CCN2 activates ERK-signaling via integrin α_v and enhances the interaction of ERK and DUSP6 in lymphatic endothelial cells

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Abstract

Cellular communication network factor 2 (CCN2, also known as CTGF), is a modular and matricellular protein and a well-known angiogenic factor in physiological and pathological angiogenesis. However, its roles in lymphangiogenesis and intracellular signaling in lymphatic endothelial cells (LECs) remain unclear. Here, we investigated CCN2 signaling in LECs and its effects on lymphangiogenesis. In primary cultured LECs, gene expressions of lymphatic endothelial markers *lymphatic vessel endothelial hyaluronan receptor 1* (*Lyve1*), *Podoplanin* and *prospero homeobox 1* (*Prox1*) and lymphangiogenic factors *vascular endothelial cell growth factor c* (*Vegfc*), *vascular endothelial cell growth factor d* (*Vegfd*) and *fms-related tyrosine kinase 4* (*Flt4*, also known as *Vegfr3*) were upregulated by CCN2. Subsequently, we found that CCN2 induced phospho-ERK and that was decreased by suppression of integrin α_v . CCN2 slightly decreased the growth of LECs due to enhancement of the interaction of ERK and dual specific protein phosphatase 6 (DUSP6), and knockdown of DUSP6 increased CCN2-induced phospho-ERK levels. In *in vivo* Matrigel plug assays, the number of Podoplanin-positive vessels was increased by exogenous CCN2, and phospho-ERK-positive LEC and DUSP6-positive LEC were detected in CCN2 plugs. These results suggest that CCN2-related lymphangiogenesis is regulated by DUSP6, which enables negative modulation of ERK-signaling.

Introduction

Cellular communication network factor 2 (CCN2), also known as connective tissue growth factor (CTGF), was originally isolated as a platelet-derived growth factor (PDGF)-like factor in HUVEC (1). CCN2 is a member of the CCN family of modular matricellular proteins, which contains six family members (CCN1–6) (2). CCN family proteins contains four structural domains: the IGF binding domain, the von Willebrand factor C repeat domain, the thrombospondin type 1 (TSP) domain and the cysteine knot C-terminal domain (3). CCN2 is a multifunctional molecule, with roles in development, tumorigenesis, fibrotic disease and wound healing (4). Of note, CCN2 has been also considered to be one of the most important regulators of both physiologic and pathologic angiogenesis (5-7). CCN2/CTGF is highly expressed in vasculature, including in the coronary vessels of the heart and vasculature of the kidneys, lung and liver (8-10), and in certain tumors to promote tumor angiogenesis (5,11).

With regards to the CCN2 signaling cascades, CCN2 activates MAPKs such as p38 MAPK, ERK, and JNK in HUVEC and chondrocytes, either directly via cell-surface receptors or indirectly via other extracellular cofactors. (12,13) Integrins are cell surface adhesion receptors for extracellular matrix proteins and immunoglobulin superfamily proteins, and conformation change of the extracellular domain triggers activation of intracellular signaling pathways, including ERK and MAPK signaling(14-17). The TSP domain in CCN2 interacts with integrin $\alpha 6\beta 1$ (18), and the C-terminal motif of CCN2 interacts with integrins

$\alpha v\beta 3$, $\alpha 5\beta 1$ and $\alpha 6\beta 1$ (19-24). In vascular endothelial cells, CCN2/CTGF functions through direct binding to integrin $\alpha v\beta 3$ (19,25) to promote proliferation and induce chemotaxis and formation of tubules (26,27).

The lymphatic vascular system has essential roles in the regulation of tissue pressure, immune surveillance and absorption of dietary fat in the intestine (28). Lymphangiogenesis is necessary not only for the development of lymphatic system during embryogenesis, but also for tissue repair and inflammatory reactions in most organs (29). Several angiogenic factors are involved in growth of lymphatic vessels, such as the Tie/angiopoietin system, neuropilin-2 and integrin $\alpha 9$, which regulate lymphangiogenesis (30-32). VEGF-C-VEGFR-3 signaling is also crucial for the development of lymphatic vessels (33-36). Impairment of lymphatic function results in various diseases that are characterized by the inadequate transport of interstitial fluid, edema, impaired immunity, and fibrosis (37,38). Abnormal proliferation of lymphatic endothelial cells (LECs) causes lymphangiomas, lymphangiosarcomas and Kaposi's sarcoma (39,40).

Previous studies in renal, peritoneal dialysis-related and liver fibrosis mouse models showed that CTGF enhanced lymphangiogenesis (41-44). Furthermore, CTGF is involved in fibrosis-associated renal lymphangiogenesis through its interaction with VEGF-C (41,45). These reports show that CCN2 is closely associated with lymphangiogenesis in fibrous diseases. However, CCN2 signaling in LECs in lymphangiogenic signaling has not been elucidated. In this

study, we investigated the downstream signaling of CCN2 in primary cultured LECs.

Results

CCN2 increases the gene expression levels of LEC markers and *Vegfc*, *Vegfd* and *Flt4* (*Vegfr3*) in LECs

To investigate the roles of CCN2 in lymphangiogenic signaling, we examined the gene expression levels of LEC markers, *lymphatic vessel endothelial hyaluronan receptor 1* (*Lyve1*), *Podoplanin* (*Pdpn*) and *prospero homeobox 1* (*Prox1*), and lymphangiogenic factors, *vascular endothelial cell growth factor c* (*Vegfc*), *vascular endothelial cell growth factor d* (*Vegfd*), *fms-related tyrosine kinase 4* (*Flt4*, also referred to as *Vegfr3*) and *kinase insert domain receptor* (*Kdr* also referred to as *Vegfr2*), in primary cultured LECs treated with CCN2. LECs were stimulated with 0, 12.5, 25, 50, 100 or 200 ng/mL of CCN2 for 3 h, and the expression levels of the LEC markers *Lyve1*, *Pdpn* and *Prox1* were analyzed by quantitative RT-PCR. The expression levels of *Lyve1*, *Pdpn* and *Prox1* were increased by CCN2 in a dose-dependent manner up to 100 ng/mL; at 200 ng/mL, their expression levels dramatically decreased compared with levels at 100 ng/mL (Fig. 1A). We further found that CCN2 increased the expression levels of the lymphangiogenic factors *Vegfc*, *Vegfd* and *Flt4* but did not affect *Kdr* expression (Fig. 1B). Phosphorylation of the receptor tyrosine kinases (RTKs) VEGFR2 and VEGFR3 were investigated using a phospho-RTK array. Our results showed that VEGFR2 and VEGFR3 phosphorylations were unchanged by CCN2 (Fig. S1). In contrast, phosphorylation levels of the RTKs EGFR and EphB4 were increased by CCN2,

while phosphorylation of ErbB4 and PDGFR α were decreased compared with controls ($p<0.001$).

CCN2 enhances the phosphorylation of ERK via integrin α_v in LECs.

To determine the intracellular signaling downstream of CCN2, we next analyzed the expression of various phosphorylated proteins that are known to be activated by CCN2 stimulation (27). While the phosphorylation levels of p38 MAPK, JNK, AKT, NF κ B and Smad2, -3, and -1/5 were unchanged in response to CCN2 (Fig. S2A–C), phospho-ERK was significantly increased in response to CCN2 ($p<0.001$) (Fig. 2A, 2B). Total ERK protein levels were not affected by CCN2 treatment (Fig. 2C).

Although Vegfrs are upstream of ERK (46,47), our RTK assay results showed that VEGFR2 and VEGFR3 were not activated by CCN2 in LECs. Previous studies showed that CCN2 promotes vascular endothelial cell growth, migration, adhesion, and survival partly through its interactions with integrins (25). Therefore, we next investigated whether CCN2-mediated effects on ERK may involve its interaction with integrins. We first examined the expression levels of *Itgav*, *Itga9*, *Itga2b*, *Itgb1*, *Itgb2* and *Itgb3* mRNA in primary LECs treated with CCN2. *Itgav*, *Itga9* and *Itgb1* were predominantly expressed in LECs. Furthermore, the expression levels of these mRNAs were increased by CCN2 stimulation (Fig. 3A). *Itga2b*, *Itgb2* and *Itgb3* were not induced by CCN2. Knockdown of integrin α_v by siRNA partially decreased phosphorylated ERK induced by CCN2 compared with non-target siRNA ($p=0.0040$) (Fig. 3B), which

suggests that CCN2 increases phospho-ERK via integrin α v in LECs. In contrast, knockdown of integrin α 9 resulted in a slight increase of CCN2-induced phospho-ERK compared with levels in cells transfected with non-target siRNA (Fig. 3C). Interestingly, phospho-ERK was increased by suppression of integrin β 1 ($p=0.05$) but was not altered by CCN2 stimulation in integrin β 1-suppressed LECs (Fig. 3D). *Itgb2* and *Itgb3* mRNA levels were not changed by suppression of integrin β 1, which suggests that integrins β 2 and β 3 may not compensate for the decrease in integrin β 1 (Fig. S3).

CCN2 promotes the binding of ERK and DUSP6 in LECs.

CCN2 promotes cell proliferation in vascular endothelium (26,48-50). Therefore, we next analyzed the effects of CCN2 on the cell growth of LECs. Treatment of LECs with 10, 50 and 100 ng/mL of CCN2 for 24 h suppressed cell growth compared with cells at 0 h ($p = 0.22$, 0.92 and > 0.99 , respectively) (Fig. 4A). In addition, treatment with 50 and 100 ng/mL of CCN2 suppressed cell growth at 24 h compared with vehicle-treated LECs ($p = 0.003$ and 0.02). However, no changes in proliferation were observed at 48 and 72 h of treatment. These results indicate CCN2 has a weak growth inhibitory effect on LECs.

Time-course experiments showed that phospho-ERK was significantly increased in LECs at 15 min after treatment with CCN2 and continued to increase up to 4h, followed by a decrease at 8 h, and increase after 48 h (Fig. 4B). The phosphorylation of ERK induced by CCN2 was non-persistent and

transient, suggesting the presence of a concomitant mechanism for suppressing ERK.

The inactivation of MAPKs by dephosphorylation of threonine and/or tyrosine residues of the T-X-Y motif within the kinase activation loop is mediated by serine/threonine phosphatases, tyrosine phosphatase, and dual-specificity phosphatases (DUSPs) (51). The DUSP family phosphatases are the largest group of protein phosphatases that specifically regulate MAPK activity in mammalian cells (51). Quantitative RT-PCR revealed that *Dusp1*, -2, -4, -5, -6, -7, -8, -9 and -10 are expressed in LECs; *Dusp6* was predominant, but *Dusp16* was undetected (Fig. 5A).

Binding of activated ERK to the kinase interaction motif of DUSP6 results in a conformational change, prompting phosphatase activation of the DUSP6 catalytic domain, leading to dephosphorylation of ERK (52). To investigate whether ERK and DUSP6 directly interact, we performed immunoprecipitation assays. The results showed that DUSP6 co-precipitated with ERK, and their interaction was enhanced by CCN2 (Fig. 5B). In addition, in LECs transfected with siRNA targeting DUSP6 (Fig. 5C), phospho-ERK levels were increased compared with levels in cells transfected with non-target siRNA at 15 min and 4, 24 and 48 h after CCN2 treatment. ($p < 0.001$ for 15 m, 4 h,; $p = 0.04$ for 24 h,; $p = 0.004$ for 48 h) (Fig. 5D). These results indicate that while CCN2 enhances the phosphorylation of ERK, CCN2 also enhances the binding between ERK and DUSP6, and DUSP6 induces dephosphorylation of ERK in LECs.

CCN2 promotes lymphangiogenesis in *in vivo* Matrigel plug assay.

To investigate the effects of CCN2 on lymphangiogenesis *in vivo*, recombinant CCN2 was mixed with Matrigel and subcutaneously injected onto the backs of mice. At 7 days after injection, blood vessels were macroscopically observed on the surface of plugs in the CCN2 group mice, but not in the control group (Fig. 6A). Histological analyses revealed that the cells in the CCN2 gel were mostly Podoplanin-positive, and tubular structures were observed (Fig. 6B and 6C). The number of Podoplanin-positive vessels and percentage of Podoplanin-positive areas were increased in CCN2-supplemented gels compared with controls, which suggests that CCN2 positively modulated lymphangiogenesis. CCN2 also increased the number of CD31-positive cavities and β 3-Tubulin-positive peripheral nerves in the CCN2 plugs (Fig. S4). Immunostaining revealed that total ERK was similarly expressed in both the control gel and the CCN2 gel; however, while phosphorylated ERK was barely present in the control gel, it was detected in the CCN2 gel and localized in the nucleus and cytosol of Podoplanin-positive cells (Fig. 7A and 7C). DUSP6 was also rarely detected in the control gel, but it was detected in the cytosol of Podoplanin-positive cells in the CCN2 gel (Fig. 7B). Phosphorylated ERK and DUSP6 expression in Podoplanin-positive cells were significantly increased in the CCN2 gel compared with the control gel ($p < 0.05$) (Fig. 7A and 7B).

Discussion

The molecular mechanism underlying the cell proliferation of LECs involves the activation of ERK signaling stimulated by autocrine or paracrine VEGF-C and VEGFR3 signaling (53). We demonstrated that CCN2 upregulates *Vegfc*, *Vegfd* and *Flt4* and increases the phosphorylation of ERK in primary cultured LECs. However, the phosphorylation of VEGFR2 (KDR) and VEGFR3 (FLT4) was not increased by CCN2, which suggests that CCN2 and endogenous VEGF-C secreted from LECs do not activate VEGF receptor signaling upstream of ERK in primary cultured LECs.

Integrins play key roles in endothelial cell migration and survival during angiogenesis and lymphangiogenesis. A previous study showed that integrin $\alpha v \beta 3$ enhanced angiogenesis induced by basic FGF and $TNF\alpha$, and integrin $\alpha v \beta 5$ enhanced VEGF-induced angiogenesis and tumor metastasis (54). Integrin $\alpha 9 \beta 1$ is required for proper development of the lymphatic system (54). In agreement with these studies, integrin αv , $\alpha 9$ and $\beta 1$ were detected in LECs, and CCN2-induced phospho-ERK was reduced by suppression of integrin αv , whereas suppression of integrin $\alpha 9$ did not decrease phosphorylated ERK. These results indicate that CCN2-induced phospho-ERK is partially mediated by integrin αv in LECs. Recently, Kumaravel et al. showed that integrin $\beta 1$ was important in lymphangiogenesis (55). However, phospho-ERK was increased by suppression of integrin $\beta 1$ without CCN2 stimulation. The loss of $\beta 1$ was not compensated for by increases in integrins $\beta 2$ and $\beta 3$. In LECs, integrin $\alpha 5$ is increased by suppression of integrin $\beta 1$ (55). We did not investigate the

expression level of *Itga5* mRNA because the interaction between CCN2 and integrin $\alpha 5$ has not been reported in LECs. The increase in phospho-ERK without CCN2 stimulation might be due to integrin $\alpha 5$ supplementation. However, phospho-ERK was not altered by CCN2 stimulation in integrin $\beta 1$ suppression, suggesting that integrin $\beta 1$ might be involved in CCN2 signaling in LECs.

Lymphangiogenesis in adults involves the cell proliferation, budding and migration of LECs. Our *in vitro* cell proliferation assay results showed that CCN2 did not increase the proliferation of LECs but rather weakly suppressed proliferation. In HUVECs, DUSP1 and DUSP5 regulate the MAPK signaling pathways that are activated by VEGF (56), and DUSP5 modulates ERK1/2 downstream of the Ang1/Tie2 receptors (57). We showed DUSP6 is highly expressed in LECs, and immunoprecipitation assays showed that CCN2 enhanced the interaction of ERK and DUSP6 in LECs. Moreover, suppression of DUSP6 by siRNA increased phospho-ERK levels compared with control cells, which suggests that DUSP6 functions as a negative modulator of ERK-signaling in LECs. In a previous study, Forkhead box C1 (FOXC1) and Forkhead box C2 (FOXC2) were identified as negative regulators of ERK (58). In mice, lymphatic endothelial cell-specific deletion of FOXC1, FOXC2, or both resulted in lymphatic hyperplasia via hyperactivation of ERK (58). Here we identified DUSP6 as a suppressor of ERK in LECs; however, the relationship between FOXC1, FOXC2 and DUSP6 has not yet been analyzed.

Our results showed that CCN2 enhanced lymphangiogenesis in *in vivo* Matrigel assays, similar to findings in fibrosis model studies (43). The expression of phosphorylated ERK and DUSP6 was similarly strong in Podoplanin-positive cells in the CCN2 gel, which suggests that DUSP6 might suppress the excessive activation of ERK, as shown *in vitro*. CCN2 also enhanced angiogenesis and peripheral nerve cell migration. Previous angiogenesis studies showed that CCN2 is sufficient to induce angiogenesis, directly or indirectly, under *in vitro* and *in vivo* experimental conditions (19,26,59). CTGF expression increases after central nervous system trauma in rodent models (60-62). In zebrafish spinal cord injury models, injury-induced *ctgfa* directs glial cell bridging and neuronal regeneration (63). Our results are consistent with these previous studies. The discrepancy observed between the *in vitro* cell proliferation and *in vivo* Matrigel assays may be due to effects of ECM and matrix metalloproteinase (MMP) in Matrigel. Since CCN2-signaling is mediated by ECM and activated through proteolytic processing by MMP-2,3 (6,64-67), lymphangiogenesis in *in vivo* Matrigel assays was promoted. The results between these assays may not be comparable, and our present results are not contradictory.

A recent study found significant expression of CTGF in the epithelium and connective tissue of oral submucous fibrosis, which was classified as a potentially malignant oral disease, as well as oral squamous cell carcinoma (OSCC) cases, increasing with disease progression, whereas healthy buccal mucosa had no CTGF expression (68). Moreover, MSC-derived CCN2

promoted tongue squamous cell carcinoma progression *in vitro* and *in vivo*.

CCN2 is expressed at higher levels in tongue squamous cell carcinoma tissues than in paraneoplastic tissues, and its expression correlated with lymph node metastasis (69). A study of patient-derived tumor samples in the Cancer Genome Atlas database showed that *CCN2* expression was higher in head and neck cancers, and high expression of *CCN2* and *MMP3* was associated with worse prognosis in head and neck cancers (64). Lysine-specific demethylase 1 (LSD1) is a nuclear histone demethylase that functions as an epigenetic regulator to promote cancer initiation, progression, and relapse. LSD1 inhibitors markedly downregulated *CTGF* mRNA and protein expression and inhibited further xenograft growth in a tonsillar OSCC patient-derived xenograft mouse model (70).

We demonstrated that CCN2 activates ERK signaling via integrin α_v and promotes lymphangiogenesis. In addition, CCN2 enhances the binding of DUSP6 to ERK, and DUSP6 suppresses excessive CCN2-induced phosphorylation of ERK. In OSCC, CCN2-signaling is associated with disease progression and lymph node metastasis. Our results suggest that integrin α_v , ERK and DUSP6-mediated lymphangiogenesis may be therapeutic targets of OSCC.

Experimental procedures

Cell culture and proliferation assay

C57BL/6 mouse primary cultured LECs (CellBiologics, Chicago, IL, USA) were cultured in 0.1% gelatin-coated culture dishes at 37°C with 5% CO₂. LECs were cultured using the EGM2-Endothelial cell growth medium-2 bullet kit (Lonza, Basel, Switzerland). Cells up to 6 passages were used in *in vitro* experiments. Cell proliferation was assessed by crystal violet staining method. Cells were plated in 24-well plates at 2×10^4 cells per well and cultured overnight. The medium was changed to medium containing 0, 10, 50 or 100 ng/mL recombinant rat CCN2/CTGF protein, Carrier Free (R&D Systems, Minneapolis, MN, USA). At 0, 24, 48 and 72 h after treatment, cells were fixed with methanol, stained with 0.5% crystal violet (Merck, Darmstadt, Germany) in 25% methanol for 5 min, washed with water five times and dried. Absorbance at 590 nm was measured using the SPARK 10M plate reader (TECAN, Männedorf, Switzerland). Cell proliferation was expressed as the ratio of the absorbance of each time point to the absorbance at 0 h.

Quantitative RT-PCR

Total RNA was prepared from cells using the Purelink RNA purification Kit (Invitrogen, Carlsbad, CA, USA). cDNA was prepared from 1 µg of total RNA using the QuantiTect RT Kit (Qiagen, Venlo, the Netherlands). Quantitative RT-PCR was performed using TB Green Premix ExTaq II (Takarabio, Otsu, Japan) and a LightCycler96 (Roche Basel, Switzerland). Primers used for qPCR are listed in Table S1.

Gene silencing by siRNA

Expression of integrins and DUSP6 was suppressed by siRNA. siRNAs were designed by an siRNA design site, siDirect ver.2 (<http://sidirect2.rnai.jp/>). Sense and complementary strand RNAs having a two-mer overhang were synthesized and annealed. The siRNA sequences for each mRNA target are listed in Table S2. LECs (1.5×10^5) were transfected with 25 pmol of target siRNA or non-target siRNA (MISSION siRNA universal control#1, Merck) using Lipofectamine RNAiMAX (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions.

Western blot analysis

LECs were pre-cultured with FBS-free and supplement-free EGM2 medium (Lonza) for 24 h. Cells were then treated with CCN2-containing FBS-free and

supplement-free EGM2 medium and cultured for the appropriate time. Cells were washed with PBS and lysed in RIPA Buffer (Merck) containing cOmplete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Nacalai, Kyoto, Japan). Protein concentration was determined with the BCA protein assay kit (Thermo). Protein samples (5–20 μ g) were separated with SDS-PAGE and transferred to PVDF membranes (Invitrogen). The membrane was blocked in Blocking-one (Nacalai) for 30 min and then incubated with primary antibodies at 4°C overnight. The primary antibodies are listed in Table S3. After three washes with PBS containing 0.1% Tween-20, the membrane was incubated with HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-rat IgG secondary antibody (Cell Signaling Technology, Danvers, MA, USA) for 2 h. After three washes with PBS containing 0.1% Tween-20, signals were visualized using ECL Prime (GE Healthcare, Chicago, IL, USA) and an image analyzer 680 (GE Healthcare). Signals were quantified using ImageQuant TL 8.1 software (GE Healthcare).

Phospho-receptor tyrosine kinase array

LECs were pre-cultured with FBS-free and supplement-free basal medium for 24 h and then treated with medium containing 100 ng/mL of recombinant rat CTGF (R&D Systems) for 5 min. The phosphorylation levels of RTK were analyzed using a Proteome profiler mouse phospho-RTK array kit (R&D Systems) according to the manufacturer's instructions. Briefly, 200 μ g of total protein was incubated with the receptor-spotted membrane at 4°C for 24 h. After washing, the membrane was incubated with anti-phospho tyrosine kinase antibody, and the phosphorylation levels of receptor tyrosine kinase were detected with the image analyzer 680 (GE Healthcare).

Immunoprecipitation assay

Cells were lysed in Lysis Buffer (CST) containing cOmplete protease inhibitor cocktail and phosphatase inhibitor cocktail. Protein concentration was determined with the BCA protein assay kit (Thermo). Cell lysates containing 200 μ g of total protein were incubated with anti-ERK (CST) or anti-DUSP6 (Abcam, Cambridge, UK) antibody at 4°C overnight. Protein A magnetic beads (New England Biolabs, Ipswich, MA, USA) were then added to the cell lysate and antibody mixture, and the samples were mixed for 20 min. Magnetic beads were separated and washed with lysis buffer five times; the samples were denatured in 20 μ l of 3xSDS Sample Buffer and subjected to Western blotting.

***In vivo* Matrigel plug assay**

Male 8-week-old C57BL/6 mice were purchased from Charles River Japan. Mice were kept under specific pathogen-free conditions and used at 9 weeks of age. All experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by the Animal Care and Use Committee of Fukuoka University (approval number: 2008024). We mixed 450 μ L of growth factor reduced Matrigel (Corning, Corning, NY, USA) and 8.33 μ g of recombinant rat CCN2/CTGF (R&D Systems)(n=4) or 50 μ L of PBS as control (n=3) and subcutaneously injected the Matrigel mixture on the back of each mouse. At day 7 after injection, mice were euthanized. Matrigel plugs were removed, fixed in 4% paraformaldehyde in PBS, and embedded in paraffin. Sections (4 μ m thick) were deparaffinized and stained with hematoxylin-eosin (HE). Primary antibodies used for immunohistochemistry are listed in Table S3. The following secondary antibodies were used: Alexa594 goat anti-Syrian hamster IgG, Alexa488 donkey anti-rat IgG and Alexa488 donkey anti-rabbit IgG (Jackson Immunolaboratory, West Grove, PA, USA). Morphology was observed using a fluorescence microscope (BZ-710X; Keyence, Kyoto, Japan), and samples were examined using a confocal microscope (LSM710; Carl Zeiss, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 8. All experiments were performed more than three times, except the RTK assay (n=2), and data are expressed as the mean $\pm$ standard error (S.E.). Comparative analysis was performed by Student's t-test, one-way and repeated measures analysis of variance (ANOVA), or two-way ANOVA. For multiple comparisons, we performed Bonferroni or Sidak analysis. Statistical significance was set at a *p*-value of less than 0.05.

Data availability

All data are contained within the article.

Supporting information

This article contains supporting information.

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Author contributions

S. H. and T. T. conducted and performed all experiments and wrote the manuscript. R.M. conducted preliminary experiments on Ccn2. Se. K. and Sh. K. supervised and arranged the experiments and reviewed and edited manuscript.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

References

1. Bradham, D. M., Igarashi, A., Potter, R. L., and Grotendorst, G. R. (1991) Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol* **114**, 1285-1294
2. Perbal, B., Tweedie, S., and Bruford, E. (2018) The official unified nomenclature adopted by the HGNC calls for the use of the acronyms, CCN1-6, and discontinuation in the use of CYR61, CTGF, NOV and WISP 1-3 respectively. *J Cell Commun Signal* **12**, 625-629
3. Holbourn, K. P., Perbal, B., and Ravi Acharya, K. (2009) Proteins on the catwalk: modelling the structural domains of the CCN family of proteins. *J Cell Commun Signal* **3**, 25-41
4. Kubota, S., and Takigawa, M. (2015) Cellular and molecular actions of CCN2/CTGF and its role under physiological and pathological conditions. *Clin Sci (Lond)* **128**, 181-196
5. Wells, J. E., Howlett, M., Cole, C. H., and Kees, U. R. (2015) Deregulated expression of connective tissue growth factor (CTGF/CCN2) is linked to poor outcome in human cancer. *Int J Cancer* **137**, 504-511
6. Chintala, H., Liu, H., Parmar, R., Kamalska, M., Kim, Y. J., Lovett, D., Grant, M. B., and Chaqour, B. (2012) Connective tissue growth factor regulates retinal neovascularization through p53 protein-dependent transactivation of the matrix metalloproteinase (MMP)-2 gene. *J Biol Chem* **287**, 40570-40585
7. Ivkovic, S., Yoon, B. S., Popoff, S. N., Safadi, F. F., Libuda, D. E., Stephenson, R. C., Daluiski, A., and Lyons, K. M. (2003) Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. *Development* **130**, 2779-2791
8. Hall-Glenn, F., and Lyons, K. M. (2011) Roles for CCN2 in normal physiological processes. *Cell Mol Life Sci* **68**, 3209-3217
9. Friedrichsen, S., Heuer, H., Christ, S., Winckler, M., Brauer, D., Bauer, K., and Raivich, G. (2003) CTGF expression during mouse embryonic development. *Cell Tissue Res* **312**, 175-188

10. Surveyor, G. A., and Brigstock, D. R. (1999) Immunohistochemical localization of connective tissue growth factor (CTGF) in the mouse embryo between days 7.5 and 14.5 of gestation. *Growth Factors* **17**, 115-124
11. Kondo, S., Kubota, S., Shimo, T., Nishida, T., Yosimichi, G., Eguchi, T., Sugahara, T., and Takigawa, M. (2002) Connective tissue growth factor increased by hypoxia may initiate angiogenesis in collaboration with matrix metalloproteinases. *Carcinogenesis* **23**, 769-776
12. Yosimichi, G., Kubota, S., Nishida, T., Kondo, S., Yanagita, T., Nakao, K., Takano-Yamamoto, T., and Takigawa, M. (2006) Roles of PKC, PI3K and JNK in multiple transduction of CCN2/CTGF signals in chondrocytes. *Bone* **38**, 853-863
13. Kubota, S., Kawaki, H., Kondo, S., Yosimichi, G., Minato, M., Nishida, T., Hanagata, H., Miyauchi, A., and Takigawa, M. (2006) Multiple activation of mitogen-activated protein kinases by purified independent CCN2 modules in vascular endothelial cells and chondrocytes in culture. *Biochimie* **88**, 1973-1981
14. Damsky, C. H., and Ilic, D. (2002) Integrin signaling: it's where the action is. *Curr Opin Cell Biol* **14**, 594-602
15. Kumar, C. C. (1998) Signaling by integrin receptors. *Oncogene* **17**, 1365-1373
16. Larsen, M., Artym, V. V., Green, J. A., and Yamada, K. M. (2006) The matrix reorganized: extracellular matrix remodeling and integrin signaling. *Curr Opin Cell Biol* **18**, 463-471
17. Schwartz, M. A. (2001) Integrin signaling revisited. *Trends Cell Biol* **11**, 466-470
18. Heng, E. C. K., Huang, Y., Samuel, A. B., and Trackman, P. C. (2006) CCN2, connective tissue growth factor, stimulates collagen deposition by gingival fibroblasts via module 3 and alpha 6-and beta 1 integrins. *Journal of Cellular Biochemistry* **98**, 409-420
19. Babic, A. M., Chen, C. C., and Lau, L. F. (1999) Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and

- migration through integrin $\alpha(v)\beta(3)$, promotes endothelial cell survival, and induces angiogenesis in vivo. *Molecular and Cellular Biology* **19**, 2958-2966
20. Gao, R. P., and Brigstock, D. R. (2004) Connective tissue growth factor (CCN2) induces adhesion of rat activated hepatic stellate cells by binding of its C-terminal domain to integrin $\alpha(v)\beta(3)$ and heparan sulfate proteoglycan. *Journal of Biological Chemistry* **279**, 8848-8855
 21. Chen, C. C., Chen, N. Y., and Lau, L. F. (2001) The angiogenic factors Cyr61 and connective tissue growth factor induce adhesive signaling in primary human skin fibroblasts. *Journal of Biological Chemistry* **276**, 10443-10452
 22. Gao, R., and Brigstock, D. R. (2005) Connective tissue growth factor (CCN2) in rat pancreatic stellate cell function: integrin $\alpha5\beta1$ as a novel CCN2 receptor. *Gastroenterology* **129**, 1019-1030
 23. Gao, R., and Brigstock, D. R. (2006) A novel integrin $\alpha5\beta1$ binding domain in module 4 of connective tissue growth factor (CCN2/CTGF) promotes adhesion and migration of activated pancreatic stellate cells. *Gut* **55**, 856-862
 24. Hoshijima, M., Hattori, T., Inoue, M., Araki, D., Hanagata, H., Miyauchi, A., and Takigawa, M. (2006) CT domain of CCN2/CTGF directly interacts with fibronectin and enhances cell adhesion of chondrocytes through integrin $\alpha5\beta1$. *FEBS Lett* **580**, 1376-1382
 25. Lau, L. F. (1999) The CCN Family of Angiogenic Regulators: The Integrin Connection. *Experimental Cell Research* **248**, 44–57
 26. Shimo, T., Nakanishi, T., Nishida, T., Asano, M., Kanyama, M., Kuboki, T., Tamatani, T., Tezuka, K., Takemura, M., Matsumura, T., and Takigawa, M. (1999) Connective tissue growth factor induces the proliferation, migration, and tube formation of vascular endothelial cells in vitro, and angiogenesis in vivo. *J Biochem* **126**, 137-145
 27. Jun, J. I., and Lau, L. F. (2011) Taking aim at the extracellular matrix: CCN proteins as emerging therapeutic targets. *Nat Rev Drug Discov* **10**, 945-963

28. Cueni, L. N., and Detmar, M. (2008) The lymphatic system in health and disease. *Lymphat Res Biol* **6**, 109-122
29. Alitalo, K., Tammela, T., and Petrova, T. V. (2005) Lymphangiogenesis in development and human disease. *Nature* **438**, 946-953
30. Gale, N. W., Thurston, G., Hackett, S. F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M. H., Jackson, D., Suri, C., Campochiaro, P. A., Wiegand, S. J., and Yancopoulos, G. D. (2002) Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. *Developmental Cell* **3**, 411-423
31. Yuan, L., Moyon, D., Pardanaud, L., Bréant, C., Karkkainen, M. J., Alitalo, K., and Eichmann, A. (2002) Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* **129**, 4797-4806
32. Huang, X. Z., Wu, J. F., Ferrando, R., Lee, J. H., Wang, Y. L., Farese, R. V., Jr., and Sheppard, D. (2000) Fatal bilateral chylothorax in mice lacking the integrin $\alpha 9 \beta 1$. *Mol Cell Biol* **20**, 5208-5215
33. Achen, M. G., Jeltsch, M., Kukk, E., Mäkinen, T., Vitali, A., Wilks, A. F., Alitalo, K., and Stacker, S. A. (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci U S A* **95**, 548-553
34. Jeltsch, M., Kaipainen, A., Joukov, V., Meng, X., Lakso, M., Rauvala, H., Swartz, M., Fukumura, D., Jain, R. K., and Alitalo, K. (1997) Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* **276**, 1423-1425
35. Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N., and Alitalo, K. (1996) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *Embo j* **15**, 290-298
36. Kaipainen, A., Korhonen, J., Mustonen, T., van Hinsbergh, V. W., Fang, G. H., Dumont, D., Breitman, M., and Alitalo, K. (1995) Expression of the *fms*-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci U S A* **92**, 3566-3570

37. Yuan, Y., Arcucci, V., Levy, S. M., and Achen, M. G. (2019) Modulation of Immunity by Lymphatic Dysfunction in Lymphedema. *Front Immunol* **10**, 76
38. Ghanta, S., Cuzzzone, D. A., Torrisi, J. S., Albano, N. J., Joseph, W. J., Savetsky, I. L., Gardenier, J. C., Chang, D., Zampell, J. C., and Mehrara, B. J. (2015) Regulation of inflammation and fibrosis by macrophages in lymphedema. *Am J Physiol Heart Circ Physiol* **308**, H1065-1077
39. Dimaio, T. A., and Lagunoff, M. (2012) KSHV Induction of Angiogenic and Lymphangiogenic Phenotypes. *Front Microbiol* **3**, 102
40. Alitalo, K., and Carmeliet, P. (2002) Molecular mechanisms of lymphangiogenesis in health and disease. *Cancer Cell* **1**, 219-227
41. Kinashi, H., Falke, L. L., Nguyen, T. Q., Bovenschen, N., Aten, J., Leask, A., Ito, Y., and Goldschmeding, R. (2017) Connective tissue growth factor regulates fibrosis-associated renal lymphangiogenesis. *Kidney Int* **92**, 850-863
42. Kinashi, H., Kok, H. M., Falke, L. L., Broekhuizen, R., Nguyen, T. Q., and Goldschmeding, R. (2015) Ctgf Silencing Suppresses Lymphangiogenesis in Kidney Disease. *Nephrol Dial Transpl* **30**
43. Kinashi, H., Toda, N., Sun, T., Nguyen, T. Q., Suzuki, Y., Katsuno, T., Yokoi, H., Aten, J., Mizuno, M., Maruyama, S., Yanagita, M., Goldschmeding, R., and Ito, Y. (2019) Connective tissue growth factor is correlated with peritoneal lymphangiogenesis. *Sci Rep* **9**, 12175
44. Montford, J. R., and Furgeson, S. B. (2017) A new CTGF target in renal fibrosis. *Kidney Int* **92**, 784-786
45. Kinashi, H., Ito, Y., Sun, T., Katsuno, T., and Takei, Y. (2018) Roles of the TGF-beta(-)VEGF-C Pathway in Fibrosis-Related Lymphangiogenesis. *Int J Mol Sci* **19**
46. Shin, M., Beane, T. J., Quillien, A., Male, I., Zhu, L. J., and Lawson, N. D. (2016) Vegfa signals through ERK to promote angiogenesis, but not artery differentiation. *Development* **143**, 3796-3805
47. Shin, M., Male, I., Beane, T. J., Villefranc, J. A., Kok, F. O., Zhu, L. J., and Lawson, N. D. (2017) Correction: Vegfc acts through ERK to induce

- sprouting and differentiation of trunk lymphatic progenitors.
Development **144**, 531
48. Yang, Z., Sun, Z., Liu, H., Ren, Y., Shao, D., Zhang, W., Lin, J., Wolfram, J., Wang, F., and Nie, S. (2015) Connective tissue growth factor stimulates the proliferation, migration and differentiation of lung fibroblasts during paraquat-induced pulmonary fibrosis. *Mol Med Rep* **12**, 1091-1097
 49. Nakanishi, T., Nishida, T., Shimo, T., Kobayashi, K., Kubo, T., Tamatani, T., Tezuka, K., and Takigawa, M. (2000) Effects of CTGF/Hcs24, a product of a hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture. *Endocrinology* **141**, 264-273
 50. Nishida, T., Nakanishi, T., Asano, M., Shimo, T., and Takigawa, M. (2000) Effects of CTGF/Hcs24, a hypertrophic chondrocyte-specific gene product, on the proliferation and differentiation of osteoblastic cells in vitro. *J Cell Physiol* **184**, 197-206
 51. Caunt, C. J., and Keyse, S. M. (2013) Dual-specificity MAP kinase phosphatases (MKPs): shaping the outcome of MAP kinase signalling. *FEBS J* **280**, 489-504
 52. Ahmad, M. K., Abdollah, N. A., Shafie, N. H., Yusof, N. M., and Razak, S. R. A. (2018) Dual-specificity phosphatase 6 (DUSP6): a review of its molecular characteristics and clinical relevance in cancer. *Cancer Biol Med* **15**, 14-28
 53. Potente, M., and Mäkinen, T. (2017) Vascular heterogeneity and specialization in development and disease. *Nat Rev Mol Cell Biol* **18**, 477-494
 54. Avraamides, C. J., Garmy-Susini, B., and Varner, J. A. (2008) Integrins in angiogenesis and lymphangiogenesis. *Nat Rev Cancer* **8**, 604-617
 55. Kumaravel, S., Abbey, C. A., Bayless, K. J., and Chakraborty, S. (2020) The beta1-integrin plays a key role in LEC invasion in an optimized 3-D collagen matrix model. *Am J Physiol Cell Physiol* **319**, C1045-C1058

56. Bellou, S., Hink, M. A., Bagli, E., Panopoulou, E., Bastiaens, P. I., Murphy, C., and Fotsis, T. (2009) VEGF autoregulates its proliferative and migratory ERK1/2 and p38 cascades by enhancing the expression of DUSP1 and DUSP5 phosphatases in endothelial cells. *Am J Physiol Cell Physiol* **297**, C1477-1489
57. Echavarria, R., and Hussain, S. N. (2013) Regulation of angiopoietin-1/Tie-2 receptor signaling in endothelial cells by dual-specificity phosphatases 1, 4, and 5. *J Am Heart Assoc* **2**, e000571
58. Fatima, A., Wang, Y., Uchida, Y., Norden, P., Liu, T., Culver, A., Dietz, W. H., Culver, F., Millay, M., Mukouyama, Y. S., and Kume, T. (2016) Foxc1 and Foxc2 deletion causes abnormal lymphangiogenesis and correlates with ERK hyperactivation. *Journal of Clinical Investigation* **126**, 2437-2451
59. Campochiaro, P. A., and Hackett, S. F. (2003) Ocular neovascularization: a valuable model system. *Oncogene* **22**, 6537-6548
60. Hertel, M., Tretter, Y., Alzheimer, C., and Werner, S. (2000) Connective tissue growth factor: a novel player in tissue reorganization after brain injury? *The European journal of neuroscience* **12**, 376-380
61. Liu, Y., Liu, Z., Li, X., Luo, B., Xiong, J., Gan, W., Jiang, M., Zhang, Z., Schluesener, H. J., and Zhang, Z. (2014) Accumulation of connective tissue growth factor+ cells during the early phase of rat traumatic brain injury. *Diagnostic pathology* **9**, 141
62. Conrad, S., Schluesener, H. J., Adibzadeh, M., and Schwab, J. M. (2005) Spinal cord injury induction of lesional expression of profibrotic and angiogenic connective tissue growth factor confined to reactive astrocytes, invading fibroblasts and endothelial cells. *J Neurosurg Spine* **2**, 319-326
63. Mokalled, M. H., Patra, C., Dickson, A. L., Endo, T., Stainier, D. Y., and Poss, K. D. (2016) Injury-induced ctgfa directs glial bridging and spinal cord regeneration in zebrafish. *Science* **354**, 630-634
64. Okusha, Y., Eguchi, T., Tran, M. T., Sogawa, C., Yoshida, K., Itagaki, M., Taha, E. A., Ono, K., Aoyama, E., Okamura, H., Kozaki, K. I.,

- Calderwood, S. K., Takigawa, M., and Okamoto, K. (2020) Extracellular Vesicles Enriched with Moonlighting Metalloproteinase Are Highly Transmissive, Pro-Tumorigenic, and Trans-Activates Cellular Communication Network Factor (CCN2/CTGF): CRISPR against Cancer. *Cancers (Basel)* **12**
65. Kubota, S., and Takigawa, M. (2011) The role of CCN2 in cartilage and bone development. *J Cell Commun Signal* **5**, 209-217
 66. Kubota, S., and Takigawa, M. (2007) CCN family proteins and angiogenesis: from embryo to adulthood. *Angiogenesis* **10**, 1-11
 67. Kaasboll, O. J., Gadicherla, A. K., Wang, J. H., Monsen, V. T., Hagelin, E. M. V., Dong, M. Q., and Attramadal, H. (2018) Connective tissue growth factor (CCN2) is a matricellular preproprotein controlled by proteolytic activation. *J Biol Chem* **293**, 17953-17970
 68. Shah, A. M., Jain, K., Desai, R. S., Bansal, S., Shirsat, P., Prasad, P., and Bodhankar, K. (2021) The Role of Increased Connective Tissue Growth Factor in the Pathogenesis of Oral Submucous Fibrosis and its Malignant Transformation-An Immunohistochemical Study. *Head Neck Pathol*
 69. Wu, Y. L., Li, H. Y., Zhao, X. P., Jiao, J. Y., Tang, D. X., Yan, L. J., Wan, Q., and Pan, C. B. (2017) Mesenchymal stem cell-derived CCN2 promotes the proliferation, migration and invasion of human tongue squamous cell carcinoma cells. *Cancer Sci* **108**, 897-909
 70. Alsaqer, S. F., Tashkandi, M. M., Kartha, V. K., Yang, Y. T., Alkheriji, Y., Salama, A., Varelas, X., Kukuruzinska, M., Monti, S., and Bais, M. V. (2017) Inhibition of LSD1 epigenetically attenuates oral cancer growth and metastasis. *Oncotarget* **8**, 73372-73386

Abbreviations

ERK	Extracellular signal-regulated kinase
IGF	Insulin-like growth factor
Tie	Tyrosine kinase with immunoglobulin-like and EGF-like domains
EGFR	Epidermal growth factor receptor
EphB4	Erythropoietin-producing hepatocellular receptor B4
ErbB4	Epidermal growth factor receptor 4
PDGFR α	Platelet-derived growth factor receptor alpha
MAPK	Mitogen-activated protein kinase
JNK	Jun N-terminal kinase
HSP	Heat shock protein
FGF	Fibroblast growth factors
TNF α	Tumor necrosis factor alpha
HUVEC	Human umbilical vein endothelial cell
ECM	Extracellular matrix
MSC	Mesenchymal stem cell
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
BCA	Bicinchoninic acid
HRP	Horseradish peroxidase

Figure 1. CCN2 upregulates lymphangiogenesis markers and *Vegfc*, *Vegfd* and *Flt4* (*Verfr3*) in lymphatic endothelial cells (LECs). Mouse primary cultured LECs were treated with 0, 12.5, 25, 50, 100 or 200 ng/mL CCN2 for 3 h. (A) mRNA expression levels of *Lyve1*, *Podoplanin* and *Prox1* were analyzed by quantitative RT-PCR. (B) *Vegfc*, *Vegfd*, *Kdr* (*Vegfr2*) and *Flt4* (*Vegfr3*) mRNA levels were analyzed by quantitative RT-PCR. *Actb* was used as an internal control, and gene expression levels were expressed relative to *Actb* mRNA levels. One-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$ vs. 0 ng/mL Ccn2. ††† $p < 0.01$ vs. 100 ng/mL CCN2.

Figure 2. Phosphorylation of ERK is increased by CCN2. (A) LECs were stimulated with 100 ng/mL CCN2 for 0, 5, 15 or 30 min, and phosphorylated ERK, total ERK and HSP90 were detected by Western blotting. (B) Quantification of protein bands from (A); phospho-ERK and total ERK levels were normalized with HSP90. One-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001 vs. 0 min.

Figure 3. CCN2 induces phospho-ERK levels via integrin α v. (A) LECs were treated with or without 100 ng/mL CCN2 and mRNA expression levels of integrin family members were evaluated by quantitative RT-PCR. Gene expressions were normalized to levels of *Actb* mRNA. *Itgav*, *Itga9* and *Itga2b* mRNA levels and *Itgb1*, *Itgb2* and *Itgb3* results are shown in left and right panels, respectively. Two-way ANOVA, * $p < 0.05$, *** $p < 0.001$. Suppression of integrin α v (B), α 9 (C) and β 1 (D) by siRNA. mRNA expression levels of integrin (left), Student t-test, *** $p < 0.001$ vs. non-target siRNA. Western blot in LEC

transfected with siRNA and treated with CCN2 (middle). Phospho-ERK levels in the middle were quantified and normalized with HSP90 expression levels (right), two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns; not significant.

Figure 4. Weak inhibitory effects of CCN2 on the proliferation of LECs and transient phosphorylation of ERK induced by CCN2. (A) LECs were cultured in medium containing 0, 10, 50 or 100 ng/mL of CCN2 for 24, 48 and 72 h. Cell proliferation was analyzed by crystal violet staining. Cell proliferation was expressed as the ratio to the value at 0 h. Two-way ANOVA, * $p < 0.05$, ** $p < 0.01$. ns; not significant. (B) LECs were treated with 100 ng/mL CCN2 for 0, 0.25, 1, 2, 4, 8, 12, 24 or 48 h, and phospho-ERK, ERK and HSP90 were detected by Western blot. Phospho-ERK levels at each time point were normalized to those of HSP90. One-way ANOVA, * $p < 0.05$, *** $p < 0.001$ vs. 0 h. ns; not significant vs. 0 h.

Figure 5. DUSP6 interacts with ERK and suppression of DUSP6 increases phospho-ERK levels in LECs. (A) Expression levels of *Dusp1*, *-2*, *-4*, *-5*, *-6*, *-7*, *-8*, *-10* and *-16* in LECs. ND, not detected. (B) Cell lysates from LECs stimulated with 100 ng/mL of CCN2 for 5 min or control cells were immunoprecipitated with anti-ERK antibody (ERK) or normal rabbit IgG (IgG) and analyzed by Western blot with antibodies against DUSP6 or ERK. (C) Suppression of DUSP6 by siRNA. mRNA expression levels (left) and protein levels (middle) in LECs transfected with non-target siRNA or DUSP6 siRNA. Quantification of protein bands from middle; DUSP6 level was normalized with HSP90. (D) Phospho-ERK levels in LECs transfected with non-target siRNA or DUSP6 siRNA, and treated with 100 ng/mL CCN2 for 0, 0.25, 4, 24 and 48 h. phospho-ERK levels were quantified and normalized with HSP90 levels. Two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. non-target siRNA. ns, not significant.

Figure 6. CCN2 enhances lymphangiogenesis in *in vivo* Matrigel plug assay. Matrigel and recombinant CCN2 were mixed and injected subcutaneously back of the mice. Control mice were injected with Matrigel and PBS mixture. (A) Images and HE-stained sections of Matrigel removed 7 days after injection. Scale bars: 3 mm (images) and 50 μ m (HE-stained sections). (B) Immunostaining of Podoplanin. Podoplanin-positive cells on the surface side of control gel and inside of the CCN2 gel. Arrowheads indicate Podoplanin-positive vessels in the CCN2 gel. Bars: 50 μ m. (C) Number of Podoplanin-positive vessels per mm² and the ratio of the Podoplanin-positive area to the whole area. Student t-test, *p < 0.05.

Figure 7. Enhanced expression of phospho-ERK and DUSP6 in the CCN2 matrigel. Expression of phospho-ERK, DUSP6 and ERK in Matrigel plugs detected by immunostaining (left) and quantification of the ratio of the phospho-ERK, DUSP6 or ERK-positive area to the Podoplanin-positive area for each gel (right). (A) Phospho-ERK-positive cells were localized on the surface side of the CCN2 gel. Arrowheads indicate Podoplanin-positive/phospho-ERK-positive cells. DUSP6-positive cells (B) and ERK-positive cells (C) were detected in both

the CCN2 gel and control gel. Scale bars: 50 μ m. Student t-test, *p < 0.05, **p < 0.01. ns; not significant.

Supporting Information

CCN2 activates ERK-signaling via integrin α v and enhances the interaction of ERK and DUSP6 in lymphatic endothelial cells

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Table S1. List of primers used for quantitative RT-PCR.

Table S2. List of siRNA sequences.

Table S3. List of antibodies used for Western blotting, immunoprecipitation assay and immunohistochemistry.

Figure S1. Phospho-RTK array.

Figure S2. Phosphorylation levels of p38 MAPK, JNK, AKT, NF κ B, Smad2, 3, 1/5/9 in LECs treated with CCN2.

Figure S3. Itgb2 and Itgb3 expression levels in integrin β 1-suppressed LECs.

Figure S4. CD31positive or β 3-Tubulin positive tubular structure was induced by CCN2 Matrigel plug.

Table S1. List of primers used for quantitative RT-PCR.

Target	GenBank Acc.	Sense 5'→3'	Antisense 5'→3'
Actb	NM_007393.5	CATCCGTAAAGACCTCTATGCCA	ATGGAGCCACCGATCCACA
Lyve1	NM_053247.4	AGCAGCCTTGTGGCTGAGA	CTGTCTACGTGCTGCAGATCAATTA
Podoplanin	NM_001290822.1	GGCTTAATGAATCTACTGGCAAG	ATCATGGTCTGAGGTGCTGAGG
Prox1	NM_001360827.1	CTTGCCCACTCTTGGAGCAC	CATATGCAAATGAGCCATTCCTG
Vegfc	NM_009506.2	CAGTGCATGAACACCAGCACA	TAGACATGCACCGGCAGGAA
Vegfd	NM_001308489.1	TGCTGTGGGATAACACCAAATGTAA	AGACGCACTCACAGCGATCTTC
Kdr (Vegfr2)	NM_001363216.1	ACAGACAGTGGGATGGTCCTTG	GTAGCCACTGGTCTGGTTGGAG
Flt4 (Vegfr3)	NM_008029.3	GCAGGACAGGCGACCATACA	AGCTTGACACTCAATCCACAGAC
Itgav	NM_008402.3	GGAACCTCAACTGCTCGCTGG	ACGGCGAGGTCAACCTTGTA
Itga9	NM_001113514.1	GCTGTCTGGGAGGAGGCTAA	GTCTGGCCCTGAGGAGAACC
Itga2b	NM_010575.2	CATGGAAGCGTGTCCATCGT	CTGAGGTGCAAGAGCAACGG
Itgb1	NM_010578.2	CCATGCCAGGGACTGACAGA	GAGCTTGATTCCAATGGTCCAGA
Itgb2	NM_008404.5	GCATCTGTGGGCAGTGTGTA	ATTTGCCACAGTTGCAGGA
Itgb3	NM_016780.2	GAAGTTCAACCGGGGAACGC	TGCCAGTATCCGTCAGCTCT
Dusp1	NM_013642.3	CTTCTTCGCTTTCAACGCCG	TCAGCGTTGGGCACGATATG
Dusp2	NM_010090.2	CCTGCTGGGGCCGAAAATAG	TGCAGGCCCTGAAGATCTGA
Dusp4	NM_176933.4	CTGGCAGTGAACAGCACTC	GTACAAGTGCATCCCCGTCG
Dusp5	NM_001085390.2	CCCGCGGGTCTACTTCCTTA	AGGCTTCTCTCGCCTTCGAT
Dusp6	NM_026268.3	TCTGTTTGAGAATGCGGGCG	CCAAGCAATGCACCAGGACA
Dusp7	NM_153459.4	GCCAGGCCTACTACCTCCAA	AGGACGAGCTGTCCACGTTA
Dusp8	NM_008748.3	CAGGGCAAGCGAGAGCC	GAGTGACCCCTGAAGTGAGGA
Dusp9	NM_029352.3	TACGACCAGGGTAGCAGTGG	CCGCTTCGTTCTCCGCTTC
Dusp10	NM_022019.6	GTGTTGCTGCTGGGGATCAAT	GAGGAGGCTGAAAACCTGGCAA
Dusp16	NM_001048054.2	CACCCTTCAGAGGAGAAGCCC	GCCCTCACTCCAACAGCTTTA

Table S2. List of siRNA sequences.

Target gene	Sequence 5'->3'	Complementary strand 5'->3'
Itgav	AAUGAUUUAAAAUCUAAGUC	CUUAGAUUUUAAUAUCAUUUA
Itga9	AAAUAUGAAAGAUACAAAGAU	CUUUGUAUCUUUCAUAUUUGG
Itgb1	UUAAACAUCUAUUUUUAUCUG	GAUAAAAAUAGAUGUUUAAAA
Dusp6	AGGAAUUUGCUUGUAUUUGAA	CAAAUACAAGCAAAUUCUUAU

Table S3. List of antibodies used for Western blotting, immunoprecipitation assay and immunohistochemistry.

Target	Antibody	Supplier/ catalogue number
phospho-ERK	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody	Cell Signaling Technology / 9101
ERK (for WB)	p44/42 MAPK (Erk1/2) Antibody	Cell Signaling Technology / 9102
ERK (for IP and IHC)	p44/42 MAPK (Erk1/2) (137F5) rabbit mAb	Cell Signaling Technology / 4695
phospho-p38	Phospho-p38 MAPK (Thr180/Tyr182) (3D7) rabbit mAb	Cell Signaling Technology / 9215
p38	p38 MAPK Antibody	Cell Signaling Technology / 9212
phospho-JNK	Phospho-SAPK/JNK (Thr183/Tyr185) Antibody	Cell Signaling Technology / 9251
JNK	SAPK/JNK Antibody	Cell Signaling Technology / 9252
phospho-AKT	Phospho-Akt (Ser473) (D9E) XP rabbit mAb	Cell Signaling Technology / 4060
AKT	Akt (pan) (C67E7) rabbit mAb	Cell Signaling Technology / 4691
phospho-NFκB	Phospho-NF-κB p65 (Ser536) (93H1) rabbit mAb	Cell Signaling Technology / 3033
NFκB	NF-κB p65 (D14E12) XP rabbit mAb	Cell Signaling Technology / 8242
HSP90	HSP90 Antibody	Cell Signaling Technology / 4874
GAPDH	GAPDH (14C10) rabbit mAb	Cell Signaling Technology / 2118
phospho-Smad1/5/9	Phospho-Smad1 (Ser463/465)/ Smad5 (Ser463/465)/ Smad9 (Ser465/467) (D5B10) rabbit mAb	Cell Signaling Technology / 13820
Smad1	Smad1 (D59D7) XP rabbit mAb	Cell Signaling Technology / 6944
Smad5	Smad5 (D4G2) rabbit mAb	Cell Signaling Technology / 12534
phospho-Smad2	Phospho-Smad2 (Ser465/467) (138D4) rabbit mAb	Cell Signaling Technology / 3108
Smad2	Smad2 (D43B4) XP rabbit mAb	Cell Signaling Technology / 5339
phospho-Smad3	Phospho-Smad3 (Ser423/425) (C25A9) rabbit mAb	Cell Signaling Technology / 9520
Smad3	Smad3 (C67H9) rabbit mAb	Cell Signaling Technology / 9523
Integrin αv	Anti-Integrin alpha V antibody [EPR16800]	abcam / ab179475
Integrin α9	Anti-Integrin alpha 9 antibody [EPR9722]	abcam / ab140599
Integrin β1	Anti-Integrin β1 Antibody, clone MB1.2	R&S systems / MAB1997
DUSP6	Anti-DUSP6 antibody [EPR129Y]	abcam / ab76310

Fig. S1 Phospho-RTK array. Cell lysate containing 200 μ g of protein prepared from LECs treated with 100 ng/mL CCN2 or PBS as (control) for 15 min was incubated with antibody-spotted membrane at 4°C overnight. Membrane was washed with wash buffer, incubated with anti-phospho-tyrosine antibody for 2hr. (A) Phosphorylated RTK was visualized with a image analyzer. (B) Pixel density of each RTK was measured by ImageQuant TL 8.1 software, and subtracted by that of reference. Two-way ANOVA, ***p < 0.001.

Fig. S2 Phosphorylation levels of p38 MAPK, JNK, AKT, NFκB, Smad2, 3, 1/5/9 in LECs treated with CCN2. LECs were treated with 100 ng/mL of CCN2 for 0, 5 or 7.5, 10 and 30 min and lysed in RIPA buffer. 5-10 μg of total protein was subjected to Western blotting. (A) phospho-p38 and phospho-JNK, (B) phospho-AKT and phospho-NFκB, (C) phospho-Smad2, phospho-Smad3 and phospho-Smad1/5/9. Cell lysate of LECs treated with TGFβ1 for 30 min was used for control for phospho-Smad3.

Fig. S3. *Itgb2* and *Itgb3* expression levels in integrin β 1-suppressed LECs. LECs were transfected with siRNA for *Itgb1* or non-target siRNA, cultured for 24 hour, expression levels of *Itgb2* and *Itgb3* were analyzed with quantitative RT-PCR. Two-way ANOVA, *** $p < 0.001$. ns; not significant.

Fig. S4. CD31 positive or β 3-Tubulin positive tubular structure was induced by CCN2 in Matrigel plug. Matrigel and recombinant CCN2 were mixed and injected subcutaneously back of the mice. Control mice were injected with Matrigel and PBS mixture. Seven days after injection, Matrigel plug was removed, and expression of CD31 and β 3-Tubulin was assessed by immunohistochemistry. (A) CD31 positive vessels were detected in CCN2 plug. (B) β 3-Tubulin positive bundles detected in CCN2 plug. Scale bars: 50 μ m.