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FGF-1 AND FGF-2 REGULATE THE EXPRESSION OF E-CADHERIN AND CATENINS IN PANCREATIC ADENOCARCINOMA

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E-cadherin is a transmembrane protein that mediates Ca^{2+}-dependent cell-cell adhesion and is implicated in a number of biologic processes, including cell growth and differentiation, cell recognition and cell sorting during development. We have previously demonstrated that both cell-cell adhesion and invasion are modulated by fibroblast growth factor (FGF)-1 and FGF-2 in a panel of pancreatic adenocarcinoma cell lines (BxPc3, T3M4 and HPAF). Here, we examine further the role of FGFs in the expression and activation of the E-cadherin/catenin system. We demonstrate that both FGF-1 and FGF-2 upregulate E-cadherin and β-catenin at the protein level in the BxPc3 and HPAF cell lines and modestly in T3M4 cells. FGF-1 and FGF-2 facilitate the association of E-cadherin and α-catenin with the cytoskeleton, as demonstrated by the increase in the detergent-insoluble fraction of E-cadherin in BxPc3 and HPAF cells. Since the correct function of the E-cadherin/catenin complex requires its association with the cytoskeleton, our data suggest that FGF-1 and FGF-2 contribute to the integrity and thus the function of the complex. Furthermore, FGFs facilitate the assembly of the E-cadherin/catenin axis. The effect is associated with elevation of tyrosine phosphorylation of E-cadherin, α-catenin, β-405 1μcatenin and γ-catenin, but not $p120^{ctn}$. These findings indicate that the E-cadherin/catenin system is a target of the FGF/FGFR system and that coordinated signals from both systems may determine the ultimate biologic responses.

Key words: *E-cadherin; catenins; FGF; FGFR; pancreatic adenocarcinoma*

Evidence is compelling that a loss of function of E-cadherin and/or one or more of the associated catenins contributes to increased proliferation, invasion and metastasis in a wide variety of solid tumours.[1] Supporting observations include an inverse correlation between E-cadherin expression and increased invasiveness *in vitro*,[2] as well as an association between loss of E-cadherin expression and dedifferentiation, advanced tumour grade, metastasis and shortened survival.[3,4]

The integrity of the entire E-cadherin-catenin-actin network is indispensable for E-cadherin-mediated cell-cell adhesion. Post-translational modification such as tyrosine phosphorylation is widely recognised as the prevalent mechanism in regulating cadherin/catenin expression and/or function.[5] The catenins (β-catenin, γ-catenin and $p120^{ctn}$) are the major targets of protein tyrosine kinases, which are heavily tyrosine phosphorylated in *Src*-transformed cells[6] and in response to growth factors such as EGF,[7,8] TGF-α, PDGF[9,10] and HGF.[11] Furthermore, β-catenin and γ-catenin directly associate with at least two receptor tyrosine kinases: EGFR[7] and c-erbB-2.[12]

There is mounting evidence that E-cadherin expression can be modulated, either positively or negatively, by growth factors and cytokines. However, the mechanism of regulation of expression remains largely speculative. For instance, TGF-β induces dedifferentiation of normal mammary epithelial cells from the epithelial to fibroblastic phenotype, which correlates with decreased expression of E-cadherin ZO-1 and reorganisation of actin fibres.[13] EGF perturbs E-cadherin function in a dose-dependent fashion and increases the detergent solubility of E-cadherin.[8] HGF induces a decrease in the levels of membrane-associated E-cadherin and severs the link between E-cadherin and the cytoskeleton in pancreatic cell lines.[14] While IGF-I restores the adhesive and invasion-suppressor functions of the E-cadherin/catenin complex in human breast cancer MCF-7/6 cells,[15] it promotes the inactivation of the E-cadherin/catenin system in colorectal cell lines by inducing tyrosine phosphorylation[16] and disrupting β-catenin from the E-cadherin complex.[17] Finally, FGF-2 and other growth factors such as PDGF-BB, EGF and IGF downmodulate T-cadherin levels in a dose- and time-dependent fashion.[18] These observations suggest that cell-cell adhesion proteins are common targets for various growth factors; their effects are most likely tissue-specific and cadherin-specific.

We previously demonstrated that both the expression of E-cadherin and catenins and the assembly of the complex are altered in pancreatic adenocarcinoma cell lines, with a subsequent impairment of the functionality of the system. We also demonstrated that FGF-1 and FGF-2 enhance E-cadherin-mediated cell-cell adhesion and decrease the *in vitro* invasion in these cell lines.[19] In this study, we set out to explore the possibility that FGF-1 and FGF-2 may regulate the expression and the activity of E-cadherin and catenins upon formation of cell-cell contacts following restoration of Ca^{2+} in a panel of adenocarcinoma cell lines. Here we report an increase in the tyrosine phosphorylation and upregulation of expression of E-cadherin and catenins following stimulation by FGF-1 and FGF-2.

MATERIAL AND METHODS

Cell lines

A panel of human pancreatic adenocarcinoma cell lines (BxPc3, T3M4 and HPAF) that express various combinations of FGFRs[20] and exhibit different grades of differentiation were used in this study. The BxPc3 cell line (ATCC, Rockville, MD) is a moderately to well-differentiated cell line derived from a primary tumour[21] and expresses FGFR-1; the T3M4 cell line is a moderately differentiated cell line derived from a lymph node metastasis[22] and expresses FGFR-3; and the HPAF cell line, moderately to poorly differentiated cell line that was derived from ascitic fluid,[23] is FGFR-3 and FGFR-4 positive. The human colorectal adenocarci-

Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; ERM, ezrin/radixin/moesin; FGF, fibroblast growth factor; FGFR, FGF receptor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; IP, immunoprecipitation; OD, optical density; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulfonyl fluoride; TGF, transforming growth factor.

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noma cell line HT29, which is E-cadherin positive, was used as a positive control cell line. Cells were grown as confluent monolayers and were routinely cultured in RPMI (pancreatic cells) or DMEM (colorectal cells) media supplemented with 10% FCS. For this study, cells in confluent cultures were used to ensure high cell density, which is expected to minimise cell-substrate interactions at focal adhesions and thus decrease tyrosine phosphorylation resulting from the formation of these structures.[24]

Growth factors and antibodies

Human recombinant FGF-1 and FGF-2 (Upstate Biotechnology, Lake Placid, NY) were used for stimulation of the cell lines at various concentrations between 1 and and 50 ng/ml. Heparin was added at 10 and 1 μg/ml with FGF-1 and FGF-2, respectively, as it is required for FGFR activation by FGFs.

Mouse monoclonal anti-E-cadherin (clone 36), anti-α-catenin (clone 5), anti-β-catenin (clone 14), anti-γ-catenin (clone 15) and anti-$p120^{ctn}$ (clone 98) antibodies were purchased from Transduction Laboratories (Exeter, UK). Mouse monoclonal anti-phosphotyrosine antibody (clone 4G10) was obtained from Upstate Biotechnology. Other antibodies were peroxidase-conjugated anti-mouse monoclonal antibody (Dako, Bucks, UK), FITC-conjugated anti-mouse antibodies (Dako) and purified mouse immunoglobulin (Sigma).

Immunofluorescence staining

Cells were removed from culture dishes with trypsin, washed twice with PBS and allowed to grow onto Permanox plastic slides (Life Technologies, Bethesda, MD) at 2×10^4 cells/well in 100 μl standard medium for 24 hr. Cells were serum-starved for 24 hr and cell-cell contacts were disrupted by treatment with 4 mM EGTA for 20 min. Thereafter, cell-cell contacts were allowed to reestablish in the presence of fresh medium supplemented with either 1% FCS alone (control) or 20 ng/ml FGF-1 or FGF-2 (stimulated) and maintained for a further 24 hr at 37°C. Immunostaining for E-cadherin and catenins was done by fixing the cells in 3.7% (v/v) formaldehyde in Tris-buffered saline (TBS) for 15 min followed by permeabilisation with solubilisation buffer (0.5% [v/v] TX-100, 50 mM NaCl, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 300 mM sucrose, 1.2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 15 min at 4°C. After blocking with 1% (w/v) BSA for 1 hr at 4°C, cells were incubated overnight at 4°C with anti-E-cadherin (1 μg/ml) or anti-catenin antibodies (α-catenin [4 μg/ml]; β-catenin [1 μg/ml]; γ-catenin [1 μg/ml]) and $p120^{ctn}$ (1 μg/ml).

As a positive control, the HT29 colorectal cell line was used for the membranous expression of E-cadherin. As a negative control, primary antibody was replaced by mouse IgG. After washing in TBS-T, slides were incubated with FITC-conjugated anti-mouse antibody for 1 hr at room temperature and finally mounted in Vectashield. Cells were examined with a fluorescence microscope. The change in the level of expression of E-cadherin and catenins was evaluated by comparing the degree of fluorescent intensity of the stimulated to unstimulated cells (control). An arbitrary scale was used for the change in the level of expression, as follows: −, no change in the intensity of staining; −/+, equivocal; and +, expression increased relative to the control. The slides were also assessed for the subcellular localisation of E-cadherin and catenins (membranous, cytoplasmic, mixed membranous and cytoplasmic).

ELISA

Measurements of cellular content of E-cadherin were also performed. Cells were harvested from confluent monolayer cultures using 4 mM EGTA and then plated in 96-well microtitre plates (Immulon, Dynatech, Chantilly, VA) at 2×10^4 cells/well suspended in 100 μl of standard medium. Cells were incubated for 24 hr at 37°C, after which fresh serum-free medium was added alone or supplemented with various doses of FGF-1 or FGF-2 (5, 10, 50, 100 ng/ml); the cells were then kept for a further 24 hr at 37°C. Cells were washed in PBS/0.1% BSA (washing buffer) and fixed in glutaraldehyde (0.1%) for 15 min. Nonspecific protein binding was blocked with 3% BSA in PBS (100 μl/well) for 1 hr at room temperature and the wells were then incubated with anti-E-cadherin antibody for 2 hr at room temperature. As a control, nonimmune mouse IgG (control) was used.

Subsequently, cells were incubated with 50 μl peroxidase-conjugated secondary anti-mouse antibody (1:1,000 dilution) for 1 hr at room temperature. After washing, colour development was achieved by incubation (100 μl/well) with peroxidase substrate solution (2 mg/ml 1,2′-phenylenediamine [OPD, Dako] in 0.1 M citrate buffer, pH 6.0 and 5 μl 30% hydrogen peroxide) for 1–5 min at room temperature and kept in the dark. The hydrolysis reaction was stopped by adding 50 μl/well of the quenching solution (2 N sulphuric acid). The OD was measured at 492 nm on an ELISA-plate reader (Dynex Technologies Inc, Chantilly, VA). All experiments were performed in triplicate and repeated at least twice.

Immunoprecipitation and Western blotting

Briefly, cell lysates solubilised in either RIPA buffer or TX-100 solubilisation buffer were precleared and incubated with anti-E-cadherin, anti-α-catenin, anti-β-catenin, anti-γ-catenin and $p120^{ctn}$ antibodies overnight at 4°C with rotation. Immune complexes were precipitated with 50 μl/ml protein G-sepharose and the immunoprecipitates were resolved by SDS-PAGE (8%) and transferred onto nitrocellulose membranes (Millipore, Herts, UK) for immunoblotting. After nonspecific binding was blocked with TBS-T, membranes were probed overnight with *(i)* anti-E-cadherin, 1 mgr;g/ml; *(ii)* anti-α-catenin, 4 μg/ml; *(iii)* anti-β-catenin, 1 μg/ml; *(iv)* anti-γ-catenin, 1 μg/ml and *(v)* $p120^{ctn}$, 1 μg/ml. Membranes were incubated with secondary antibodies for 2 hr at room temperature, washed, incubated with enhanced chemiluminescence reagent (ECL; Amersham Life Sciences, Bucks, UK) and exposed to Hyperfilm-MP film (Amersham). Cell lysates immunoprecipitated with IgG were used as a control for antibody specificity.

Protein concentrations were quantified after cell extraction with a Bio-Rad (Herts, UK) protein assay kit. Densitometry of the ECL signals, performed in duplicate, was performed with the NIH image software package. Results were presented in arbitrary units of the integrated OD. Equal immunoprecipitation and loading of proteins was further assessed by immunoblotting membranes with the respective antibodies. All experiments were repeated twice.

Tyrosine phosphorylation of E-cadherin and catenins

Experiments were carried out as above except for the following modifications. The extraction buffer was supplemented with phosphatase inhibitors (200 μM sodium orthovanadate [Na_3VO_4] and hydrogen peroxide [200 μM]). Tyrosine phosphorylation of E-cadherin and catenins was determined by blotting the membranes with anti-phosphotyrosine antibody (1 μg/ml). The membranes were then stripped and reprobed with the relevant primary antibody used for immunoprecipitation.

RESULTS

FGF-1 and FGF-2 upregulate E-cadherin and catenins and enhance E-cadherin-mediated cell-cell contacts

We were interested to study the effect of FGF-1 and FGF-2 stimulation on E-cadherin and catenin expression and subcellular localisation in monolayer culture by immunofluorescence staining of the total cellular proteins, *i.e.*, fixation in formaldehyde before extraction in TX-100. Since the formation and the maintenance of adherens junctions in areas of cell-cell contacts depends on E-cadherin activation in the presence of Ca^{2+}, chelation of Ca^{2+} and then reinitiation of cell-cell contacts by subsequent restoration of Ca^{2+} represents a useful tool for studying E-cadherin biology.[25]

Thus, cells were serum-starved for 24 hr, after reaching confluence to optimise cell-cell contacts and minimise the influence of

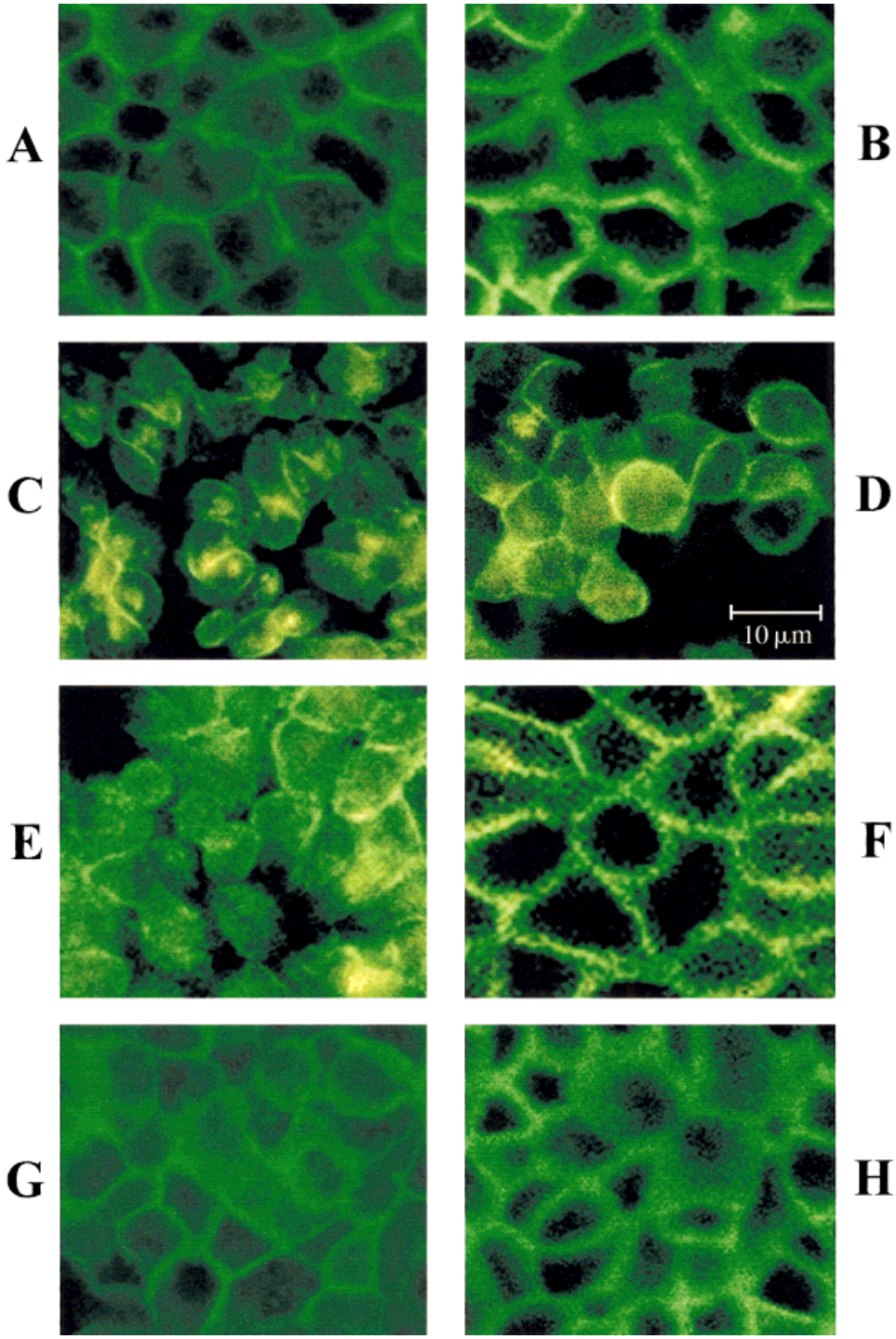

FIGURE 1 – FGF-1 and FGF-2 increase E-cadherin and catenin expression. Confluent cell monolayers were disrupted by incubating with EGTA and cells were treated with medium only as controls (*a*,*c*,*e*,*g*) or with 20 ng/ml FGF-1 (*d*,*f*,*h*) or FGF-2 (*b*) for 24 hr and were then probed with the anti-E-cadherin or anti-catenin antibodies overnight. Bound antibodies were visualised with FITC-conjugated secondary antibodies. *(a–f):* E-cadherin immunolocalisation in BxPc3 cells (*a*,*b*), T3M4 cells (*c*,*d*) and HPAF cells (*e*,*f*). (*g*,*h*): β-Catenin immunolocalisation in HAPF cells. Scale bar = 10 μm.

extracellular matrix interactions and cell-cell contacts were abrogated by EGTA treatment and then allowed to re-form in standard medium with or without FGF-1 or FGF-2. In BxPc3 cells, FGF-1 and FGF-2 increased E-cadherin (Fig. 1*b*), β-catenin and γ-catenin expression. Both FGFs marginally increased E-cadherin expression in T3M4 cells (Fig. 1*d*) but had no apparent effect on catenin expression. In both cell lines, there was no change in the subcellular distribution of E-cadherin or catenins. In the HPAF cell line, FGF-1 (Fig. 1*f*) and FGF-2 induced a change in the cell morphology that coincided with the translocation of E-cadherin from mixed membranous and cytoplasmic expression in untreated cells to exclusively membranous expression, with the characteristic

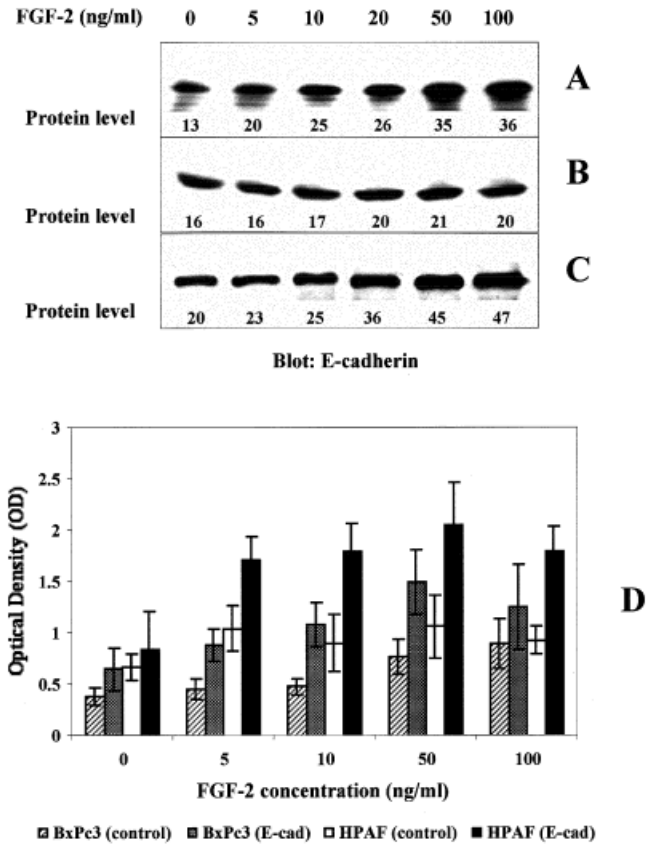

FIGURE 2 – FGF-2 upregulates the expression of E-cadherin in (*a*) BxPc3 cells, (*b*) T3M4 cells and (*c*) HPAF cells. Cells were treated with medium only (control) or incubated with FGF-2 at increasing doses (5, 10, 20, 50, 100 ng/ml) for 24 hr. Cell lysates were separated by SDS-PAGE and analysed by Western blotting with anti-E-cadherin antibody. The densities of the bands were quantified by NIH image and are represented as arbitrary units at the bottom of each panel. (*d*) Total E-cadherin protein levels in BxPc3 and HPAF cells were quantified by ELISA. Serum-starved cells were plated in 96-well microtiter plates and then stimulated with FGF-2 at increasing doses for 24 hr. After blocking of the nonspecific protein binding, cells were probed with anti-E-cadherin antibody. Control samples were incubated with mouse IgG.

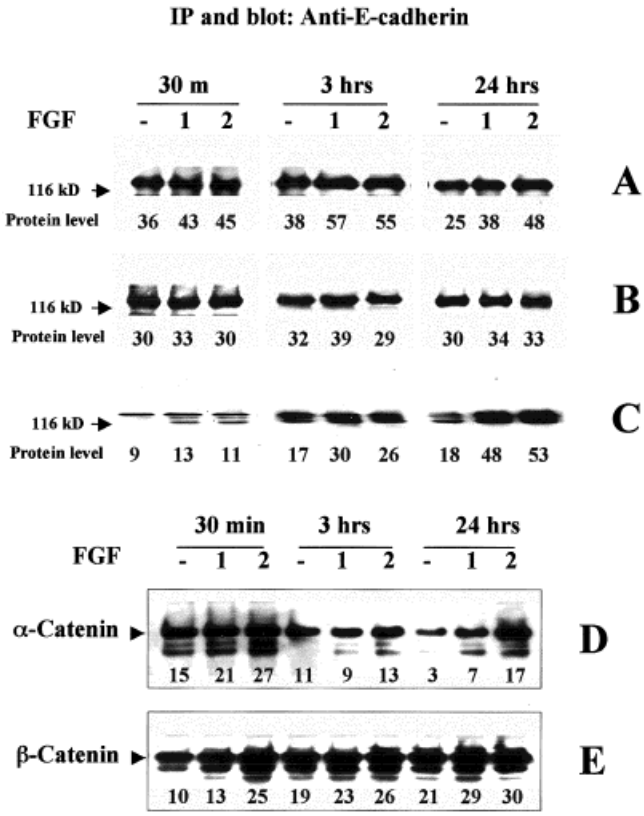

FIGURE 3 – FGF-induced expression of total E-cadherin and catenins as demonstrated by IP. Serum-starved cells were treated with FGF-1 or FGF-2 (50 ng/ml) for 30 min and 3 and 24 hr. Cells extracted in RIPA buffer were immunoprecipitated with anti-E-cadherin (*a–c*), anti-α-catenin (*d*) and anti-β-catenin antibodies (*e*), separated by SDS-PAGE (8%) and probed with the respective antibody. The protein levels are represented as arbitrary units. (*a*) BxPc3 cells. (*b*) T3M4 cells. (*c–e*) HPAF cells.

cobblestone epithelial morphology. A similar shift from a mixed cytoplasmic and membranous localisation of β-catenin to a uniform membranous expression at the cell-cell contacts was also observed with FGF-1 (Fig. 1*h*) and FGF-2. In contrast, FGFs did not affect α-catenin or $p120^{ctn}$ expression or their subcellular localisation in all cell lines (not shown). These results suggest that both FGFs enhance the reassembly of E-cadherin-mediated cell-cell contacts that coincide with increased immunoreactivity of E-cadherin or β- and/or γ-catenins.

We next investigated whether FGF-1 or FGF-2 affected the total level of E-cadherin protein. Serum-starved cells were stimulated with FGFs at increasing concentrations (5, 10, 50 and 100 ng/ml) for 24 hr, extracted in sample buffer and then subjected to SDS-PAGE and Western blotting. Both FGFs gave similar results. As shown in Figure 2, FGF-2 stimulation induced a more than 2-fold increase in the E-cadherin level with the higher doses in BxPc3 cells (Fig. 2*a*) and in HPAF cells (Fig. 2*c*). In contrast, T3M4 cells showed a marginal response to FGF-1 and FGF-2 (Fig. 2*b*). The increased expression of E-cadherin in the former two cell lines was also confirmed by ELISA experiments (Fig. 2*d*).

A kinetic study revealed that the increase in total E-cadherin expression appeared as early as 3 hr in BxPc3 cells after stimulation with FGF-1 or FGF-2 (Fig. 3*a*) and in T3M4 cells after stimulation with FGF-1 (Fig. 3*b*). In HPAF cells, both FGFs led to an early and dramatic increase in E-cadherin expression (Fig. 3*c*).

The total levels of α-catenin remained almost unchanged with FGF-1 or FGF-2 stimulation in both BxPc3 and T3M4 cells, whereas increased levels of β-catenin were observed in BxPc3 cells with both FGF-1 and FGF-2 and only with FGF-1 in the case of T3M4 cells (not shown). In contrast, the levels of α-catenin (Fig. 3*d*) and β-catenin (Fig. 3*e*) increased in HPAF cells after stimulation with FGF-2 and to some extent with FGF-1. Thus, these results show that FGF-1 and FGF-2 lead to upregulated expression of E-cadherin and catenins at the protein level.

FGF-1 and FGF-2 increase the detergent insolubility of E-cadherin

The correct function of the E-cadherin/catenin complex requires its association with the cytoskeleton, which can be demonstrated by Triton TX-100 insolubility of the components of the system. Therefore, the subcellular distribution of E-cadherin has been widely used to determine its level of activity.[26] TX-100 soluble (S) and insoluble (I) fractions of FGF-stimulated cells were prepared and the levels of E-cadherin were compared. As shown in Figure 4, E-cadherin level increased significantly in the insoluble fraction after stimulation with FGF-1 and FGF-2 for 24 hr—in order of

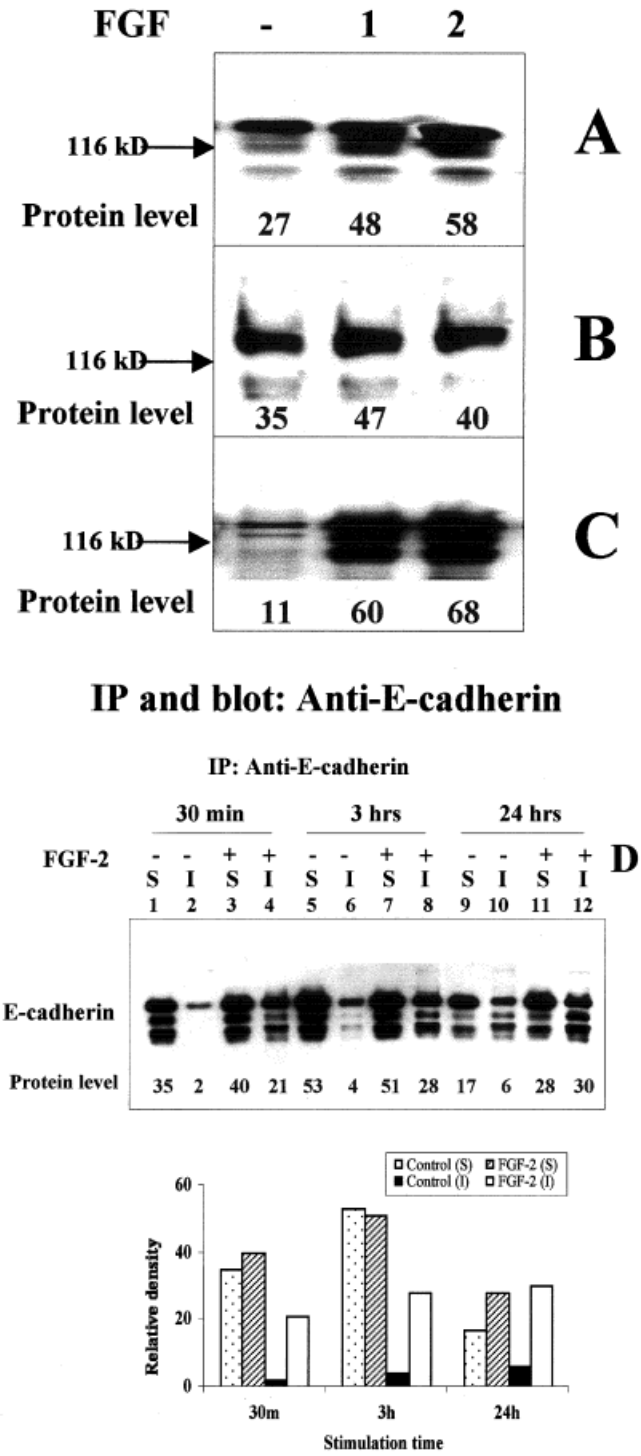

FIGURE 4 – Association of E-cadherin with the cytoskeleton in (*a*) BxPc3 cells, (*b*) T3M4 cells and (*c*) HPAF cells. Serum-starved cells were stimulated with either FGF-1 or FGF-2 (50 ng/ml) for 24 hr and TX-100-insoluble fraction was extracted by solubilisation buffer. Lysates were immunoprecipitated with anti-E-cadherin antibody and subjected to SDS/PAGE and Western blotting with anti-E-cadherin antibody. (*d*) Kinetic changes in the TX-100-soluble (S) and -insoluble (I) fractions of HPAF cells after stimulation with FGF-2 (50 ng/ml) for 30 min and 3 and 24 hr. The results were analysed by densitometry and expressed graphically.

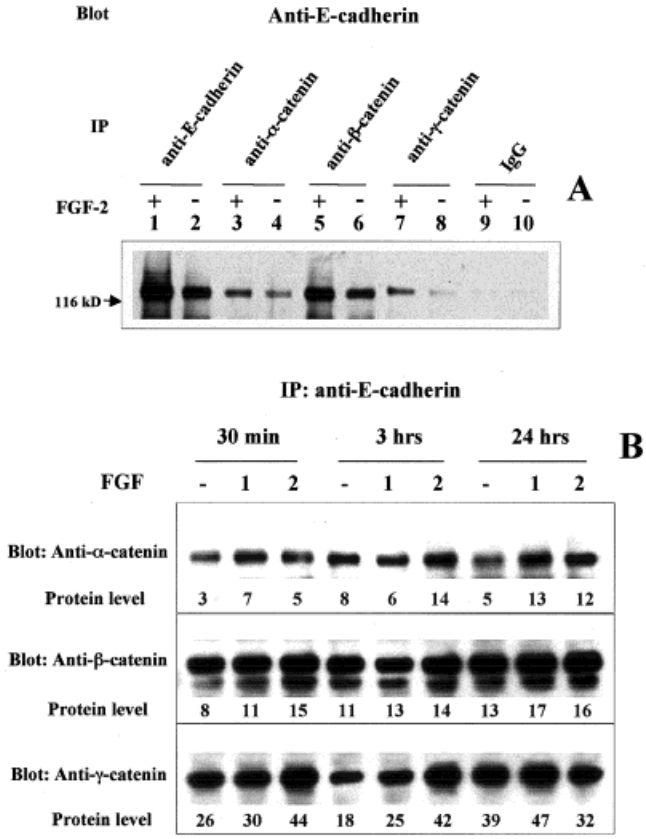

FIGURE 5 – FGF-1 and FGF-2 increase the levels of catenins associated with E-cadherin. (*a*) BxPc3 cells were treated with FGF-2 (50 ng/ml) for 24 hr, lysed in solubilisation buffer and immunoprecipitated with anti-E-cadherin (lanes 1, 2), anti-α-catenin (lanes 3, 4), anti-β-catenin (lanes 5, 6) and anti-γ-catenin antibodies (lanes 7, 8), or with IgG (lanes 9, 10) as a control for antibody specificity. Proteins were analysed by SDS-PAGE and probed with anti-E-cadherin antibody. (*b*) FGF-treated HPAF cells were immunoprecipitated with anti-E-cadherin antibody and probed with anti-α-catenin (upper panel), anti-β-catenin (middle panel) and anti-γ-catenin antibodies (lower panel). Protein levels are shown as arbitrary units.

intensity—in HPAF cells (Fig. 4*c*), followed by BxPc3 cells (Fig. 4*a*) and finally in T3M4 cells with FGF-1 but not FGF-2 (Fig. 4*b*).

As depicted in Figure 4*d*, E-cadherin was mostly found in the soluble fraction in HPAF cells. FGF-2 induced a significant increase in the insoluble fraction at 30 min (lane 4) that was maintained until 24 hr (lane 12). FGF-1 gave also similar results (not shown). Results from two independent experiments revealed that approximately 5–10% of the total E-cadherin belonged to the insoluble fraction in the control cells, which significantly increased to 30–50% with either FGF-1 or FGF-2 treatment. This means there was an approximately 5-fold increase in the insoluble fraction with FGF-1 or FGF-2 stimulation. Additionally, treatment of the serum-starved HPAF cells with FGF-2 increased the association of actin in E-cadherin immunoprecipitates (not shown). These results indicate that FGFs enhanced the association of E-cadherin with the cytoskeleton, which may promote the activation of the E-cadherin/catenin complex.

FGF-1 and FGF-2 increase catenin association with E-cadherin

The relocalisation of E-cadherin in the detergent-resistant membranous compartment raised the question of whether both FGFs affect the extent of association of catenins with E-cadherin. In the

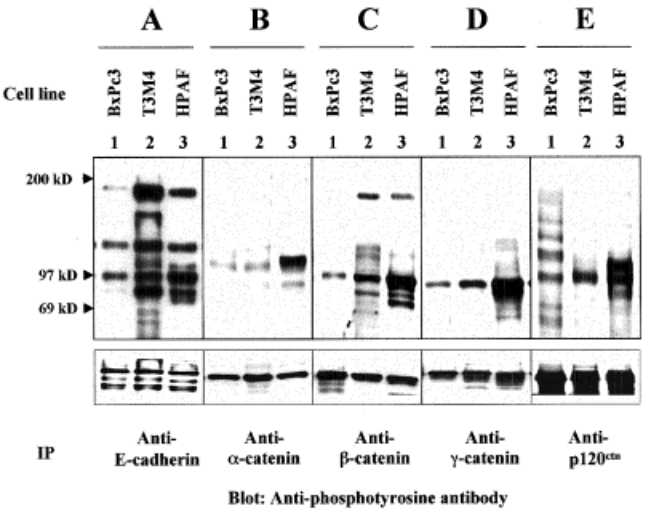

FIGURE 6 – Phosphorylation on tyrosine residues of E-cadherin and catenins. Total cell lysates from each cell line were extracted and equal aliquots were immunoprecipitated with anti-E-cadherin (*a*), anti-α-catenin (*b*), anti-β-catenin (*c*), anti-γ-catenin (*d*) and p120^{ctn} (*e*) antibodies. Samples were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. The same blots were subsequently reprobed with the respective antibody. Lane 1, BxPc3 cells; lane 2, T3M4 cells; and lane 3, HPAF cells. The position of the molecular weight markers is indicated by arrows.

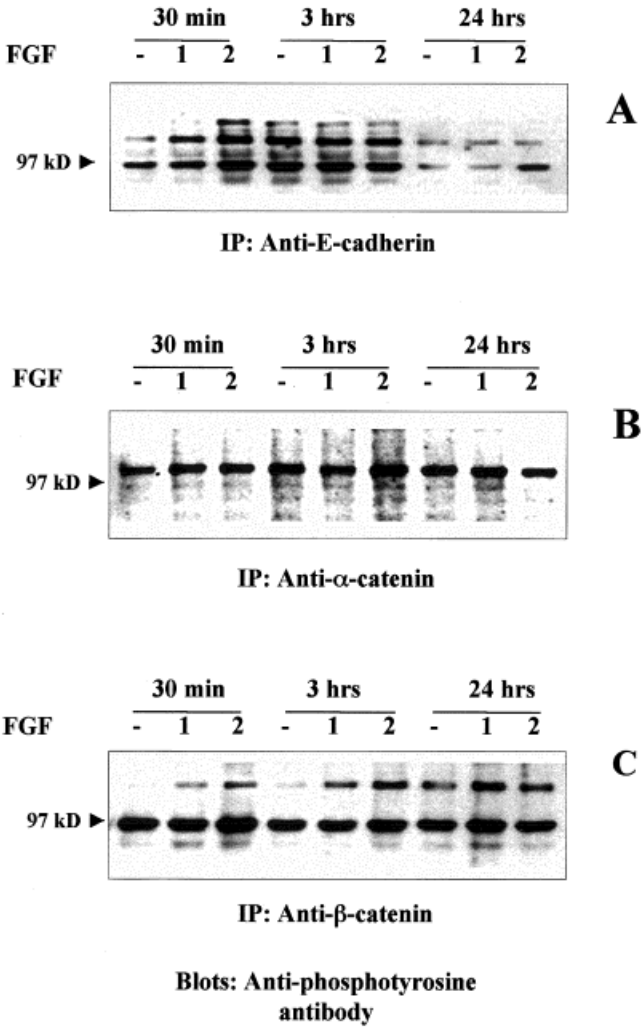

FIGURE 7 – FGF-1 and FGF-2 modify the tyrosine phosphorylation of E-cadherin, α-catenin and β-catenin in the HPAF cell line. Serum-starved cells were left untreated (−) or cells treated with either FGF-1 or FGF-2 for 30 min or 3 or 24 hr were extracted in solubilisation buffer containing phosphatase inhibitors and immunoprecipitated with anti-E-cadherin (*a*), anti-α-catenin (*b*) or anti-β-catenin antibodies (*c*). Samples were separated by SDS-PAGE and probed with anti-phosphotyrosine antibody. The position of the molecular weight markers is indicated by arrows.

first set of experiments, serum-starved BxPc3 and T3M4 cells were stimulated with FGF-1 or FGF-2 (50 ng/ml) for 24 hr, immunoprecipitated with anti-E-cadherin and anti-catenin antibodies and immunoblotted with anti-E-cadherin antibody. Relative to control cells, FGFs increased the association of E-cadherin with α-, β- and γ-catenins in BxPc3 cells (Fig. 5*a*, lanes 3, 5 and 7, respectively) and with α-catenin only in the T3M4 cells (not shown).

As expected in the HPAF cell line, FGF-2 increased the association of E-cadherin with α-catenin (Fig. 5*b*, upper panel) and modestly with β-catenin (Fig. 5*b*, middle panel), but not with p120^{ctn} (not shown). Interestingly, FGF-1 led to an enhanced association of E-cadherin with γ-catenin (Fig. 5*b*, lower panel), which developed slowly but was sustained, compared with FGF-2, that led to an early but short-term increase of γ-catenin in the E-cadherin immunoprecipitates (Fig. 5*b*, lower panel). These results indicate that FGF-1 and FGF-2 stimulation enhance the assembly of the E-cadherin/β-/γ-/α-catenin axis.

Tyrosine phosphorylation of E-cadherin and catenins at the steady state

We previously demonstrated that FGFs induce tyrosine phosphorylation of E-cadherin and catenins after short-term stimulation.[19] We evaluated the phosphorylation state of E-cadherin and the catenins under basal conditions, since it is tightly linked with system function. As shown in Figure 6*a*, the E-cadherin immunoprecipitates revealed a tyrosine-phosphorylated band of ≈120kD that corresponded to E-cadherin in Western blotting. In all cell lines, a tyrosine-phosphorylated band of ≈92 kd was identified as β-catenin, as judged by reprobing of the membranes with the specific antibody. In addition, a tyrosine-phosphorylated γ-catenin (86 kd) comigrated with E-cadherin in both T3M4 (Fig. 6*a*, lane 2) and HPAF (lane 3) cell lines but not BxPc3 cells. Furthermore, other tyrosine-phosphorylated proteins were an ≈180–190 kD protein in BxPc3, T3M4 and HPAF cell lines, an ≈110 kD protein in T3M4 and HPAF cells and a 65 kD protein, as a double band, in T3M4 cells. Only the ≈180 kD band was identified as EGFR (not shown).

Tyrosine phosphorylation of α-catenin was detected as faint bands in BxPc3 (Fig. 6*b*, lane 1) and T3M4 cells (lane 2) and as a strong signal in the HPAF cell line (lane 3). β-Catenin and γ-catenin were also tyrosine-phosphorylated in all cell lines with variable intensities (Fig. 6*c* and *d*, respectively). The same 180–190 kD band seen in the E-cadherin immunoprecipitates was also detected in β-catenin immunoprecipitates in T3M4 and HPAF cell extracts (Fig. 6*c*, lanes 2 and 3, respectively). p120^{ctn} was heavily phosphorylated in T3M4 and HPAF cells (Fig. 6*e*). In addition, multiple tyrosine-phosphorylated bands of 180, 150, 130, 120 (E-cadherin) and 65 kD were seen in the p120^{ctn} immunoprecipitates from BxPc3 cell extracts (Fig. 6*e*, lane 1). The identity of these additional bands was not determined.

FGFs increase the tyrosine phosphorylation of E-cadherin and catenins

Phosphorylation experiments were carried out to investigate the possible posttranslational modifications of E-cadherin and catenins

TABLE I – EFFECT OF FGF-1 AND FGF-2 TREATMENT ON THE EXPRESSION OF E-CADHERIN AND CATENINS[1]

Cell line	IF		WB		ELISA		IP	
	FGF-1	FGF-2	FGF-1	FGF-2	FGF-1	FGF-2	FGF-1	FGF-2
BxPc3								
E-cadherin (total)	+	+	+	+	+	+	+	+
E-cadherin (I)							+	+
α-catenin	−	−					−	−
β-catenin	+	+					+	+
γ-catenin	+	+					−/+	+
E-β/γ-catenin assembly							+	+
p-tyr (E and β)							+	+
T3M4								
E-cadherin (total)	+/−	+/−	+/−	+/−	+/−	+/−	+	−
E-cadherin (I)							+	−
α-catenin	−	−					−	−
β-catenin	−	−					+	−
γ-catenin	−	−					−	−
E-β/γ-α-catenin assembly							+/−	−
p-tyr (E and β)							+	+
HPAF								
E-cadherin (total)	+	+	+	+	+	+	+	+
E-cadherin (I)							+	+
α-catenin	−	−					+	+
β-catenin	+	+					+	+
γ-catenin	+	+					+	+
E-β/γ-catenin assembly							+	+
p-tyr (E and β)							+	+

[1]An arbitrary scale was used: −, no difference in expression level compared with untreated cells; +/−, expression is equivocal; +, increased expression. IF, immunofluorescence; WB, Western blotting; IP, immunoprecipitation; I, insoluble fraction; E, E-cadherin; α, α-catenin; β, β-catenin; γ, γ-catenin; p-tyr, tyrosine phosphorylation.

after stimulation of the cell lines with FGF-1 and FGF-2. Extracts from serum-starved cells were immunoprecipitated with anti-E-cadherin and anti-catenin antibodies, followed by immunoblotting with anti-phosphotyrosine antibody. A similar pattern was generally noticed in all cell lines and therefore, only the HPAF cell line is presented as an example. As shown in Figure 7*a*, FGF-1 and FGF-2 enhanced the phosphorylation of E-cadherin on tyrosine residues, which was brief and detected at 30 min. The anti-phosphotyrosine antibody specifically reacted to two bands at ≈135 and ≈92 kD in the HPAF cell line only (Fig. 7*a*). The 92kD band was likely to correspond to β-catenin. An increase in the level of tyrosine phosphorylation of α-catenin was modestly observed after FGF stimulation for 3 hr (Fig. 7*b*). In contrast, β-catenin was more phosphorylated on tyrosine residues with FGF-1 and FGF-2 compared with the control cells, appearing as early as 30 min (Fig. 7*c*). Neither FGF-1 nor FGF-2 induced any significant increase in γ-catenin or $p120^{ctn}$ tyrosine phosphorylation (not shown). Although phosphorylated on tyrosine residues, $p120^{ctn}$ does not regulate the linkage of E-cadherin with the cytoskeleton, as it does not associate with α-catenin.[6] These results suggest that coupling of the E-cadherin/catenin complex with the cytoskeleton and the subcellular relocalisation of E-cadherin can be accounted for—at least in part—by modification of the tyrosine phosphorylation status of E-cadherin and β-catenin. Table I is a summary of the effects of both FGFs on expression and tyrosine phosphorylation of E-cadherin and catenins by various experiments.

DISCUSSION

Increasing evidence suggests functional cross-talk exists between growth regulatory and signalling molecules, such as EGF and EGFR and the E-cadherin/catenin system that determines the fate of the cell. However, the potential involvement of the FGF/FGFR system in the regulation of E-cadherin/catenin system is less well explored. The present study examined the effect of FGF-1 and FGF-2 stimulation and possibly FGFR activation, on E-cadherin and catenin expression and the association of the E-cadherin/catenin complex with the cytoskeleton in a panel of three pancreatic adenocarcinoma cell lines that express various combinations of FGFRs.[20] These cell lines display varied phenotypes and their E-cadherin/catenin complexes were previously characterised.[27] We have previously shown that FGF-1 and FGF-2 facilitate the adhesive and decrease the invasive properties of these cell lines and that FGFRs associate with the E-cadherin/catenin system, most probably via β-catenin.[19]

In this study, we provide evidence that both FGF-1 and FGF-2 are involved not only in controlling the cell surface expression of E-cadherin and catenins but also in controlling their subcellular localisation. To ensure that these effects are E-cadherin-dependent, a "calcium switch" approach was used, whereby cell junctions are first disrupted by chelating Ca^{2+} and then allowed to re-form by adding Ca^{2+}.[25] In this way, FGFs are implicated as the possible source for the differential accumulation of E-cadherin at the cell-cell contacts in FGF-treated cells as opposed to control cells. The enhanced assembly of the E-cadherin-mediated cell junctions following FGF-1 and FGF-2 stimulation coincides with a shift to a well-differentiated phenotype, particularly in the HPAF cell line and provides further support to our previous observations of augmented adhesive properties of the E-cadherin/catenin system.[19] Taken together, both FGF/FGFR and E-cadherin/catenin systems appear to be ideal candidates playing tightly coordinated roles in differentiation, morphogenesis and thus pancreatic tissue integrity.

FGF-1 and/or FGF-2 lead to an upregulated expression of membrane-associated α-catenin in HPAF cells and β-catenin in all cell lines and facilitate the assembly of the system in all cell lines. It was previously noted that modulation of E-cadherin expression subsequently facilitates the stability of α-catenin and β-catenin in complex with E-cadherin.[28] It is possible that the association of α-catenin and β-catenin with E-cadherin may selectively protect them from rapid degradation mechanisms.[28]

A key finding in our study is the increase in the detergent insolubility of E-cadherin upon exposure of pancreatic cancer cells

to FGF-1 and FGF-2. It is well established that the insoluble pools of E-cadherin and catenins indicate their ability to anchor to the actin cytoskeleton, which is one of the hallmarks of the functionality of the system.[29] The mechanism(s) by which FGF-1 and FGF-2 increase the detergent insolubility of E-cadherin are not understood. FGFs may regulate the availability of E-cadherin molecules at the cell surface by activation of the downstream molecules such as PI 3-kinase[30] and in turn Rho and Rac.[31] Rho and Rac provide a link between growth factor signalling and reorganisation of the actin cytoskeleton[32] and are also required for the establishment of cadherin-mediated cell-cell adhesion and actin reorganisation necessary to establish the adherens junctions.[33] It is conceivable that the cell-cell contact leads to the activation of PI 3-kinase and subsequently to some conformational changes that facilitate its recruitment to E-cadherin at the level of the plasma membrane[34] and hence activation of the E-cadherin/catenin complex.

Despite the demonstration of the stimulatory effects of FGF-1 and FGF-2 on the activation of E-cadherin/catenin in all cell lines, these effects vary either in extent or amplitude and are interpreted on the basis of two observations. First, the difference in their phenotypic properties may account for the difference in response to both FGF-1 and FGF-2. Second, FGFRs are activated by FGF-1 and FGF-2 with variable affinities and in addition have differing signalling capabilities.[35,36] Whereas FGFR-1 has more affinity for FGF-2, FGFR-4 has more affinity for FGF-1. FGF-2 induces a slightly higher response in the BxPc3 cell line (FGFR-1 positive), whereas the T3M4 cell line (FGFR-3 positive) is more responsive to FGF-1 and both FGFs induce similar response in the HPAF cell line (FGFR3 and FGFR-4 positive). FGFs are required for both kinase activation and the intracellular trafficking of FGFRs that lead to discrete signalling pathways and ultimate cellular responses. Although receptor autophosphorylation and the initial signals are the same when these receptors are compared,[37] they differ significantly in their ability to sustain signals[38] and to activate key signalling pathways and cellular targets.[39,40] Therefore, although the results of our study demonstrate that the E-cadherin/catenin system is a target for FGF/FGFR activation, the ultimate effect could be modified by the altered expression of other interacting cellular proteins that reflect different cellular phenotypes.

We demonstrate that E-cadherin and the catenins are phosphorylated on tyrosine residues under basal conditions. In the T3M4 and HPAF cell lines, E-cadherin is associated with EGFR, which may account, at least in part, for the increased level of tyrosine phosphorylation of E-cadherin. Previous studies have also reported the presence of phosphotyrosine on E-cadherin, β-catenin, γ-catenin and $p120^{ctn}$.[41,42] Controversy exists as to whether tyrosine phosphorylation of catenins adversely affects their association with E-cadherin. A positive correlation has been demonstrated between tyrosine phosphorylation of $p120^{ctn}$ and its binding to E-cadherin in *ras*-transformed human breast epithelial cells.[43] In the same epithelial cells, tyrosine phosphorylation of β-catenin correlates inversely with its association to E-cadherin. Several authors have also observed that tyrosine phosphorylation of β-catenin as well as γ-catenin does not alter their binding to cadherins (both E- and N-) in cultured cells.[28,41,44] It is possible that different cell lines may have different levels of kinases or phosphatases that contribute to the overall biologic behaviour of the E-cadherin/catenin system. In support of this notion, recent data have demonstrated that other junctional proteins such as ZO-1[45] or members of the ERM family[46] play a pivotal role in E-cadherin-mediated cell adhesion and that their activities can be modulated according to whether they become phosphorylated on tyrosine or serine residues.

In this study, FGF-1 and FGF-2 facilitate the coupling of the E-cadherin/catenin complex with the cytoskeleton, which is accompanied by an early and short-term augmentation of the tyrosine phosphorylation of E-cadherin and α-catenin but with a more sustained elevation of phosphorylated β-catenin. We have previously shown that stimulation with FGF-1 and FGF-2 enhances E-cadherin-mediated cell-cell adhesion, with a concomitant increase in the level of tyrosine phosphorylation of the system.[19] Together, these results suggest a new model, whereby augmentation of the tyrosine phosphorylation of E-cadherin and catenins—upon FGF treatment—is a plausible mechanism for the increase in the detergent insolubility of the complex or its association with the cytoskeleton and hence the functionality of the system.

The association of tyrosine phosphorylation with activation and upregulated expression of the E-cadherin/catenin system is intriguing, in view of the conflicting evidence linking tyrosine phosphorylation to impaired E-cadherin/catenin expression and/or function. A theme is now emerging implicating other mechanisms in the loss of E-cadherin/catenin function. In primary mouse keratinocytes, β-catenin, γ-catenin and $p120^{ctn}$ become strongly tyrosine phosphorylated upon induction of differentiation with Ca^{2+} treatment, which is also accompanied by an increased association of ρ-catenin and $p120^{ctn}$ with E-cadherin.[47] In human thyroid carcinomas, cytoskeletal uncoupling and loss of function of the E-cadherin/catenin system is tightly associated with a loss of the basal tyrosine phosphorylation of E-cadherin, compared with normal tissues.[48,49] Similarly, loss of functionality of the E-cadherin/catenin system is associated with a lack of basal E-cadherin tyrosine phosphorylation in human bronchopulmonary carcinomas.[50] Furthermore, changes in E-cadherin localisation and function are associated with a decrease in its phosphorylation state, suggesting a role of E-cadherin phosphorylation in cell-cell interactions.[51]

Taken together, these data suggest that tyrosine phosphorylation of E-cadherin and catenins is necessary under normal conditions *in vivo* to maintain the functionality of the system. The possibility that FGF-1 and FGF-2 induce phosphorylation of new and distinct tyrosine residues on catenins provides an attractive explanation. Indeed, β-catenin contains two tyrosine residues, Tyr-654 and Tyr-86; the former is relevant for the modulation of E-cadherin/β-catenin interactions and the latter is a better substrate for $pp60^{c\text{–}src}$.[52] Alternatively, a difference in the protein kinases involved in this process could explain the FGF-mediated effects. Several studies have demonstrated that EGF/EGFR axis exerts a negative regulatory control mechanism on the E-cadherin/catenin system. We postulate that the FGF/FGFR system exerts a stimulatory cue on the E-cadherin/catenin system. FGFRs activated by FGF-1 or FGF-2 may act by competing for the same binding sites on β-catenin with EGFR, for which we have demonstrated an association with the E-cadherin/catenin system in all cell lines.

Several *in vitro* and *in vivo* observations suggest the growth-stimulatory effect of FGF-1 and FGF-2, as well as overexpression of FGFRs in various types of carcinomas, including pancreatic adenocarcinoma.[53] FGF-1 has been found to induce a dysfunctional state of E-cadherin in a rat bladder cell line[54] and to confer a growth advantage to NET-II cells.[55,56] However, these effects are most probably cell type- and tissue-specific. For instance in an *FGF-2* negative rat pancreatic cancer cell line, introduction of the *FGF-2* gene leads to a dramatic reduction of tumour growth in nude mice.[57] Similarly, FGF-2 antisense transfection in prostatic carcinoma cell lines reduces cell growth *in vitro* and *in vivo*.[58] Furthermore, retroviral infection of the FGF-2 gene into MCF-7 cells suppresses cell growth *in vitro* and in nude mice.[59] Our current results and previous observations thus demonstrate that the effects of FGF-1 and FGF-2 depend on the interplay with the E-cadherin/catenin system, enhancing its expression and function and thus they add further support to the differential effect of FGFs in the milieu of the tumour microenvironment.

In conclusion, the results presented here demonstrate that FGF-1 and FGF-2 upregulate the expression of E-cadherin and at least β-catenin in a panel of pancreatic adenocarcinoma cell lines—

upon formation of cell-cell contacts after restoration of calcium levels—and enhance the association of catenins with E-cadherin. Furthermore, FGFs increase the detergent insolubility of E-cadherin, which reflects the coupling of the E-cadherin/catenin system with the cytoskeleton. Thus, FGF-1 and FGF-2 promote the integrity and function of the E-cadherin/catenin complex. Although the molecular basis remains to be elucidated, we postulate that in this cell type, FGF-1 and FGF-2 promote activation of the E-cadherin/catenin system at the posttranslational level by inducing tyrosine phosphorylation. Modulation of the phosphorylation status of E-cadherin/catenin has significant implications for our understanding of the role of both the E-cadherin/catenin and FGF/FGFR systems in pancreatic cancer biology and it may represent a potential therapeutic target.

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