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Original Article

Integrin αVβ5/Akt/Sp1 pathway participates in matrix stiffness-mediated effects on VEGFR2 upregulation in vascular endothelial cells

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Abstract: Our previous study has validated that higher matrix stiffness obviously improves vascular endothelial growth factor (VEGF) expression in HCC cells, highlighting a linkage between matrix stiffness and HCC angiogenesis. However, the effects of matrix stiffness on vascular endothelial cells in HCC and its underlying mechanism remain largely uncharacterized. Here we further analyzed the expression of vascular endothelial growth factor receptor 2 (VEGFR2) in human umbilical vein endothelial cells (HUVECs) grown on different stiffness substrates and explored its regulatory mechanism for better understanding matrix stiffness-regulated angiogenesis in HCC. Our results revealed that increased matrix stiffness significantly upregulated the expression of VEGFR2 in HUVECs, and the expression level of VEGFR2 was positively correlated with the expression levels of COL1 and lysyl oxidase in human HCC tissues and rat HCC tissue, moreover VEGFR2 and CD34 were co-localized at blood vessel of HCC tissues, indicating an obvious regulation role of matrix stiffness in VEGFR2 expression. Simultaneously, increased matrix stiffness also elevated the phosphorylation level of Akt and the expressions of integrin αV/β5 and nuclear Sp1 in HUVECs. Inhibition of integrin αVβ5 remarkably reversed the expression of VEGFR2 and phosphorylation level of Akt in HUVECs grown on higher stiffness substrate. Except that, PI3K inhibitor also suppressed the phosphorylation level of Akt and the expressions of VEGFR2 and nuclear Sp1 evidently. Taken together, higher matrix stiffness increased VEGFR2 expression in HUVECs, and integrin αVβ5/Akt/Sp1 pathway participated in stiffness-mediated effects on VEGFR2 upregulation. This study combining with our previous report discloses a new paradigm in which higher matrix stiffness as an initiator drives HCC angiogenesis via upregulating both VEGFR2 expression in vascular endothelial cells and VEGF expression in HCC cells.

Keywords: Hepatocellular carcinoma, matrix stiffness, angiogenesis

Introduction

Increasing evidence has suggested that higher matrix stiffness signal as an initiator can obviously strengthen malignant characteristics of HCC cells and contribute to HCC invasion and metastasis. Our previous researches also demonstrate that higher matrix stiffness significantly upregulates invasion/metastasis-associated gene expression [1], enhances stemness characteristics [2], triggers the occurrence of epithelial-mesenchymal transition [3], induces lung pre-metastasis niche formation [4], and ultimately facilitates HCC metastasis. Besides, our data also supports that higher matrix stiffness improves VEGF expression in HCC cells, highlighting a close linkage between higher matrix stiffness and HCC angiogenesis [5]. However, the effects of matrix stiffness on vascular endothelial cells in HCC and its underlying mechanism remain largely unknown. Given that the expression of VEGFRs in vascular endothelial cells is essential for the occurrence of angiogenesis, we speculate that higher matrix stiffness may exert great impact on VEGFRs expression in vascular endothelial cells in favor of

sensing the stimulation of VEGF derived from HCC cells.

Angiogenesis, which is manifested as the formation of new blood vessels and capillaries from the growth of preexisting vessels, frequently occurs during wound healing and embryonic development, and is also required for sustained tumor growth and metastasis formation [6]. In general, tumors have the ability to induce more neo-vascularization to offer oxygen and nutrients for the growth and survival of tumor. The angiogenic process involves the activation, proliferation, and migration of endothelial cells toward angiogenic stimuli [7]. A fine balance between pro- and anti-angiogenic factors controls cascade molecular events of angiogenesis and influences the formation of new blood vessels. Such identified biochemical stimuli in tumor microenvironment as VEGF, TGFβ, FGF, EGF have validated to trigger angiogenesis and facilitate tumor growth and metastasis [8-10]. VEGFs and their receptors, as the most important pathway in the regulation of angiogenesis [11], execute the angiogenesis program by inducing proliferation, survival, migration and sprouting of endothelial cells [17]. Of all VEGF receptors, VEGFR2 is the primary transducer of VEGF signal in vascular endothelial cells, which plays a dominant role in VEGF-dependent angiogenesis [10, 11]. In addition to biochemical stimuli, mechanical stiffness signal, a typical physical attribute of solid tumor, may participate in the modulation of the blood vessel formation [12, 13]. Although our previous results also confirm a significant role of matrix stiffness in promoting HCC angiogenesis [5], it remains unclear about how higher matrix stiffness influences vascular endothelia cells in HCC and modulates HCC angiogenesis. In the study, we further clarified the underlying mechanisms of matrix stiffness-mediated effects on VEGFR2 expression in vascular endothelial cells, and this study combining with our previous reports supply a complete theory that matrix stiffness modulates HCC angiogenesis by upregulating both VEGF expression in HCC cells and VEGFR2 expression in vascular endothelial cells.

Materials and methods

Cell and cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell Research Laboratories, Inc. HUVECs were cultured in endothelial cell medium (ECM, ScienCell) with 5% fetal bovine serum (FBS, ScienCell), 1% penicillin/streptomycin and 1% endothelial cell growth supplement (ECGS, ScienCell) at 37°C in a humidified atmosphere with 5% CO_2. Medium was changed every 3 days. When they grew to reach 80%-90% confluence, the cells were collected for further experiments. The used HUVECs in the experiment were between passage 3 and passage 6.

In vitro system of COL1-coated polyacrylamide gel with tunable stiffness

An in vitro system of COL1-coated polyacrylamide gel with tunable stiffness was established according to the method described previously [5]. Approximately 3×10^5 HUVECs in 0.3 ml medium were spread onto a thin layer of COL1-coated polyacrylamide gels with a stiffness of 6 kPa, 10 kPa and 16 kPa, respectively, and cultured for 2 h at room temperature. Subsequently 12 ml of culture medium was added into the dish, and the cells were transferred into an incubator for further culture.

Biological behavior analysis of HUVECs grown on different stiffness substrates

The proliferation and migration abilities of HUVECs grown on 6, 10, and 16 kPa stiffness substrates were measured by a real-time cell monitoring system containing a Cell-IQ cell culturing platform (Chip-Man Technologies, Tampere, Finland), a phase-contrast microscope (Nikon CFI Achromat phase contrast objective with 10× magnification) and a camera. HUVECs were cultured on different stiffness substrates for 12 h. Cells were imaged and recorded every 10 min for 24 h with a controlled environmental chamber (37°C and 5% CO_2).

Cell proliferation and migration were analyzed by Image J and Chemotaxis tool. Cell populations were counted from at least 10 images for each experimental condition to calculate their proliferation rate. Time-lapse images and movies were assembled using Image J software, individual cell tracking was performed using the "Manual tracking" plugin, which enables the selection and tracking of a cell and its position in each frame. All data were analyzed using "Chemotaxis tool", which provides graphical and statistical analysis of the dataset.

RNA preparation and quantitative real-time PCR

Total RNA was extracted from HUVECs using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA was first reverse-transcribed into cDNA using the Superscript First-Strand Synthesis System (Thermo Scientific), and then the resulting cDNA was used as templates for subsequent target gene PCR amplification (SYBR Green PCR Master Mix, Invitrogen). Relative gene expression was normalized to GAPDH and reported as $2^{-\Delta Ct}$ [ΔCt=Ct (target gene)-Ct (GAPDH)]. The primer sequences for VEGFR1, VEGFR2 and VEGFR3 were as follows: VEGFR1: 5'-CTGTCATGCTAATGGTGTCCC-3' (forward) and 5'-TGCTGCTTCCTGGTCCTAAAATA-3' (reverse); VEGFR2: 5'-CACCACTCAAACGCTGACATGTA-3' (forward) and 5'-CCAACTGCCAATACCAGTGGA-3' (reverse); VEGFR3: 5'-CTCGGCTCACGCAGAACTT-3' (forward) and 5'-GCTGCACAGATAGCGTCCC-3' (reverse). All the primers were synthesized by Sangon Biotech Co. Ltd.

A HCC tissue microarray and immunohistochemistry

A HCC tissue microarray derived from rat HCC models with different liver stiffness backgrounds was constructed previously [5]. Immunohistochemistry staining was performed as the procedure in our previous work [1, 5].

Extraction of nuclear proteins and Western blot

Nuclear protein of HUVECs was purified by Affymetrix Nuclear Extraction Kit (Santa Clara, CA). The concentration of nuclear protein was measured by BCA Protein Assay (Beyotime, China). Total Protein extraction and Western blot analysis were done as previously described [14]. Primary antibodies were diluted with 3% TBSA as follows: VEGFR2 (1:1000, Cell Signal Technology), Akt (1:1000, Cell Signal Technology), p-Akt (Ser473) (1:1000, Cell Signal Technology), Sp1 (1:1000, Cell Signal Technology), Integrin antibody sampler Kit (1:1000, Cell Signal Technology) and GAPDH (1:1000, Cell Signal Technology). Secondary antibodies were diluted with 3% TBSA (1:4000, Proteintech). The target protein band was visualized using an electrochemiluminescence kit (Thermo).

Inhibitors

SB273005, an inhibitor of αVβ3 and αVβ5 was obtained from Selleck Chemicals LLC, STI, and LY294002, the PI3K/Akt inhibitor, was purchased from Cell Signal Technology. HUVECs grown on 16 kPa stiffness substrates were treated with SB273005 at a final concentration of 0.1 μM, and treated with LY294002 at a final concentration of 50 μM.

siRNA against integrin αV/or integrin β5

siRNA oligonucleotides against integrin αV and integrin β5 were respectively synthesized by Sangon Biotec, Shanghai, China. The siRNA sequences were shown in **Table 1**. Approximately 5×10^5 HUVECs were seeded on 16 kPa stiffness substrates in 100 mm culture dish and cultured overnight, then they were transfected with 50 nM integrin αV-siRNA or 50 nM integrin β5-siRNA using Lipofectamine2000 Transfection Agent (Invitrogen, USA) according to the manufacturer's instructions. These transfected cells were subsequently harvested at 24 h after transfection for the following analysis.

Dual-luciferase reporter assay

A dual-luciferase reporter assay was implemented to evaluate interaction between SP1 and VEGFR2 promoter. HEK293T cells were co-transfected with pGL3-basic-VEGFR2-promotor and pLenti-CMV-SP1-3Flag-PGK-Puro as the subject, and the cells with pGL3-basic-VEGFR2-promotor and pLenti-CMV-EGFP-3Flag-PGK-Puro vector were as the control. The relative luciferase activity was assessed by the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega, USA). The experiment was carried out in collaboration with OBiO Technology (Shanghai) Corp., Ltd.

TCGA data analysis

We downloaded the gene expression data and clinical information of 361 HCC patients from The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov) using GDC Data Transfer Tool, and then selected 137 HCC patients with HBV (+) for further analysis. We set the median expression levels of COL1A1 and LOX as the cutoff to classify HCC patients

Table 1. The sequences of siRNA

siRNA	Sense Primer Sequence (5'→3')	Anti-sense Primer Sequence (5'→3')
NC siRNA	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
Integrin αV	CGACAAAGCUGAAUGGAUUTT	AAUCCAUUCAGCUUUGUCGTT
Integrin β5	GCAACUUCCGGUUGGGAUUTT	AAUCCCAACCGGAAGUUGCTT

into LOX^{high}/$COL1A1^{high}$ group (high matrix stiffness group, 45 patients) and LOX^{low}/$COL1A1^{low}$ group (low matrix stiffness group, 44 patients), and comparatively analyzed their VEGFR2, CD31 and CD34 expressions.

Clinical specimens

The clinicopathological characteristics of 20 HCC patients, who underwent curative hepatectomy at Fudan University Shanghai Cancer Center from 2017 to 2018, were retrospectively analyzed including gender, age, tumor size, serum alpha fetoprotein (AFP), HBV infection, microvascular invasion, histological grade, Metavir's G grade, and Metavir's S grade. The diagnosis of HCC patients was confirmed by pathological examinations. Tumor differentiation was evaluated according to the Edmondson grading system. Microscopic vascular invasion was defined as the presence of tumor emboli within the central vein, the portal vein, or large capsular vessels or the lobar or segmental branches of the portal vein or the hepatic veins. Histological grading of inflammation (G0-G4) and staging of fibrosis (S0-S4) for non-tumoral liver were evaluated according to the Metavir scoring criteria. The present study was approved by the Clinical Research Ethics Committee of Fudan University Shanghai Cancer Center (Shanghai, China), and written informed consent was obtained from all participants.

Statistical analysis

All data represented the mean value of at least 3 independent experiments and were analyzed using SPSS software (version 22.0). Continuous variables were expressed as the mean ± standard deviation (SD), and statistical analysis was performed by one-way ANOVA and Student's t-test. Categorical data were displayed as absolute number (n) and calculated using Fisher's exact test. Pearson correlation analysis was used to determine the linear relationship between two quantitative variables. For all cases, differences were considered statistically significant when *p*-value was less than 0.05.

Results

Migration and proliferation analysis of HUVECs grown on different stiffness substrates

We constructed 6 kPa (L), 10 kPa (M) and 16 kPa (H) COL1-coated gel substrates as reported previously [1, 4, 5] to mirror the stiffness of normal liver, fibrotic liver and cirrhotic liver, and then comparatively analyzed the biological behavior changes of HUVECs cultured on three stiffness substrates such as cell growth and migration [15]. Taking use of a real-time cell-monitoring system, we respectively recorded the position tracking of HUVECs grown on different stiffness substrates (**Figure 1A**), and measured their motility velocities (**Figure 1B**). The average motility velocities of HUVECs under 6 kPa, 10 kPa and 16 kPa stiffness stimulations were 64.9 ± 4.2, 96.6 ± 8.5, and 117.2 ± 10.5 μm/h. HUVECs cultured on higher stiffness substrate migrated faster than the cells cultured on lower stiffness substrate ($P<0.05$), meaning that increased matrix stiffness obviously strengthens the motility ability of HUVECs. Except that, increased rate of total cell number under higher stiffness stimulation was also significantly higher than that under lower stiffness stimulation ($P<0.01$), indicating that higher stiffness stimulation can remarkably enhance proliferation ability of HUVECs, and the proliferation of HUVECs may be stiffness-dependent (**Figure 1C**). All these results above suggest that increased matrix stiffness exerts considerable impacts on the biological behaviors of HUVECs, implying it may alter biological function of vascular endothelial cells.

Comparative analysis of VEGR2 and CD34 expressions in HCC tissues with different liver stiffness backgrounds

Previously we found that VEGF and CD31 highly expressed in HCC tissue with higher liver stiffness background [5]. We further used same HCC tissue microarray, derived from three groups of rat HCC models with different liver stiffness backgrounds, to detect the expressions of CD34 and VEGFR2. The results demon-

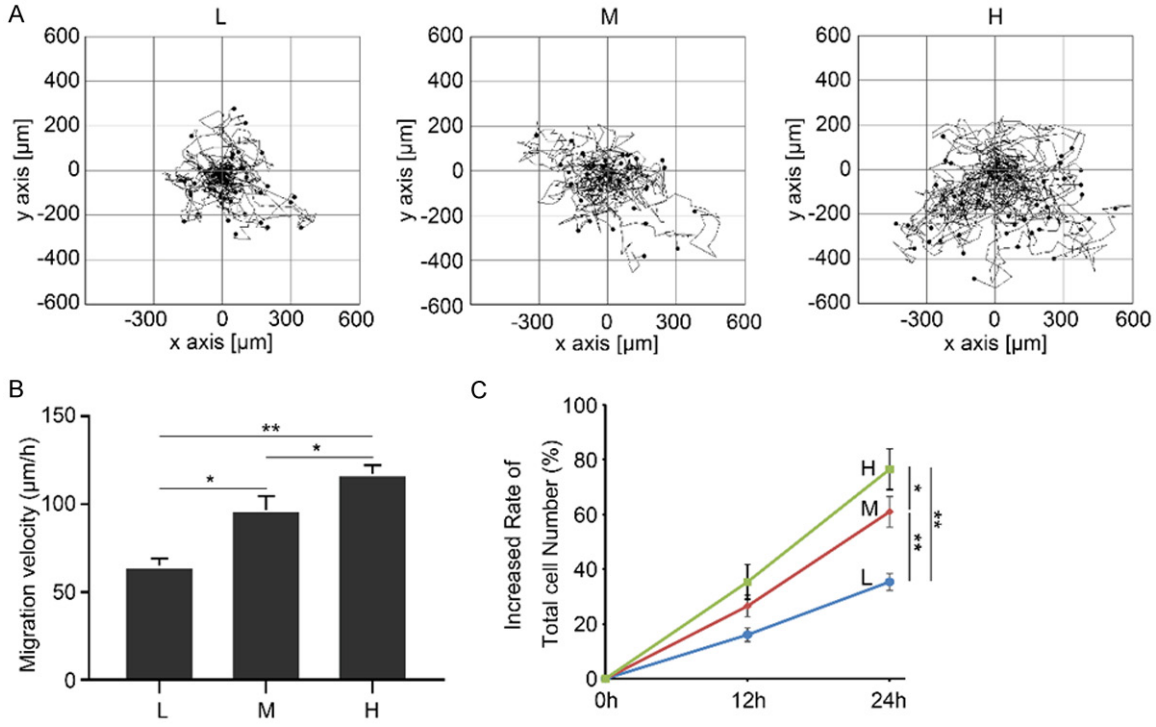

Figure 1. Migration and proliferation analysis of HUVECs under different stiffness stimulations. A. Migratory trajectories of HUVECs grown on 6 kPa stiffness substrate (L), 10 kPa stiffness substrate (M) and 16 kPa stiffness substrate (H). B. The average motility velocities of HUVECs grown on 6 kPa stiffness substrate (L), 10 kPa stiffness substrate (M) and 16 kPa stiffness substrate (H). C. Proliferation curves of HUVECs grown on 6 KPa stiffness substrate (L), 10 kPa stiffness substrate (M) and 16 kPa stiffness substrate (H). Data are expressed as the mean ± SD of triplicate samples or at least three independent experiments. At least 8 images were analyzed per sample. **P*<0.05, ***P*<0.01. ****P*<0.001.

strated that the expressions of CD34 and VEGFR2 were also remarkably increased in HCC tissues with higher liver stiffness background as compared to HCC tissues with normal liver stiffness background (**Figure 2A**, **2B**). Additionally, both VEGFR2 and CD34 also co-localized at blood vessel of HCC tissues. Taken together, higher liver stiffness background upregulated both expression of VEGFR2 and VEGF in HCC tissues, implying that there exists an obvious linkage between liver matrix stiffness and HCC angiogenesis, and VEGF/VEGFR2 pathway participates in matrix stiffness-induced angiogenesis.

Increased matrix stiffness actives Akt/Sp1 pathway and upregulates the expressions of VEGFR2 in HUVECs

Increased matrix stiffness upregulated the expression of VEGF in HCC cells in our previous work [5]. VEGF and VEGFR2 also highly expressed in HCC tissues with higher liver stiffness background. Thereby, we speculated that higher matrix stiffness might promote the expression of VEGF receptors in vascular endothelial cells in favor of sensing the secreted VEGF from HCC cells. We first analyzed the expression patterns of VEGFR in HUVECs grown on different stiffness substrates, and found that mRNA expressions of VEGFR1, VEGFR2 and VEGFR3 were all upregulated in HUVECs as matrix stiffness increased (**Figures 3A** and S1). Because VEGFR2 was the most important subtype of VEGF receptor family in response to VEGF stimulation during angiogenesis [11, 16], the underlying mechanism of matrix stiffness-upregulated VEGFR2 in HUVECs became a priority problem to be solved in this study. We further confirmed high expression of VEGFR2 at protein level in HUVECs grown on higher stiffness substrate (**Figure 3B**), and simultaneously discovered that increased matrix stiffness obviously improved phosphorylation level of Akt and nuclear Sp1 expression in HUVECs (**Figure**

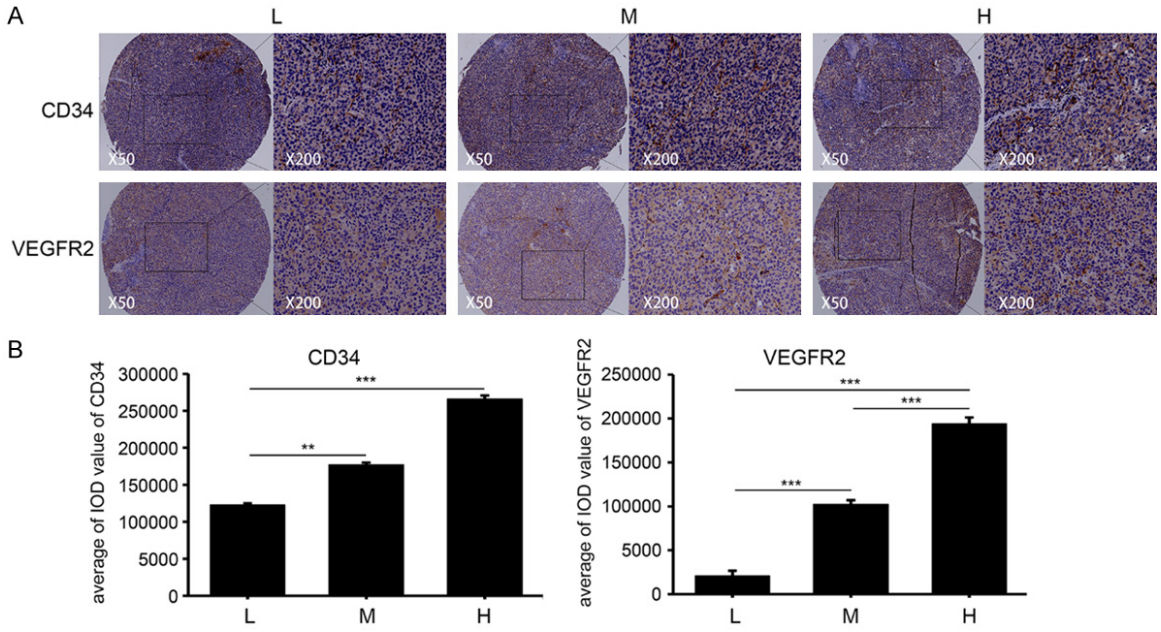

Figure 2. Comparative analysis of VEGR2 and CD34 expressions in HCC tissues with different liver stiffness backgrounds. A. The expression levels of CD34 and VEGFR2 in HCC tissues with normal liver stiffness background, medium liver stiffness background and high liver stiffness background. B. A comparative analysis of the average IOD value of CD34 and VEGFR2 expression in three groups of HCC tissues with different liver stiffness backgrounds by image pro-plus 6.0 software. In each case, error bars represent SD, **P*<0.05, ***P*<0.01, ****P*<0.001.

3C, **3D**). Moreover, the luciferase assay showed that there was an interaction between transcription factor Sp1 and the promoter of gene VEGFR2 (**Figure 3E**). All these results suggest that the activated Akt/Sp1 pathway may be associated with matrix stiffness-upregulated VEGFR2 in HUVECs.

Integrin αVβ5/Akt/Sp1 pathway participates in matrix stiffness-upregulated VEGFR2 in HUVECs

In order to screen which integrin subtype as the most important bridge molecule delivers stiffness signal into HUVECs, we comparatively analyzed the expression patterns of integrin subtypes in HUVECs grown on 6, 10, 16 kPa stiffness substrates. As shown in **Figure 4A**, integrin β5 and αV exhibited obvious alteration in expression level under higher stiffness stimulation. We applied an antagonist SB273005 to inhibit integrin αVβ5 of HUVECs and further detected the expressions of p-Akt and VEGFR2. The results showed that antagonist SB273005 obviously reversed the phosphorylation level of Akt and VEGFR2 expression in HUVECs grown on higher stiffness substrate (**Figure 4B**). Subsequently, we applied siRNA against integrin αV or integrin β5 to clarify which integrin subtype delivered matrix stiffness signal into cells for VEGFR2 upregulation (**Figure 4C** and **4D**). Our results revealed that knockdown of integrin αV or integrin β5 presented similar reversal effects on the expression of p-Akt and VEGFR2 in vascular endothelial cells grown on higher stiffness substrate. Therefore, integrin αVβ, as an important stiffness sensor molecule, mediated matrix stiffness-induced VEGFR2 upregulation in HUVECs. Afterwards, we used a PI3K inhibitor LY294002 to treat HUVECs grown on higher stiffness substrate, and discovered that the treated HUVECs presented a significant decrease in the phosphorylation level of Akt and the expressions of VEGFR2 and nuclear Sp1 as compared to the control (**Figure 4E**), confirming that activation of Akt is required for matrix stiffness-induced VEGFR2 upregulation. Taken together, integrinαVβ5/Akt/Sp1 pathway participated in matrix stiffness-upregulated VEGFR2 in HUVECs.

TCGA analysis and validation of VEGFR2 expression in HCC tissues

TCGA analysis revealed that VEGFR2 (**Figure 5A**), CD34 (**Figure 5D**) and CD31 (Figure S2A) all highly expressed in HCC tissues in high matrix stiffness group (LOX^{high}/$COL1A1^{high}$,

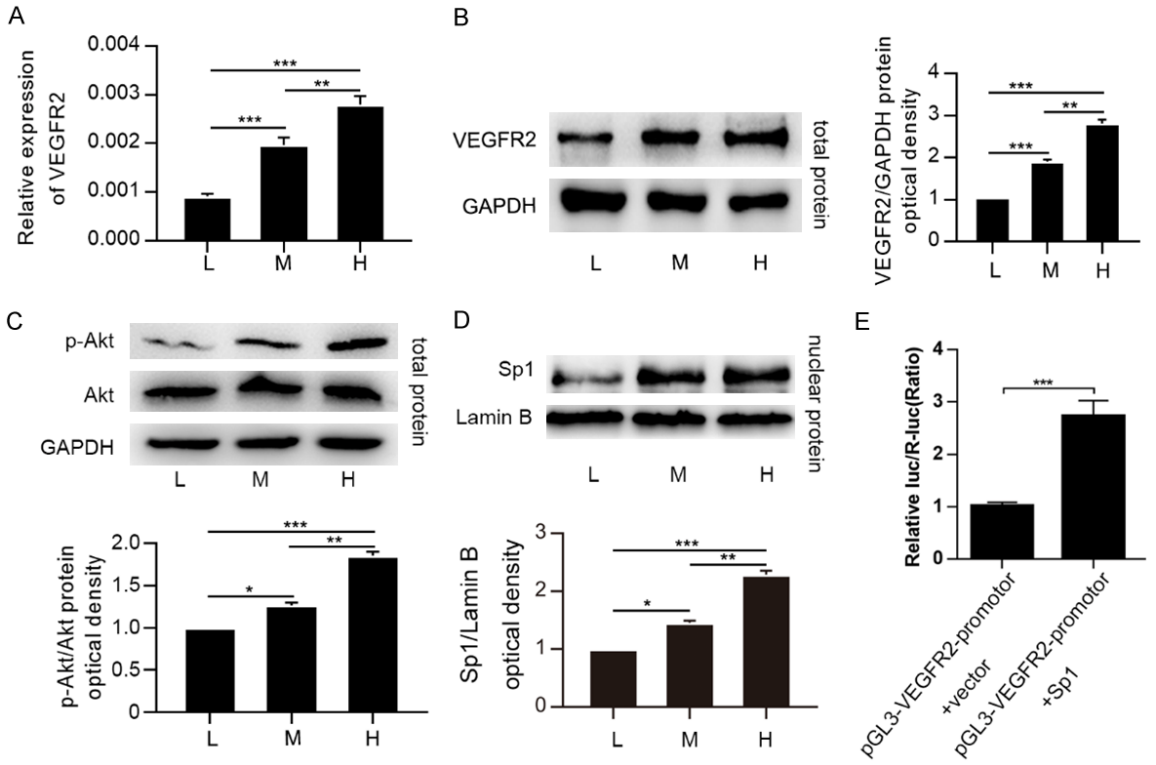

Figure 3. Increased matrix stiffness actives AKT/Sp1 pathway and upregulates the expressions of VEGFR2 in HUVECs. A. mRNA expression level of VEGFR2 in HUVECs grown on 6 kPa stiffness substrate (L), 10 kPa stiffness substrate (M) and 16 kPa stiffness substrate (H). B. Protein expression level of VEGFR2 in HUVECs grown on 6 kPa stiffness substrate (L), 10 kPa stiffness substrate (M) and 16 kPa stiffness substrate (H). C. Higher matrix stiffness obviously elevates phosphorylation level of Akt in HUVECs. D. Higher matrix stiffness remarkably increased the expression of transcription factors Sp1 in HUVECs. E. Dual luciferase assay reveals an interaction between Sp1 and the promoter of gene VEGFR2.

n=45) compared with those in low matrix stiffness group ($LOX^{low}/COL1A1^{low}$, n=44). Pearson correlation analysis demonstrated that the expression level of VEGFR2 was positively correlated with the expression level of COL1A1 (**Figure 5B**, *P*=0.0001) but not LOX (**Figure 5C**, *P*=0.0669), and the expression level of CD34 or CD31 was positively correlated with that of COL1A1 (**Figures 5E** and S2C) and LOX (**Figures 5F** and S2B). In addition, the expression level of CD34 was positively correlated with that of CD31 (Figure S2D). The above results suggested that there was a close linkage between VEGFR2 expression and matrix stiffness, and increased matrix stiffness may promote HCC angiogenesis.

We defined median expression level of VEGFR2 in HCC tissues as a threshold to classify 20 HCC patients into VEGFR2 low expression group and VEGFR2 high expression group for assessing the association of VEGFR2 expression and clinicopathological variables in HCC. Our results showed that VEGFR2 expression level was positively associated with higher matrix stiffness (*P*=0.040) and Metavir's S grade (*P*=0.005) (**Table 2**), supporting that increased matrix stiffness promotes the expression of VEGFR2. On the other hand, VEGFR2 and CD34 also exhibited obviously higher expression in $LOX^{high}/COL1A1^{high}$ group compared to in $LOX^{low}/COL1A1^{low}$ group, further validating a positive correlation between VEGFR2/CD34 expression and matrix stiffness level (**Figure 6A** and **6B**), in agreement with our results in vitro.

Discussion

Matrix stiffening frequently accompanies with HCC development and progression [17] and currently becomes a typical biomechanical feature of HCC for indicating its unfavorable outcome and poor prognosis [18, 19]. Hyper-

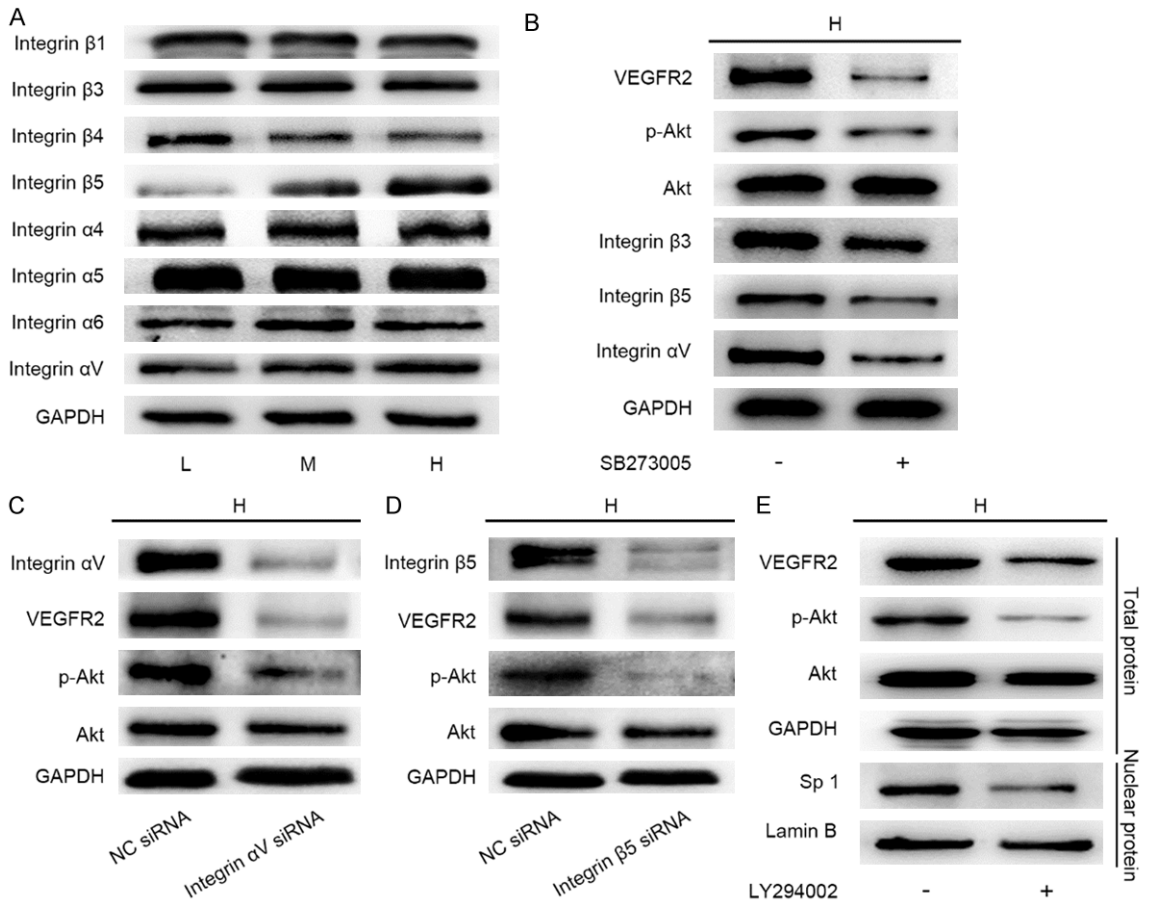

Figure 4. Integrin αV/β5/Akt/Sp1 pathway participates in matrix stiffness-mediated effects on VEGFR2 upregulation in HUVECs. A. Analysis of integrin subtypes in HUVECs under different stiffness stimulation. B. Antagonist SB273005 suppresses the phosphorylation level of Akt and VEGFR2 expression in HUVECs grown on 16 kPa stiffness substrate (H). C, D. siRNA against integrin αV or integrin β5 partially reverses the phosphorylation level of Akt and VEGFR2 expression in HUVECs grown on 16 kPa stiffness substrate (H). E. PI3K inhibitor LY294002 downregulates the phosphorylation level of Akt and the expressions of VEGFR2 and nuclear Sp1 in HUVECs grown on 16 kPa stiffness substrate (H).

vascularity, another pathological characteristic of HCC, also influences and determines tumor progression and survival. However, little is known about biomechanical signal-regulated neovascularization in HCC and its underlying mechanism. Several lines of evidence have demonstrated that increased matrix stiffness participates in the regulation of HCC invasion and metastasis in different ways such as strengthening invadopodial activity [20, 21], upregulating invasion and metastasis-associated gene expression [1], enhancing stemness characteristics [2], triggering EMT occurrence [3], and facilitating pre-metastatic niche formation [4]. Additionally, matrix stiffness also alters and influences the proliferation and chemotherapeutic response of HCC cells [17]. Previously, we found an obvious upregulation in VEGF expression in higher stiffness-stimulated HCC cells and in HCC tissues with higher liver stiffness background, suggesting a regulation role of matrix stiffness in HCC angiogenesis [7]. In this study, we further discovered that there was an upregulation in VEGFR2 expression in HCC tissues with higher liver stiffness background, moreover VEGFR2 and CD34 co-localized at blood vessel of HCC tissues. These findings hinted that higher matrix stiffness might also promote the expression of VEGF receptors in vascular endothelial cells to sense the released VEGF from HCC cells. We firstly observed the changes of biological behaviors in vascular endothelial cells grown on different stiffness substrates, and found that HUVECs

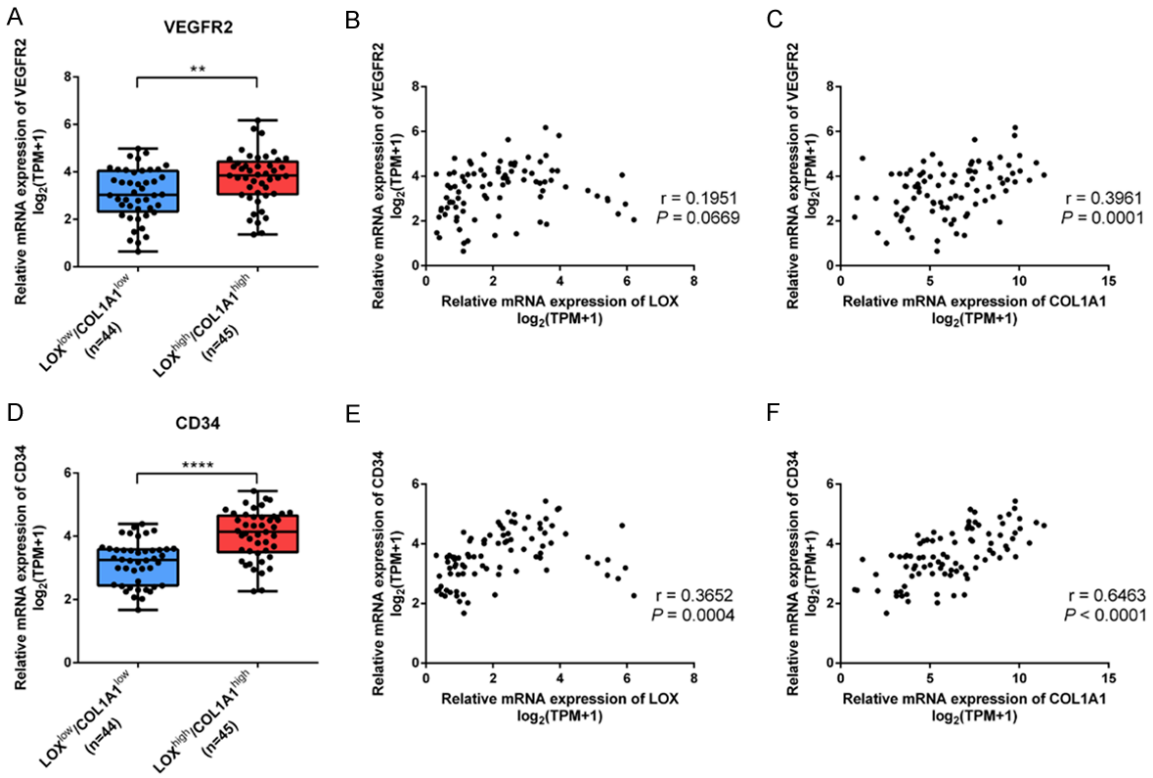

Figure 5. VEGFR2 and CD34 expressions in TCGA-HCC tissues and their correlation with matrix stiffness. A. We defined the median expression of COL1A1 and LOX in TCGA-HCC tissues as the threshold to classify HCC patients into $LOX^{high}/COL1A1^{high}$ group (45 patients) and $LOX^{low}/COL1A1^{low}$ group (44 patients), The mRNA expression level of VEGFR2 in TCGA-HCC tissues in $LOX^{high}/COL1A1^{high}$ group (n=45) is obviously higher than that in $LOX^{low}/COL1A1^{low}$ group (n=44). B and C. Pearson correlation analysis indicates that the mRNA level of VEGFR2 in TCGA-HCC cohort is positively correlated with the mRNA level of COL1A1, while uncorrelated with that of LOX. D. The expression level of CD34 in HCC tissues $LOX^{high}/COL1A1^{high}$ group (n=45) is significantly higher than that in $LOX^{low}/COL1A1^{low}$ group (n=44). E and F. Pearson correlation analysis indicates that the mRNA level of CD34 in TCGA-HCC cohort is positively correlated with that of both COL1A1 and LOX. $^{**}P<0.01$, $^{****}P<0.0001$.

exhibited stronger migration and faster growth abilities under higher stiffness stimulation. Subsequently, our results further confirmed high expression state of VEGFR2 in HUVECs grown on higher matrix substrate at mRNA and protein level. TCGA data also revealed that there existed a close linkage between VEGFR2 expression and matrix stiffness. VEGFR2 and CD34 presented obviously higher expression in HCC tissues with $LOX^{high}/COL1A1^{high}$, and VEGFR2 expression level was positively correlated with higher matrix stiffness (P=0.040) and Metavir's S grade (P=0.005), all supporting that increased matrix stiffness may promote the expression of VEGFR2. Based on these evidences, we inferred that matrix stiffening not only promoted VEGF expression in HCC cells, but also upregulated VEGFR2 expression in HUVECs.

Hypervascularity characteristic of HCC underlines a unique role of angiogenesis in development and progression of HCC. Many proangiogenic factors and antiangiogenic factors have well characterized to drive or inhibit HCC angiogenesis including VEGF, FGF, PDGF, angiopoietins, HGF, endoglin, angiostatin, endostatin, thrombospondin-1 [11, 22]. These factors generally stimulate the activation/inactivation of endothelial cell tyrosine kinase and downstream intracellular signal, then regulate vessel growth and maturation, ultimately determine the fate of angiogenesis in HCC [11, 23]. Among these factors, VEGF binding with its high-affinity receptor VEGFR2 are the key mediator of tumor angiogenesis, this binding results in the receptor dimerization, protein kinase activation, auto-phosphorylation, then activates PI3K/Akt and other signal pathways to modu-

Table 2. Associations of VEGFR2 expression with clinicopathologic characteristics in HCC patients

Characteristics	Expression of VEGFR2		*P* value
	Low expression (n=10)	High expression (n=10)	
Gender			
Male	10 (50.0%)	10 (50.0%)	
Female	0	0	
Age			
≤50	4 (44.4%)	5 (55.6%)	1.000
>50	6 (54.5%)	5 (45.5%)	
HBsAg			
Negative	0	0	
Positive	10 (50.0%)	10 (50.0%)	
AFP (ng/ml)			
≤20	7 (53.8%)	6 (46.2%)	1.000
>20	3 (42.9%)	4 (57.1%)	
Tumor size (cm)			
<3*3	3 (42.9%)	4 (57.1%)	1.000
≥3*3	7 (53.8%)	6 (46.2%)	
Microvascular invasion			
No	3 (60.0%)	2 (40.0%)	1.000
Yes	7 (46.7%)	8 (53.3%)	
Tumor differentiation			
Edmondson I-II	3 (42.9%)	4 (57.1%)	1.000
III-IV	7 (53.8%)	6 (46.2%)	
Metavir's G grade			
0-1	8 (72.7%)	3 (27.3%)	0.070
2-3	2 (22.2%)	7 (77.8%)	
Metavir's S grade			
0-2	8 (88.9%)	1 (11.1%)	0.005
3-4	2 (18.1%)	9 (81.9%)	
Expression of			
$LOX^{high}/COL1A1^{high}$	1 (12.5%)	7 (87.5%)	0.040
$LOX^{low}/COL1A1^{low}$	6 (75.0%)	2 (25.0%)	

late endothelial cell survival, proliferation and migration [24-26]. A recent study reports that PI3K/Akt pathway is involved in VEGFR2 expression and contributes to HCC angiogenesis [24]. In addition, some transcription factors such as NF-κB [27], GATA2 [28], TFII-I [29] and Sp1 [30], have also been identified to participate in regulation of VEGFR2 expression. Transcription factors GATA2 and TFII show to change matrix stiffness-mediated VEGFR2 expression in Human Dermal Microvascular Endothelial [12]. This finding prompts us to speculate that activation of target signal pathway and nuclear translocation of transcription factor may mediate matrix stiffness-upregulated VEGFR2 in HUVECs. In our study, higher matrix stiffness significantly upregulated the expression of VEGFR2 and the phosphorylation levels of Akt in HUVECs. Inhibition of integrin αVβ5 obviously reversed VEGFR2 expression and Akt phosphorylation level in HUVECs grown on higher stiffness substrate. Given that PI3K/Akt pathway activates Sp1 and causes its nuclear translocation in some cancer cells [31, 32], we used PI3K inhibitor to inactive PI3K/Akt pathway for further observing the change of Sp1 expression in nuclear. Our results showed that PI3K inhibitor suppressed the expression nuclear Sp1 significantly, simultaneously attenuated the phosphorylation level of Akt and the expressions of VEGFR2. Taken all together, higher matrix stiffness upregulates VEGFR2 expression in HUVECs via activating αVβ5/Akt-Sp1 pathway. Here, it should be noted that the data provided here is unable to exclude the roles of other signal pathways in stiffness-mediated effects on

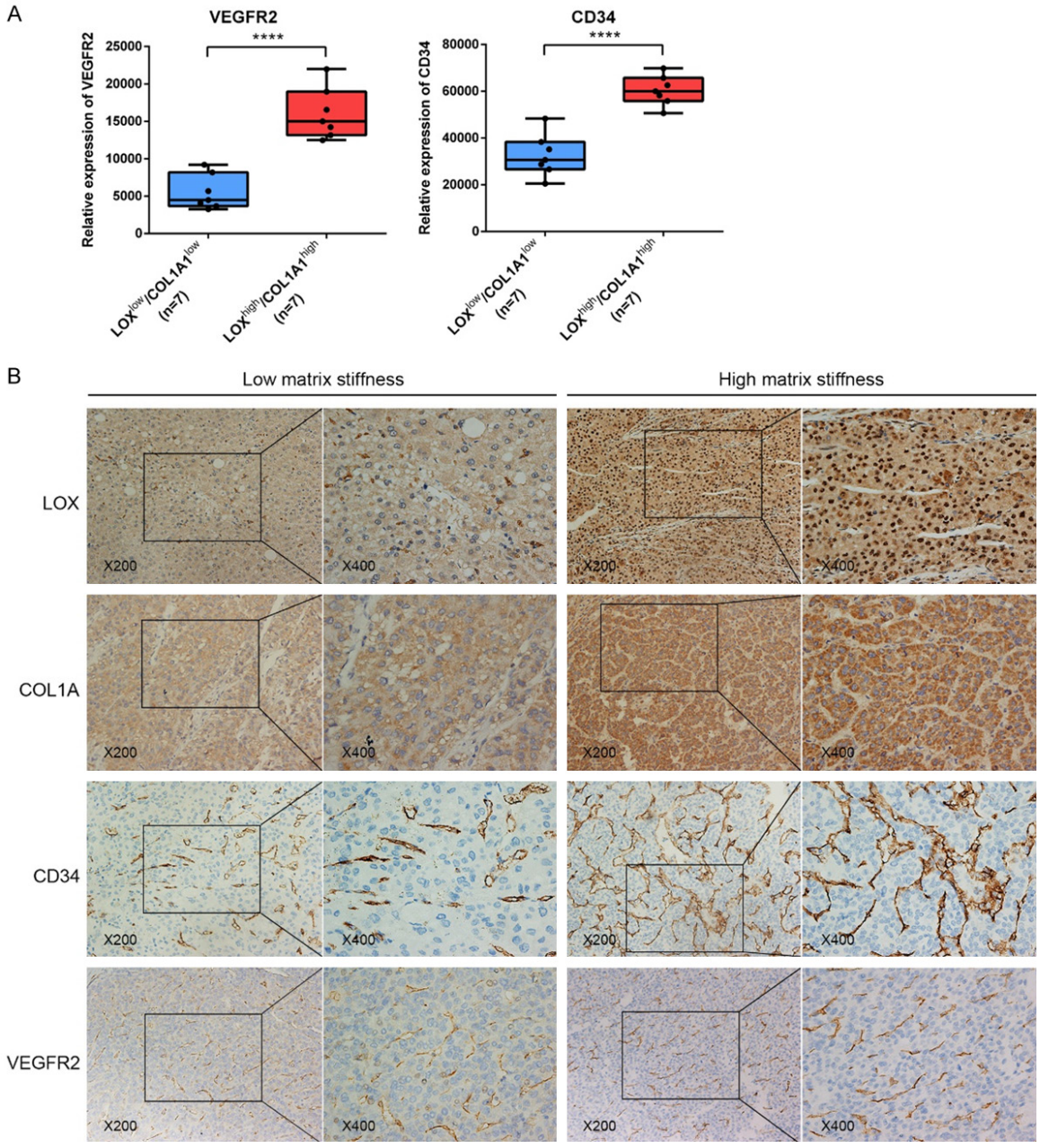

Figure 6. Correlation analysis between liver matrix stiffness and the VEGFR2/CD34 expression in HCC tissues. A. VEGFR2 and CD34 all exhibit obviously higher expression in $LOX^{high}/COL1A1^{high}$ group compared to in $LOX^{low}/COL1A1^{low}$ group. ****P<0.0001. B. Representative images of immunohistochemistry staining of COL1A1, LOX, VEGFR2 and CD34.

VEGFR expression in HUVECs. The associated target signal pathway merit to be further investigated in the future study by high throughput signal pathway screening.

In summary, integrin αVβ5/Akt/Sp1 pathway participated in matrix stiffness-mediated effects on VEGFR2 upregulation. This study combining with our previous report discloses a new paradigm in which higher matrix stiffness as an initiator drives HCC angiogenesis via upregulating both VEGFR2 expression in vascular endothelial cells and VEGF expression in HCC cells (**Figure 7**).

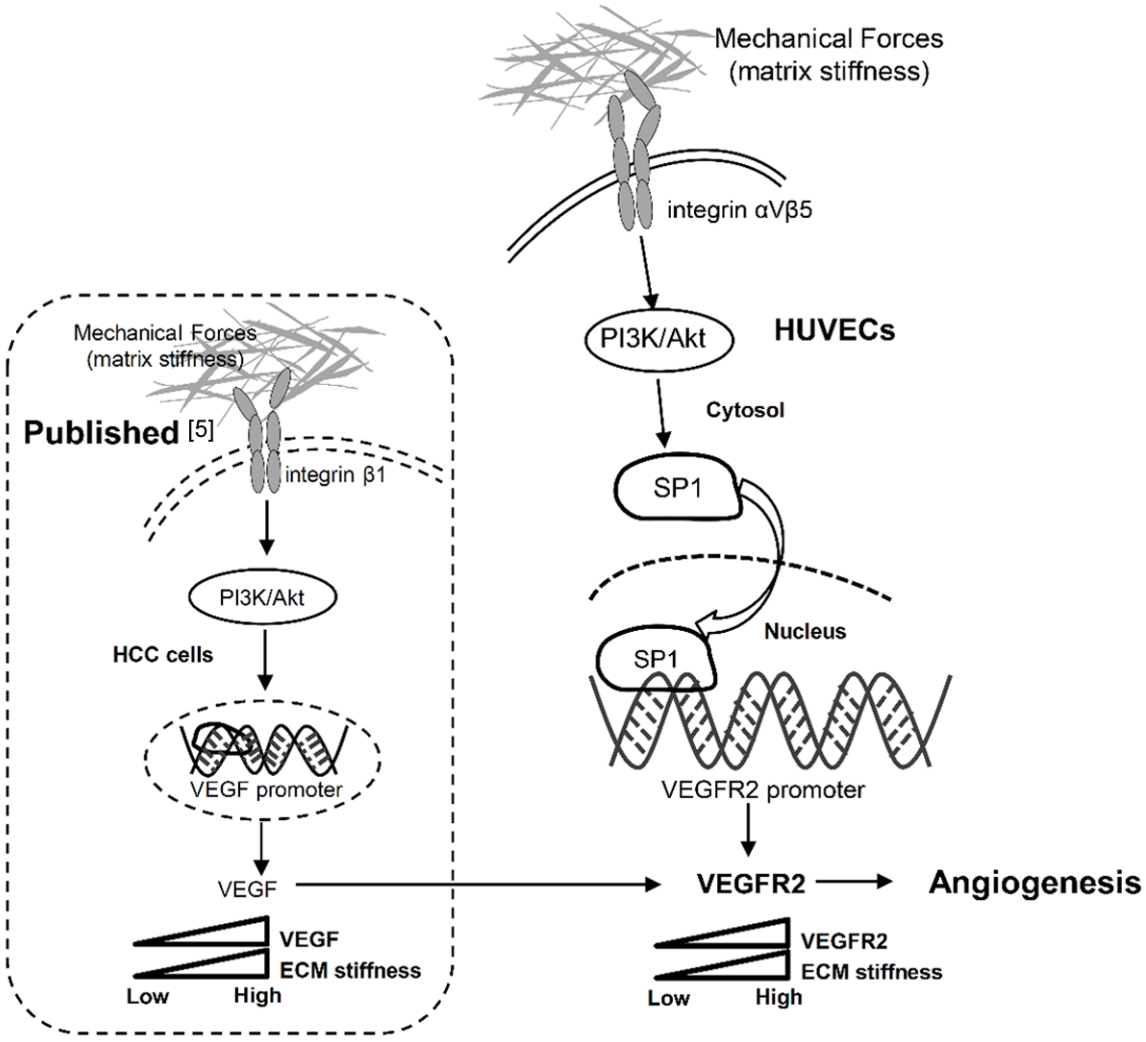

Figure 7. The proposed mechanism by which matrix stiffness upregulates the expression of VEGFR2 in HUVECs via activating integrin αVβ5/Akt/Sp1 pathway.

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Disclosure of conflict of interest

None.

Abbreviations

HCC, hepatocellular carcinoma; HUVECs, human umbilical vein endothelial cells; VEGFR2, vascular endothelial growth factor receptor 2; COL1, collagen 1; LOX, lysyl oxidase; VEGF, vascular endothelial growth factor.

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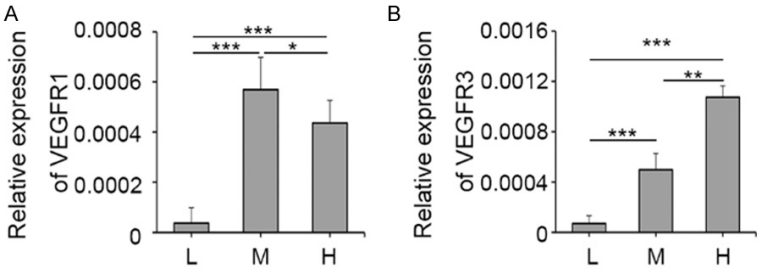

Figure S1. Increased matrix stiffness upregulates the expressions of VEGFR1 and VEGFR2 in HUVECs. The mRNA expression level of VEGFR1 (A) and VEGFR3 (B) in HUVECs grown on 6 kPa stiffness substrate (L), 10 kPa stiffness substrate (M) and 16 kPa stiffness substrate (H).

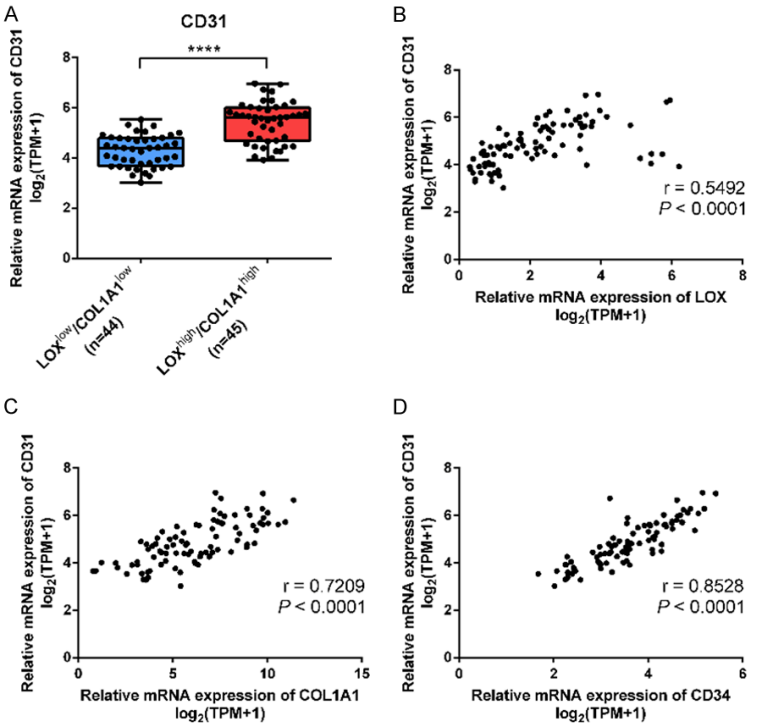

Figure S2. CD31 expression in TCGA-HCC patients and its correlation with matrix stiffness and CD34 expression. A-C. In TCGA-HCC patients, CD31 is significantly increased in LOX^{high}/$COL1A1^{high}$ group (n=45) compared to in LOX^{low}/$COL1A1^{low}$ group (n=44). Pearson correlation analysis reveals that the mRNA level of CD31 is positively correlated with the mRNA level of both LOX and COL1A1. D. Pearson correlation analysis indicates that the mRNA level of CD31 is positively correlated with the mRNA level of CD34. ****P<0.0001.