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Tumour-derived fibroblast growth factor-2 exerts lymphangiogenic effects through Akt/mTOR/p70S6kinase pathway in rat lymphatic endothelial cells

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ABSTRACT

Fibroblast growth factor-2 (FGF-2) has been shown to induce both angiogenesis and lymphangiogenesis in the mouse corneum; however, the signalling mechanism underlying FGF-2-induced lymphangiogenesis remains unknown. Here we investigated the effect of FGF-2 on newly developed temperature-sensitive rat lymphatic endothelial (TR-LE) cells. The supernatant of PC-3 prostate cancer cells facilitated tube-like formation in TR-LE cells, and formation was inhibited by neutralising antibodies against FGF-2. The addition of FGF-2 stimulated tube-like formation as well as proliferation and chemotactic migration of TR-LE cells. Blockade of the Akt signalling pathway by LY294002 abolished the elongation of tubes induced by FGF-2, whereas inhibition of the extracellular signal-regulated kinase (ERK) signalling pathway had no effect. Rapamycin abrogated the phosphorylation of p70S6kinase and consistently inhibited the formation of tubes induced by FGF-2. Furthermore, tube-like formation induced by the supernatant of PC-3 cells was inhibited by LY294002 or rapamycin. These data suggest that FGF-2 exerts lymphangiogenic effects by activating the Akt/mammalian target of rapamycin (mTOR)/p70S6kinase pathway in lymphatic endothelial cells, and that the pathway provides a potent target for tumour lymphangiogenesis.

1. Introduction

The lymphatic vasculature is an important route for the metastatic spread of human cancer. Clinico-pathological studies have revealed that lymphangiogenesis can occur adjacent to or within human cancers, and that it correlates with metastasis to lymph nodes in many human tumours including prostate cancer.[1,2]

Fibroblast growth factor-2 (FGF-2) is a heparin-binding protein that induces the proliferation or differentiation of a variety of cell types.[3] In the corneal neovascularisation assay, FGF-2 can induce both angiogenesis and lymphangiogenesis.[4] FGF-2-induced lymphangiogenesis is blocked by neutralising vascular endothelial growth factor receptor-3 (VEGFR-3) antibodies, indicating that FGF-2 promotes lymphatic growth via the VEGF-C/VEGFR-3 signalling system in the corneum.[5,6] However, the corneal stroma does not express heparin sulfate, which is critical for efficient binding and signalling of heparin-binding growth factors such as FGF-2. Therefore, an indirect effect of FGF-2 mediated by non-heparin-binding factors such

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as VEGF-C may be predominant in promoting the lymphangiogenesis. Thus, the direct lymphangiogenic effect of FGF-2 in the tumour microenvironment remains controversial.

A culture of lymphatic endothelial cells is important to obtain a better understanding of their respective roles in vascular physiology and pathology including lymphangiogenesis. Recently, we succeeded in establishing a rat lymphatic endothelial cell line (TR-LE) from the thoracic duct of a transgenic rat harbouring a temperature-sensitive simian virus 40 (SV40) large T-antigen and enhanced green fluorescent protein (EGFP).[7,8] TR-LE cells maintain cobblestone-like morphology and express lymphatic endothelial markers VEGFR-3 (vascular endothelial growth factor receptor), LYVE-1, Prox-1, and podoplanin, together with endothelial markers CD31, Tie-2, and VEGFR-2. This lymphatic endothelial cell line enables lymphangiogenesis experiments *in vitro*.

In this study, we used TR-LE cells to identify the possible signalling targets regulating the lymphangiogenic effects of FGF-2, and to assess the involvement of FGF-2 in the interaction between cancer cells and lymphatic endothelial cells in tumour lymphangiogenesis.

2. Materials and methods

2.1. Materials

LY294002, SU4984, U0126, SP600125, rapamycin, and VEGF-C were purchased from Calbiochem (Darmstadt, Germany). Wortmannin and anti-FGF-2 antibody were purchased from Alomone Laboratories (Jerusalem, Israel) and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively. Reagents were dissolved in dimethyl sulfoxide, PBS supplemented with 0.1% bovine serum albumin w/v or distilled water and stored at −20 or −80 °C.

2.2. Cells

TR-LE cells, a conditionally immortalised rat lymphatic endothelial cell line, were maintained on culture dishes pre-coated with 10 μg/mL fibronectin (Iwaki Glass, Tokyo, Japan), in HuMedia-EG2 (Kurabo, Osaka, Japan) supplemented with 20% foetal bovine serum (FBS) v/v at a permissive temperature (33 °C).[7,8] PC-3 human prostate cancer cells were kindly provided by Dr. M. Nakajima (Novartis Pharma K.K., Takarazuka), and maintained in RPMI-1640 (Invitrogen Corp., Carlsbad, CA) supplemented with 10% FBS v/v.

2.3. Preparation of conditioned media (CM)

PC-3 cells were seeded in 10-cm culture dishes in 10 mL of RPMI1640 supplemented with 10% FBS v/v for 48 h to reach sub-confluence. The supernatants were centrifuged to remove debris and were used immediately in the tube formation assay.

2.4. Tube formation assay

TR-LE cells were cultured in DMEM (Invitrogen Corp.) supplemented 0.2% v/v FBS for 12 h, and were harvested with trypsin/EDTA. A cell suspension (1.1×10^4/ 200 μL) was prepared in CM supplemented with antibodies or DMEM supplemented with 0.2% FBS v/v. Heparin (Sigma; 10 μg/mL) was added to the suspension. After 5 min of treatment with inhibitors, the suspension was seeded in a 96-well plate that had been coated with 40 μl of 10 mg/ml Matrigel. After a 3-h incubation at 37 °C, cultures were photographed with a NIKON inverted microscope, and the length of the tube-like network was measured using MAPMETER PJ Type (UCHIDA YOKO, Tokyo, Japan).

2.5. Cell proliferation assay

TR-LE cells (6×10^3 cells/well) were seeded in 100 μl of DMEM containing 5% FBS v/v in 96-well plates pre-coated with 10 μg/mL laminin. Cells were allowed to adhere for 3 h, and incubated with human recombinant FGF-2 (Kaken Pharmaceutical Co., Ltd.) and heparin (10 μg/mL) for an additional 48 h at 37 °C. The cells were fixed with 2.5% glutaraldehyde v/v and stained with crystal violet (0.1% w/v in a 20% methanol solution v/v). After several washes, crystal violet was extracted with 30% acetic acid v/v, and the absorbance was measured at 595 nm.

2.6. Cell migration assay

The chemotactic migration of TR-LE cells was measured using Transwell cell culture chambers (Costar 3422, Cambridge, MA, USA) as described previously[9] with some modifications. The filter's lower surface was precoated with 5 μg of Matrigel (Collaborative Research Co., Bedford, MA). After a 12-h pre-incubation in DMEM supplemented with 0.2% FBS v/v, TR-LE cells were harvested with trypsin/EDTA. After several washes, a TR-LE cell suspension (1×10^5/100 μL) was added to the upper compartment of the chamber; FGF-2 and heparin (10 μg/mL) were added to the lower compartment. After a 9-h incubation at 37 °C, the filters were fixed with methanol, and cells that had migrated to the lower surface were counted by crystal violet assay.

2.7. Western blot analysis

After the indicated treatment, the cells were rinsed with ice-cold PBS and lysed in sample buffer [24 mM Tris-HCl (pH 6.8), 5% glycerol w/v, 1% SDS w/v, and 0.05% bromophenol blue w/v]. Cell lysates were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were incubated with Block Ace (Dainipponseiyaku, Suita, Japan) and probed with the indicated primary antibodies (1:1000). Protein content was visualised using horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Phospho-Akt, phospho-ERK, phospho-p38 and phospho-p70S6kinase antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA), and FGFR-1 from SantaCruz (Santa Cruz, CA).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA expression of FGFRs by TR-LE cells and normal rat liver tissue (male 19-week-old Wistar rat; Japan SLC,

Hamamatsu, Japan) was evaluated by semi-quantitative RT-PCR. In brief, total RNA was extracted using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's directions. First-strand complementary DNA (cDNA) was prepared from the RNA template (1 μg) using Random Primers and Super-Script II reverse transcriptase (Invitrogen Corp.). The RT-reaction profile was 42 °C for 50 min, followed by 70 °C for 15 min. PCR amplification was performed by denaturation at 94 °C for 30 s, annealing at 56 °C or 60 °C for 1 min, and extension at 72 °C for 1 min and 45 s, using template cDNA and a TaKaRa Ex Taq (Takara-Bio Co., Ltd.). The sequences of primers were as follows: FGFR-1 (sense 5′-CCGTGACCTCACCTCTG-3′ and anti-sense 5′-GCTCATATTCAGAGACGCCA-3′ predicted product 294 bp)[10]; FGFR2 mRNA (sense 5′-CTGTGCCGAATGA-AGAACACGACC-3′ and anti-sense 5′-CCCAAAGTCTGCTAT-CTTCATCAC-3′ predicted product 743 bp); FGFR3 mRNA (sense 5′-GGAGGACGTGGCTGAAGACACAGG-3′ and antisense 5′-TCTGCCGGATGCTGCCAAACTTGT-3′ predicted product 295 bp), and FGFR4 mRNA (sense 5′-CTCACGGGCCTTGTGAA-TCTAGAC-3′ and anti-sense 5′-CCCAAAGTCTGCTATCTTCAT-CAC-3′ predicted product 560 bp).[11,12] The PCR products were electrophoresed on 1.5% agarose gels w/v and detected with ethidium bromide staining.

2.9. Statistical analysis

Statistical comparisons were carried out using Student's two-tailed *t*-test with Bonferroni correction. $P < 0.05$ was considered significant.

3. Results

3.1. PC-3 cells induce tube-like formation of TR-LE cells via FGF-2-mediated mechanism

PC-3 prostate cancer cells produce FGF-2 in the supernatant,[13] and induce peri-tumoural lymphangiogenesis by orthotopic implantation.[14] To investigate the involvement of FGF-2 in lymphangiogenesis, we first evaluate the length of tubes formed by TR-LE lymphatic endothelial cells in conditioned media (CM) obtained from PC-3 cells. As shown in Fig. 1A, tubes in tumour-derived CM were dramatically elongated compared with non-cultured CM. Tumour-induced elongation was significantly inhibited by treatment with neutralising antibodies against FGF-2, compared with control IgG antibodies (Fig. 1B). These data suggest that FGF-2 derived from PC-3 cells leads to the formation of capillary-like tubes of TR-LE cells.

3.2. FGF-2 promotes lymphangiogenesis in vitro

We next examined the direct effect of FGF-2 on TR-LE cells. As shown in Fig. 2A, FGF-2 induced elongation of the tube-like network formed by TR-LE cells. The lymphangiogenic ability of FGF-2 is comparable to that of VEGF-C (Fig. 2B). The proliferation of TR-LE cells was enhanced in the presence of FGF-2 (Fig. 2C). When FGF-2 was added to the lower compartment of the Transwell chamber, chemotactic migration of TR-LE cells was stimulated (Fig. 2D). These data indicate that FGF-2 has direct lymphangiogenic activities in TR-LE cells *in vitro*.

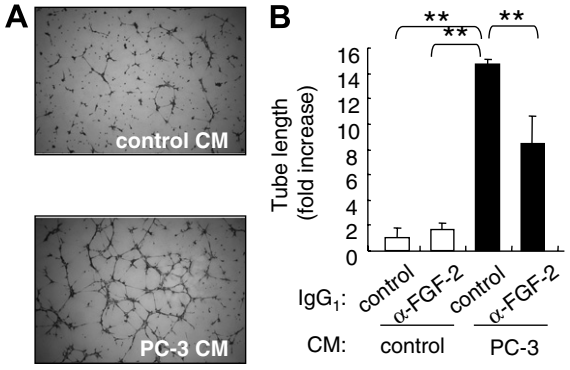

Fig. 1 – Involvement of FGF-2 on TR-LE cell tube formation induced by supernatants from PC-3 prostate cancer cells. PC-3 cells were cultured in RPMI-1640 supplemented with 10% FBS v/v for 48 h. Serum-starved TR-LE cells were suspended in conditioned medium (CM) (*A*) and seeded onto Matrigel-coated wells in the presence of anti-FGF2 antibody (*B*; 10 ng/mL) or control IgG (*B*; 10 ng/mL). After a 3-h incubation, the length of tube-like formation was evaluated. A representative experiment is shown with the mean ± SD of four wells. $^{**}P < 0.01$ compared with CM-treated groups. Similar results were obtained in three independent experiments.

3.3. Akt and ERK pathways are activated by FGF-2 in TR-LE cells

We next examined the effect of FGF-2 on signalling transduction pathways in TR-LE cells. As shown in Fig. 3, phosphorylation of Akt and extracellular signal-regulated kinase (ERK) was induced by FGF-2, whereas that of p38 was not affected. Phosphorylation of Akt and ERK reached a peak at 5 min, and declined gradually (Fig. 3A). The maximal phosphorylation appeared with FGF-2 treatment at 100 ng/mL (Fig. 3B), suggesting that activation of TR-LE cells by FGF-2 shows a bell-shaped dose-response for FGF-2 concentrations.

3.4. FGFR tyrosine kinases play an important role in FGF-2-induced lymphangiogenesis

FGF-2 exerts its angiogenic activities by binding to FGF receptors (FGFRs), especially FGFR-1 and FGFR-2, on the surface of endothelial cells.[15] Recent study revealed that inhibition of FGFR-3 represses proliferation of primary lymphatic endothelial cells induced by FGF-2.[16] Function of FGFR-4 in angiogenesis and lymphangiogenesis has not been revealed. To investigate the role of FGFRs in the FGF-2-induced tube-like formation, we assess the expression of FGFRs mRNA in TR-LE cells by RT-PCR analysis (Fig. 4A). TR-LE cells express high levels of FGFR-1 and low levels of FGFR-2, compared with normal rat liver extracts as a positive control. In contrast, expression of FGFR-3 and FGFR-4 was not detected in lymphatic endothelial cells. By Western blotting analysis, expression of FGFR-1 at protein level was confirmed (Fig. 4B). The receptor tyrosine kinase inhibitor SU4984 inhibited the phosphorylation of Akt and ERK induced by FGF-2 (Fig. 4C). Treatment with SU4984 resulted in the repression of tube formation induced

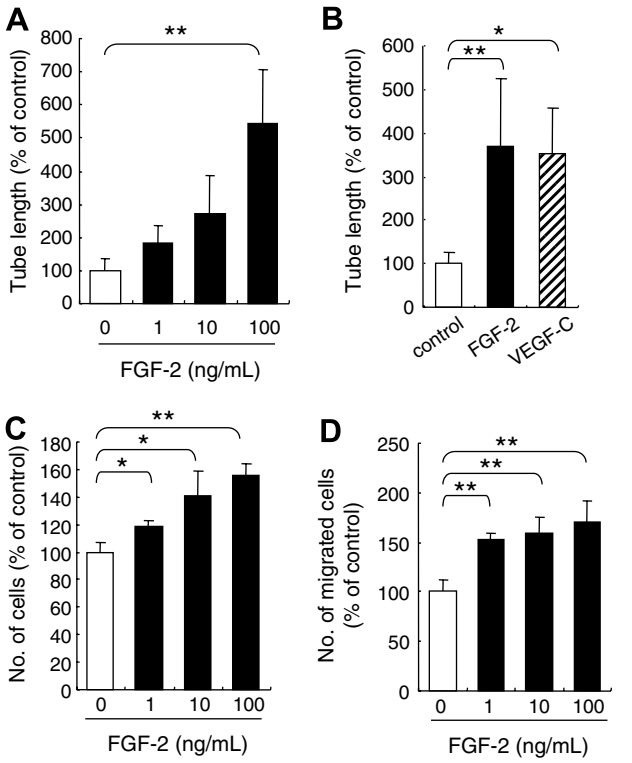

Fig. 2 – Effect of FGF-2 on lymphangiogenesis *in vitro*. (A,B) Effect of FGF-2 on tube formation. Serum-starved TR-LE cells were seeded onto 96-well plates precoated with Matrigel in the presence of FGF-2 (B, 100 ng/mL) or VEGF-C (B, 100 ng/mL). After a 3-h incubation, the length of tube-like formation was evaluated. (C) Effect of FGF-2 on cell proliferation. TR-LE cells were incubated with indicated concentrations of FGF-2 for 48 h, and cell proliferation was determined by crystal violet assay. (D) Effect of FGF-2 on cell migration. Serum-starved TR-LE cells were seeded onto filters precoated with Matrigel on the lower surface, and FGF-2 was added to the lower compartment of Transwell chambers. After a 9-h incubation, cells that had migrated to the lower surface of the filters were counted by crystal violet staining. A representative experiment is shown with the mean ± SD of four wells. *P< 0.05 and **P< 0.01 compared with vehicle-treated groups. Similar results were obtained in three independent experiments.

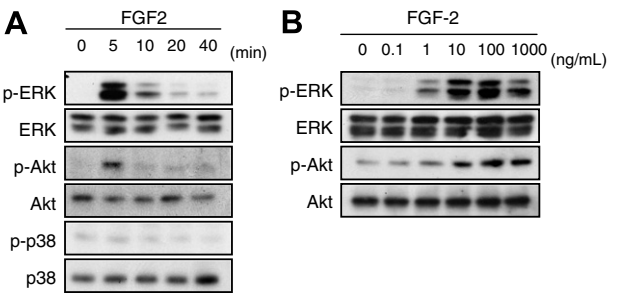

Fig. 3 – Induction of ERK and Akt phosphorylation by FGF-2 in TR-LE cells. (A) Time course of ERK, Akt, and p38 phosphorylation. Serum-starved TR-LE cells were stimulated with 100 ng/ml of FGF-2 for the indicated time. (B) Dose-dependent increase in ERK and Akt phosphorylation. Serum-starved TR-LE cells were treated with the indicated concentrations of FGF-2 for 10 min. Phospho-ERK, phospho-p38, and phospho-Akt were determined by Western blotting using phospho-ERK (Thr202 and Tyr204), phospho-Akt (Ser473), and phospho-p38 (Thr180, Tyr182) antibodies, respectively. Similar results were obtained in three independent experiments.

by FGF-2 (Fig. 4D). These data suggest that FGFR tyrosine kinases, potentially FGFR-1 and FGFR-2, play an important role in tube-like formation by FGF-2.

3.5. *Akt/mTOR/p70S6kinase signalling pathway is essential for the formation of capillary-like tubes of TR-LE cells induced by FGF-2*

To address the mechanism of FGF-2-induced lymphangiogenesis, we examined the effect of the phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002 and the MAPK/ERK kinase (MEK) inhibitor U0126 on tube-like formation of TR-LE cells. As shown in Fig. 5A and B, inhibition of Akt by LY294002 resulted in suppression of the tube-like formation of TR-LE cells by FGF-2. Similar results were obtained by using other type of the PI3K inhibitor wortmannin, which inhibited phosphorylation of Akt and tube-formation induced by FGF-2 (data not shown). In contrast, complete inhibition of ERK by U0126 did not affect FGF-2-induced tube-like formation. These data suggest that lymphatic tube formation induced by FGF-2 depends on Akt signalling pathway, but not the ERK pathway.

To identify the downstream pathway of Akt, we examined the effect of rapamycin on tube-like formation (Fig. 6). Rapamycin completely inhibited the phosphorylation of p70S6kinase and the elongation of tubes induced by FGF-2 without affecting basal tube-forming ability. These data suggest that the Akt/mammalian target of rapamycin (mTOR)/p70S6kinase pathway plays a pivotal role in capillary-like formation of lymphatic endothelial cells stimulated by FGF-2.

3.6. *Tumour-induced tube-like formation is inhibited by SU4984, LY294002, and rapamycin*

To evaluate the potency of molecular target in tumour lymphangiogenesis, we examine the effect of SU4984, LY294002, and rapamycin on PC3-induced tube-like formation of TR-LE cells. As shown in Fig. 7, SU4984, LY294002, and rapamycin significantly inhibited the tumour-induced elongation of tubes. These data suggest that FGFR and the Akt/mTOR/p70S6kinase pathway have an important role in PC-3 cells-mediated lymphangiogenesis, and provide the potent target signalling pathway in tumour lymphangiogenesis.

4. Discussion

In our previous study, a conditionally immortalised lymphatic endothelial cell line (TR-LE) was established from the thoracic duct of a transgenic rat harbouring a temperature-sensitive SV40 large T-antigen and EGFP.[7] Using TR-LE cells, present study demonstrated the direct lymphangiogenic effect of FGF-2 on lymphatic endothelial cells, and indicated that the

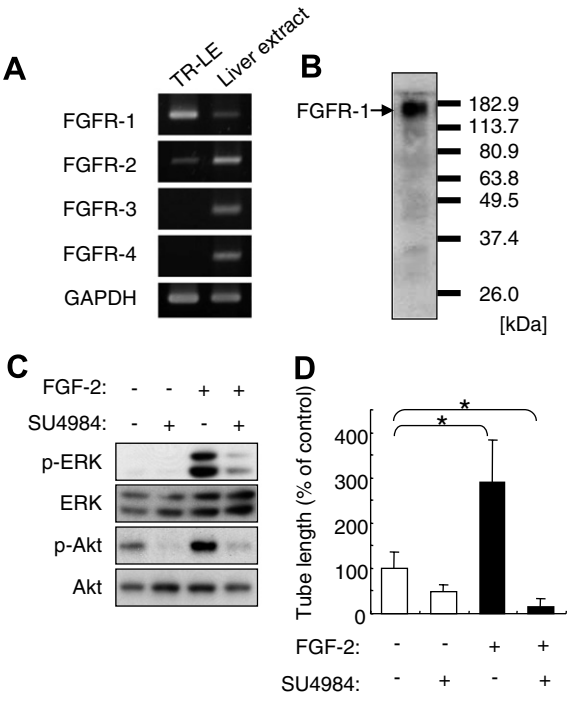

Fig. 4 – SU4984 inhibits FGF-2-induced signalling and tube formation of TR-LE cells. (*A*) RT-PCR analyses of FGFR1, 2, 3, and 4 in TR-LE cells. GAPDH (D-glyceraldehyde-3-phosphate dehydrogenase) served as a loading control. Amplification cycles are 28 for FGFR-1 and GAPDH and 32 for the others. (*B*) Western blot analysis of FGFR-1 in TR-LE cells. Positions of molecular weight markers are shown in kilodaltons (*kDa*). Effect of the tyrosine kinase inhibitor SU4984 on signalling transduction pathways (*C*) and tube formation (*D*). (*C*) Serum-starved TR-LE cells were pre-treated with 20 μM of SU4984, followed by the addition of FGF-2 (100 ng/mL). Phospho-ERK and phospho-Akt were determined by Western blotting using phospho-ERK (Thr202 and Tyr204) and phospho-Akt (Ser473) antibodies, respectively. (*D*) Serum-starved TR-LE cells were pre-treated with 20 μM of SU4984. The cells were seeded onto Matrigel-coated plates in the presence of FGF-2 (100 ng/mL). After a 3-h incubation, the length of tube-like formation was evaluated. A representative experiment is shown with the mean ± SD of four wells. $^{*}P < 0.05$ compared with vehicle-treated groups. Similar results were obtained in three independent experiments.

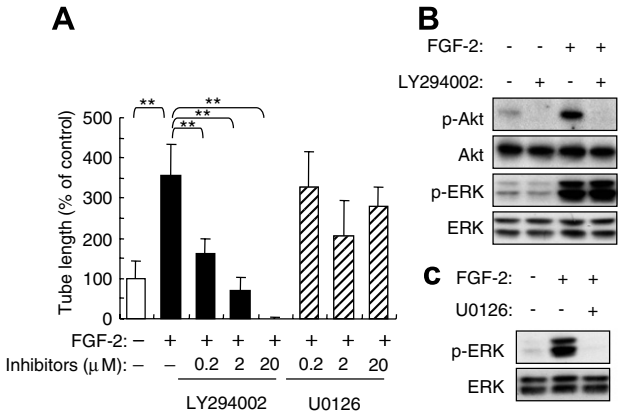

Fig. 5 – Involvement of Akt pathway, but not ERK, on TR-LE cell tube-like formation induced by FGF-2. (*A*) Serum-starved TR-LE cells were pre-treated with the PI3K inhibitor LY294002 or MEK inhibitor U0126. The cells were seeded onto Matrigel-coated plates in the presence of FGF-2 (100 ng/mL). After a 3-h incubation, the length of tube-like formation was evaluated. A representative experiment is shown with the mean ± SD of four wells. $^{**}P < 0.01$ compared with FGF-2-treated groups. (*B*,*C*) Serum-starved TR-LE cells were pre-treated with LY294002 (*B*, 20 μM) or U0126 (*C*, 20 μM), followed by the addition of FGF-2 (100 ng/mL). Phospho-ERK and phospho-Akt were determined by Western blotting using Phospho-ERK (Thr202 and Tyr204) and phospho-Akt (Ser473) antibodies. Similar results were obtained in three independent experiments.

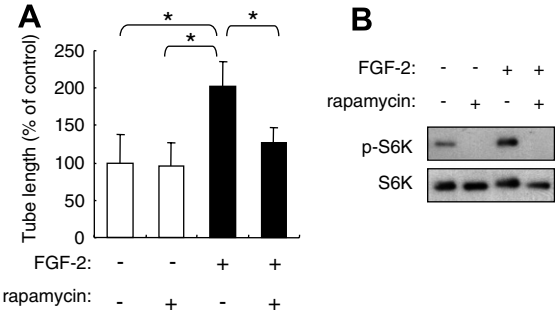

Fig. 6 – Involvement of mTOR/p70S6kinase pathway on TR-LE cell tube-like formation induced by FGF-2. (*A*) Serum-starved TR-LE cells were pre-treated with rapamycin (10 ng/mL). The cells were seeded onto Matrigel-coated plates in the presence of FGF-2 (100 ng/mL). After a 3-h incubation, the length of tube-like formation was evaluated. A representative experiment is shown with the mean ± SD of four wells. $^{*}P < 0.05$ compared with FGF-2-treated groups. (*B*) Serum-starved TR-LE cells were pre-treated with rapamycin (10 ng/mL), followed by the addition of FGF-2 (100 ng/mL). Phospho-S6K was determined by Western blotting using phospho-S6K (Thr389) antibodies. Similar results were obtained in three independent experiments.

Akt/mTOR/p70S6kinse pathway plays a pivotal role in tube-forming ability. In addition, our results suggested that the conditioned medium of PC-3 accelerates tube-like formation of TR-LE cells through an FGF-2-mediated mechanism.

PI3K/Akt plays a crucial role in the tube-like formation of vascular endothelial cells.[17] The downstream targets of Akt in tube-like formation are reported to be the Rho family of G proteins,[18] p21-activated kinase,[19] girdin,[20] endothelial nitric oxide synthase (eNOS),[21] and p70S6kinase.[22] In this study, we found that PI3K inhibitors, LY294002 and wortmannin, and rapamycin abolished FGF-2-induced tube-like formation of TR-LE cells. The mechanism underlying that the PI3K inhibitor suppresses basal tube-like formation (Fig. 5A) might be due to inhibition of the constitutive activation of Akt substrates such as the above-mentioned molecules. In contrast, rapamycin did not affect basal tube-like

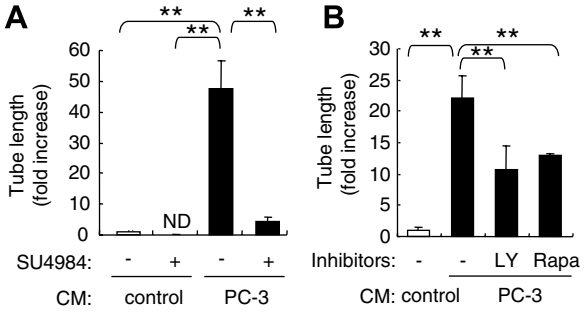

Fig. 7 – Effect of SU4984, LY294002, and rapamycin on TR-LE cell tube formation induced by supernatants from PC-3 prostate cancer cells. PC-3 cells were cultured in RPMI-1640 supplemented with 10% FBS v/v for 48 h. Serum-starved TR-LE cells were suspended in conditioned medium (CM), and seeded onto Matrigel-coated wells in the presence of SU4984 (*A*; 20 μM), LY294002 (*B*; 20 μM), or rapamycin (*B*; 10 ng/mL). After a 3-h incubation, the length of tube-like formation was evaluated. A representative experiment is shown with the mean ± SD of three (*A*) or four (*B*) wells. ND, not detectable. $^{**}P < 0.01$ compared with CM-treated groups. Similar results were obtained in three independent experiments.

formation, suggesting that FGF-2-induced lymphangiogenesis preferentially depends on the activation of mTOR/p70S6kinase signalling. Further dissection of the PI3K/Akt/mTOR/p70S6kinase pathway and elucidation of downstream effector molecules will lead to a better understanding of physiological and pathological lymphangiogenesis, and may provide avenues for the development of a novel therapeutic strategy for cancer treatment.

This study demonstrated that PC-3 cells induced the tube-like formation of TR-LE cells. Although the concentration of FGF-2 in the supernatant of PC-3 cells is estimated at around 10 pg/mL,[13] tumour-induced lymphangiogenesis was partly blocked by neutralising FGF-2 antibodies (Fig. 1), indicating that PC-3 cells promote tube-like formation via the FGF-2 signalling system. In inducible angiogenesis, the combination of FGF-2 and other types of growth factors such as VEGF-A and platelet-derived growth factor(PDGF)-BB has a potent synergistic effect.[23,24] Likewise, it is reported that lymphatics induced by tumours that solely overexpress VEGF-C or PDGF-BB exhibit an abnormal draining function.[25] Moreover, we found that FGF-2 induced the tube-like formation of TR-LE cells on Matrigel, extracted from EHS mouse sarcoma (Fig. 2A), but not on the synthetic peptide PuraMatrix (BD Biosciences, Bedford, MA) or PBS-washed Matrigel to reduce soluble factors (data not shown). These observations suggest that FGF-2 might need unknown tumour-derived soluble factor(s) to effectively induce lymphangiogenic activity in TR-LE cells. A recent study by Zeng and colleagues using the co-culture system with PC-3 cells demonstrated that VEGFR-2 blockade significantly reduced the tumour-induced formation of tubes by primary lymphatic endothelial cells.[14] Thus, combination therapy targeted at plural growth factor signalling pathways such as VEGFs and FGF-2 might result in more effective anticancer thrapeutics.

The homeodomain protein Prox1 plays an essential role in the lymphatic system development during embryogenesis as a master regulator that induces lymphatic lineage-specific differentiation. Shin and colleagues revealed that expression of FGFR-3 is modulated by a direct binding of Prox1 to FGFR-3 promoter, and FGFR-3 is strongly and specifically expressed in the newly formed lymphatic vessels but not in developing blood vessels.[16] As shown in Fig. 4A, unexpectedly, FGFR-3 mRNA was not detected in TR-LE cells. Our previous study revealed that TR-LE cells express weak levels of Prox-1 by western blotting.[7] Thus, the mechanism underlying negative expression of FGFR-3 might be due to weak expression of Prox1 in TR-LE cells.

SU4984 is a derivative of indolinones that bind the ATP binding pocket of receptor tyrosine kinases and inhibits their activities.[26] SU4984 has a relatively broad spectrum of inhibition, being effective against PDGF receptor and FGFR-1, in comparison with other indolinone-derivatives.[27] Therefore, we cannot rule out the possibility that the inhibitory effect of SU4984 in PC-3-induced tube-like formation might be partly mediated by PDGF receptor, FGFR-2, -3, and/or -4. Of note, the inhibitory activity of SU4984 in tumour-induced tube-like formation was greater than that of LY294002 and rapamycin (Fig. 7). These observations might support advantage of combination therapy targeted at plural growth factor signalling pathways.

To assess the participation of FGFR-1 in FGF-2-induced response, we performed siRNA experiments against FGFR-1 (data not shown). Unfortunately, TR-LE cells almost completely died during incubation with siRNA targeting FGFR-1, suggesting that FGFR-1 plays an essential role in survival of TR-LE cells. This observation is consistent with a finding that expression of dominant-negative FGFR-1 inhibits MAPK phosphorylation and subsequently induces apoptosis in human umbilical vein endothelial cells.[28] Since identifying the subtype of FGFRs is informative to figure out the mechanism underlying FGF-2-induced lymphangiogenesis, we might need other approaches to elucidate the role of FGFRs, especially FGFR-1 and -2.

In summary, we found lymphangiogenic activity of FGF-2 in lymphatic endothelial cells mainly through Akt/mTOR/p70S6kinase, and active molecular interactions between the tumour and lymphatic endothelium via FGF-2, underling prostate cancer lymphatic metastasis. Further histochemical analysis using an *in vivo* lymphangiogenic model is required to support these findings.

Conflict of interest statement

None declared.

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