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Increasing matrix stiffness upregulates vascular endothelial growth factor expression in hepatocellular carcinoma cells mediated by integrin β1

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ABSTRACT

Matrix stiffness as a novel regulation factor involves in modulating the pathogenesis of hepatocellular carcinoma (HCC) invasion or metastasis. However, the mechanism by which matrix stiffness modulates HCC angiogenesis remains unknown. Here, using buffalo rat HCC models with different liver matrix stiffness backgrounds and an in vitro cell culture system of mechanically tunable Collagen1 (COL1)-coated polyacrylamide gel, we investigated the effects of different matrix stiffness levels on vascular endothelial growth factor (VEGF) expression in HCC cells and explored its regulatory mechanism for controlling HCC angiogenesis. Tissue microarray analysis showed that the expression levels of VEGF and CD31 were gradually upregulated in tumor tissues with increasing COL1 and lysyl oxidase (LOX) expression, indicating a positive correlation between tumor angiogenesis and matrix rigidity. The expression of VEGF and the phosphorylation levels of PI3K and Akt were all upregulated in HCC cells on high-stiffness gel than on low-stiffness gel. Meanwhile, alteration of intergrin β1 expression was found to be the most distinctive, implying that it might mediate the response of HCC cells to matrix stiffness simulation. After integrin β1 was blocked in HCC cells using specific monoclonal antibody, the expression of VEGF and the phosphorylation levels of PI3K and Akt at different culture times were accordingly suppressed and downregulated in the treatment group as compared with those in the control group. All data suggested that the extracellular matrix stiffness stimulation signal was transduced into HCC cells via integrin β1, and this signal activated the PI3K/Akt pathway and upregulated VEGF expression. This study unveils a new paradigm in which matrix stiffness as initiators to modulate HCC angiogenesis.

1. Background

Angiogenesis is a physiological process not only in growth and development but also in wound healing and granulation tissue formation. This process is also involved in the malignant transformation, spread, and metastasis of tumors. Angiogenesis expands the surface of endothelial cells, allowing more tumor cells to enter the circulation for metastasis [1,2] and secrets more matrix-degrading proteinases/growth factors to facilitate metastasis [3,4]. Tumor angiogenesis is complex and tightly regulated by the balance between endogenous angiogenic stimulators and inhibitors [2,5]. Increased secretion of angiogenic factors and/or downregulation of angiogenic inhibitors can trigger angiogenesis and further change the number of capillaries in a defined network. Vascular growth is modulated by key stimulation factors [6], including VEGF, fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), angiogenin, angiopoietin (Ang), endostatin, and thrombospondin-1. VEGF is a major contributor and regulator of angiogenesis [7]. The downstream molecular events of VEGF in endothelial cells have been characterized to date. The secreted VEGF binds to VEGF receptor-2 and triggers a tyrosine

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kinase signaling cascade that increases the production of proangiogenic factors, which promote vessel permeability (endothelial nitric oxide synthase producing nitric oxide), proliferation/survival (basic FGF), and migration (intercellular adhesion molecules/vascular cell adhesion molecules/matrix metalloproteinases), and finally differentiate these vessels into mature blood vessels [8–12]. Meanwhile, the upstream molecular events of VEGF driving its expression in cancer cells have been well documented, involving oncogenic gene mutation, hormones, cytokines, and various signaling molecules (nitric oxide, MAPK) [13–16]. Other microenvironmental factors, such as hypoxia and acidosis, also regulate the expression of VEGF [17,18]. However, the mechanism by which matrix stiffness modulates angiogenesis remains unclear.

HCC is one of the most frequent malignant tumors characterized by hypervascularity, high aggressiveness/metastasis, and poor prognosis. Approximately 80% of HCC patients emerge on a background of advanced fibrosis or liver cirrhosis [19], and their liver stiffness measurement has become a strong predictor of HCC development and progress in clinic [20]. Recent studies have suggested that the extracellular matrix (ECM) stiffness of HCC can regulate the proliferation and chemotherapeutic response of HCC cells [21], as well as increase integrin β1 expression, which is correlated with HCC invasion/metastasis [22]. Therefore, as a novel regulation factor, matrix stiffness may be involved in modulating the pathogenesis of HCC invasion or metastasis. However, little is known about the mechanism by which matrix stiffness regulates HCC angiogenesis. In the present study, we demonstrated that matrix stiffness and angiogenesis are positively correlated in liver tumor tissues and that increasing matrix stiffness upregulates VEGF expression in HCC cells via integrin β1 and further facilitates HCC angiogenesis.

2. Materials and methods

2.1. In vitro system of mechanically tunable COL1-coated polyacrylamide gel

An in vitro system of mechanically tunable COL1-coated polyacrylamide gel was established according to the method described by Pelham and Wang with some modifications [23]. For more details, see the Supplementary Materials and methods with this article.

2.2. Cells and cell culture

The highly metastatic HCC cell line MHCC97H established in the Liver Cancer Institute of Fudan University [24] was cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Biowest, South America Origin) and 1% penicillin/streptomycin (Gibco, USA). The lowly metastatic HCC cell line Hep3B (ATCC, USA) was cultured in minimum essential medium (Gibco, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. Approximately 3×10^5 HCC cells in 0.1 ml medium were spread onto a thin layer of COL1-coated polyacrylamide gel with tunable stiffness for 2 h at room temperature, then the cells were incubated for 24 h at 37 °C after adding 3 ml culture medium.

2.3. Establishment of buffalo rat HCC models with different liver matrix stiffness backgrounds

Buffalo rats (Charles River Laboratories, USA) with different liver stiffness levels were first induced by long-term intraperitoneal injection of different doses of carbon tetrachloride (CCl4) according to the method described by Mu with some modifications [25]. The rats were divided into three groups (six rats in each group): group 1 (control), subcutaneous injection of saline; group 2, subcutaneous injection of 100% CCl4 (3 ml/kg) followed by 50% CCl4 olive solution (2 ml/kg) once a week for 12 weeks; and group 3, subcutaneous injection of 100% CCl4 (3 ml/kg) followed by 50% CCl4 olive solution (2 ml/kg) twice a week for 12 weeks. These groups represented rat models with different liver stiffness levels. Subsequently, a subcutaneous tumor sample (2 mm × 2 mm × 2 mm) derived from the HCC cell line McA-RH7777 was orthotopically transplanted into these rat models. After 25 days, HCC tumor-bearing rats with different liver stiffness backgrounds were formed. The rats were executed, and their liver tumor tissues were collected, fixed in 4% formaldehyde for pathological analysis, and then snap-frozen in liquid nitrogen to store for gene/protein analysis. All animal protocols were approved by the Ethical Committee on Animal Experiments of the University of Fudan Animal Care Committee, Shanghai, China.

2.4. Tissue microarray and immunohistochemistry

A tissue microarray slide was constructed in collaboration with Shanghai Superchip Company, Ltd. Basing on the results of hematoxylin and eosin-stained tumor tissue slides, two cores containing optimal tumor content were positioned and taken by punch cores from a formalin-fixed, paraffin-embedded tumor tissue.

Immunohistochemical staining was performed as described in our previous work [4]. The detailed protocol can be found in the Supplementary Materials and methods.

2.5. Western blot

For the detailed procedures of Western blot, see in the Supplementary Materials and methods.

2.6. Integrin β1 antibody blocked integrin β1 receptor in HCC cells

HCC cells were preincubated with an antibody against integrin β1 (50 μg/ml, Cell Signal Technology, Danvers, MA) for 2 h to block integrin β1 subtype. Subsequently, the cells were seeded on COL1-coated polyacrylamide gels with tunable stiffness to assess the changes in VEGF and its related signal molecules.

2.7. Statistical analysis

Data were analyzed using SPSS software (version 16.0). Data were expressed as the mean ± SD. Statistical analysis was performed by one-way ANOVA and multiple linear regression. $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Expression levels of CD31, VEGF, LOX, and COL1 in HCC tissue with different matrix stiffness backgrounds

Three groups of rat HCC models with different matrix stiffness backgrounds were established by intraperitoneal long-term injection of doses of CCl4 combining with orthotopic implantation of subcutaneous tumor. We collected these tumor tissue samples and constructed a HCC tissue microarray to define the relationship between matrix stiffness and VEGF expression/HCC angiogenesis. The expression levels of COL1 and LOX were obviously different among three groups, moreover its expression in groups 3 and 2 were remarkably higher than that in group 1. Representative samples of immunohistochemical staining are shown in Fig. 1A. This finding suggested that there existed a distinct change in the degree of matrix stiffness among the three groups, and the established rat

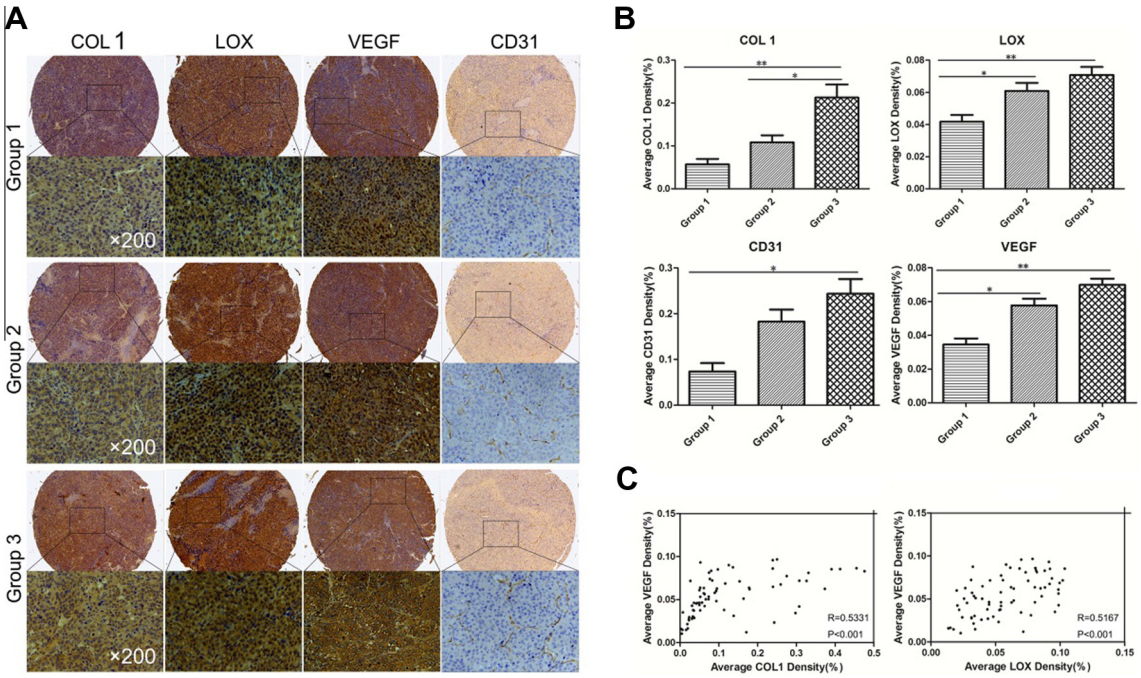

Fig. 1. Expression of COL1, LOX, VEGF and CD31 in HCC tissue with different matrix stiffness backgrounds. (A) Representative HCC tumor samples show the expression levels of COL1, LOX, VEGF, and CD31. Groups 1, 2, and 3 are defined in Section 2. (B) Graphs showing the densities of COL1, LOX, VEGF and CD31 expression in the three groups. In each case, error bars represent SD, $^{*}p < 0.05$ and $^{**}p < 0.01$. (C) Multiple linear regression indicates that the levels of VEGF are positively correlated with the expression levels of LOX and COL1.

HCC models with different matrix stiffness backgrounds were successful. Additionally, the expression levels of VEGF and CD31 in groups 3 and 2 were also significantly higher than that in group 1. Multiple linear regression analysis showed that the expression levels of VEGF were positively correlated with the levels of COL1 ($r = 0.533$) and LOX ($r = 0.517$). These data described above reveal that increasing matrix stiffness may be involved in the regulation of VEGF expression and thus affect angiogenesis.

3.2. Increasing matrix stiffness upregulates VEGF expression and activates the PI3K/Akt signaling pathway in vitro

An in vitro system of mechanically tunable COL1-coated polyacrylamide gel was established to mirror the pathological matrix stiffness of different liver diseases. Three polyacrylamide gels (see Table 1) designated as high-stiffness gel (HSG, 16 kPa), medium-stiffness gel (MSG, 10 kPa), and low-stiffness gel (LSM, 6 kPa), which represent the stiffness levels of cirrhosis, fibrosis, and normal liver tissue [26], respectively, were selected to determine the effect of matrix stiffness on the expression of VEGF in HCC cells. The morphologies of MHCC97H and Hep3B cells on COL1-coated polyacrylamide gels with different stiffness levels are shown in Fig. 2, and their morphologies changed from small and round (on LSM) to well spread and flat (on HSG). The expression level of VEGF was significantly higher in HCC cells on HSG than on LSG and MSG. As the gel stiffness was increased, the expression of VEGF was gradually upregulated in both MHCC97H and Hep3B cells (Fig. 3A), suggesting that increasing matrix stiffness upregulates VEGF expression in HCC cells. Additionally, the phosphorylation

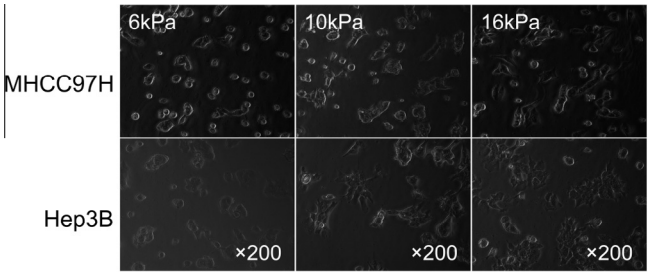

Fig. 2. Morphological changes in HCC cells cultured on COL1-coated polyacrylamide gel with tunable stiffness. Morphology of MHCC97H and Hep3B cells cultured on high-stiffness gel (16 kPa), medium-stiffness gel (10 kPa), and low-stiffness gel (6 kPa).

levels of PI3K and Akt in HCC cells were significantly upregulated with increasing stiffness (Fig. 3A). It indicates that the PI3K/ Akt signaling pathway can be activated under high matrix stiffness stimulation.

3.3. Integrin subtypes in HCC cells cultured on mechanically tunable Col1-coated polyacrylamide gel

All available antibodies against different integrin subtypes were used to screen which subtype of integrin was responsible for the response of HCC cells to different matrix stiffness levels. Minimal difference in the expression patterns of integrin subtypes was found between the MHCC97H and Hep3B cells on the same stiffness gel (Fig. 3B). Integrin subtypes α5, β1, αV, and β4 were all

Table 1
Stiffness value of polyacrylamide gel substrates.

Bis (%)	30% Acr (μl)	2% Bis (μl)	1 M HEPES (μl)	10% APS (μl)	TEMED (μl)	H_2O (μl)	Total volume (μl)	Stiffness value (Pa)
0.09	1000	135	30	30	3	1802	3000	5623.704
0.19	1000	285	30	30	3	1652	3000	9952.764
0.50	1000	750	30	30	3	1187	3000	16032.990

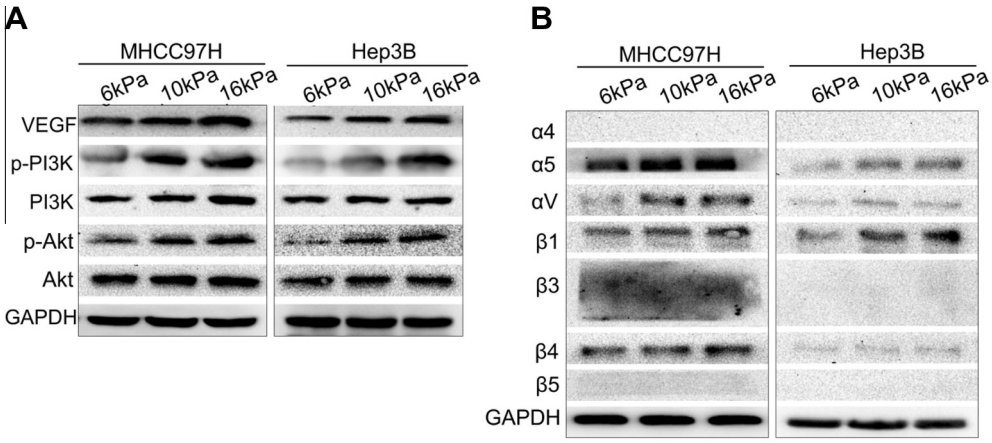

Fig. 3. Analysis of VEGF expression, activation of the PI3K/Akt signaling pathway and intergrin subtype in HCC cells cultured on COL1-coated polyacrylamide gel with tunable stiffness. (A) Increasing matrix stiffness upregulates VEGF expression and activates the PI3K/Akt signaling pathway in both MHCC97H and Hep3B cells. (B) Analysis of integrin subtypes in HCC cells cultured on high-stiffness gel (16 kPa), medium-stiffness gel (10 kPa), and low-stiffness gel (6 kPa).

highly expressed as matrix stiffness was increased in the highly metastatic MHCC97H cells; the expression levels of integrin subtypes β1 and αV were remarkably changed, but those of integrin subtypes β5, β3, and α4 were undetectable. Only integrin subtypes α5, β1, and αV were highly expressed in the lowly metastatic Hep3B cells; particularly, the upregulation of integrin β1 and α5 was distinct, whereas integrin subtypes β5, β3, and α4 were undetectable. Thus, the high expression of integrin β1, a major mechanotransducer, might mediate extracellular mechanical stimulation signals into HCC cells.

3.4. Matrix stiffness modulates VEGF expression through the integrin β1/PI3K/Akt signaling pathway

According to the detected VEGF and integrin β1 expression levels in HCC cells cultured on COL1-coated polyacrylamide gels with different stiffness levels (Fig. 3), we selected HSG as the stimulator to clarify the detailed mechanism by which integrin β1 mediates VEGF expression. As shown in Fig. 4A, the expression of VEGF in the treated MHCC97H and Hep3B cells with an antibody blocking against integrinβ1 was all suppressed at different culture times

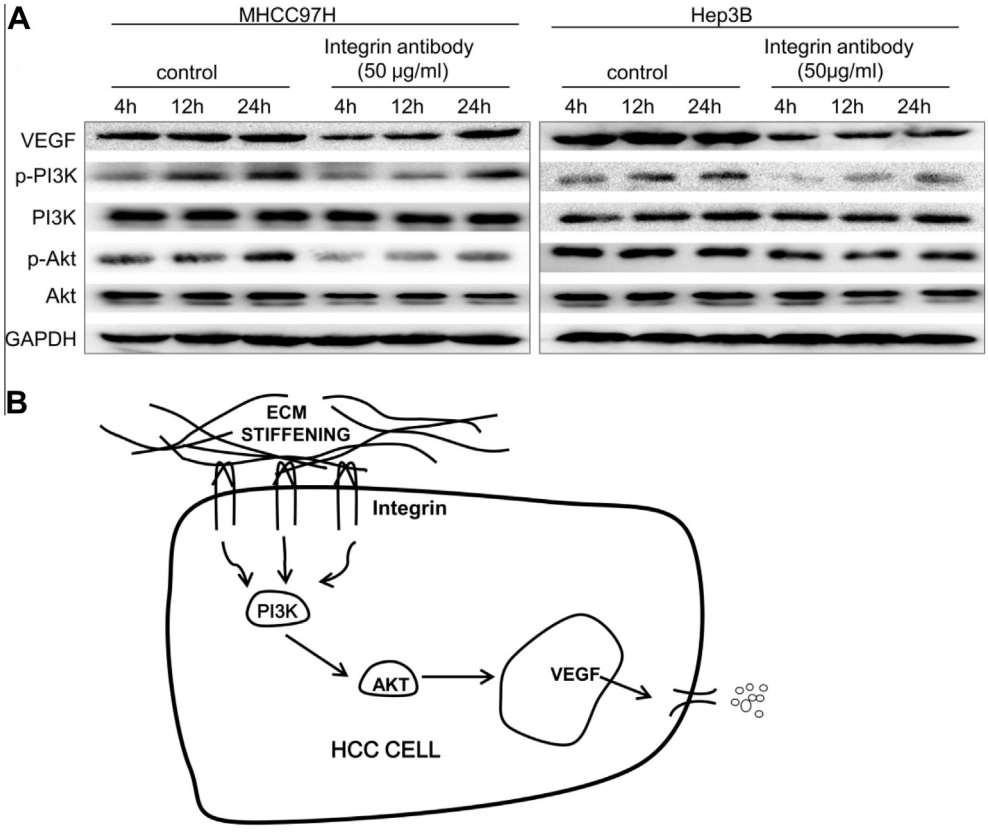

Fig. 4. Increasing matrix stiffness upregulates VEGF expression through the integrin β1/PI3K/AKT pathway. (A) The expression of VEGF in the treated MHCC97H and Hep3B cells with an antibody blocking against integrin β1 was suppressed at different culture times as compared with the corresponding controls. Moreover, the phosphorylation levels of PI3K and AKT were also all downregulated at different culture times. (B) Schematic of the proposed mechanism by which matrix stiffness initiates the integrin β1/PI3K/AKT pathway to modulate VEGF expression.

compared with the corresponding controls. Moreover, the phosphorylation levels of PI3K and Akt were also downregulated at different culture times. These data illuminated that ECM stiffness stimulation signal was transduced into the HCC cells via integrin β1 and that increasing matrix stiffness activated the PI3K/Akt pathway and upregulated VEGF expression.

4. Discussion

The growth and development of HCC cells depend on their ability to recruit blood vessels by forming new vessels through angiogenesis. The neovasculature in liver tumor is highly abnormal, and abnormalities in neovascularization and angioarchitecture indirectly reflect the pathological progression of HCC [2,8,27]. Many known chemical stimulators, such as VEGF, FGF, PDGF, Ang1, and Ang2, have been documented to be involved in the regulation of angiogenesis in tumor [8]. Moreover, the degrees of some angiogenic factors increase with pathological histology grade, portal venous thrombosis, and tumor capsular invasion [28]. VEGF is the most important regulator of the growth and migration of endothelial cells. It also induces the capillary into the tumor to supply the required nutrients. High VEGF expression contributes to the progression from small dysplastic nodules through neoplastic lesions to large HCC [8], also appears in tumor tissues and serum of most HCC patients. The expression of VEGF is correlated with tumor stage, vascular invasion, and metastasis [8]. VEGF level is a useful index for predicting the overall survival and for defining prognosis [29]. Some specific agents targeting VEGF in HCC angiogenesis, including VEGF receptors (sorafenib and sunitinib) or VEGF binders (bevacizumab), have entered clinic or clinical trials and shown a promising novel treatment for this disease [27,28,30]. However, the mechanism by which matrix stiffness modulates VEGF expression in HCC angiogenesis remains dismal partly because of the lack of an ideal animal model and an in vitro cell experimental platform. Most established HCC animal models do not have underlying cirrhosis. This study is the first to establish novel buffalo rat HCC models with different liver matrix stiffness backgrounds and to make an HCC tissue microarray to clarify the relationship between matrix stiffness and VEGF expression/HCC angiogenesis. COL1 is one of the most abundant ECM scaffolding proteins in the stroma, and LOX catalyzes the cross-linking of collagens or elastin and regulates the tensile strength of tissue. Both COL1 and LOX can better indicate the degree of matrix stiffness or fibrosis stage of liver tumor. The matrix stiffness accordingly increased as the expression levels of COL1 and LOX were gradually increased in tumor tissues (Fig. 1). Meanwhile, the expression levels of VEGF and CD31 in tumor tissue were also upregulated, indicating that angiogenesis was enhanced. Correlation analysis showed a positive correlation between VEGF expression in tumor exografts and matrix rigidity. These findings raise the possibility that increasing matrix stiffness may be involved in the regulation of VEGF expression and thereby facilitate angiogenesis. Other studies also demonstrated that the overexpression of markers for microvessel density implicate in the development and progression of HCC, and affecting the prognosis of patients [31]. But, the effects of matrix stiffness on HCC angiogenesis were little mentioned in these studies.

Subsequently, we established an in vitro system of COL1-coated polyacrylamide gel with tunable stiffness to explore the molecular mechanism of matrix stiffness modulating angiogenesis. Gels with different degrees of stiffness (16, 10, and 6 kPa) represented the pathological matrix stiffness levels of cirrhosis, fibrosis, and normal liver tissue, respectively. As gel stiffness increased, the expression of VEGF was significantly upregulated in both MHCC97H and Hep3B cells (Fig. 3A), and the phosphorylation levels of PI3K and Akt were remarkably elevated in these two HCC cells (Fig. 3A). The expression level of VEGF increased with increasing matrix stiffness, which is consistent with the results found in liver tumor tissues with high liver matrix stiffness backgrounds. These results adequately prove that changes in matrix stiffness stimulate and modulate the expression of VEGF in HCC cells. Simultaneously, increasing matrix stiffness activates the PI3K/Akt signaling pathway. PI3K/Akt is one of the major signaling pathways in the regulation of VEGF expression [32]. Genetic alteration (Ras, Src, Her2/Neu) or binding of some growth factors (epidermal growth factor, hepatocyte growth factor, and insulin-like growth factor) to their tyrosine kinase receptors activates Ras and subsequently the Raf/MEK/MAPK and PI3K/Akt pathways. This activation enhances the transcriptional activities of transcription factors (AP-1, HIF-1, Sp-1, or STAT3) and eventually controls VEGF gene transcription [32]. The activation of the PI3K/Akt signaling pathway is correlated with the regulation of VEGF expression in HCC cells [33], ovarian cancer cells [34,35], and breast cancer cells [36,37]. Combining these previous and present findings, we can conclude that high matrix stiffness stimulation activates the PI3K/Akt signaling pathway and further upregulates VEGF expression. Subsequently, we further investigated which molecule passes the information about the mechanical status of ECM stiffness into the cell and activates the PI3K/Akt pathway. Integrins are transmembrane receptors that can mediate the attachment of a cell to its surrounding cells and matrix; they also transduce cues from the ECM to initiate biochemical signaling and regulate cell behavior [38]. Either exogenous or endogenous force can activate integrins, facilitate their nucleation and clustering, and drive their maturation into focal adhesions [39]. Increased stiffness of the ECM as observed in tumors in vivo may promote integrin clustering [40]. Furthermore, tissue stiffness could drive the expression of malignant phenotypes through force-dependent regulation of integrin expression, activity, or adhesion [41]. Integrin levels and signaling are altered in "stiff" tumors [42]. In the present study, we compared the expression levels of integrin subtypes in HCC cells under different matrix stiffness stimulation. The upregulation of integrin β1 with increasing gel stiffness was the most distinct among all subtypes in the two HCC cell lines. Previous reports also revealed that integrin β1 is overexpressed in liver tumors with hepatic cirrhosis and that the level of integrin β1 increases in HepG2 cells cultured on stiff substrates as compared with those culture on control substrates. Integrin β1 expression is higher in epithelia on rigid 2D substrata than on a compliant 3D matrix [43], and matrix rigidity increases integrin β1 expression [44]. The present study showed that the expression of integrin β1 in HCC tissues with high matrix stiffness background was significantly higher than that in HCC tissues with low matrix stiffness background (data not shown). This result supports that increasing matrix stiffness upregulates integrin β1 expression. Accordingly, integrin β1 may be the most important integrin subtype in this event and mediates the signal transduction of mechanical stiffness of the ECM into HCC cells.

We used a specific antibody to block integrin β1 in HCC cells and then plated on HSG to clarify whether or not integrin β1 mediates VEGF expression in HCC cells. The expression of VEGF in the treated HCC cells was suppressed at different culture times as compared with that of the corresponding controls. Moreover, the phosphorylation levels of PI3K and Akt were also downregulated. Integrin β1 blocking not only inhibits the activation of PI3K/Akt in HCC cells but also downregulates VEGF expression. These findings suggest that integrin β1 specifically transduces the stimulation signal from matrix stiffness into HCC cells and influences intracellular PI3K/Akt activation and VEGF expression. To the best of our knowledge, the study is the first to report that matrix stiffness modulates VEGF expression in HCC cells via the integrin β1/PI3K/Akt pathway.

In summary, this study provides new insights into the mechanism by which matrix stiffness regulates VEGF expression in HCC cells via the integrin β1/PI3K/Akt pathway. This study reveals the functions of matrix stiffness in HCC angiogenesis and also provides a potential target on the integrin β1/PI3K/Akt pathway for HCC angiogenesis to prevent HCC growth and development.

Competing interests

The authors declared that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.079.

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