft agar colony formation assay

Cells were plated at 20 000 cells/35 mm dish in 0.25% agar as described (Quarto *et al.*, 1991). For each cell types, colonies in 10 microscopic fields were counted at a 40-fold magnification 15 days after seeding.

Matrigel assay

Twenty-four well-plates were coated with 250 μl /well of 10 mg/ml Matrigel (Becton Dickinson, Milan, Italy) at 4°C. After gelling at 37°C, cells were seeded onto Matrigel layers at 150 000 cells/well. Cultures were observed after 48 h for

the formation of 'capillary-like' structures (Grant *et al.*, 1989).

3D-fibrin gel assay

Fibrinogen (2.5 mg/ml) was dissolved in calcium-free medium. Then, cell aggregates (Gualandris *et al.*, 1996) were resuspended in the fibrinogen solution and clotting was started by addition of thrombin (250 mU/ml). The mixture was transferred into 24 well-plates and allowed to gel at 37°C. Trasylol (200 KIU/ml) was added to the gel and to the culture medium to prevent the dissolution of the substrate. Cultures were maintained for 2–3 days in DMEM and observed for the generation of endothelial sprouts (Gualandris *et al.*, 1996). When indicated, fibrinogen and culture medium were added with a 1:10 dilution (v:v) of pZipFGF2 or pZipFGF4 MAE cell conditioned medium as described (Ribatti *et al.*, 1999b).

Cell injection into the chick embryo allantoic sac

Fertilized chicken eggs were incubated under routine conditions, and a square window was opened in the egg shell at the third day of incubation after removal of 2–3 ml of albumen to detach the shell from the developing CAM. The window was sealed with a glass and the eggs returned to the incubator. Then, 200 µl of a cell suspension containing 6 × 10⁶ cells per ml of PBS were injected twice into the allantoic sac at day 8 and day 9. Eggs were examined daily

for the formation of macroscopic hemangiomas until day 12 (Ribatti *et al.*, 1999b).

Cell delivery onto the CAM and quantification of the angiogenic response

Gelatin sponges (Gelfoam, Upjohn Company) were cut to the size of 1 mm³ and placed on top of the CAM at day 8 (Ribatti *et al.*, 1997). Then sponges were absorbed with 3 µl of cell suspension (18 000 cells per sponge). Sponges containing vehicle alone were used as negative controls. CAMs were examined daily, photographed *in ovo* and processed for light microscopy at day 12. The angiogenic response was assessed histologically by a planimetric method of 'point counting' (Ribatti *et al.*, 1999b).

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References

- Basilico C and Moscatelli D. (1992). *Adv. Cancer Res.*, **59**, 115–165.
- Bastaki M, Nelli EE, Dell'Era P, Rusnati M, Molinari-Tosatti MP, Parolini S, Auerbach R, Ruco LP, Possati L and Presta M. (1997). *Arterioscler. Thromb. Vasc. Biol.*, **17**, 454–464.
- Besser D, Presta M and Nagamine Y. (1995). *Cell Growth Differ.*, **6**, 1009–1017.
- Bikfalvi A, Klein S, Pintucci G, Quarto N, Mignatti P and Rifkin DB. (1995). *J. Cell Biol.*, **129**, 233–243.
- Chellaiyah AT, McEwen DG, Werner S, Xu J and Ornitz DM. (1994). *J. Biol. Chem.*, **269**, 11620–11627.
- Chomczynski P and Sacchi N. (1987). *Anal. Biochem.*, **162**, 156–159.
- Coltrini D, Rusnati M, Zoppetti G, Oreste P, Grazioli G, Naggi A and Presta M. (1994). *Biochem. J.*, **303**, 583–590.
- Couderc B, Prats H, Bayard F and Amalric F. (1991). *Cell Reg.*, **2**, 709–718.
- Delli Bovi P, Curatola AM, Kern FG, Greco A, Ittmann M and Basilico C. (1987). *Cell*, **50**, 729–737.
- Delli Bovi P, Curatola AM, Newman KM, Sato Y, Moscatelli D, Hewick RM, Rifkin DB and Basilico C. (1988). *Mol. Cell Biol.*, **8**, 2933–2941.
- Dionne CA, Crumley G, Bellot F, Kaplow JM, Searfoss G, Ruta M, Burgess WH, Jaye M and Schlessinger J. (1990). *EMBO J.*, **9**, 2685–2692.
- Dong QG, Bernasconi S, Lostaglio S, Wainstock De Calmanovici R, Martin-Padura I, Breviaro F, Garlanda C, Ramponi S, Mantovani A and Vecchi A. (1997). *Arterioscler. Thromb. Vasc. Biol.*, **17**, 1599–1604.
- Ensoli B, Gendelman R, Markham P, Fiorelli V, Colombini S, Raffeld M, Cafaro A, Chang HK, Brady JN and Gallo RC. (1994a). *Nature*, **371**, 674–680.
- Ensoli B, Markham P, Kao V, Barillari G, Fiorelli V, Gendelman R, Raffeld M, Zon G and Gallo RC. (1994b). *J. Clin. Invest.*, **94**, 1736–1746.
- Escaffit F, Estival A, Bertrand C, Vaysse N, Hollande E and Clemente F. (2000). *Int. J. Cancer*, **85**, 555–562.
- Florkiewicz RZ and Sommer A. (1989). *Proc. Natl. Acad. Sci. USA*, **86**, 3978–3981.
- Gaubert F, Escaffit F, Bertrand C, Korc M, Pradayrol L, Clemente F and Estival A. (2001). *J. Biol. Chem.*, **276**, 1545–1554.
- Grant DS, Tashiro K-I, Segui-Real B, Yamada Y, Martin GR and Kleinman HK. (1989). *Cell*, **58**, 933–943.
- Grinspan JB, Stephen NM and Levine EM. (1983). *J. Cell. Physiol.*, **114**, 328–338.
- Gualandris A, Rusnati M, Belleri M, Nelli EE, Bastaki M, Molinari-Tosatti MP, Bonardi F, Parolini S, Albini A, Morbidelli L, Ziche M, Corallini A, Possati L, Vacca A, Ribatti D and Presta M. (1996). *Cell Growth Differ.*, **7**, 147–160.
- Gualandris A, Urbinati C, Rusnati M, Ziche M and Presta M. (1994). *J. Cell Physiol.*, **161**, 149–159.
- Guimond S, Maccarana M, Olwin BB, Lindahl U and Rapraeger AC. (1993). *J. Biol. Chem.*, **268**, 23906–23914.
- Isacchi A, Statuto M, Chiesa R, Bergonzoni L, Rusnati M, Sarmientos P, Ragnotti G and Presta M. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 2628–2632.
- Johnson DE and Williams LT. (1993). *Adv. Cancer Res.*, **60**, 1–41.
- Keegan K, Johnson DE, Williams LT and Hayman MJ. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 1095–1099.
- Kiuru-Kuhlefelt S, Sarlomo-Rikala M, Larramendy ML, Soderlund M, Hedman K, Miettinen M and Knuutila S. (2000). *Mod. Pathol.*, **13**, 433–437.

- Klagsbrun M and Baird A. (1991). *Cell*, **67**, 229–231.
- Lee PL, Johnson DE, Cousens LS, Fried VA and Williams LT. (1989). *Science*, **245**, 57–60.
- Mansukhani A, Dell'Era P, Moscatelli D, Kornbluth S, Hanafusa H and Basilico C. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 3305–3309.
- Mignatti P, Morimoto T and Rifkin DB. (1992). *J. Cell Physiol.*, **151**, 81–93.
- Moscatelli D. (1987). *J. Cell Physiol.*, **131**, 123–130.
- Moscatelli D and Quarto N. (1989). *J. Cell Biol.*, **109**, 2519–2527.
- Oliver RT. (1999). *Curr. Opin. Oncol.*, **11**, 236–241.
- Ornitz DM, Xu J, Colvin JS, McEwen DG, MacArthur CA, Coulier F, Gao G and Goldfarb M. (1996). *J. Biol. Chem.*, **271**, 15292–15297.
- Partanen J, Makela TP, Eerola E, Korhonen J, Hirvonen H, Claesson-Welsh L and Alitalo K. (1991). *EMBO J.*, **10**, 1347–1354.
- Plotnikov AN, Hubbard SR, Schlessinger J and Mohammedi M. (2000). *Cell*, **101**, 413–424.
- Quarto N, Talarico D, Florkiewicz R and Rifkin DB. (1991). *Cell Reg.*, **2**, 699–708.
- Renko M, Quarto N, Morimoto T and Rifkin DB. (1990). *J. Cell Physiol.*, **144**, 108–114.
- Ribatti D, Gualandris A, Bastaki M, Vacca A, Iurlaro M, Roncali L and Presta M. (1997). *J. Vasc. Res.*, **34**, 455–463.
- Ribatti D, Gualandris A, Belleri M, Massardi L, Nico B, Rusnati M, Dell'Era P, Vacca A, Roncali L and Presta M. (1999b). *J. Pathol.*, **189**, 590–599.
- Ribatti D, Leali D, Vacca A, Giuliani R, Gualandris A, Roncali L, Nolli ML and Presta M. (1999a). *J. Cell Sci.*, **112**, 4213–4221.
- Sakaguchi K, Lorenzi MV, Bottaro DP and Miki T. (1999). *Mol. Cell Biol.*, **19**, 6754–6764.
- Sato Y and Rifkin DB. (1988). *J. Cell Biol.*, **107**, 1199–1205.
- Shimon I, Hinton DR, Weiss MH and Melmed S. (1998). *Clin. Endocrinol.*, **48**, 23–29.
- Spivak-Kroizman T, Lemmon MA, Dikic I, Ladbury JE, Pinchasi D, Huang J, Jaye M, Crumley G, Schlessinger J and Lax I. (1994). *Cell*, **79**, 1015–1024.
- Takahashi K, Mulliken JB, Kozakewich HP, Rogers RA, Folkman J and Ezekowitz RA. (1994). *J. Clin. Invest.*, **93**, 2357–2364.
- Talarico D and Basilico C. (1991). *Mol. Cell Biol.*, **11**, 1138–1145.
- Theillet C, Le Roy X, De Lapeyriere O, Grosgeorges J, Adnane J, Raynaud SD, Simony-Lafontaine J, Goldfarb M, Escot C and Birnbaum D. (1989). *Oncogene*, **4**, 915–922.
- Twigg SR, Burns HD, Oldridge M, Heath JK and Wilkie AO. (1998). *Hum. Mol. Genet.*, **7**, 685–691.
- Uematsu F, Kan M, Wang F, Jang JH, Luo Y and McKeehan WL. (2000). *Biochem. Biophys. Res. Commun.*, **272**, 830–836.
- Yoshida T, Ishimaru K, Sakamoto H, Yokota J, Hirohashi S, Igarashi K, Sudo K and Terada M. (1994). *Cancer Lett.*, **83**, 261–268.