Brief Report

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Sushi repeat-containing protein X-linked 2 promotes angiogenesis through the urokinase-type plasminogen activator receptor dependent integrin αvβ3/focal adhesion kinase pathways

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Summary **Sushi repeat-containing protein X-linked 2 (SRPX2) is a newly identified chondroitin sulfate proteoglycan that is markedly elevated in multiple solid tumors. It is also suggested that SRPX2 is associated with angiogenesis. A conditioned medium of SRPX2 overexpressing colorectal cancer (CRC) cells and SRPX2 recombinant protein was used to evaluate the effect of secretory SRPX2 on the angiogenesis ability of human umbilical vein endothelial cells (HUVECs) and the involved molecular mechanisms. It was revealed that the activity of SRPX2 is dependent on the urokinase-type plasminogen activator receptor and cooperation of the integrin αvβ3 co-receptor. Subsequent studies showed that both PI3K/Akt and Ras/MAPK pathways and phosphorylation of focal adhesion kinase is involved in the intracellular signaling pathway of SRPX2/uPAR. This study suggests that SRPX2 promotes angiogenesis of HUVECs through the cooperation of the uPAR and integrin/FAK pathway.**

Keywords: SRPX2, extracellular matrix, angiogenesis, proteoglycan

1. Introduction

Proteoglycans represent a large and diverse family of macromolecules composed of a specific core protein with covalently linked glycosaminoglycan (GAG) chains as important components of extracellular matrix (ECM). Proteoglycans have important effects on various aspects of tumor angiogenesis. For example, the V2 isoform of versican, a member of chondroitin sulfate proteoglycan (CSPG), promotes the adhesion of glioblastoma cells to endothelial cells and facilitates the formation of tube-like structures (*1*); NG2, a member of CSPG, is involved in the interaction of pericytes with endothelial cells, and the knockdown of NG2 in pericytes leads to increased permeability of endothelial cell layers (*2*); YKL-40, a secreted heparin-binding glycoprotein, promotes angiogenesis ability in both breast cancer and colorectal cancer tumor cells through syndecan 1 and integrin αvβ3 (*3*) and upregulates VEGF expression in glioblastoma cells (*3,4*).

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Sushi repeat-containing protein X-linked 2 (SRPX2) is a newly demonstrated secretory CSPG (5) that is markedly increased in multiple cancers, such as gastric cancer, colorectal cancer (CRC), pancreatic cancer, and glioblastoma and correlates with a poor outcome or advanced tumor stage (*5-10*). SRPX2 is also believed to have a role in angiogenesis. Miljkovic-Licina *et al*. (*14*) found that *SRPX2* is a markedly upregulated gene in the mice angiogenic phenotype t.End.1V cell line, and transfection of siRNAs against *SRPX2* markedly inhibits the migration and angiogenic sprout formation of these cells. However, the exact effect of molecular mechanism of human SRPX2 in tumor angiogenesis is still elusive.

In this study, the angiogenic ability of SRPX2 in human umbilical endothelial cells (HUVECs) was investigated. First, conditioned medium (CM) of colorectal cancer (CRC) SW480 cells were prepared and the effect of secretory SRPX2 from SW480 on HUVECs was examined. Next, SRPX2 recombinant protein was used to explore the signaling pathways involved. Our findings provide new insights into the role of proteoglycans in ECM in tumor angiogenesis and sheds light on the development of new anti-tumor angiogenesis therapy.

2. Materials and Methods

2.1. *Cell culture*

Human CRC cell lines SW480 purchased from ATCC (Manassas, VA, USA) was maintained in a humidified atmosphere of 5.0% CO_2 at 37°C with Dulbecco's modified Eagle medium/F12 medium (Neuronbc Co., Ltd., Beijing, China). HUVEC, a gift from the Institute of Materia Medica, Chinese Academy of Medical Sciences, was maintained in HuMedia-EG2 (KURABO, Tokyo, Japan) medium with 1.0% fetal bovine serum (FBS) with epidermal growth factor and fibroblast growth factor.

2.2. *SRPX2 recombinant protein and SRPX2-conditioned medium*

SRPX2 human recombinant protein prepared in an *in vitro* wheat germ expression system was purchased from Abnova (H00027286-P01), Taipei, Taiwan. The overexpression vector of pcDNA3.1(+)-SRPX2 plasmid and empty plasmid vector as control were constructed and transfected into SW480 (ATCC, Manassas, VA, USA) as previously described (*8*). SRPX2-CM was prepared according to Tanaka *et al*. (*10*). The medium in which subconfluent SW480 cells were being cultured was replaced with an Opti-MEM reduced serum medium (Gibco Life Technologies Inc., Grand Island, New York, USA), after which the cells were cultured for an additional 24 h and the CM collected. The CM was centrifuged, filtered, and stored at – 80°C.

2.3. *Cell proliferation assay*

HUVECs were cultured with prepared CM from pcDNA 3.1(+)-SRPX2 plasmid-introduced SW480 (SRPX2-CM) or control plasmid-introduced SW480 (negative control, NC) for 72 h and incubated with Cell Counting Kit (CCK)-8 for 4.0 h. Cell viability was measured at the designated time by the amount of absorbance at 450 nm.

2.4. *Transwell migration assay*

In Transwell assays, 5.0×10^3 HUVECs were planted into the top chamber of a Transwell chamber (Corning Cabelcon, Vordingborg, Denmark) lined with a non-coated membrane. SRPX2 human recombinant protein (50 ng/μL) or SW480 cells transfected with SRPX2 overexpressing plasmid were added to the lower chamber. After incubation at 37°C in 5.0% CO_2 for 24 h, the cells remaining in the top chamber were removed and those attached to the underside of the membrane were fixed and stained with 0.1% crystal violet. Each insert was counted using a microscope (Olympus Corp., Tokyo, Japan) on three random fields.

2.5. *Wound-healing assay*

In wound-healing assays, HUVECs were seeded on 24-well plates at a density of 1×10^5 cells/well. After adhesion to the plate, the cell monolayer was scratched with a 10-μL tip. After 24 h at 37°C, photos were taken with an Olympus camera (Olympus Corporation, Tokyo, Japan) under a light microscope (40×) at 0, 6, and 12 h, and analyzed using Image J (National Institudes of Health, Bethesda, MD, USA).

2.6. *Tube formation assay*

Fifty microliters of Matrigel (50 mg/L, BD Biosciences, San Jose, CA, USA) were added to 96-well plates and incubated at 37°C for 1.0 h. HUVECs (5.0×10^5) were seeded into a 96-well plate precoated with Matrigel at a density of 3.0×10^4 cells/well. Capillary tube structures were observed and the representative images were captured using an inverted microscope (4×) equipped with a camera. The tube-forming-structures were counted and analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

2.7. *Neutralizing antibodies and small molecular inhibitors*

Before investigating the intracellular signaling pathway, urokinase plasminogen activator receptor (uPAR) neutralizing antibody (20 μg/mL, MAB807, R&D Systems, Minnesota, USA), integrin αvβ3 neutralizing antibody (20 μg/mL, MAB3050, R&D Systems, Minnesota, USA), or immunoglobulin G (20 μg/mL, MAB002, R&D Systems, Minnesota, USA) as a control were added to HUVECs for 2.0 h. To evaluate the role of the PI3K/Akt and MAPK pathways, 10 μM PI3K inhibitor LY294002 and 10 μM MEK inhibitor PD98059 (both Selleck Chemicals, LLC, Houston, TX, USA) in dimethyl sulfoxide (DMSO) or blank DMSO were added to the HUVECs for 2.0 h.

2.8. *Western blots*

Western blots were performed as previously described (*8*). The primary antibodies involved were as following: 1:1,000 phospho-Akt (Ser473, 4060), 1:1,000 Akt (9272), 1:1,000 phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199, 4228), 1:1,000 PI3 Kinase p85 (42571), 1:1,000 phospho-ERK1/2 (Thr202/Tyr204, 43771), 1:1,000 ERK1/2 (4695), and the focal adhesion kinase (FAK) antibody kit (9330) including phospho-FAK (Tyr576/577) antibody, phospho-FAK (Tyr925) antibody, phospho-FAK (Tyr397) 1:2,000 antibody, FAK antibody, and 1:10,000 glyceraldehyde 3-phosphate dehydrogenase (5174). All antibodies were purchased from Cell Signaling Technology, Danvers, MA, USA.

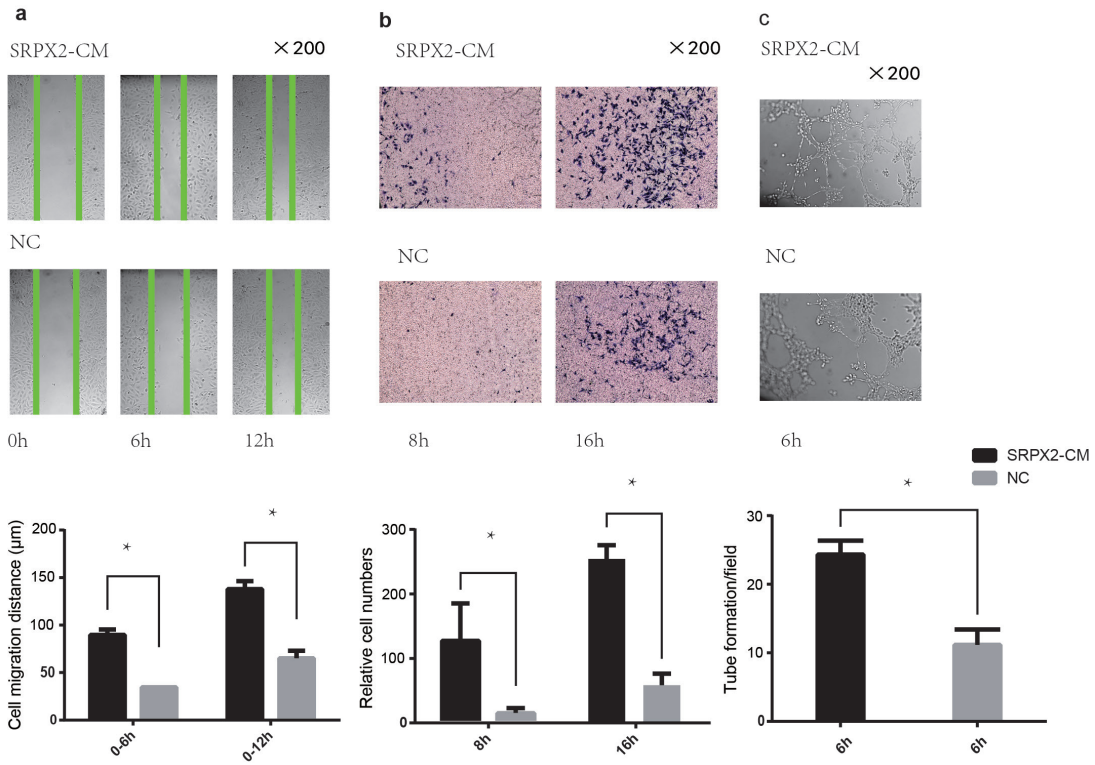

Figure 1. SRPX2 conditional media (CM) significantly promoted migration of HUVECs in scratching assays (a) and Transwell assays (b) respectively compared with negative control (NC) group; (c) in matrigel assays, tube formation ability of HUVECs was also markedly promoted in SRPX2-CM group. *$p < 0.001$.

2.9. *Statistical analyses*

Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All data were expressed as the means ± SEM. Statistical analyses were done using the Student's *t*-test or one-way analysis of variance. The differences were considered statistically significant when $p < 0.05$.

3. Results and Discussion

Since *SRPX2* has three sushi domains encoding three sushi motifs and one hyaline domain mediating specific protein-protein and protein-carbohydrate interaction (*7*), it was predicted that SRPX2 has a role in cellular motility. Hence, the effect of SRPX2 on the migration of HUVECs was first investigated. In wound-healing assays, SRPX2-CM obviously accelerated scratch healing at both 6 and 12 h (both $p < 0.001$; Figure 1a). Similarly, in Transwell migration assays, SRPX2-CM markedly increased the number of HUVECs migrating through the membrane at both 8 and 16 h (both $p < 0.001$; Figure 1b). In addition, the number of tube-like structures per field on Matrigel was also significantly increased by SRPX2-CM ($p < 0.001$, Figure 1c). However, in CCK-8 assays, SRPX2-CM did not promote the proliferation of HUVECs, suggesting that cell proliferation was not involved in the pro-angiogenic effects of SRPX2 on HUVECs. Taken together, we demonstrated that a conditioned medium of SRPX2 from SRPX2 overexpressing SW480 cells significantly increases the angiogenic ability of HUVECs.

The detailed molecular mechanism of the proangiogenic effect of SRPX2 remains unknown. As referenced above, Miljkovic-Licina *et al*. (*11*) proved that mouse SRPX2 directly binds to vascular uPAR on t.End.1V cells. SRPX2 is a proven ligand of cell surface uPAR located on the cell membranes of both tumor cells and HUVECs (*12*); therefore, the role of uPAR in the pro-angiogenic effects of SRPX2 in HUVECs was evaluated using SRPX2 recombinant protein and uPAR-neutralizing antibody. As shown in Figure 2a, in Transwell assays, the number of HUVECs that migrated through the membrane per field was markedly increased in the SRPX2 group compared with that of the control group ($p < 0.001$), while treating HUVECs with uPAR-neutralizing antibody before the Transwell assay significantly attenuated the number of migrating cells compared with the SRPX2 group ($p < 0.01$). Similarly, this effect was also observed in tube-formation assays (Figure 2b). The number of tube-formation structures was significantly increased in the SRPX2 group compared with control group ($p < 0.001$), while significantly decreased after pretreatment with uPAR-neutralizing antibody ($p < 0.001$). These results suggest that the binding of SRPX2 to uPAR is crucial for the proangiogenic effect of SRPX2 on HUVECs.

As is known, uPAR is a GPI-anchored receptor

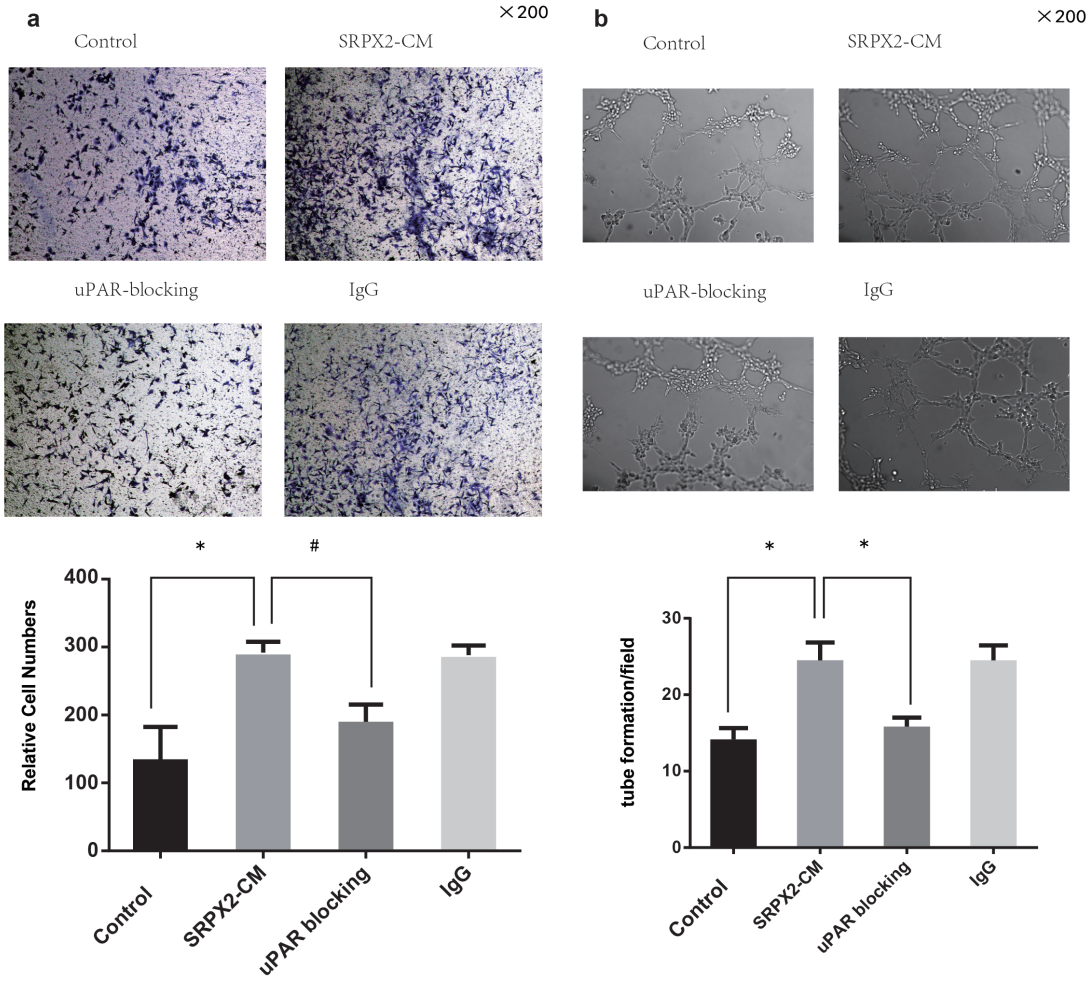

Figure 2. SRPX2 recombinant protein promoted both migration through Transwell chamber and tube structure formation of HUVECs on matrigel compared to control group, while pretreatment of uPAR neutralizing antibody in HUVECs (uPAR blocking group) markedly blocked this effect both in Transwell assay (a) and matrigel tube formation assay (b). IgG group:pretreatment of HUVECs with control mouse IgG antibody .*$p < 0.001$, #$p < 0.01$.

lacking transmembrane and intracellular domains, intracellular signaling of uPAR is dependent on the interaction of uPAR and multiple integrin molecules as co-receptors (*13*). Integrin receptors, an important family of ECM receptors essential in cancer biology, are major co-receptors of uPAR (*14,15*). Because integrin αvβ3 is strongly implicated in uPAR signaling (*16*) and also involved in YKL-40/S1 ligand-receptor interaction mediating angiogenesis (*3*), integrin αvβ3-neutralizing antibody was used to demonstrate that cooperation of uPAR and integrin αvβ3 is necessary for the SRPX2/uPAR complex. In tube-formation assays, the number of tube-like structures of HUVECs in the SRPX2 group was significantly increased compared to control group ($p < 0.001$), while pretreatment with the integrin αvβ3-neutralizing antibody significantly inhibited this effect ($p < 0.001$, Figure 3a), confirming integrin αvβ3 is a crucial mediator for the SRPX2/uPAR complex.

In addition, FAK is a cytoplasmic tyrosine kinase that plays a critical role in integrin-mediated signal transductions as well as in angiogenesis and tumor progression (*17,18*). Tanaka *et al*. (*7*) reported that SRPX2 increases FAK phosphorylation levels in several gastric cancer cells. Gao *et al*. (*9*) also found that the increase in SRPX2 is associated with FAK phosphorylation in pancreatic cancer tissue. In this study, FAK phosphorylation in HUVECs after addition of SRPX2 recombinant protein for 4.0 h was investigated. Similarly, compared to the control group, FAK phosphorylation (Tyr 397 and Tyr 576/577) in the SRPX2 group was significantly increased, while Tyr 925 was not affected (Figure 3b). These results demonstrated that the integrin αvβ3/FAK pathway is involved in the pro-angiogenic effects of SRPX2 on HUVECs and Tyr397 on FAK is indeed a crucial component in angiogenesis (*19-21*).

Because the PI3K/Akt and Ras/MAPK pathways are known as downstream signaling pathways of FAK (*3,19,20,22*), PI3K inhibitor LY294002 and MEK inhibitor PD98059 were used to further clarify the intracellular signaling of SRPX2/uPAR in HUVECs. In tube-formation assays, tube like–structure formation of HUVECs promoted by SRPX2 was significantly inhibited after pretreatment of LY294002 and PD98059 (both $p < 0.001$). Furthermore, phosphorylation of PI3K/Akt and ERK1/2 in HUVECs after addition of SRPX2

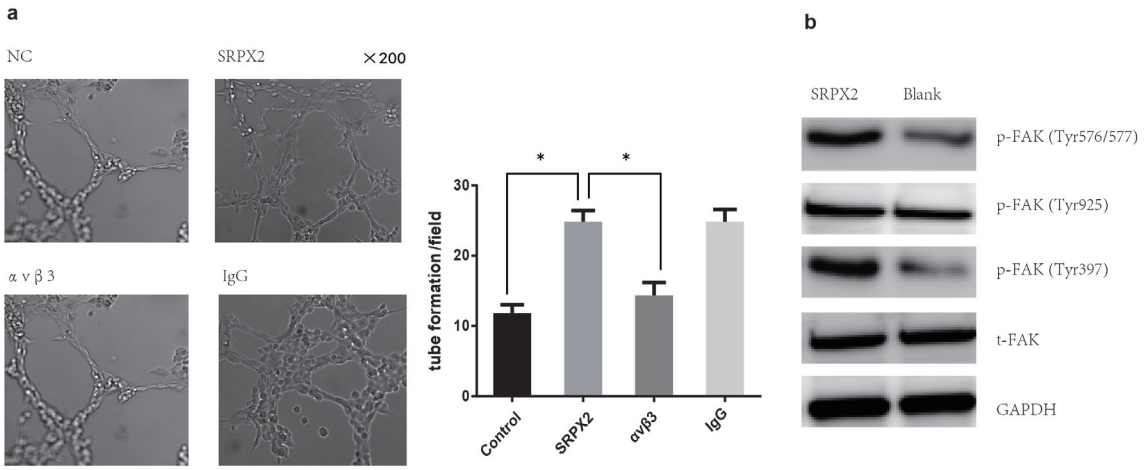

Figure 3. SRPX2 recombinant protein promoted tube formation of HUVECs on matrigel and pretreatment of integrinαvβ3 neutralizing antibody in HUVECs (αvβ3 group) significantly attenuated this effect (a). IgG group: pretreatment of HUVECs with control mouse IgG antibody. **SRPX2 recombinant protein increased phosphorylation of FAK at sites of Tyr 397 and Tyr 576/577 in HUVECS after 4h culture compared with NC and Blank group (b).** *$p < 0.001$.

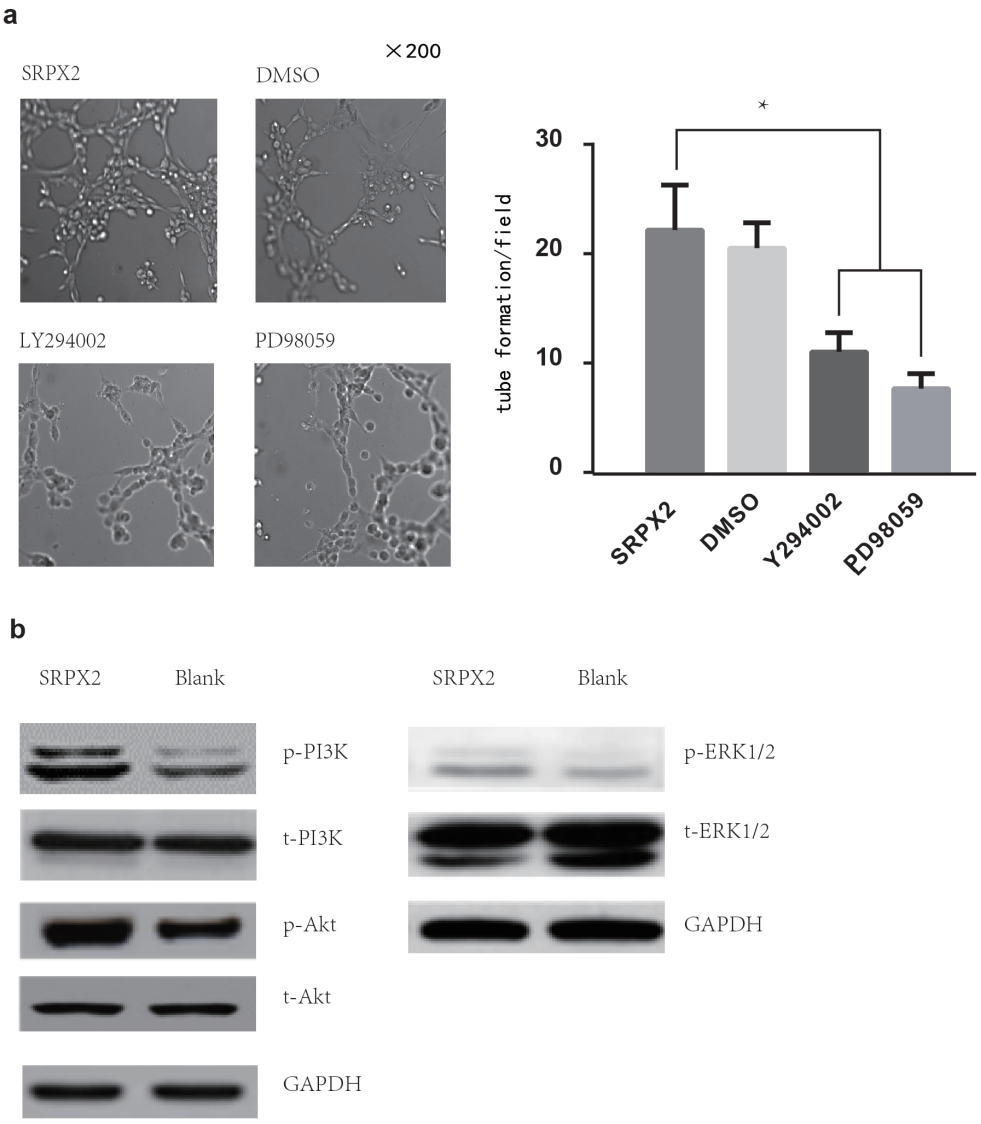

Figure 4. Pretreatment wiht PI3K inhibitor LY294002 and MEK inhibitor PD98059 in HUVECs both significantly inhibited the effect of enhancing tube formation of HUVECs in matrigel by SRPX2 recombinant protein (SRPX2 group) compared with DMSO group (only DMSO was added to HUVECs as control of small molecular inhibitors) (a). *$p < 0.001$. **SRPX2 recombinant protein activated phosphrylation of PI3K, Akt and ERK1/2 in HUVECs compared with blank group (b).**

recombinant protein for 4.0 h was increased compared with control group. All of these findings supported the role of the ERK and PI3K pathways in the angiogenesis effect of SRPX2 on HUVECs.

In this study, we found that SRPX2 from CRC cells promoted migration and tube formation of HUVECs and further molecular mechanisms were investigated. As far as we know, this is the first study to explore the effects and molecular mechanisms of secretory SRPX2 in human vascular endothelial cells. However, in this study, only the direct effect of SRPX2 on HUVECs was investigated. As a secretory proteoglycan in ECM, the interaction of SRPX2 with other growth factors, such as vascular endothelial growth factor and hepatocyte growth factor, both of which are also involved in tumor angiogenesis, can be presumed. Further research should focus on the complex interactions between SRPX2 and other ECM components in tumor angiogenesis. The clarification of the function of SRPX2 in tumor angiogenesis might hold promise for new therapeutic agents targeting overexpressed SRPX2 in various tumors.

Acknowledgements

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References

1. Yang W, Yee AJ. Versican V2 isoform enhances angiogenesis by regulating endothelial cell activities and fibronectin expression. FEBS Lett. 2013; 587:185-192.
2. You WK, Yotsumoto F, Sakimura K, Adams RH, Stallcup WB. NG2 proteoglycan promotes tumor vascularization via integrin-dependent effects on pericyte function. Angiogenesis. 2014; 17:61-76.
3. Shao R, Hamel K, Petersen L, Cao QJ, Arenas RB, Bigelow C, Bentley B, Yan W. YKL-40, a secreted glycoprotein, promotes tumor angiogenesis. Oncogene. 2009; 28:4456-4468.
4. Shao R, Hamel K, Petersen L, Cao QJ, Arenas RB, Bigelow C, Bentley B, Yan W. YKL-40, a secreted glycoprotein, promotes tumor angiogenesis. Oncogene. 2009; 28:4456-4468.
5. Tanaka K, Arao T, Tamura D, Aomatsu K, Furuta K, Matsumoto K, Kaneda H, Kudo K, Fujita Y, Kimura H, Yanagihara K, Yamada Y, Okamoto I, Nakagawa K, Nishio K. SRPX2 is a novel chondroitin sulfate proteoglycan that is overexpressed in gastrointestinal cancer. PloS one. 2012; 7:e27922.
6. Yamada T, Oshima T, Yoshihara K, Sato T, Nozaki A, Shiozawa M, Ota M, Yoshikawa T, Akaike M, Numata K, Rino Y, Kunisaki C, Tanaka K, Imada T, Masuda M. Impact of overexpression of Sushi repeat-containing protein X-linked 2 gene on outcomes of gastric cancer. J Surg Oncol. 2014; 109:836-840.
7. Tanaka K, Arao T, Maegawa M, Matsumoto K, Kaneda H, Kudo K, Fujita Y, Yokote H, Yanagihara K, Yamada Y, Okamoto I, Nakagawa K, Nishio K. SRPX2 is overexpressed in gastric cancer and promotes cellular migration and adhesion. Int J Cancer. 2009; 124:1072-1080.
8. Liu KL, Wu J, Zhou Y, Fan JH. Increased Sushi repeat-containing protein X-linked 2 is associated with progression of colorectal cancer. Med Oncol. 2015; 32:99.
9. Liu KL, Wu J, Zhou Y, Fan JH. Increased Sushi repeat-containing protein X-linked 2 is associated with progression of colorectal cancer. Med Oncol. 2015; 32:99.
10. Tang H, Zhao J, Zhang L, Zhao J, Zhuang Y, Liang P. SRPX2 Enhances the epithelial-mesenchymal transition and temozolomide resistance in glioblastoma cells. Cell Mol Neurobiol. 2016; 36:1067-1067.
11. Miljkovic-Licina M, Hammel P, Garrido-Urbani S, Bradfield PF, Szepetowski P, Imhof BA. Sushi repeat protein X-linked 2, a novel mediator of angiogenesis. FASEB J. 2009; 23:4105-4116.
12. Royer-Zemmour B, Ponsole-Lenfant M, Gara H, Roll P, Lévêque C, Massacrier A, Ferracci G, Cillario J, Robaglia-Schlupp A,Vincentelli R, Cau P, Szepetowski P. Epileptic and developmental disorders of the speech cortex: Ligand/receptor interaction of wild-type and mutant SRPX2 with the plasminogen activator receptor uPAR. Hum Mol Genet. 2008; 17:3617-3630.
13. Smith HW, Marshall CJ. Regulation of cell signalling by uPAR. Nat Rev Mol Cell Biol. 2010; 11:23-36.
14. Xiong J, Balcioglu HE, Danen EH. Integrin signaling in control of tumor growth and progression. Int J Biochem Cell Biol. 2013; 45:1012-1015.
15. Rathinam R, Alahari SK. Important role of integrins in the cancer biology. Cancer Metastasis Rev. 2010; 29:223-237.
16. Wei C, Möller CC, Altintas MM, *et al*. Modification of kidney barrier function by the urokinase receptor. Nat Med. 2008; 14:55-63.
17. Zhao X, Guan JL. Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. Adv Drug Deliv Rev. 2011; 63:610-615.
18. Tai YL, Chen LC, Shen TL. Emerging roles of focal adhesion kinase in cancer. Biomed Res Int. 2015; 2015:690690.
19. Byun HJ, Lee JH, Kim BR, Kang S, Dong SM, Park MS, Lee SH, Park SH, Rho SB. Anti-angiogenic effects of thioridazine involving the FAK-mTOR pathway. Microvasc Res. 2012; 84:227-234.
20. Desgrosellier JS, Cheresh DA. Integrins in cancer: Biological implications and therapeutic opportunities. Nat Rev Cancer. 2010; 10:9-22.
21. Corsi JM, Houbron C, Billuart P, Brunet I, Bouvrée K, Eichmann A, Girault JA, Enslen H. Autophosphorylation-independent and -dependent functions of focal adhesion kinase during development. J Biol Chem. 2009; 284:34769-34776.
22. Kaczmarek E, Erb L, Koziak K, Jarzyna R, Wink MR, Guckelberger O, Blusztajn JK, Trinkaus-Randall V, Weisman GA, Robson SC. Modulation of endothelial cell migrationby extracellular nucleotides: Involvement of focal adhesion kinase and phosphatidylinositol 3-kinase-mediated pathways. Thromb Haemost. 2005; 93:735-742.

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