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T-Cell Factor-4-Dependent Up-Regulation of Fibronectin Is Involved in Fibroblast Growth Factor-2-Induced Tube Formation by Endothelial Cells

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Abstract Deletion of fibronectin or its receptor, α_5 integrin, interferes with the formation of a functional circulation in mice. We hypothesized that $\alpha_5\beta_1$ integrin/fibronectin interaction may be involved in differentiation of endothelial cells during angiogenesis. We examined the effect of blocking antibody against $\alpha_5\beta_1$ integrin in fibroblast growth factor-2 (FGF-2)-induced angiogenesis by Matrigel plug assay. Although the antibody did not inhibit the recruitment of endothelial cells into plugs, it inhibited organization of lumen-containing capillaries. The antibody also inhibited FGF-2-induced tube formation by murine brain capillary endothelial cells (IBE cells) cultured in type I collagen gels. We previously showed that FGF-2 failed to induce tube formation by IBE cells expressing kinase-dead c-Fyn (KDFyn cells). Association with β-catenin enhances the transcriptional activity of T-cell factor-4 (TCF-4), which up-regulates the expression of fibronectin. FGF-2 induced association of β-catenin with TCF-4 and up-regulation of fibronectin in IBE cells, but not in KDFyn cells. Expression of mutant TCF-4, which does not associate with β-catenin, inhibited FGF-2-induced tube formation and expression of fibronectin in IBE cells. FGF-2-induced tyrosine phosphorylation of β-catenin, and association with TCF-4 was increased in IBE cells, but not in KDFyn cells. Taken together, interaction of $\alpha_5\beta_1$ integrin and fibronectin is involved in FGF-2-induced tube formation by endothelial cells and up-regulation of fibronectin through TCF-4 seemed to be involved in this process. J. Cell. Biochem. 94: 835–847, 2005.

Key words: FGF-2; T-cell factor-4; fibronectin; tube formation; endothelial cells

Angiogenesis is prerequisite for many pathophysiological conditions, such as solid tumor growth, diabetic retinopathy, and wound healing [Hanahan and Folkman, 1996; Ferrara and Alitalo, 1999; Carmeliet and Jain, 2000]. Angiogenesis is strictly regulated by proangiogenic growth factors and extracellular matrix proteins. Signals by these proteins are transduced via receptor tyrosine kinases and integrins [Mustonen and Alitalo, 1995; Ruoslahti, 1999; Eliceiri and Cheresh, 2001; Hynes, 2002].

Recent studies have shown that an interaction between fibronectin and its receptor, $\alpha_5\beta_1$ integrin, plays pivotal roles in angiogenesis and vascular development. Inhibition of $\alpha_5\beta_1$ integrin activity resulted in FGF-2-induced angiogenesis in vivo [Kim et al., 2000]. α_5 integrin-negative teratocarcinoma grew slowly in mice in association with reduced blood vessel formation [Taverna and Hynes, 2001]. Engagement of $\alpha_5\beta_1$ integrin caused NF-κB-dependent program of gene expression that coordinately regulated angiogenesis promoted by FGF-2 [Klein et al., 2002]. These studies indicate that the interaction of $\alpha_5\beta_1$ integrin and fibronectin is required in angiogenesis. During development, blood islands were formed in fibronectin-null and α_5 integrin-null mouse embryos. However, later phase of forming a functional circulation was impaired, resulting in fetal

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death [George et al., 1993; Yang et al., 1993]. These observations may support the hypothesis that fibronectin and α_5 integrin may regulate the differentiation of endothelial cells.

Wnt proteins regulate several signaling pathways; a β-catenin pathway, a Ca^{2+} pathway, a Jun N-terminal kinase pathway, and a focal adhesion kinase pathway [Dale, 1998; Polakis, 2000; Kikuchi, 2003]. β-catenin associates with glycogen synthase kinase-3β (GSK-3β), Axin, adenomatous polyposis coli, and casein kinase I (CKI) in the cytoplasm. After the phosphorylation by GSK-3β and CKI, β-catenin is ubiquitinated, followed by the degradation with proteasome. Wnt signaling stabilize β-catenin, which allows β-catenin to move into nucleus, followed by the association with high mobility group box transcription factors of T-cell factor (TCF)/lymphoid-enhancer factor family. TCF proteins by themselves, have poor transcriptional activities. When β-catenin binds to and form complex with TCF, it exhibits a potent transcriptional activity. β-catenin is also phosphorylated at tyrosine residues [Ilan et al., 1999; Roura et al., 1999; Danilkovitch-Miagkova et al., 2001]. Dephosphorylation of tyrosine residues of β-catenin by protein tyrosine phosphatase SHP-1 decreased the β-catenin/TCF-dependent transcription [Duchesne et al., 2003]. However, it has not been reported whether transcriptional activity of TCF was regulated by Src family kinases or β-catenin was tyrosine phosphorylated by c-Fyn to date.

In the vascular system, several reports provide evidence that Wnt signaling regulated vascular development as well as pathological angiogenesis in certain tissues [Goodwin and D'Amore, 2002]. Expression of genes encoding Wnt and their receptor Frizzled in endothelial cells has been demonstrated [Wright et al., 1999]. Mutations of Wnt and Frizzled genes result in defect of vascular development [Monkley et al., 1996; Ishikawa et al., 2001; Robitaille et al., 2002]. Inhibitors of Wnt signaling, a secreted Frizzled related protein, inhibits proliferation of endothelial cells [Duplaa et al., 1999]. Wnt signaling up-regulated cyclooxygenase 2, resulting in migration of endothelial cells and angiogenesis in vivo [Howe et al., 1999]. TCF-4 induced expression of proangiogenic factor, vascular endothelial growth factor [Zhang et al., 2001]. Another potent proangiogenic factor, fibroblast growth factor-2 (FGF-2), promoted TCF-dependent transcription in human umbilical vein endothelial cells [Holnthoner et al., 2002]. In the study, cyclin D1, a target protein of TCF, was involved in FGF-2-induced angiogenesis in mice. It has also been shown that TCF-4 up-regulated the expression of fibronectin [Gradl et al., 1999; Sen et al., 2002]. Thus, TCF-4 may contribute to FGF-2-induced angiogenic responses of endothelial cells by up-regulation of fibronectin.

Src family kinases play important roles in angiogenesis. c-Src is involved in vascular endothelial growth factor-A (VEGF-A)-induced survival of endothelial cells and VEGF-A-mediated vascular permeability [Eliceiri et al., 1999]. VEGF-A-mediated migration of endothelial cells is also regulated by c-Src [Abu-Ghazaleh et al., 2001]. FGF-2-induced chemotaxis and tube formation was regulated through c-Src [Klint et al., 1999; Shono et al., 2001]. Compared to c-Src, a role of c-Fyn in angiogenesis has been poorly examined. c-Fyn is required for thrombospondin-induced apoptosis of endothelial cells [Jimenez et al., 2000]. Our previous studies showed that c-Fyn was activated by FGF-2- and angiopoietin 2-treatment in murine brain capillary endothelial cells, denoted IBE cells [Tsuda et al., 2002; Mochizuki et al., 2002]. In these studies, expression of kinase-inactive c-Fyn exhibited dominant negative effect on FGF-2- and angiopoietin 2-induced tube formation. However, the downstream signaling pathways of c-Fyn were unknown. Since fibronectin and $\alpha_5\beta_1$ integrin may regulate tube formation (a part of differentiation) by endothelial cells, we hypothesized that c-Fyn can play roles in FGF-2-induced tube formation through modulating fibronectin and $\alpha_5\beta_1$ integrin functions. In the present study, we show that FGF-2 promoted the association of β-catenin with TCF-4, resulted in up-regulation of fibronectin and tube formation of cultured endothelial cells. These responses were not observed in cells expressing kinase-inactive c-Fyn, suggesting that the association of β-catenin with TCF-4 may be a downstream signaling pathway of c-Fyn.

MATERIALS AND METHODS

Reagents

Anti-β-catenin antibody, anti-His tag antibody, anti-Myc antibody, anti-phosphotyrosine antibody (PY99), and GSK-3β inhibitor SB216763 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TCF-3/4,

ant-TCF-4, and anti-phospholipase C-γ mixed monoclonal antibodies were from Upstate Biotechnologies, Inc. (Lake Placid, NY). Anti-mouse $\alpha_5\beta_1$ integrin antibody and anti-fibronectin antibody were from Chemicon, International, Inc., Temecula, CA. Anti-FLAG polyclonal antibody was obtained from Sigma Chemical Company (St. Louis, MO). GRGDS peptide was purchased from Peptide Institute, Inc., Osaka, Japan.

Matrigel Plug Assay

Matrigel plug assay was performed as described previously [Kibbey et al., 1992; Passaniti et al., 1992]. The animal experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University Graduate School of Biomedical Science. Growth factor-reduced Matrigel supplemented with either normal rat IgG or anti-mouse $\alpha_5\beta_1$ integrin antibody (50 μg/ml), and FGF-2 at 100 ng/ml or phosphate buffered saline were injected into Balb/c nu/nu mice subcutaneously. Seven days later, Matrigel plug was excised, fixed in formalin, and embedded in paraffin. Sections were deparaffinized and stained with Masson-trichrome. Several sections were examined in each plug and the number of endothelial cells and their organization were similar in all sections from each plug. The section with a maximal diameter of each plug was further examined and migrated endothelial cells into plugs and lumen-containing vessels were counted from four to six random fields (×20 objectives) per section by an observer in a blinded procedure and the mean number of endothelial cells or vessels per a field was quantified. Finally, mean values ± SD of each group were calculated.

Cell Culture

Parental IBE cell line established from brain capillaries of tsA58 large T transgenic mouse was cultured in Ham's F-12 medium containing fetal bovine serum, endothelial cell growth supplement, insulin, and epidermal growth factor as has been described previously [Kanda et al., 1996]. A stable IBE cell line expressing kinase-inactive c-Fyn (denoted KDFyn-8 cells) was described elsewhere [Tsuda et al., 2002]. In these cells, kinase-inactive c-Fyn exerted dominant negative effect on FGF-2-induced activation of endogenous c-Fyn and tube formation [Tsuda et al., 2002].

Plasmids and Transfection

cDNA encoding FLAG-tagged, N-terminally deleted mutant human TCF-4 [ΔNTCF-4; Molenaar et al., 1996] was a kind gift from Dr. Hans Clevers (Department of Immunology and Center for Biomedical Genetics, University, Utrecht, The Netherlands). It was subcloned into pcDNA3.1/Hygro expression plasmid and was transfected into IBE cells as has been described [Kanda et al., 2000]. Stable cell lines expressing FLAG-tagged (ΔNTCF-4 were obtained by the screening with hygromycin (0.25 mg/ml) resistance, followed by the expression of FLAG-tagged (ΔNTCF-4. cDNA encoding kinase-inactive mutant GSK-3β [Foltz et al., 2002] was a kind gift from Dr. Daniel R. Foltz at the Ludwig Institute for Cancer Research, University of California San Diego, CA and was subcloned into pcDNA3.1/Myc.His expression plasmid. Stable cell lines expressing kinase-inactive GSK-3β were obtained by the screening with G418 (0.4 mg/ml) resistance, followed by the expression of Myc.His-tagged GSK-3β.

Tube Formation Assay

IBE cells were cultured between two layers of type I collagen gels as described previously [Kanda et al., 1996]. In brief, wells of 24-well plates were coated with type I collagen gels. After the gelation, cells suspended in Ham's F-12 medium containing 0.25% bovine serum albumin (BSA) were seeded with or without indicated samples. Four hours later, medium was aspirated and the second layer of collagen gels was overlaid. After the gelation, Ham's F-12 medium containing 0.25% BSA was added and the culture was continued for overnight. To examine the effect of anti-$\alpha_5\beta_1$ integrin antibody or GRGDS peptide, cells were incubated with normal rat IgG (50 μg/ml), anti-$\alpha_5\beta_1$ integrin antibody (50 μg/ml), or GRGDS peptide (50 or 100 μM) for 1 h and then either stimulated or left unstimulated with FGF-2. Cells were also treated with either SB216763 or vehicle (0.1% dimethyl sulfoxide) for 1 h and then either stimulated or left unstimulated with FGF-2. Three hours later, the second layer of collagen gels was overlaid onto the cells and the culture was continued for 16 h. Tube-like structure was observed as a fused, continuous cell aggregates. To quantify the length of tubes, three phase-contrast photomicrographs (×10 objectives) per well were taken, and the length of each tube was

measured using NIH image soft ware (version 1.64). Tube length obtained from FGF-2-stimulated cells was set to 100.

Immunoblotting

To examine the association of β-catenin with TCF, cells were suspended in Ham's F-12 medium containing 0.25% BSA, seeded onto dishes pre-coated with type I collagen gels [Kanda et al., 1999a], and cultured for 2 h in the presence or absence of 20 ng/ml FGF-2. Cells were washed with Tris-buffered saline and lysed in buffer containing 1% Nonidet P-40 [Kanda et al., 1996]. Proteins were immunoprecipitated with anti-β-catenin antibody and separated by SDS–polyacrylamide gel electrophoresis. After the transfer onto polyvinylidene difluoride membranes, the blots were probed with indicated antibodies.

To examine the ectopic expression of proteins by transfection, cells grown on 6 cm dishes (approximately 2×10^6 cells) were washed and immunoprecipitated with antibody against FLAG, followed by the detection with immunoblotting.

To determine the expressed amount of cell-associated fibronectin, cells were seeded into wells of 24-well plate precoated with type I collagen gels and cultured for 2 or 6 h with or without 20 ng/ml FGF-2. Two sets of cultured cells were prepared for each treatment. One set of cells was washed with Tris-buffered saline, and cell-associated fibronectin was extracted by the treatment with 0.1M Tris/HCl, pH 7.5, 2M urea, 10 mM EDTA, 10 mM sodium disulfite, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml of leupeptin, and 100 U/ml of aprotinin on ice [Danker et al., 1993]. After the centrifugation to remove insoluble materials, proteins were separated by SDS–polyacrylamide gel electrophoresis. Then expression of fibronectin was determined by immunoblotting. Another set of cells was lysed in SDS-sample buffer and immunoblotting with anti-β-actin antibody was performed to assess the loaded amount of proteins.

To prepare cytosolic and nuclear protein separately, cells grown on collagen gels were incubated with 20 mM HEPES/KOH, pH 7.4, 250 mM sucrose, 100 mM potassium acetate, 1 mM ethyleneglycol-*bis* (b-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 2 mM magnesium acetate, 2 mM dithiothreitol, 10 μg/ml leupeptin, 1 μg/ml elastinol, and 50 μg/ml digitonin [Adam et al., 1990] for 5 min on ice. Lysate was recovered from the dishes and centrifuged. Supernatant was supplemented with 1% Nonidet P-40 and cytosolic TCF-4 was immunoprecipitated. Nuclear pellets were lysed with 1% Nonidet P-40 and nuclear fraction of TCF-4 was immunoprecipitated. To examine the β-catenin/TCF ratio, the density of particular protein band was measured by Densitopattern Analyzer [Kanda et al., 1999b] and the ratio of cytosolic fraction in untreated Mock cells was set to 1.00.

Statistical Analysis

Data are presented with mean values ± SD. Differences between the two groups were examined by Scheffe's test or Mann–Whitney's *U*-test.

RESULTS

Blocking Antibody Against $\alpha_5\beta_1$ Integrin Inhibits Organization of Endothelial Cells in Matrigel Plug

To examine the role of $\alpha_5\beta_1$ integrin in FGF-2-induced angiogenesis in vivo, we employed Matrigel plug assay. As shown in Figure 1, a few endothelial cells invaded into growth factor-reduced Matrigel plug in the absence of FGF-2. FGF-2 markedly enhanced the endothelial cell migration into the plugs and the antibody did not significantly affect the number of migrated endothelial cells. In plugs containing FGF-2 and normal rat IgG (control IgG), endothelial cells lined in parallel to form lumens (arrow heads). In the plug supplemented with FGF-2 and anti-$\alpha_5\beta_1$ integrin antibody, organization of lumen-containing capillary structure was significantly reduced. These results suggest that $\alpha_5\beta_1$ integrin may function to organize lumen-containing, capillary formation of endothelial cells in vivo.

Blocking Antibody Against $\alpha_5\beta_1$ Integrin or GRGDS Peptide Inhibits Tube Formation by Cultured Endothelial Cells

To understand the role of $\alpha_5\beta_1$ integrin in angiogenesis in vitro, we tested the effect of anti-$\alpha_5\beta_1$ integrin antibody on FGF-2-induced tube formation by IBE cells. As shown in Figure 2A, the antibody inhibited FGF-2-induced tube formation. FGF-2 could induce cell aggregates. However, fusion of aggregates was impaired, resulting in the impaired tube formation. The

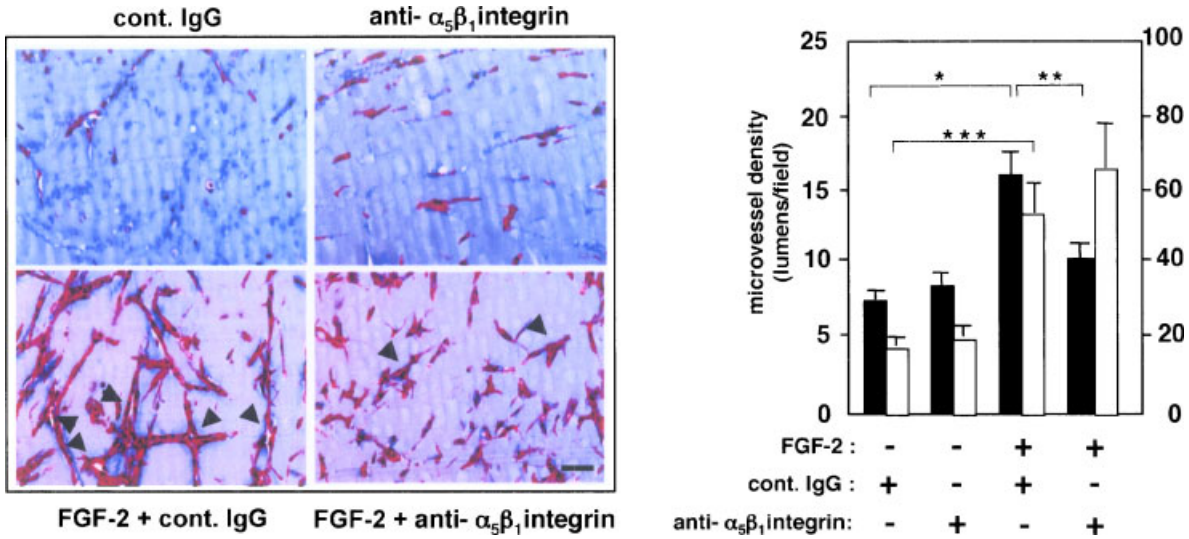

Fig. 1. Blocking antibody against mouse $\alpha_5\beta_1$ integrin inhibits capillary organization in vivo. Growth factor-reduced Matrigels supplemented with indicated IgG (50 μg/ml) and/or FGF-2 at 100 ng/ml were injected into Balb/c nu/nu mice subcutaneously. Seven days later, Matrigel plug was excised, fixed, and stained with Masson-trichrome. Migrated endothelial cells and lumen containing vessels were counted under a light microscopic observation, as has been described in the ''Materials and Methods.'' Arrowhead indicates a lumen-containing vessel. White bar indicates the number of migrated cells per field and black bar indicates the number of lumen-containing vessels per field. Bar, 50 μm. Values are expressed as mean ± SD for five independent specimens. Statistical analyses were performed by Scheffe's test *, $P = 0.0005$; **, $P = 0.0108$; ***, $P = 0.0012$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

result suggests that FGF-2-promoted tube formation of IBE cells seems to require the function of $\alpha_5\beta_1$ integrin. GRGDS peptide contains a sequence essential for the binding of fibronectin to $\alpha_5\beta_1$ integrin [Ruoslahti and Pierschbacher, 1986]. We also examined the effect of this peptide on tube formation. As shown in Figure 2B, the peptide dose-dependently inhibited FGF-2-induced tube formation. This result suggests that the interaction between fibronectin and $\alpha_5\beta_1$ integrin is dependent on the RGD sequence of fibronectin.

FGF-2 Induces Association of β-Catenin and TCF-4, Resulting in Up-Regulation of Fibronectin

It has been shown that FGF-2 induces TCF-dependent transcription in endothelial cells [Holnthoner et al., 2002]. Expression of fibronectin, a major ligand for $\alpha_5\beta_1$ integrin, is regulated by TCF-4 [Gradl et al., 1999; Sen et al., 2002], and the transcriptional activity of TCF-4 is enhanced when it is associated with β-catenin. Our previous study showed that kinase-inactive c-Fyn exerted the dominant negative effect on FGF-2-induced tube formation [Tsuda et al., 2002]. To clarify the hypothesis that dominant negative effect of c-Fyn may be due to the inhibition of β-catenin/TCF-4 association and resultant decrease in fibronectin production, we first examined the effect of FGF-2 on the association of β-catenin with TCF-4 in IBE cells cultured on collagen gels. As shown in Figure 3A, FGF-2 stimulated the association in Mock IBE cells. In a cell line stably expressing kinase-inactive c-Fyn (KDFyn-8 cells), co-precipitated TCF-4 with β-catenin was not remarkably increased by FGF-2-treatment. We next examined the expression of cell-associated fibronectin in these cells. As shown in Figure 3B, FGF-2 time-dependently increased the expression of fibronectin in Mock IBE cells, but not in KDFyn-8 cells. These data suggest that FGF-2-promoted up-regulation of fibronectin in IBE cells correlated to the association of β-catenin with TCF-4 in a manner dependent on c-Fyn activity.

FGF-2 Induced Tyrosine Phosphorylation and Association of β-Catenin With TCF-4 in IBE Cells, but not in KDFyn Cells

c-Fyn is a protein tyrosine kinase and our data showed that its kinase activity seemed to affect the association of β-catenin with TCF (Fig. 3A). We immunoprecipitated the tyrosine phosphorylated proteins from Mock IBE cells and KDFyn-8 cells, and particular proteins

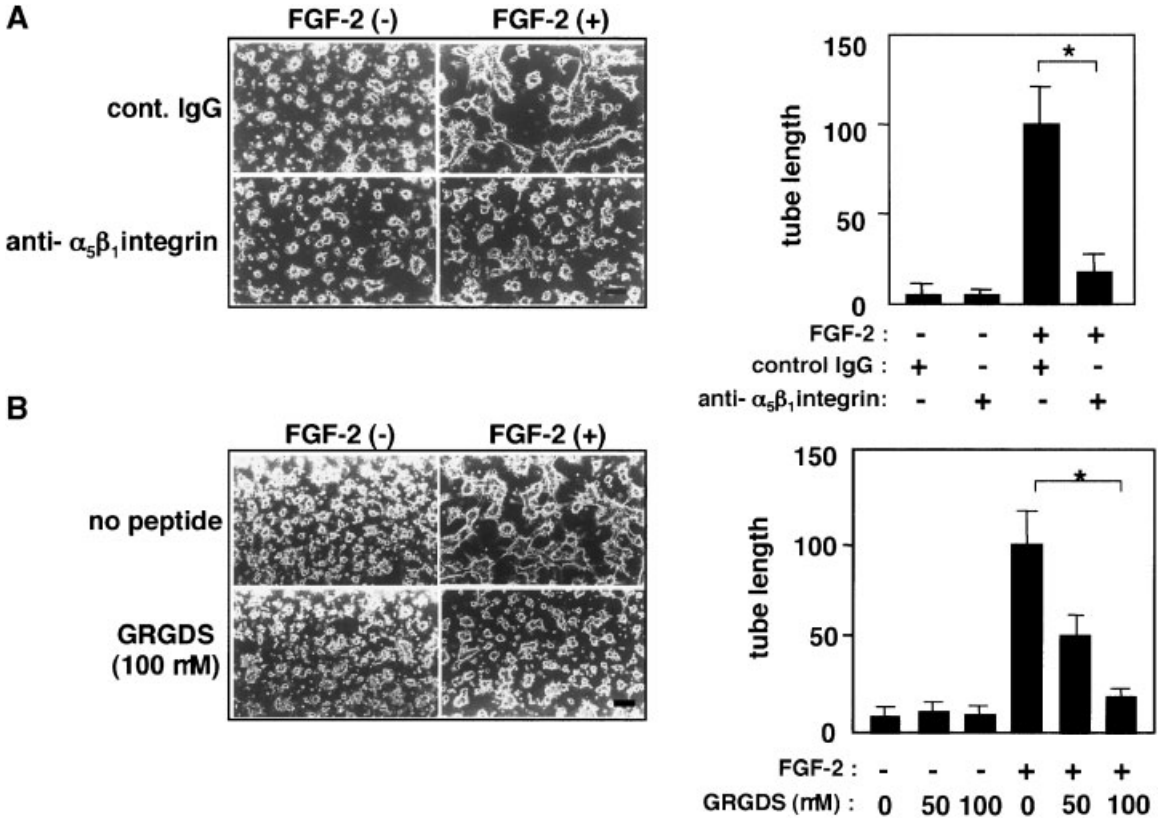

Fig. 2. **A**: Anti-$\alpha_5\beta_1$ integrin antibody inhibits FGF-2-promoted tube formation of IBE cells. Cells suspended in Ham's F-12 medium containing BSA were seeded onto collagen gels in the presence of either control rat IgG or anti-$\alpha_5\beta_1$ integrin antibody and cultured for 1 h. FGF-2 (20 ng/ml) was added to the indicated cells and the culture was continued. After 3 h, collagen was overlaid and tube formation was examined on the next day. Tube length obtained from FGF-2-stimulated cells with rat IgG was set to 100. **B**: GRGDS peptide inhibits FGF-2-promoted tube formation of IBE cells. Cells suspended in Ham's F-12 medium containing BSA were seeded onto collagen gels in the presence of peptide at indicated concentrations and cultured for 1 h. FGF-2 (20 ng/ml) was added to the indicated cells and the culture was continued. After 3 h, collagen was overlaid and tube formation was examined on the next day. Tube length obtained from FGF-2-stimulated cells without peptide was set to 100. Bar, 100 μm. Reproducible data were obtained from two independent experiments. Statistical analyses were performed by Mann–Whitney's *U*-test. *, $P < 0.01$.

were detected by immunoblotting. As shown in Figure 4A, FGF-2 increased the tyrosine phosphorylated β-catenin in Mock cells, but not in KDFyn-8 cells, suggesting that FGF-2-induced activation of c-Fyn may be responsible for tyrosine phosphorylation of β-catenin. Phospholipase C-γ is tyrosine phosphorylated by activated FGF receptor tyrosine kinase [Mohammadi et al., 1992; Peters et al., 1992], but not by c-Fyn. Tyrosine phosphorylated phospholipase C-γ was increased in both Mock and KDFyn-8 cells, indicating that signaling via FGF receptor tyrosine kinase was not affected by the expression of kinase-inactive c-Fyn. We next examined the association and localization of β-catenin/TCF-4 complex in these cells. Cells were cultured on collagen gels with or without FGF-2 and cell lysate was separated into digitonin-eluted fraction (cytosolic) and the remaining Nonidet P-40-soluble fraction, which contains nuclear proteins. As shown in Figure 4B, the amount of TCF-4 was greater in nuclei than in cytosol in these cells and its localization was not affected by FGF-2-treatment. In Mock cells, FGF-2 increased the association of β-catenin with TCF-4 at both cytosol and nuclei. In KDFyn-8 cells, FGF-2 failed to increase the association at both cytosol and nuclei. Tyrosine phosphorylation of TCF-4 was not affected by FGF-2-treatment or c-Fyn activity. Considered collectively, c-Fyn possibly affected the tyrosine phosphorylation of β-catenin, which in turn promoted the association with TCF-4.

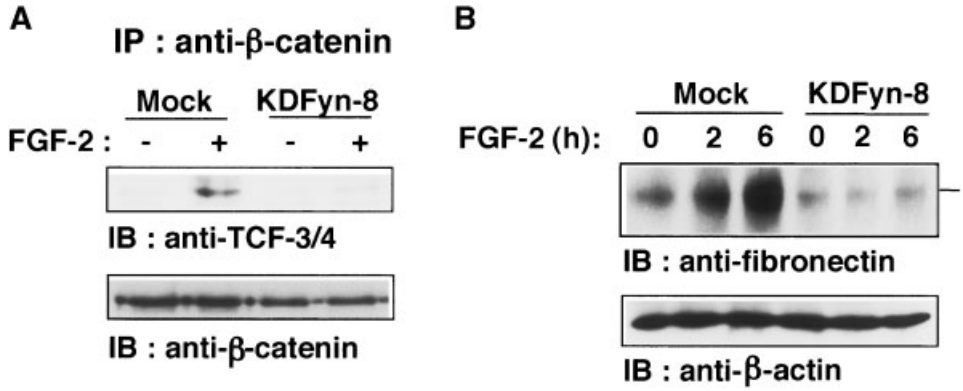

Fig. 3. **A**: Association of β-catenin with TFC-4 is increased by FGF-2-treatment in IBE cells, but not in KDFyn-8 cells. Cells were suspended in Ham's F-12 medium containing BSA, then seeded onto 6 cm dishes pre-coated with type I collagen gels in the presence or absence of 20 ng/ml FGF-2 (IBE cells, 2.4×10^6 cells/dish; KDFyn-8 cells, 1.8×10^6 cells/dish). After 2 h, cells were lysed and β-catenin was immunoprecipitated. Proteins were separated by SDS–polyacrylamide gels followed by the transfer onto membranes. β-catenin and coprecipitated TCF-4 were examined by immunoblotting. Reproducible data were obtained from two independent experiments. **B**: FGF-2 up-regulates the expression of cell-associated fibronectin in IBE cells, but not in KDFyn-8 cells. Cells suspended in Ham's F-12 medium containing BSA were seeded into wells of 24-well plates pre-coated type I collagen gels and cultured for 2 h in the presence or absence of 20 ng/ml FGF-2 (IBE cells, 6×10^5 cells/well; KDFyn-8 cells, 5.5×10^5 cells/well). Two sets of cells were prepared and one set of cells was used for the examination of cell-associated fibronectin and another set of cells was used for monitoring the loaded proteins. Expression of particular proteins was determined by immunoblotting. Reproducible data were obtained from two independent experiments.

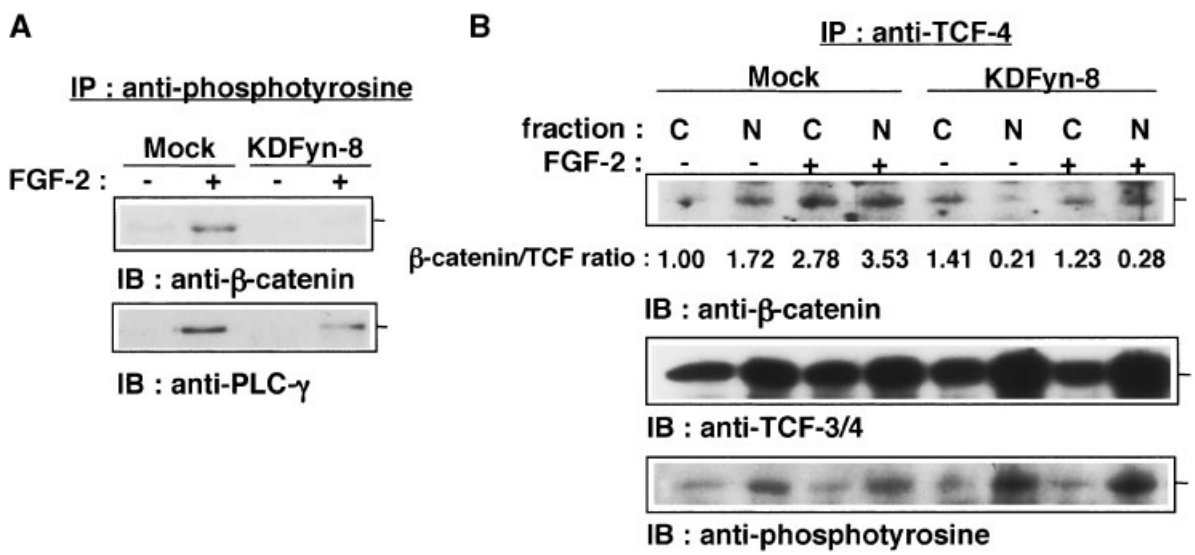

Fig. 4. **A**: Tyrosine phosphorylated β-catenin is increased by FGF-2-treatment in Mock IBE cells, but not in KDFyn-8 cells. Cells were cultured in 6 cm dishes pre-coated with collagen gels for 2 h in the presence or absence of 20 ng/ml FGF-2 (IBE cells, 2.2×10^6 cells/dish; KDFyn-8 cells, 1.4×10^6 cells/dish). Cells were washed, lysed, and tyrosine phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibody (PY99), followed by the separation with SDS–PAGE. β-catenin and phospholipase-Cγ were visualized by immunoblotting. Similar results were obtained by two independent experiments. **B**: FGF-2 promotes the association of β-catenin with TCF-4 in both cytosol and nuclei of Mock cells, but not of KDFyn-8 cells. Cells were cultured in 6 cm dishes pre-coated with collagen gels for 2 h in the presence or absence of FGF-2 (IBE cells, 2.4×10^6 cells/dish; KDFyn-8 cells, 2.3×10^6 cells/dish). Cells were treated with digitonin at 50 μg/ml to extract cytosolic (C) proteins. The remaining cells were lysed in buffer containing 1% Nonidet P-40 (nuclear extract; N) and TCF-4 was immunoprecipitated from both cytosolic and nuclear extracts. Coprecipitated β-catenin with TCF-4 was examined by immunoblotting with indicated antibodies. Reproducible data were obtained by two independent experiments.

Dominant Negative TCF Inhibits FGF-2-Induced Tube Formation in Association With Down-Regulation of Fibronectin Production

To clarify the link between TCF-4 and tube formation, we examined the effect of mutant human TCF-4 (ΔNhTCF-4), which lacks N-terminal 31 amino acids to abrogate the interaction with β-catenin, but maintains the ability to bind target DNA sequence, on FGF-2-induced expression of fibronectin and tube formation. After the transfection, 16 hygromycin-resistant clones were obtained, and clone 3 and 4 were found to express FLAG-tagged ΔNhTCF-4 (denoted ΔNhTCF-4-3 and -4 cells, respectively; Fig. 5A). IBE cells transfected with empty pcDNA3.1/Hygro was also obtained (denoted Mock/Hygro cells). As shown in Figure 5B, the amount of ΔNhTCF-4 was higher than that of endogenous TCF-4 (3.8-fold in (ΔNhTCF-4-3 cells and 4.3-fold in ΔNhTCF-4-4 cells, respectively), suggesting that ΔNhTCF-4 may efficiently exert dominant negative effect. We next examined the FGF-2-induced expression of fibronectin in these cells. As shown in Figure 5C, expression of ΔNhTCF-4 abolished FGF-2-induced up-regulation of fibronectin. We then examined the tube formation by these cells. Cells stably expressing ΔNhTCF-4 failed to form tubes by FGF-2-treatment (Fig. 5D).

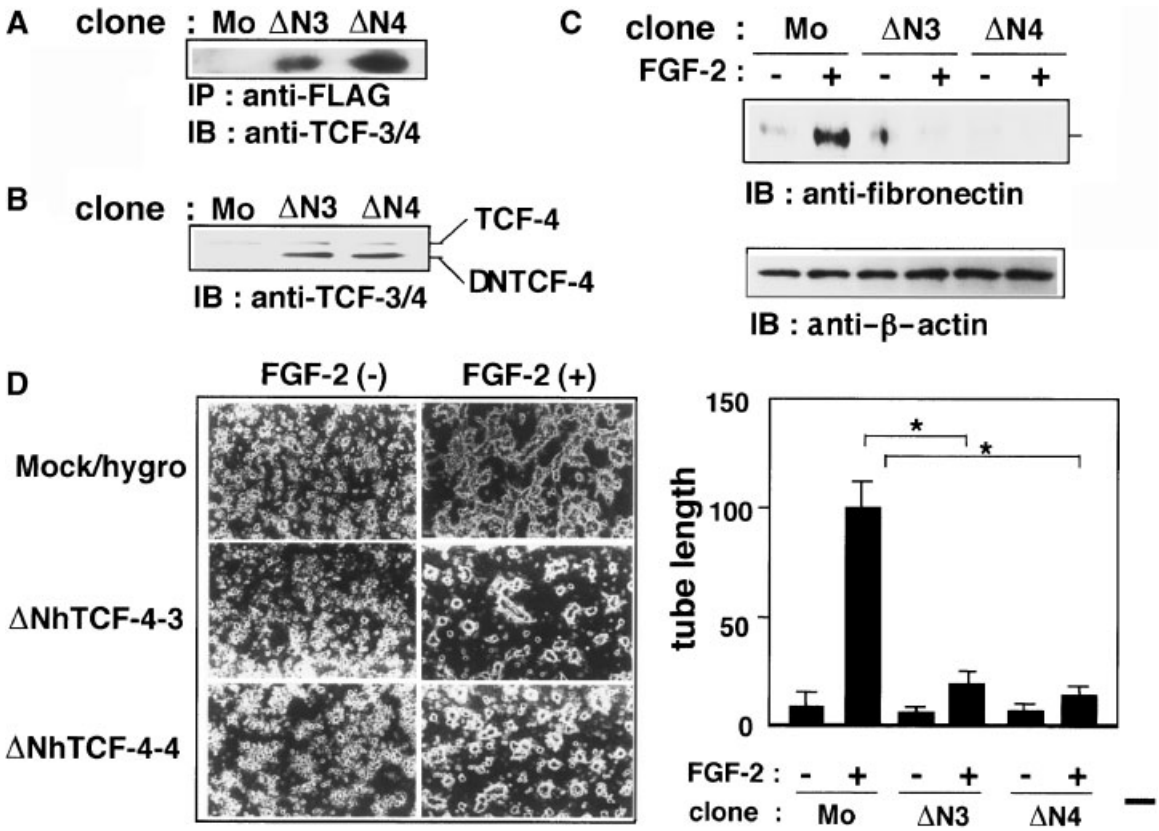

Fig. 5. **A**: Stable expression of N-terminally deleted human TCF-4 (ΔNhTCF-4) in IBE cells. Cells were grown to confluent in 6 cm dishes and FLAG-tagged ΔNhTCF-4 was immunoprecipitated and expression was estimated by immunoblotting. **B**: Expression of endogenous and mutant TCF-4 in IBE cell lines. Total cell extracts were electrophoresed, transferred onto membranes and immunoblotted with anti-TCF 3/4 antibody. **C**: FGF-2-induced up-regulation of fibronectin is not observed in cells stably expressing ΔNhTCF-4 (denoted ΔNhTCF-4-3 and -4 cells). Cells suspended in Ham's F-12 medium containing BSA were seeded into wells of 24-well plate pre-coated type I collagen gels and cultured for 6 h in the presence or absence of 20 ng/ml FGF-2. Two sets of cells were prepared and expression of cell-associated fibronectin or β-actin was examined by immunoblotting as described in the legend of Figure 3B. **D**: ΔNhTCF-4-3 and -4 cells fail to form tub-like structure by FGF-2-treatment. Cells were cultured between two layers of collagen gels with or without 20 ng/ml FGF-2. Tube length obtained from FGF-2-stimulated Mock/Hygro cells was set to 100. Bar, 100 μm. Reproducible data were obtained from two independent experiments.

Treatment With GSK-3β Inhibito or Expression of Kinase-Inactive GSK-3β Induces FGF-2-Independent Tube Formation by IBE Cells

GSK-3β phosphorylates serine residues located at N-terminal region of β-catenin, resulting in degradation of β-catenin by proteasome. Decrease in the amount of β-catenin attenuate TCF-dependent transcriptional regulation, which is a canonical Wnt/β-catenin signaling pathway. To examine the alteration of β-catenin protein during FGF-2-treatment, IBE cells were treated with a pharmacological inhibitor for GSK-3β, SB216763 [Coghlan et al., 2000], in the presence or absence of FGF-2. After 2 h, total cell extract was prepared and immunoblot analyses were performed. As shown in Figure 6A, FGF-2 did not increase the amount of β-catenin, whereas β-catenin was increased in SB216763-treated cells. Subsequently, association of β-catenin with TCF-4 was increased by SB216763-treatment (Fig. 6B). Accordingly, SB216763 induced FGF-2-independent tube formation (Fig. 6C). FGF-2 further stimulated tube formation, suggesting that distinct signaling pathways beside TCF-4 also cooperatively acted for FGF-2-induced tube formation. We next examined the effect of the expression of kinase-inactive GSK-3β in IBE cells on FGF-2-induced tube formation. Twenty four

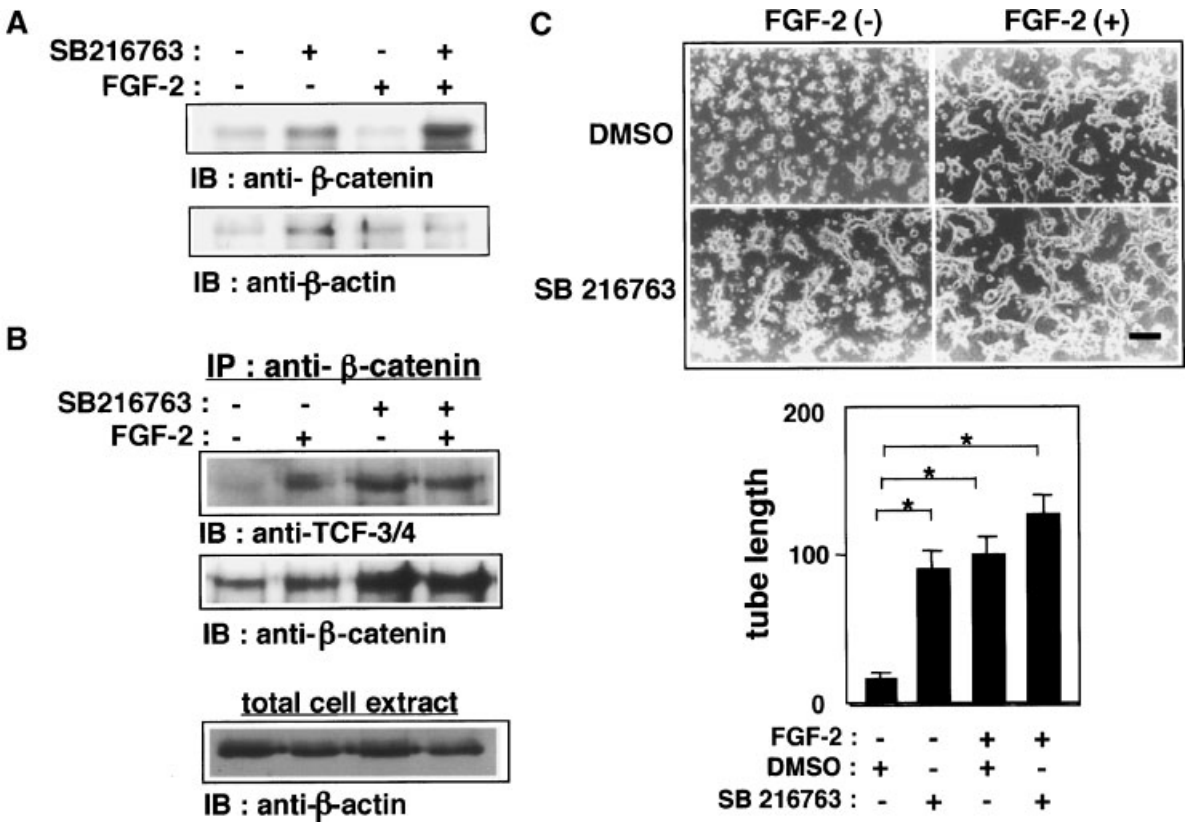

Fig. 6. **A**: GSK-3β inhibitor SB216763 increases the amount of β-catenin. IBE cells were seeded onto the surface of collagen gels with or without indicated samples and cultured for 2 h. Cells were lysed in boiled SDS-sample buffer, clarified by centrifugation, and proteins were separated by SDS–PAGE, followed by the transfer onto PVDF membranes. The amount of β-catenin or β-actin was examined by immunoblotting. **B**: SB216763 induces FGF-2-independent association of β-catenin with TCF-4. Cells suspended in Ham's F-12 medium containing BSA were seeded onto collagen gels in the presence of either 0.1% dimethyl sulfoxide or 20 μM of SB216763. FGF-2 (20 ng/ml) was added to indicated cells and cultured for 2 h. Association of β-catenin with TCF-4 was examined as described in the legend of Figure 3A. Ten percent of each cell lysate was also electrophoresed, transferred onto membranes and immunoblotted by anti-β-actin to assess loaded proteins. Reproducible data were obtained from two independent experiments. **C**: SB216763 induces FGF-2-independent tube formation. Cells suspended in Ham's F-12 medium containing BSA were seeded onto collagen gels in the presence of either 0.1% dimethyl sulfoxide or 20 μM of SB216763 and cultured for 1 h. FGF-2 (20 ng/ml) was added to the indicated cells and the culture was continued. Tube length obtained from FGF-2-stimulated cells with Me_2SO_4 was set to 100. Statistical analyses were performed by Mann–Whitney's *U*-test. *, $P < 0.01$. Bar, 100 μm. Reproducible data were obtained from two independent experiments.

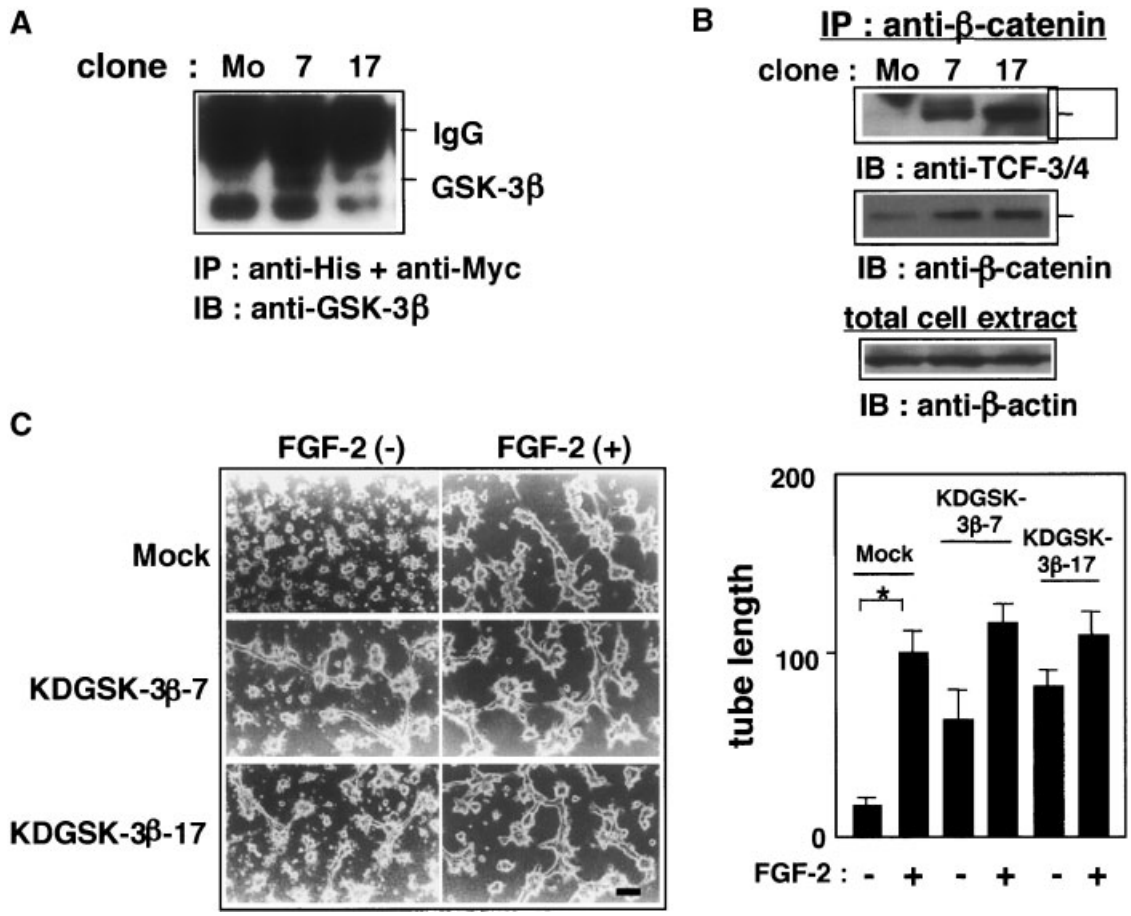

Fig. 7. **A**: Stable expression of Myc/His-tagged kinase-inactive GSK-3β in IBE cells. Cells were grown to confluent in 6 cm dishes and Myc/His-tagged kinase-inactive GSK-3β was immunoprecipitated with anti-tag antibodies and expression was estimated by immunoblotting **B**: Expression of kinase-inactive GSK-3β in IBE cells increases association of β-catenin with TCF-4 in the absence of FGF-2. Cells suspended in Ham's F-12 medium containing BSA were seeded onto collagen gels in the absence of FGF-2 and cultured for 2 h. Immunoblot analysis of the association of β-catenin with TFC was performed as described in Figure 3A. Expression of actin was examined to monitor loaded proteins. Ten percent of each cell lysate was also electrophoresed, transferred onto membranes and immunoblotted by anti-β-actin to assess loaded proteins. Reproducible data were obtained from two independent experiments. **C**: Expression of kinase-inactive GSK-3β in IBE cells (denoted KDGSK-3β-7 and-17 cells) promotes FGF-2-independent tube formation. Cells were cultured between two layers of collagen gels with or without 20 ng/ml FGF-2. Tube length obtained from FGF-2-stimulated Mock cells was set to 100. Statistical analyses were performed by Mann–Whitney's *U*-test. *, $P < 0.01$. Bar, 100 μm. Reproducible data were obtained from two independent experiments.

G418-resistant clones were screened by the expression of Myc/His-tagged kinase- inactive GSK-3β. As shown in Figure 7A, two clones were found to express kinase-inactive GSK-3β, denoted KDGSK-3β-7 and -17 cells, respectively. In these cells, TCF-4 was coprecipitated with β-catenin in the absence of FGF-2 (Fig. 7B). The association correlated to the amount of β-catenin. Using these cell lines, tube formation was examined. As shown in Figure 7C, KDGSK-3β-7 and -17 cells formed tubes in the absence of FGF-2. Similarly, FGF-2 further stimulated tube formation by KDGSK-3β cells.

DISCUSSION

In the present study, blocking the interaction between fibronectin and $\alpha_5\beta_1$ integrin by a specific antibody or GRGDS peptide inhibited FGF-2-induced tube formation both in vivo and in vitro. We have previously shown that kinase-inactive c-Fyn exhibited dominant negative effect on FGF-2-induced activation of c-Fyn and tube formation by IBE cells [Tsuda et al., 2002]. However, the downstream signaling pathway of c-Fyn was not identified. In the present study, we found that FGF-2 induced the

association of β-catenin with TCF-4 in a manner dependent on c-Fyn activity in IBE cells. Tyrosine phosphorylation of β-catenin was dependent on c-Fyn activity. It has been shown that β-catenin was a substrate of c-Src [Behrens et al., 1993]. Thus, it seems plausible that FGF-2-activated c-Fyn induced tyrosine phosphorylation of β-catenin, followed by the increase in association with TCF-4. Subsequently, activated TCF-4 induced up-regulation of fibronectin, which seemed to contribute FGF-2-induced tube formation by IBE cells.

Previous study showed that down-regulation of GSK-3β activity by FGF-2-treatment increased the association of β-catenin with TCF-4, resulted in the enhanced cyclin D1 promoter activity [Holnthoner et al., 2002]. However, we could not detect the up-regulation of cyclin D1 in FGF-2-treated IBE cells cultured on collagen gels (data not shown). More recently, it has been shown that Wnt-1 signaling inhibited the proliferation of human umbilical vein endothelial cells [Cheng et al., 2003]. In this report, authors noted that Wnt-1 signaling induced cellular aggregation. We have previously shown that IBE cells cultured on collagen gels formed tubes in response to FGF-2-treatment without cellular proliferation [Kanda et al., 1999a]. Kinetic study revealed that the initial event of tube formation was aggregation of cells [Kanda et al., 1999a]. Our data and the report by Cheng suggest that association of β-catenin with TCF-4 may be involved in tube formation other than proliferation.

According to the canonical Wnt/β-catenin pathway, Wnt signaling stabilizes the β-catenin to prevent from serine phosphorylation by GSK-3β and CKI. FGF-2-mediated association of β-catenin with TCF-4 may be regulated by the inactivation of GSK-3β. It has been shown that FGF inactivated GSK-3β in a manner depending on the phosphoinositide 3-kinase/c-Akt pathway [Hashimoto et al., 2002]. c-Akt phosphorylates Ser 9 of GSK-3β, which in turn reduce its kinase activity [Cross et al., 1995]. Inhibition of GSK-3β increases unphosphorylated β-catenin, which in turn stabilizes and promotes the association with TCF. However, FGF-2-induced association of β-catenin with TCF in the present study did not seem to be promoted by the inhibition of GSK-3β, because we never observed the increase in the amount of β-catenin by FGF-2-treatment (Figs. 3A and 6A). In addition, we did not observe the increase in serine phosphorylation of β-catenin by FGF-2-treatment, which was assessed by immunoblotting (data not shown). On the contrary, two stable cell lines expressing kinase-inactive GSK-3β as well as IBE cells treated with SB216763 exhibited the increase in β-catenin (Figs. 6 and 7). Thus, FGF-2 does not seem to stabilize β-catenin in IBE cells during tube formation.

How FGF-2 regulates the association of β-catenin with TCF-4 in a manner dependent on c-Fyn β-catenin is tyrosine phosphorylated by c-Src, hepatocyte growth factor receptor, or vascular endothelial growth factor receptor [Ilan et al., 1999; Roura et al., 1999; Danilkovitch-Miagkova et al., 2001]. In the present study, FGF-2 increased tyrosine phosphorylated β-catenin in IBE cells, but not KDFyn-8 cells, suggesting that c-Fyn may tyrosine phosphorylate the β-catenin. It has been shown that v-Src-mediated tyrosine phosphorylation of β-catenin did not affect its stability [Papkoff, 1997]. On the other hand, dephosphorylation of β-catenin by protein tyrosine phosphatase SHP-1 decreased the β-catenin/TCF-dependent transcription [Duchesne et al., 2003]. They did not mention the stability of β-catenin. Another report has shown that tyrosine phosphorylation of β-catenin was involved in the association between β-catenin and TATA-binding protein [Piedra et al., 2001]. Importantly, increase in association of β-catenin with TATA-binding protein correlated with the increased association between β-catenin and TCF [Piedra et al., 2001]. Thus, it seems likely that FGF-2-induced, c-Fyn-dependent tyrosine phosphoryaltion of β-catenin may enhance its association with TCF.

Wnt signaling is involved in carcinogenesis [Polakis, 2000]. In addition, present study provides the possibility that Wnt-independent increase in β-catenin/TCF-4 association and subsequent tube formation promoted by FGF-2 also support the tumor growth by angiogenesis. Targeting TCF-4 would thus be a potential anticancer strategy.

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