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# Biological activity of substrate-bound basic fibroblast growth factor (FGF2): recruitment of FGF receptor-1 in endothelial cell adhesion contacts

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**Substrate-bound FGF2 promotes endothelial cell adhesion by interacting with $\alpha_v\beta_3$ integrin. Here, endothelial GM7373 cells spread and organize focal adhesion plaques on immobilized FGF2, fibronectin (FN), and vitronectin (VN). $\alpha_v\beta_3$ integrin, paxillin, focal adhesion kinase, vinculin and pp60$^{src}$ localize in cell-substratum contact sites on FGF2, FN or VN. However, only immobilized FGF2 induces a long-lasting activation of extracellular signal-regulated kinases$_{1/2}$ (ERK$_{1/2}$) and cell proliferation that was inhibited by the ERK$_{1/2}$ inhibitor PD 098059 and the tyrosine kinase (TK) inhibitor tyrphostin 23, pointing to the engagement of FGF receptor (FGFR) at the basal side of the cell. To assess this hypothesis, GM7373 cells were transfected with a dominant negative TK$^-$-ΔFGFR1 mutant (GM7373-ΔFGFR1 cells) or with the full-length receptor (GM7373-FGFR1 cells). Both transfectants adhere and spread on FGF2 but GM7373-ΔFGFR1 cells do not proliferate. Also, parental and GM7373-FGFR1 cells, but not GM7373-ΔFGFR1 cells, undergo morphological changes and increased motility on FGF2-coated plastic. Finally, FGFR1, but not TK$^-$-ΔFGFR1, localizes in cell adhesion contacts on immobilized FGF2. In conclusion, substrate-bound FGF2 induces endothelial cell proliferation, motility, and the recruitment of FGFR1 in cell-substratum contacts. This may contribute to the cross talk among intracellular signaling pathways activated by FGFR1 and $\alpha_v\beta_3$ integrin in endothelial cells.** *Oncogene* (2002) **21,** 3889–3897. DOI: 10.1038/sj/onc/1205407

**Keywords:** cell adhesion; cell proliferation; angiogenesis; integrin; FGF; tyrosine kinase; FGF receptor

## Introduction

Angiogenesis is a multi-step process that plays a key role in different physiological and pathological conditions, including embryonic development, wound repair, inflammation, and tumor growth (Carmeliet and Jain, 2000). It begins with the degradation of the basement membrane by activated endothelial cells that will migrate and proliferate, leading to the formation of solid endothelial cell sprouts into the stromal space. Then, vascular loops are formed and capillary tubes develop with deposition of new basement membrane and accessory cell recruitment (Carmeliet, 2000). A close interaction exists among cell-adhesive proteins of the extracellular matrix (ECM), their integrin receptors, and soluble angiogenesis growth factors during each step of the angiogenesis process (Ingber and Folkman, 1989a,b; Davis *et al*., 1993; Brooks *et al*., 1994; Plopper *et al*., 1995).

Basic fibroblast growth factor (FGF2) is one of the best characterized modulators of angiogenesis. FGF2 induces neovascularization *in vivo* in different experimental models (Basilico and Moscatelli, 1992) and is implicated in the growth of new blood vessels during wound healing and chick embryo development (Broadley *et al*., 1989; Ribatti *et al*., 1995). *In vitro*, FGF2 induces cell proliferation, migration, and production of proteases in endothelial cells (Moscatelli *et al*., 1986) by interacting with specific tyrosine-kinase (TK) receptors (FGFRs) and with heparan sulfate proteoglycans (HSPGs) of the cell surface (Johnson and Williams, 1993). Also, FGF2 modulates integrin expression in endothelium (Enenstein *et al*., 1992; Klein *et al*., 1993).

Integrins are a family of transmembrane, heterodimeric adhesion receptors comprised of $\alpha$ and $\beta$ subunits. The combination of different subunits originates distinct integrin molecules that mediate cell adhesion to a variety of adhesive proteins of the ECM such as fibronectin (FN), vitronectin (VN), thrombospondin, laminin and collagens (Albelda and Buck, 1990; Hynes, 1992). Besides mediating cell adhesion, the interaction of integrins with cell-adhesive proteins plays a crucial role in regulating the response of endothelial cells to soluble growth factors, including FGF2 (Ingber and Folkman, 1989a,b; Stromblad and Cheresh, 1996). Also, $\alpha_v\beta_3$ integrin is highly expressed by endothelial cells during angiogenesis and is required to sustain neovascularization induced by FGF2

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(Brooks *et al*., 1994; Friedlander *et al*., 1995; Eliceiri *et al*., 1998). Interestingly, FGFRs, integrins, and intracellular transducers may co-localize in focal adhesion contacts (Plopper *et al*., 1995, Miyamoto *et al*., 1996). Despite these observations, the molecular mechanism(s) underlying the relationship between the FGF2/FGFR system and the cell adhesion machinery are not fully elucidated.

Large amounts of FGF2 are present in ECM both *in vivo* and *in vitro* (Vlodavsky *et al*., 1987; Folkman *et al*., 1988). Collagen-bound FGF2 is mitogenically active *in situ* for BALB/c-3T3 fibroblasts (Smith *et al*., 1982) and FGF2 immobilized onto heparin-coated surfaces promotes endothelial cell adhesion (Baird *et al*., 1988) and PC12 cell adhesion and differentiation (Schubert *et al*., 1987). Thus, ECM-bound FGF2 may induce endothelial cell adhesion and act at the same time as a localized, persistent stimulus for angiogenesis by interacting with different cell-surface molecules. Indeed, previous data obtained in our laboratory had shown that FGF2 interacts also with $\alpha_v\beta_3$ integrin and that this interaction mediates the capacity of the angiogenic growth factor to induce cell adhesion, mitogenesis, and urokinase-type plasminogen activator upregulation in endothelial cells (Rusnati *et al*., 1997). Interestingly, the endothelial cell-adhesive capacity of FGF2 does not require the interaction of the growth factor with FGFRs and/or HSPGs that are instead able to mediate a FGF2-dependent cell-cell interaction via the formation of a ternary FGFR/FGF2/HSPG complex (Richard *et al*., 1995).

In the present study we investigated the molecular mechanisms mediating the mitogenic activity of substrate-bound FGF2 in endothelial cells. The results demonstrate that immobilized FGF2 induces focal adhesion plaque formation and the activation of extracellular signal-regulated kinases ($ERK_{1/2}$) in bovine aortic endothelial GM7373 cells. This is paralleled by a significant increase in the proliferation rate of adherent cells. GM7373 cells transfected with a dominant negative, truncated $TK^-$ FGFR1 mutant adhere and spread but have lost the capacity to proliferate on immobilized FGF2. Intact FGFR1, but not the truncated receptor, localizes in the focal adhesion contacts induced by FGF2. These data shed a new light on the cross-talk between endothelial cell adhesion and proliferative events during angiogenesis.

## Results

### *Substrate-bound FGF-2 promotes focal adhesion plaque formation in endothelial cells*

Previous observations had shown that substrate-bound FGF2 promotes endothelial cell adhesion via $\alpha_v\beta_3$ integrin engagement (Rusnati *et al*., 1997). Accordingly, fetal bovine aortic endothelial GM 7373 cells adhere onto non-tissue culture plates coated with native or heat-inactivated FGF2, FN, or VN (Figure 1A). Also, the distribution and organization of F-actin and the localization of vinculin (Figure 1B) and paxillin (not shown) demonstrate that immobilized FGF2 induces the formation of focal adhesion contacts in adherent GM7373 cells. Similar results were obtained for cells adherent to FN or VN (data not shown). Under the same experimental conditions, GM 7373 cells attach (Figure 1A) but do not spread (not shown) on polylysine (PLL)-coated plastic.

To confirm these observations, GM7373 cells were seeded on FGF2, FN, VN, or PLL, allowed to adhere for 6 h, and stripped by PBS/EDTA washes (Culp, 1976; Del Rosso *et al*., 1992). Cell-substratum contact sites, that represent 3–4% of the surface membrane of monolayered cells (Del Rosso *et al*., 1992), were then extracted and analyzed by Western blotting. As shown in Figure 2, FGF2-adherent plasma membrane remnants contain paxillin, $\alpha_v\beta_3$ integrin, focal adhesion kinase (FAK), and $pp60^{src}$. Similar results were obtained with cells adherent to FN and VN but not with cells adherent to PLL.

Taken together the results demonstrate that immobilized FGF2 promotes focal adhesion plaque formation in endothelial cells with the recruitment of signal transducing molecules including FAK and $pp60^{src}$.

### *Immobilized FGF2 induces endothelial cell proliferation*

Next, we evaluated the capacity of plastic-bound FGF2 to promote cell proliferation in endothelial GM 7373 cells. To this purpose, cells were allowed to adhere for 2 h on different substrata, including native or heat-inactivated FGF2, FN, or VN. Then, non-adherent cells were removed and adherent cells were incubated in low serum. After a 24 h incubation, cells were trypsinized and counted. As shown in Figure 3, only immobilized native FGF2 is able to exert a rapid and significant mitogenic response in adherent cells that

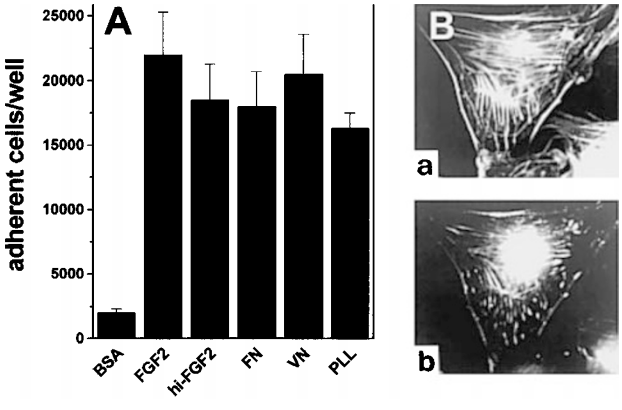


**Figure 1** GM 7373 cell adhesion to FGF2-coated plastic. (**A**) Non tissue culture plastic plates were incubated with carbonate buffer containing 20 $\mu$g/ml of BSA, FGF2, heat-inactivated FGF2 (hi-FGF2), FN, VN, or PLL. GM 7373 cells were seeded onto coated plates and allowed to adhere for 2 h at 37°C. Then, the number of adherent cells was evaluated. Each point is the mean $\pm$ s.e.m. of three determinations in duplicate. (**B**) GM 7373 cells adherent to immobilized FGF2 were stained with rodaminate-phalloidin (a) or anti-vinculin antibody (b)

continue to proliferate for further 24 h. In contrast, FN adherent cell cultures showed a significant increase in cell number only 72 h after seeding (Figure 3B). No proliferation was observed for cells seeded on plastic coated with heat-inactivated FGF2 (not shown) or BSA. It must be pointed out that no significant differences in the levels of vascular endothelial growth factor (ranging between 16 and 30 pg/ml) were detected by ELISA in the conditioned medium of GM 7373 cells grown on the different substrata.

Downstream signaling triggered by the binding of FGF2 to its $TK^+$ FGFRs encompasses the activation of mitogen-activated protein kinase kinase (MEK) with consequent phosphorylation of ERKs (Giuliani *et al*., 1999). Accordingly, a slow but long-lasting increase in $ERK_{1/2}$ phosphorylation was observed in GM7373 cells seeded on immobilized FGF2 but not on immobilized FN (Figure 4A). Indeed, integrin engagement by FN is known to cause a rapid but transient activation of this signaling pathway (Miyamoto *et al*., 1996). The MEK inhibitor PD 098059 (Alessi *et al*., 1995) prevented $ERK_{1/2}$ activation whereas SB 210313, a selective inhibitor of p38 kinase (Cuenda *et al*., 1995), was ineffective (Figure 4B). Accordingly, PD 098059 inhibited the proliferation of GM7373 cells adherent to FGF2-coated plastic whereas SB 210313 was ineffective (Figure 4D). The mitogenic response triggered by immobilized FGF2 was inhibited also by the TK inhibitor tyrphostin 23 (Boyer and Thiery, 1993), but not by tyrphostin 63 (Figure 4D) here used as a negative control (Gazit *et al*., 1989). None of the compounds tested was able to affect the adhesion of GM7373 cells to immobilized FGF2 (Figure 4C).

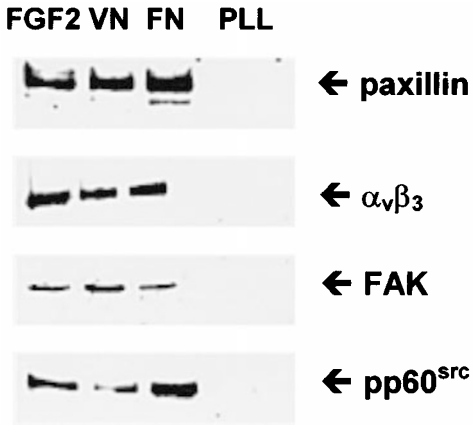


**Figure 2** Western blot analysis of cell-substratum contact sites. GM 7373 cells were seeded onto plates coated with FGF2, FN, VN, or PLL and allowed to adhere for 6 h at 37°C. Then cells were detached from the plastic with 3 mM EDTA/PBS washes. Plasma membrane remnants were washed three times with PBS and extracted. Aliquots (30 $\mu$g) of the extracted material were analysed by Western blotting with the indicated antibodies

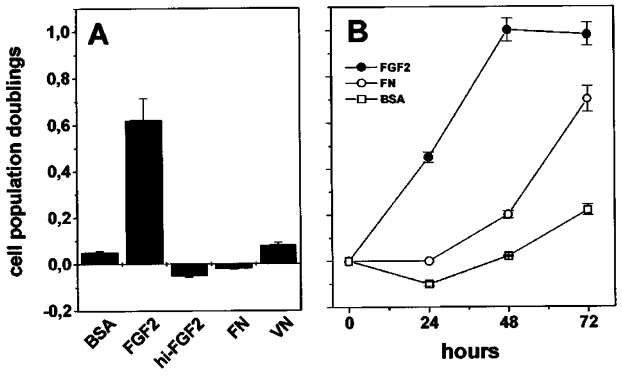


**Figure 3** Mitogenic activity of immobilized FGF2. GM 7373 cells were seeded onto plates coated with FGF2, heat-inactivated FGF2 (hi-FGF2), FN, or VN and allowed to adhere for 2 h at 37°C ($T_0$). Then, non-adherent cells were removed and adherent cells were incubated in fresh medium containing 0.4% FCS. Cells were trypsinized and counted 24 h (**A**) or 24, 48 and 72 h (**B**) after seeding. Data represent the mean ± s.e.m. of three determinations in duplicate and are expressed as cell population doublings in respect to cells adherent at $T_0$

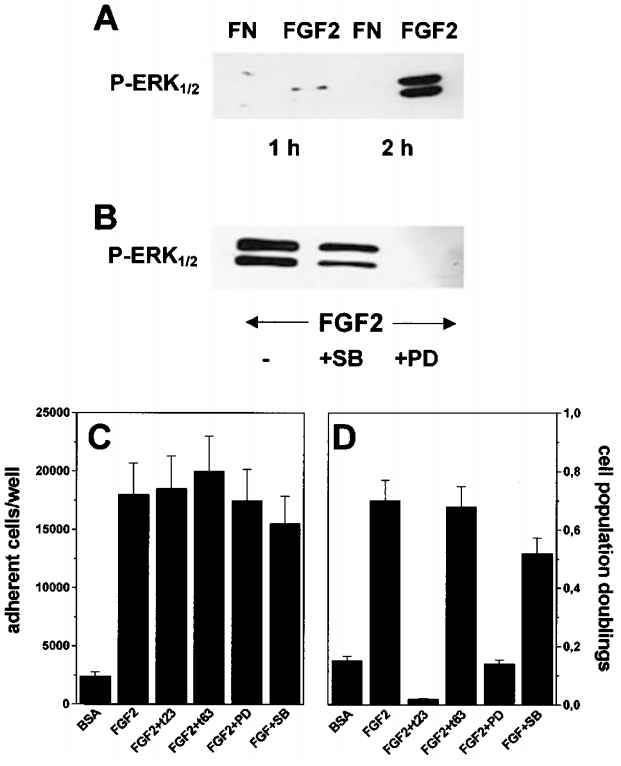


**Figure 4** $ERK_{1/2}$ phosphorylation by immobilized FGF2. GM 7373 cells were seeded onto FN- or FGF2-coated plastic. Western blot analysis of the cell extracts was performed 1 and 2 h after seeding using anti-phospho-$ERK_{1/2}$ antibodies (**A**). In **B**, cells were seeded on FGF2-coated plastic in the absence or in the presence of 50 $\mu$M SB 210313 (SB) or PD 098059 (PD). Western blot analysis of the cell extracts was performed 2 h after seeding using anti-phospho-$ERK_{1/2}$ antibodies. In parallel experiments, GM 7373 cells were seeded onto FGF2-coated plates in the absence or in the presence of 100 $\mu$M tyrphostin 23 (t23), 100 $\mu$M tyrphostin 63 (t63), 50 $\mu$M SB 210313 (SB) or 50 $\mu$M PD 098059 (PD) and allowed to adhere for 2 h at 37°C ($T_0$). Then, non-adherent cells were removed and adherent cells were counted immediately (**C**) or after a 24 h-incubation in fresh medium containing 0.4% FCS (**D**). Data represent the mean ± s.e.m. of three determinations in duplicate. In **D**, data are expressed as cell population doublings in respect to cells adherent at $T_0$

Taken together, the data indicate that substrate-bound FGF2 retains its mitogenic capacity that requires TK activity and $ERK_{1/2}$ phosphorylation. These observations raise the possibility that FGFR localized at the basal side of endothelial cells is involved in mediating the mitogenic response of adherent cells to the immobilized growth factor.

### *Dominant negative TK FGFR1 abolishes the response of endothelial cells to immobilized FGF2*

To assess the role of FGFR in transducing a mitogenic signal in endothelial cells adherent to immobilized FGF2, GM 7373 cells were transfected with a mutated FGFR1 cDNA carrying a stop codon in the juxtamembrane domain. These cells, named GM7373-ΔFGFR1 cells, will express a dominant negative, truncated $TK^-$ ΔFGFR1 devoid of its TK domain and C-terminus (Li *et al*., 1994). In parallel, distinct GM7373 cell cultures were transfected with the full length $TK^+$ FGFR1 cDNA, thus generating GM7373-FGFR1 cells.

As shown in Figure 5A, both GM7373-ΔFGFR1 and GM7373-FGFR1 cells binds $^{125}$I-FGF2 with a capacity significantly higher than that of parental cells. Western blot analysis of the cell extracts probed with a monoclonal anti-FGFR1 antibody evidenced the presence of a *Mr* 130 000 immunoreactive band in the cell extract of GM7373-FGFR1 cells (Figure 5B), corresponding to the full length receptor and comigrating with a fainter band present in the extract of parental cells. An intense *Mr* 90 000 immunoreative band, corresponding to the overexpressed truncated receptor, was instead detected in the cell extract of GM7373-ΔFGFR1 cells (Figure 5B). A significant increase of $ERK_{1/2}$ phosphorylation was detectable in parental and GM7373-FGFR1 cells adherent to tissue culture plastic and treated with soluble FGF2. No $ERK_{1/2}$ phosphorylation was instead detected in FGF2-treated GM7373-ΔFGFR1 cells, thus confirming the dominant negative effect of the truncated $TK^-$ receptor (Figure 5C).

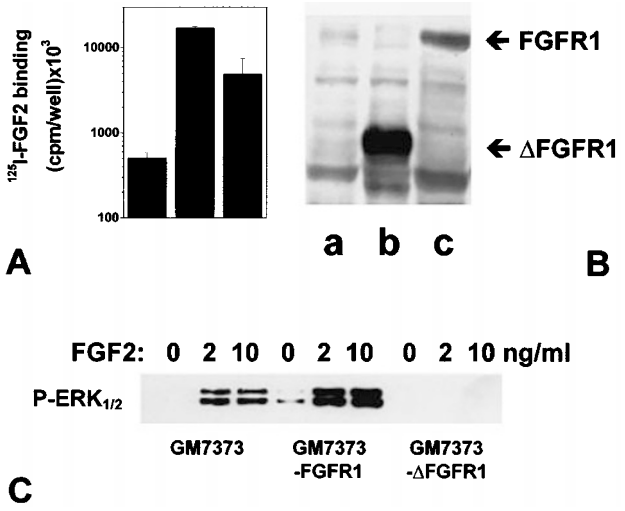


**Figure 5** Expression of dominant negative ΔFGFR1 in GM 7373 cells. GM 7373 cells were transfected with a retroviral expression vector harboring the full length $TK^+$ FGFR1 cDNA or the truncated $TK^-$ FGFR1 cDNA. Stable transfectants were isolated, generating GM7373-FGFR1 and GM7373-ΔFGFR1 cells, respectively. Then, binding of $^{125}$I-FGF2 to high affinity receptors was evaluated as described in Materials and methods (**A**). Also, cell extracts were probed with anti-FGFR1 antibodies by Western blotting (**B**). In **C**, parental, GM7373-FGFR1, and GM7373-ΔFGFR1 cells were incubated for 20 min in the presence of the indicated concentrations of soluble FGF2. Then, Western blot analysis of the cell extracts was performed using anti-phospho-$ERK_{1/2}$ antibodies. **a**, parental GM 7373 cells; **b**, GM7373-ΔFGFR1 cells; **c**, GM7373-FGFR1 cells

GM7373-ΔFGFR1 and GM7373-FGFR1 cells adhere to immobilized FGF2, FN, or VN with an efficiency similar to that shown by parental cells (Figure 6A). Also, their capacity to adhere to immobilized FGF2 was prevented by the highly specific monoclonal LM 609 antibody directed to $\alpha_v\beta_3$ (Cheresh, 1987) (Figure 6A). Accordingly, all the cell lines express similar amounts of $\alpha_v\beta_3$, as evidenced by Western blot analysis of the cell extracts (data not shown). However, GM7373-ΔFGFR1 cells have lost the ability to proliferate when seeded on FGF2-coated plastic; this ability is instead retained by GM7373-FGFR1 transfectants (Figure 6B). As observed for parental cells, GM7373-FGFR1 and GM7373-ΔFGFR1 cells do not proliferate when seeded on heat-inactivated FGF2, FN, or VN. It must be pointed out that no significant differences were observed in the proliferation rate of the three cell lines when seeded on tissue culture plastic and maintained in 10% FCS (data not shown).

Administration of FGF2 to the culture medium affects the appearance of endothelial cells that acquire an elongated, fibroblast-like morphology associated to increased cell motility (Tsuboi *et al*., 1990). Similarly, GM7373-FGFR1 cells adherent to FGF2-coated plastic took an elongated appearance with a crisscross pattern within 24–48 h after seeding (Figure 7a,b). Also, crawling cells characterized by lamellipodia and microspikes at the leading edge were frequently observed (Figure 7c–e). Similar even though less dramatic changes were observed 48–72 h after seeding in parental GM 7373 cells adherent to immobilized FGF2 (data not shown), possibly reflecting the lower number of FGFR receptors expressed by parental cells in respect to the transfectants. In contrast, no morphological changes were observed in FGF2-adherent GM7373-ΔFGFR1 cells that retained a flatter cobblestone-like appearance throughout the whole experimental period (Figure 7f,g). Specificity of the effect was demonstrated by the lack of activity of immobilized FN (Figure 7h–l) and VN (not shown) that did not influence the morphological features of adherent GM7373-FGFR1 and parental cells.

### *Immobilized FGF2 recruits FGFR1 in cell-substratum contact sites*

The above data suggest that immobilized FGF2 can interact with FGFR1 at the basal side of the adherent

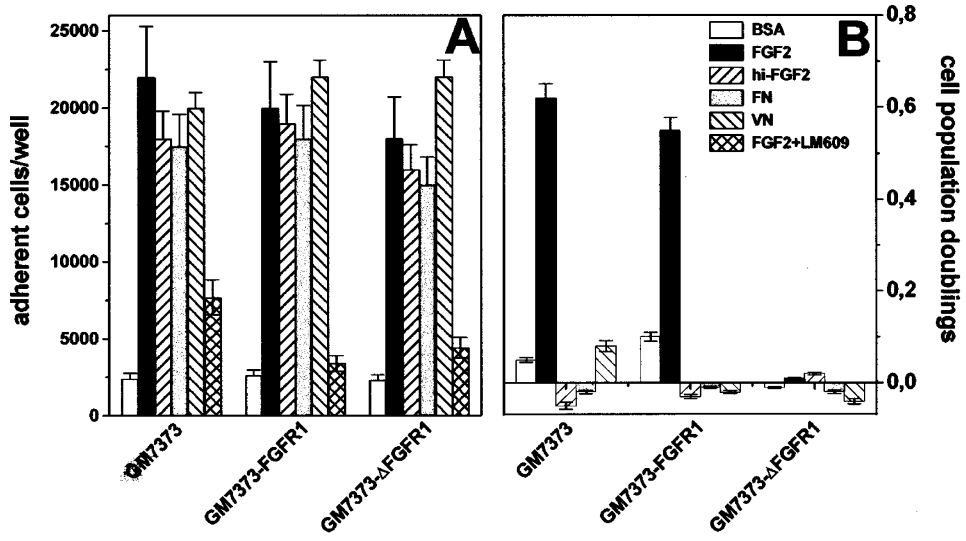


**Figure 6** Effect of dominant negative ΔFGFR1 on the mitogenic activity of immobilized FGF2. Parental, GM7373-FGFR1, and GM7373-ΔFGFR1 cells were seeded onto plastic coated with BSA, FGF2 (in the absence or in the presence of monoclonal LM609 anti-$\alpha_v\beta_3$ antibody), heat-inactivated FGF2, FN, or VN and allowed to adhere for 2 h at 37°C ($T_0$). Then, non-adherent cells were removed and adherent cells were counted immediately (**A**) or after a 24 h-incubation in fresh medium containing 0.4% FCS (**B**). Data represent the mean ± s.e.m. of three determinations in duplicate. In **B**, data are expressed as cell population doublings in respect to cells adherent at $T_0$

cells, thus triggering a mitogenic and morphogenic response. Accordingly, FGFR1 and paxillin co-localize in GM 7373 cells adherent to immobilized FGF2 (Figure 8A). Also, Western blot analysis of cell-substratum contact sites organized by GM7373-FGFR1 transfectants adherent to FGF2-coated plastic demonstrates the presence of FGFR1 in this plasma membrane fraction (Figure 8B). Semi-quantitative Western blot analysis indicates that more than 50% of the total amount of FGFR1 accumulates in this fraction. No FGFR1 was detected in contacts formed by GM7373-FGFR1 cells adherent to FN.

To confirm that the interaction with immobilized FGF2 causes a redistribution of FGFR1 at the basal side of the cell, GM7373-FGFR1 cells were seeded in 96-well plates at 75 000 cells/cm$^2$ on tissue culture plastic or on plastic coated with FGF2 or FN. After 6 h, adherent cell monolayers were incubated for 2 h at 4°C with $^{125}$I-FGF2 (10 ng/ml) and its binding to high affinity FGFRs was evaluated (Rusnati *et al*., 1996). FGF2-adherent cells showed a significant reduction in the capacity to bind $^{125}$I-FGF2 at the apical side of the cell monolayer (5 ± 2 c.p.m./well) when compared to cells adherent to FN (151 ± 20 c.p.m./well) or to tissue culture plastic (225 ± 30 c.p.m./well) ($n = 3$). This occurs in the absence of significant differences in the total amount of FGFR1 expressed by these cells under the various experimental conditions, as shown by Western blot analysis of the cell extracts with anti-FGFR1 antibodies (data not shown).

No truncated ΔFGFR1 accumulates in contacts organized by GM7373-ΔFGFR1 adherent to immobilized FGF2 (Figure 8b) despite the high levels of expression of the receptor in these cells (see Figure 5). These data indicate that the recruitment of FGFR1 by immobilized FGF2 is specific and that the interaction of FGF2 with the extracellular domain of the receptor is not sufficient for its cooption in the adhesion contacts. Accordingly, the TK inhibitor tyrphostin 23 prevented the recruitment of intact FGFR1 and caused a decrease in the amount of pp60$^{src}$ in focal contacts of GM7373-FGFR1 transfectants adherent to immobilized FGF2. No effect was instead exerted by tyrphostin 63 (Figure 9).

Taken together the data indicate that immobilized FGF2 is able to recruit FGFR1 in cell adhesion contacts of adherent GM7373 cells. The intracellular domain and/or the TK activity of the receptor are required for its cooption in these structures.

## Discussion

Immobilized FGF2 interacts with $\alpha_v\beta_3$ integrin and promotes endothelial cell adhesion and spreading (Rusnati *et al*., 1997). Our results demonstrate that substrate-bound FGF2 retains its biological activity, stimulating $ERK_{1/2}$ phosphorylation, cell proliferation, and motility in adherent endothelial GM7373 cells. This is paralleled by the recruitment of FGFR1 in cell-substratum contact sites. Overexpression of the dominant negative TK$^-$ ΔFGFR1, treatment with the TK inhibitor tyrphostin 23, or treatment with the MEK inhibitor PD 098059 inhibit the mitogenic activity of immobilized FGF2 without affecting $\alpha_v\beta_3$-mediated cell adhesion.

ECM may act as a physiological reservoir for extracellular FGF2 (Vlodavsky *et al*., 1987; Folkman *et al*., 1988). Various enzymes, including heparanase, thrombin, collagenases, plasmin, and urokinase-type

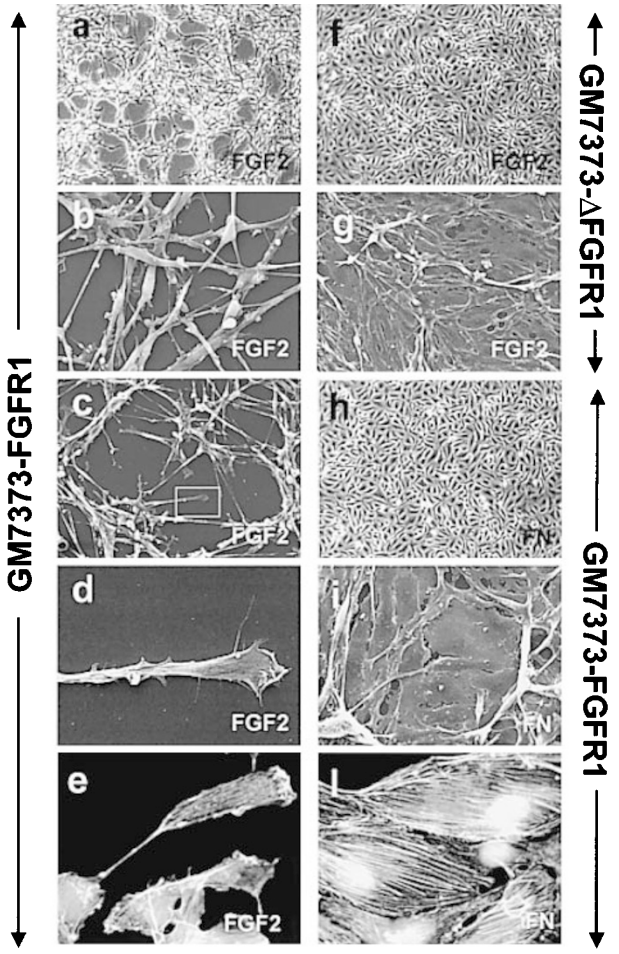


**Figure 7** Morphology of GM7373-FGFR1 cells adherent to immobilized FGF2. GM7373-FGFR1 cells (**a–e, h–l**) and GM7373-ΔFGFR1 cells (**f, g**) were allowed to adhere onto glass coverslips coated with 20 $\mu$g/ml of FGF2 (**a–g**) or FN (**i, l**). After 48 h, cells were fixed and photographed under a phase contrast inverted microscope at 40× magnification (**a, f, h**), processed for scanning electron microscopy and photographed at 800× (**b**), 300× (**c, g**), 2000× (**d**), or 600× (**i**) magnification, or stained with rodaminate-phalloidin and photographed at 630× magnification (**e, l**). Note the elongated shape and crisscross pattern of GM7373-FGFR1 cells adherent to FGF2 (**a, b, e**) when compared to the flatter and more regular appearance of FN-adherent cells (**h–l**) and of FGF2-adherent GM7373-ΔFGFR1 cells (**f, g**). In **c**, GM7373-FGFR1 cells migrate onto the FGF2-coated substratum (white boxed enlarged in **c** showing a crawling cell characterized by lamellipodia and microspikes at the leading edge, also evident in **e** after rodaminate-phalloidin staining)

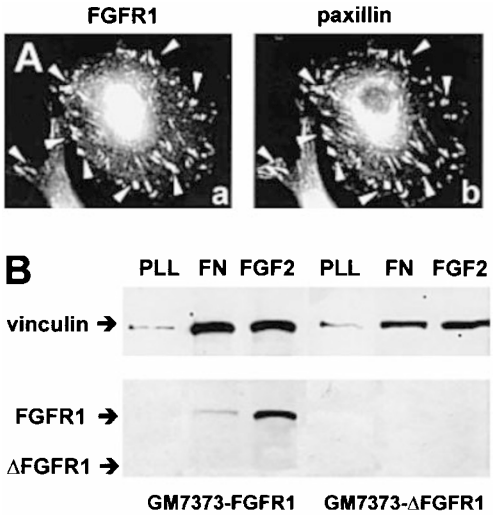


**Figure 8** FGFR1 recruitment in cell-substratum contact sites. (**A**) GM 7373 cells adherent to immobilized FGF2 were immunostained with anti-FGFR1 (**A**) and anti-paxillin (**B**) antibodies. FGFR1 co-localizes in paxillin-positive focal adhesion contacts (arrowheads). (**B**) GM7373-FGFR1 and GM7373-ΔFGFR1 cells were seeded onto plates coated with FGF2, FN, or PLL and allowed to adhere for 6 h at 37°C. Then cells were detached from the plastic with 3 mM EDTA/PBS washes. Plasma membrane remnants were washed three times with PBS and extracted. Aliquots (30 $\mu$g) of the extracted material were analysed by Western blotting with antibodies directed against vinculin or FGFR1. The anticipated position corresponding to ΔFGFR1 migration is also indicated

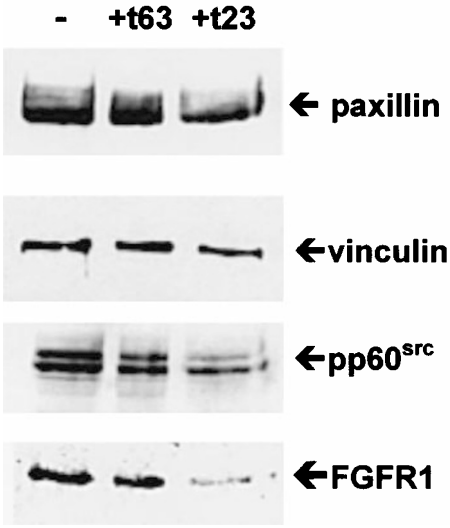


**Figure 9** TK activity is required for FGFR1 recruitment in cell-substratum contact sites. GM7373-FGFR1 cells were seeded onto FGF2-coated plates and allowed to adhere for 6 h at 37°C in the absence or in the presence of 100 $\mu$M tyrphostin 63 (t63) or tyrphostin 23 (t23). Then cells were detached from the plastic with 3 mM EDTA/PBS washes. Plasma membrane remnants were washed three times with PBS and extracted. Aliquots (30 $\mu$g) of the extracted material were analysed by Western blotting with antibodies directed against the indicated proteins

plasminogen activator release ECM-bound FGF2 (Ribatti *et al*., 1999, and references therein) suggesting that the balance between storage and release of FGF2 in ECM, as well as the integrity of the matrix, may regulate the biological effects of this growth factor on endothelium. On the other hand, collagen-bound FGF2 is mitogenically active *in situ* for BALB/c-3T3 fibroblasts (Smith *et al*., 1982) and FGF2 immobilized onto heparin-coated surfaces promotes endothelial cell adhesion (Baird *et al*., 1988) and PC12 cell adhesion and differentiation (Schubert *et al*., 1987). Our findings extend these observations and demonstrate that endothelial cells adherent to FGF2 proliferate and acquire a migra-

tory phenotype. FGF2 bound to plastic resists to extraction with urea, methanol, or ethanol and it is removed only by drastic treatment with detergents like Triton X-100 or SDS (Smith *et al*., 1982; Rusnati *et al*., 1997). Accordingly, no FGF2 internalization was observed in cells adherent to FGF2-coated plastic in which $^{125}$I-FGF2 was used as a tracer (M Rusnati, unpublished observations). These data indicate that FGF2 may represent an angiogenic stimulus also when tightly bound to the substratum.

Under our experimental conditions the amount of FGF2 bound to non-tissue culture plastic corresponds to $10^{12}$ molecules/cm$^2$ (Rusnati *et al*., 1997). Thus, each endothelial cell adheres onto approximately $10^7$ molecules of immobilized FGF2. This causes the $\alpha_v\beta_3$-mediated formation of focal adhesion contacts and actin cytoskeletal organization. The analysis of the components of the cell-substratum contact sites indicates that immobilized FGF2 activates the all series of specific stages of hierarchies of protein interactions that occur during integrin response, as observed for typical cell-adhesion proteins like FN and VN (Miyamoto *et al*., 1995). Indeed, the presence of $\alpha_v\beta_3$ integrin, FAK, vinculin, pp60$^{src}$, and paxillin in cell-substratum contact sites demonstrate that immobilized FGF2 is able to cause integrin receptor aggregation, integrin occupancy, cytoplasmic tyrosine phosphorylation, and actin cytoskeletal organization (Miyamoto *et al*., 1995).

As stated above, endothelial cell adhesion to immobilized FGF2 is followed by a rapid and significant increase in cell proliferation. However, integrin engagement is not sufficient *per sè* to trigger a mitogenic response. Indeed, GM 7373 cells adhere and spread also on heat-inactivated FGF2, FN, or VN leading to the formation of focal adhesion contacts in the absence of a significant increase of their rate of proliferation that occurs only at late time points. Conversely, overexpression of the dominant negative ΔFGFR1, tyrphostin 23, and PD 098059 completely abolish the mitogenic response to immobilized FGF2 without affecting $\alpha_v\beta_3$-mediated cell adhesion. Moreover, neutralizing anti-$\alpha_v\beta_3$ monoclonal and polyclonal antibodies do inhibit cell proliferation induced by soluble FGF2 in GM 7373 cells grown on tissue culture plastic (Rusnati *et al*., 1997). Thus, interaction of FGF2 with $\alpha_v\beta_3$ integrin is necessary but not sufficient to transduce a mitogenic signal that requires the activation of a functional TK$^+$ FGFR.

Previous observations had shown that integrin clustering at sites of contact of FN-coated beads with fibroblast cell surface is accompanied by a transient accumulation of various growth factor TK receptors, including FGFR1 (Miyamoto *et al*., 1996). Here we show that immobilized FGF2, but not immobilized FN or PLL, triggers a long-lasting accumulation of FGFR1 at the cell-substratum contact sites of adherent GM7373-FGFR1 transfectants. Six hours after adhesion, cell-substratum contact sites, that represent 3–4% of the surface membrane of monolayered cells (Del Rosso *et al*., 1992), contain more than 50% of total FGFR1 molecules, clearly indicating that the interaction with immobilized FGF2 concentrates the receptor in these cell membrane remnants. Accordingly, FGF2-adherent cells showed a dramatic reduction in the number of high affinity $^{125}$I-FGF2 binding sites present on the apical side of the cell monolayer when compared to cells adherent to FN or to tissue culture plastic, thus confirming that interaction with immobilized FGF2 causes a redistribution of FGFRs at the basal side of the cell. Interestingly, the truncated receptor overexpressed by GM7373-ΔFGFR1 cells does not accumulate in FGF2-induced contact sites, despite its ability to bind FGF2 in a manner undistinguishable from the wild-type receptor. Thus, the interaction of the extracellular domain of the receptor with the immobilized growth factor is not sufficient to guarantee a long-lasting accumulation of FGFR1 in the focal adhesion contacts that may require the activation of the intracellular TK moiety of the receptor and/or its interaction with other intracellular proteins. The ability of tyrphostin 23 to prevent FGFR1 recruitment and the capacity of FGFR1 to associate with pp60$^{src}$ and cortactin (Zhan *et al*., 1994) support this hypothesis. Recently, the vascular endothelial growth factor receptor VEGFR2/KDR has been shown to interact directly with $\alpha_v\beta_3$ integrin (Soldi *et al*., 1999). By using the same experimental approaches, we have been unable to observe a direct interaction between FGFR1 and $\alpha_v\beta_3$ integrin (E Tanghetti, unpublished data). The dissection of the molecular basis of FGFR1 recruitment in cell-substratum contact sites by immobilized FGF2 will require further investigation.

Previous observations had implicated ERK activation in FGF2 signaling (Besser *et al*., 1995; Giuliani *et al*., 1999) and angiogenesis (Eliceiri *et al*., 1998). FGF2 causes a long-lasting phosphorylation of ERK$_{1/2}$ in parental and GM7373-FGFR1 cells, but not in GM7373-ΔFGFR1 transfectants. Also, the MEK inhibitor PD 098059 prevents the mitogenic response of parental and GM7373-FGFR1 cells to immobilized FGF2. These data emphasize the role of ERK$_{1/2}$ in signal transduction activated by FGFR1 occupancy in endothelium.

Several experimental evidences support the hypothesis that integrins collaborate with TK receptors in transducing the intracellular signals triggered by growth factors in target cells (Miyamoto, 1995, 1996; Kumar, 1998; Giancotti and Rouslathi, 1999). We report here that immobilized FGF2 interacts with $\alpha_v\beta_3$ integrin and FGFR1, affecting different aspects of the angiogenic phenotype of endothelial cells, including cell adhesion, proliferation, morphogenesis, and motility. Our data indicate that FGFR1 and $\alpha_v\beta_3$ integrin may be favored in their cross-talk by the long-lasting structural vicinity that occur at the basal aspect of the endothelium where they colocalize in cell-substratum contact sites after adhesion onto immobilized FGF2.

## Materials and methods

### *Materials*

Human recombinant FGF2 was expressed and purified from transformed *E. coli* cells as described (Isacchi *et al*., 1991). Anti-$\alpha_v\beta_3$ monoclonal LM 609 antibody was from Chemicon International (Temecula, CA, USA). For Western blotting, monoclonal anti-FGFR1 antibody and polyclonal anti-pp60$^{src}$ antibody were from Upstate Biotechnology (Lake Placid, NY, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. PD 098059 and SB 210313 were from Calbiochem (San Diego, CA, USA) and RBI (Natick, MA, USA), respectively. PLL, tyrphostin 23, tyrphostin 63, rodaminate-phalloidin, and anti-vinculin antibody were from Sigma (St. Louis, MO, USA). Monoclonal anti-FAK antibody was a gift from G Tarone (University of Turin, Italy). Bovine FN was from Harbor Bio-Products (Norwood, MA, USA) and human VN was from Becton Dickinson Labware (Bedford, MA, USA).

### *Cell cultures and transfection*

Fetal bovine aortic endothelial GM 7373 cells, corresponding to the BFA-1c multilayered transformed clone (Grinspan *et al*., 1983), were grown in Eagle's minimal essential medium (MEM) containing 10% FCS, vitamins, essential and non essential amino acids.

Plasmids 91023b-*flg* (murine IIIc variant of FGFR-1 cDNA), pCEP4-*flg* 1.2 (truncated murine IIIc variant of FGFR-1 cDNA) and pCB7 (a plasmid that carries the *neo*$^r$ gene) were provided by A Mansukhani and C Basilico (New York University Medical Center, New York, NY, USA). GM7373 cells were transfected with 91023b-*flg* in association (10 : 1, wt:wt) with pCB7 according to a calcium phosphate precipitation protocol as described (Rusnati *et al*., 1996) and selected with 250 $\mu$g/ml of G418 (Sigma, St. Louis, MO, USA). Parallel cultures were transfected with pCEP4-*flg* 1.2 and selected with 200 $\mu$g/ml of hygromycin B (Boehringer Mannheim GmbH, Mannheim). For each transfection, resistant clones were tested for $^{125}$I-FGF2 binding capacity (Rusnati *et al*., 1996) and by Western blotting using anti-FGFR1 antibody (Upstate Biotechnology, Lake Placid, NY, USA).

### *Cell adhesion assay*

One hundred $\mu$l aliquots of 100 mM $NaHCO_3$, pH 9.6 (carbonate buffer), containing the adhesive molecule under test were added to polystyrene non-tissue culture microtiter plates. After 16 h of incubation at 4°C the solution was removed and wells were washed three times with cold phosphate buffered saline (PBS). Then, wells were incubated for 1 h at 37°C with 1.0 mg/ml BSA and washed extensively with PBS. For the cell-adhesion assay, confluent cultures of GM 7373 cells were trypsinized, washed and resuspended with the appropriate medium. Previous observations had indicated that low concentrations of serum were required in some experiments for optimal cell adhesion to FGF2-coated plastic (Rusnati *et al*., 1997). For this reason, 1% FCS was utilized routinely in cell-adhesion experiments. Fifty thousand GM 7373 cells were resuspended in 200 $\mu$l of medium and were immediately seeded onto wells coated with the molecule under test. Routinely, cell-adhesion was allowed to occur for 2 h at 37°C. Then, wells were washed with 2 mM EDTA/PBS. Adherent cells were trypsinized and counted.

### *Western blot analysis of cell-substratum contact sites*

Cells were seeded at 75 000 cells/cm$^2$ on plastic coated with the different substrata. After 6 h, cells were detached from the plastic with 3 mM EDTA/PBS (Del Rosso *et al*., 1992). The material remaining on the plastic, representing plasma membrane remnants, was washed three times with PBS and extracted with 0.1 M Tris-HCl (pH 8.1) containing 1 mM PMSF, 0.2% sodium-deoxycholate, 1% Triton-X100. Aliquots (30 $\mu$g) of the extracted material were analysed by Western blotting with the indicated antibodies.

### *Evaluation of the mitogenic activity of immobilized FGF2*

Parental and transfected GM7373 cells were seeded at 130 000 cells/cm$^2$ onto 96-well plates coated with the different substrata and incubated for 2 h at 37°C with MEM containing 1% FCS. Then, cells were washed with 2 mM EDTA/PBS ($T_0$). Adherent cells were incubated for further 24, 48 or 72 h in fresh MEM containing 0.4% FCS. In some experiments, medium was added with different inhibitors. At the end of incubation, cells were trypsinized and counted in a Burker chamber. Data are expressed as cell population doublings in respect to cells adherent at $T_0$.

### *Western blot analysis of ERK$_{1/2}$ phosphorylation*

Parental and transfected GM7373 cells were seeded at 42 000 cells/cm$^2$ in 35 mm tissue culture dishes. After adhesion, cells were incubated in serum-free MEM added with 10 $\mu$g/ml transferrin and 1 mg/ml BSA. After 24 h, cells were incubated for 20 min at 37°C with no addition or 2.0 or 10.0 ng/ml FGF2. Then, Western blot analysis of the cell extracts was performed as described (Besser *et al*., 1995) using anti-phospho-ERK$_{1/2}$ antibody (New England Biolabs, Inc., Beverly, MA, USA).

In some experiments, GM7373 cells were seeded at 90 000 cells/cm$^2$ in 35 mm dishes coated with FGF2 or FN in the absence or in the presence of 50 $\mu$M PD 098059 or SB 210313. After 1 or 2 h, cell extracts were analysed for ERK$_{1/2}$ phosphorylation as above.

For all the experiments, equal loading of the different lanes of the gel were confirmed by Western blotting of the stripped membranes with anti-ERK$_{1/2}$ antibody (not shown).

### *Immunocytochemistry*

Cells were seeded onto FGF2- or FN-coated glass coverslips in MEM added with 1% FCS. After 2–6 h, cells were washed, fixed in 3% paraformaldehyde, 2% sucrose in PBS, permeabilized with 0.5% Triton-X100, and saturated with 3% BSA in PBS. Then, in a first set of experiments, cells were incubated with monoclonal anti-vinculin antibody followed by fluorescinated anti-mouse IgG *plus* rodaminate-phalloidin. In a second set of experiments, cells were incubated with monoclonal anti-paxillin antibody (Transduction Laboratories, Lexington, KY, USA) followed by Alexa Fluor 594 anti-mouse IgG (Molecular Probes, Eugene, OR, USA) and polyclonal anti-FGFR1 antibody (Santa Cruz Biotechnology) followed by biotinylated swine anti-rabbit IgG (Dako, Glostrup, Denmark) and rhodol green conjugated streptavidin (Molecular Probes).

### *Scanning electron microscopy*

Glass coverslips (10 mm in diameter) were placed within 24-well tissue culture plates and coated overnight at 4°C

with carbonate buffer containing 20 $\mu$g/ml of FGF2, FN, or VN. Then, plates were washed three times with cold PBS. Cells were seeded at 130 000/cm$^2$ and allowed to adhere onto glass coverslips for 2 h in MEM added with 1% FCS. Then, cells were washed with 2 mM EDTA in PBS. Adherent cells were incubated for further 24–72 h in fresh MEM containing 0.4% FCS. Cells were then fixed with 1.5% glutaraldehyde, 1% paraformaldehyde in 0.15 M cacodylate buffer (pH 7.4) for 1 h. Coverslips were then washed, dehydrated, critical point dried with $CO_2$ and sputter coated with 3.2 nM platinum with Emitech K575 apparatus (Emitech, UK). Cells were then viewed under Hitachi scanning electron microscope find emission mod. S4000 (Hitachi, Japan) operated at 15 kV and photographed.

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