Acta Biochim Biophys Sin 2011, 43: 930–939 | © The Author 2011. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmr094.
Advance Access Publication 19 October 2011

Original Article

The influence of hyaluronic acid on vascular endothelial cell proliferation and the relationship with ezrin/merlin expression

Wei Mo[1†], Cuixia Yang[2†], Yiwen Liu[2], Yiqing He[2], Yingzhi Wang[2], and Feng Gao[2*]

[1]Department of Pathogen Biology and Immunology, Guangzhou Medical University, Guangzhou 510182, China
[2]Department of Molecular Biology, Shanghai Sixth People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200233, China
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-21-64369181-58702; Fax: +86-21-64701361; E-mail: gaofengc@online.sh.cn

It has been established that hyaluronic acid (HA) glycans (nHA) and oligosaccharide (oHA) exert different effects on the biological function of the vascular endothelial cell (EC), resulting in altered regulation of angiogenesis. However, the specific mechanism is still unclear. Our study focused on the effects of nHA and oHA on the ezrin and merlin proteins in EC. The expression of ezrin and merlin was silenced by siRNA, and the regulation on EC growth as well as the mRNA expression and activation (phosphorylation) of ezrin and merlin stimulated by oHA and nHA was investigated. The results revealed that when treated with nHA, there was no significant change in ezrin expression or activation. After being treated with oHA, the expression and activation of ezrin were definitively increased whereas there were no obvious changes in merlin expression (including its phosphorylation). With ezrin expression silenced, the expression of merlin as well as its phosphorylation levels in nHA-stimulated human umbilical vein endothelial cells were notably elevated, while there was no significant change induced by oHA. With merlin expression silenced, no obvious change was found in the expression of ezrin (including its phosphorylation) induced by nHA. Conversely, the expression of ezrin and its activation was significantly improved after being treated with oHA. The results suggest that the mechanism for the promotion of EC proliferation by oHA is likely related to the expression and activation of ezrin, and the inhibition of EC proliferation by nHA is likely related to the expression and activation of merlin.

Keywords hyaluronan; ezrin; merlin; endothelial cell; proliferation

Received: June 22, 2011 Accepted: August 8, 2011

Introduction

In recent years, there has been a considerable increase in research of the hyaluronic acid (HA) molecule because more functions of HA has been revealed in physiology and pathology [1]. HA is a ubiquitous glycosaminoglycan (GAG) and has a high molecular weight ($\sim 10^7$ Da). Unlike other GAGs, HA contains no sulfate groups or epimerized uronic acid residues. HA plays an important role in maintaining the extracellular matrix structure and regulating intercellular activities like cell–cell attachment and aggregation by engaging membrane-bound ligands, glycan, and other HA-binding proteins. Moreover, HA occupies a large hydrodynamic volume that greatly influences the hydration and physical properties of tissues. Commonly, natural HA with a high molecular weight is named as high-molecular-weight hyaluronan or native hyaluronan (nHA), and degraded HA fragments with low molecular weight is called hyaluronan oligosaccharides (oHA). It is widely believed that the activity of HA is dependent on its molecular mass. In fact, there are different numbers of disaccharide units in nHA and oHA, although they possess similar glycuronate structure, which consist of D-glycuronic acid and D-acetyl-glycosamine subunits. However, this distinction results in different, opposite regulation of cell growth and functions [2,3]. Key findings in this field showed that nHA were anti-angiogenic and anti-inflammatory in several *in vivo* assays, and inhibited endothelial cell (EC) migration, proliferation, and sprout formation [4–6]. In contrast, oHAs stimulate EC proliferation, motility, and tubule formation, and they promote inflammation, stimulate immunity, and induce angiogenesis in a variety of experimental systems. However, regardless of the mechanisms that oHAs are involved in, for the pathological and physiological processes of angiogenesis, the key effect of oHA on new blood vessel formation

might be its role in stimulating EC proliferation [5]. Notably, angiogenesis is generally considered a phenomenon that occurs during the proliferative phase and has drawn increasing attention in recent years for its biological functions in a variety of pathological processes; therefore, a role for HA oligosaccharides in regulating angiogenesis should be established to ascertain the possibility that it could be a target for clinical treatment. Furthermore, the biological function of the two kinds of HA molecules displays a strong dose-dependent effect, in other words, nHA and oHA should function over a range of certain concentrations. However, the detailed mechanism for nHA and oHA in EC function is unclear and needs more investigation [7,8].

A possible mechanism for the effect of HA on cellular function is that HA can bind to its receptors and initiate a series of downstream events [9–11], including the activation of a series of downstream molecules and the initiation of a signal transduction cascade. Elucidation of this mechanism may provide a new strategy in establishing the relationship of HA receptors to their downstream molecules and demonstrate a mechanism for the different proliferation behaviors induced by different sizes of HA products. CD44, which belongs to a family of transmembrane glycoproteins [12], is the most important HA receptor and acts as the essential target molecule in the regulation of blood vessel neogenesis and function. A number of studies and reports regarding interaction of CD44 and HA were almost about the activation of intercellular signal molecules; the function of kinases, such as Rho kinase, the activation of phosphatidase C, and the influence of this interaction on the MAPK pathway [9,10]. Moreover, there is another hotspot focusing on the cellular carboxyl terminus of CD44, which is linked with the amino terminus of ezrin protein family, involved in cell proliferation and migration, to trigger a series of cascade reactions and to display important functions [13].

The members of ezrin protein subfamily include ezrin, moesin, radixin (ERM), and merlin. Among these family members, ezrin and merlin are the important linkage proteins that interact with the cellular HA receptor CD44 and the cytoskeletal protein F-actin. Further, both of these proteins can interact with the cellular C-terminus of CD44 [14]; merlin probably competes with ezrin to bind with CD44 [15]. The absolute different properties of ezrin and merlin could influence cell proliferation and function. Ezrin is the essential molecule that stimulates proliferation and migration, whereas merlin is the molecule that inhibits cell proliferation. Several investigations revealed that the expression and activation of ezrin and merlin can be regulated by HA [16,17]. Therefore, it has been proposed that merlin and ezrin are probably involved in the different regulatory processes in vascular endothelial cells (VECs) associated with glycans and oligosaccharides [18]; however, there is no definitive evidence for this assertion.

In this study, we used human umbilical vein endothelial cell (HUVEC) based on the above theories, and observed the regulatory effect of nHA and oHA (4–20mer) on the growth of HUVEC as well as the expression and activation of ezrin and merlin. We further investigated the dependence of HUVEC proliferation on induction by distinct sizes of HA as well as the change in expression and activation of variant ezrin family members (ezrin and merlin). We also silenced ezrin and merlin genes using siRNA to determine the effect of ezrin and merlin expression on the regulation of VEC growth by nHA and oHA.

Materials and Methods

Materials

nHA and all other laboratory reagents, unless specified otherwise, were obtained from Sigma-Aldrich (St Louis, USA). oHAs (4–20mer) were prepared in our lab [7]. EBM-2 and EGM-2 media were purchased from Lonza (Cambrex, USA); Brdu kits were purchased from Chemicon (Billerica, USA); rabbit anti-human ezrin antibody, rabbit anti-phospho ezrin (Thr567) antibody, rabbit anti-human merlin antibody, and rabbit anti-phospho merlin (Ser518) were obtained from Cell Signaling Technology (Beverly, USA). siRNAs were designed and synthesized by Ambion (Austin, USA). Trizol reagents were purchased from Invitrogen (Carlsbad, USA).

Culture of HUVECs

HUVECs were isolated from umbilical cord veins. Briefly, umbilical vein was infused with 0.1% type I collagenase and then placed in a CO_2 incubator for 12 min at 37°C. After incubation, the cell suspension was collected and centrifuged at 1000 rpm for 10 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in EGM-2 media and then seeded in culture flasks coated with 0.5% gelatin and incubated at 37°C under 5% CO_2 in a humidified chamber. HUVECs grew to a confluent monolayer after 16–24 h of incubation. When 80% confluence was reached, the HUVECs were passaged at a ratio of 1:2.

Cell proliferation

The capacity of HUVEC proliferation was tested using the Brdu enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions. Cultured HUVECs were seeded into 96-well plates (1×10^4 cells/well). After a 24-h incubation, the cells were cultured in EBM-2 media and starved for 12 h. Then either 200 μl oHA [prepared in EBM-2 supplemented with 5% fetal bovine serum (FBS)] at final concentrations of 3, 5, 10, and 20 μg/ml, or 200 μl

nHA (prepared in EBM-2 supplemented with 5% FBS) at final concentrations of 50, 100, 200, and 500 μg/ml was added. As a control, 200 μl EBM-2 supplemented with 5% FBS was added. The cells were then incubated for 24, 48, 72 and 96 h and Brdu was added to each well. After incubation for 2 h, cells were fixed with methanol followed by addition of anti-Brdu monoclonal antibody for 30 min at room temperature. Then, secondary antibody [horseradish peroxidase (HRP)-conjugated polyclonal] was added. The mixture was further incubated for 30 min at room temperature, and washed three times with PBS. The coloration and absorbance were determined at 450 nm. The relative growth rate as a function of oHA or nHA was calculated using [oHA (nHA) group absorbance−control absorbance]/control absorbance×100%.

Immunofluorescence microscopy

HUVEC cells were plated at 2×10^5 cells/ml on glass coverslips and allowed to adhere overnight. The coverslips were then washed with PBS and fixed in 4% paraformaldehyde. After being permeabilized in 0.5% Triton X-100 for 15 min at room temperature, the cells were washed by PBS and incubated at 4°C overnight in PBS (pH 7.0) containing 1% bovine serum albumin with rabbit anti-human ezrin antibody, rabbit anti-human merlin antibody or 10% goat serum (Biostar, Wuhan, China). After that, the cells were washed three times and incubated at 4°C for 1 h with anti-rabbit IgG conjugated to TRITC (Biostar). Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI). After a final wash, the cells were visualized using an Olympus fluorescent microscope.

Reverse transcription-polymerase chain reaction

The total cellular RNA was exacted using Trizol reagents, and RNA was reverse-transcribed into cDNA using RNA PCR core kit (Takara, Shiga, Japan). The polymerase chain reaction (PCR) amplification reaction conditions were as follows: 94°C 1 min, 58.8°C 30 s, 72°C 1 min, and then 35 cycles. The PCR primer sequences (Invitrogen) were as follows: *ezrin* sense 5′-GTGGGATGCTCAAAGATAATGC-3′ and anti-sense 5′-CACCTCGATGGTGTCAGGCT-3′; *merlin* sense 5′-CGCACGAGGGATGAGTTG-3′ and anti-sense 5′-CAGGCTGTCACCAATGAGGT-3′; and *GAPDH* sense, 5′-CAACGAATTTGGCTACAGCA-3′ and anti-sense 5′-AGGGGTCTACATGGCAACTG-3′.

Western blot

HUVECs at log phase growth (4×10^5 cells/well) were seeded in six-well plates, washed twice using ice-cold PBS. Cells were harvested and homogenized in ice-cold sodium dodecylsulfate (SDS) lysis buffer. Total cell lysates were collected and equal quantities of protein were separated by 12% SDS-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked with Tris-buffered saline (TBS) containing 5% skimmed milk powder for 1 h, washed for 1 h in TBS, incubated with a 1/1000 dilution of primary antibodies against ezrin, phospho-ezrin (p-ezrin), merlin and phospho-merlin (p-merlin), and oscillated at room temperature for 3 h. Membranes then were washed with 1×Tris-buffered saline/Tween-20 (TBS/T) buffer for three times (5 min each) and incubated with HRP-conjugated secondary antibody for 1 h. After being washed again with 1×TBS/T buffer for three times (5 min each), the membranes were developed with the enhanced plus chemiluminescence assay (ECL+; Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's instructions; the films were exposed with an X-ray.

RNA interference

siRNAs were used to knock down ezrin and merlin in the HUVECs. Non-silenced and siControl HUVECs were used as control. The sequence of siRNAs were siRNA-ezrin1 (5′-GGACACUUGGAUUUUUUUUtt-3′), siRNA-ezrin2 (5′-GGUGGUAAAGACUAUCGGCtt-3′), siRNA-ezrin3 (5′-GGAUUUCCUACCUGGCUG tt-3′), siRNA-merlin1 (5′-GAUGAAAAGAUCUACUGCCtt-3′), siRNA-merlin2 (5′-AGAUCUACUGCCC CUCCUGAtt-3′), siRNA-merlin3 (5′-AAAGAUCUACUG CCCUCCUtt-3′). An electroporation instrument (Bio-Rad, Hercules, USA) was employed for transfection. Briefly, 400 μl of HUVECs (2.5×10^6 cells/ml cold EBM-2) together with 1.4 μg siRNA were added into a prechilled electrode gap cuvette (Bio-Rad). Then, the cuvette was inserted into an electrotransfer channel. After cells were shocked once using square wave 250 V for 1 ms, the cuvette was taken out immediately and 0.5 ml of complete medium EGM-2 pre-warmed at 37°C was added. Finally, the cells were seeded into six-well culture plates and incubated at 37°C in a humidified 5% CO_2 chamber.

Statistical analysis

Data are presented as the mean ± SD. The Student's *t*-test was used to compare the two groups. $P < 0.05$ was considered statistically significant. Reverse transcription (RT)−PCR and western blot bands were semi-quantified by the Image Pro Plus software which was purchased from Media Cybernetics Inc, USA.

Results

Effects of oHA and nHA on HUVEC proliferation

HUVECs were stimulated by various concentrations of oHA and nHA, and proliferation was detected using Brdu ELISA method. Our data showed that oHA at 3−20 μg/ml could stimulate vascular EC proliferation, and caused a

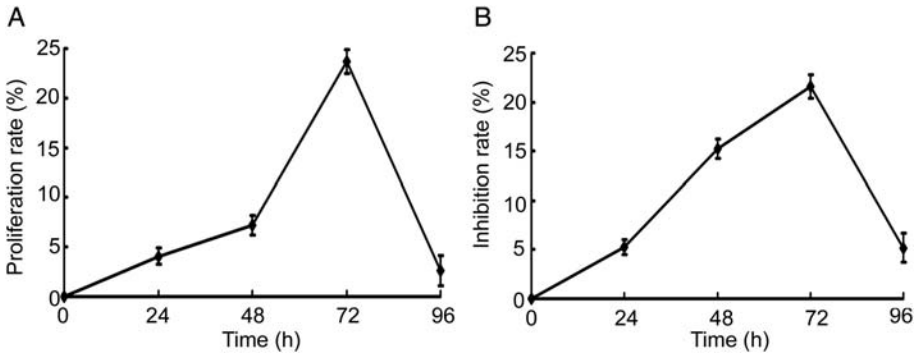

Figure 1 The effects of oHA and nHA on HUVEC proliferation at different time points After HUVECs were stimulated with 10 μg/ml oHA (A) or 200 μg/ml nHA (B) for different time points, the proliferation of HUVECs was determined using BrDu ELISA method. Data were expressed as mean ± SD from three different experiments.

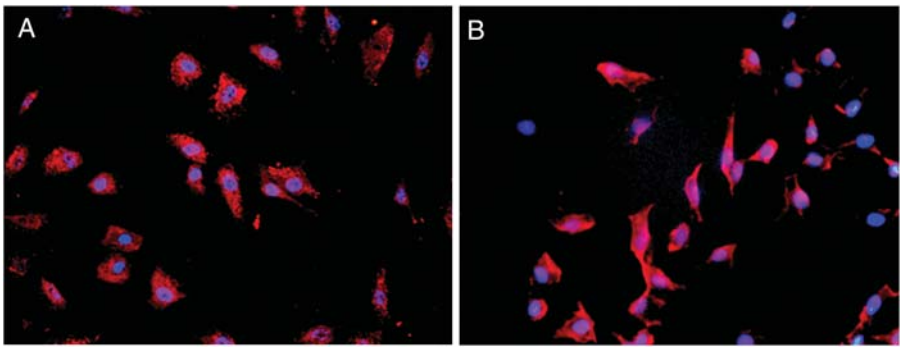

Figure 2 The expression and distribution of ezrin and merlin in HUVECs The cells were fixed and visualized by fluorescence microscopy (×400). Immunofluorescence stains was shown with red channel. (A) The expression and distribution of ezrin in HUVECs. (B) The expression and distribution of merlin in HUVECs.

maximum increase at 10 μg/ml (**Supplementary Fig. S1**), and nHA at 100–500 μg/ml could inhibit vascular EC proliferation ($P < 0.01$), and the best inhibition efficiency was reached at 200 μg/ml (**Supplementary Fig. S1**). Therefore, we chose 10 μg/ml of oHA and 200 μg/ml of nHA to investigate their stimulation mechanism on vascular EC proliferation. HUVECs were then treated with 10 μg/ml oHA or 200 μg/ml nHA for 24, 48, 72, and 96 h, and the proliferation data were recorded to calculate the relative proliferation rate. The time–proliferation rate curve is shown in **Fig. 1**. At 72 h, the best efficiency was reached. The proliferation-promoting rate of oHA reached 23.70% and the inhibition rate of nHA was 43.2%.

Expression and distribution of ezrin and merlin of HUVECs

To ascertain whether or not the isolated HUVECs express ezrin and merlin, the immunofluorescence staining method was used to detect the expression of ezrin and merlin in HUVECs. The data revealed a high expression of ezrin and merlin in HUVECs and their wide distribution in cells (**Fig. 2**).

Expression of *ezrin* and *merlin* mRNA in HUVECs stimulated by oHA and nHA

To determine whether or not the expression levels of *ezrin*/*merlin* mRNA in HUVECs stimulated by oHA/nHA are different, 10 μg/ml oHA and 200 μg/ml nHA were incubated with HUVECs for 12, 24, 48, and 72 h, and then the mRNA expression of *ezrin* and *merlin* were tested. Cells treated with PBS were treated as control. As shown in **Fig. 3**, *ezrin* mRNA expression increased 12–24 h after oHA treatment; however, there was no significant difference at other time points. The *merlin* mRNA expression showed no change at all time points under oHA treatment. Both *ezrin* and *merlin* mRNA expression in HUVECs after nHA treatment showed no significant difference (**Fig. 3**).

Expression of ezrin and merlin protein and their phosphorylation in HUVECs stimulated by oHA and nHA

HUVECs were treated with 10 μg/ml oHA and 200 μg/ml nHA for 24, 48, and 72 h, and the expression and phosphorylation levels of ezrin and merlin were analyzed. As illustrated in **Fig. 4(A,B,C)**, ezrin expression increased

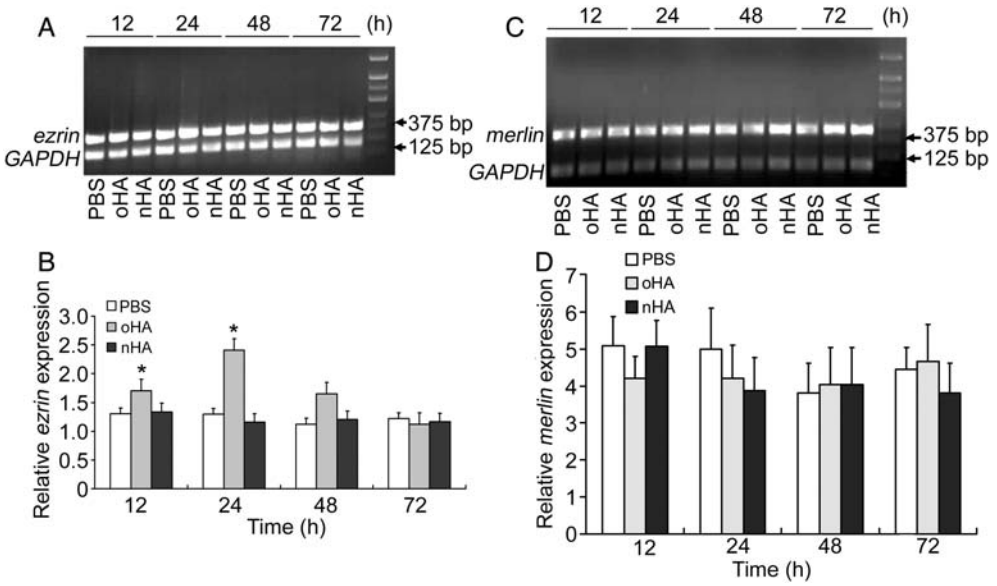

Figure 3 The effects of oHA and nHA on *ezrin*/*merlin* mRNA expression in HUVECs After treatment with 10 μg/ml oHA or 200 μg/ml nHA for different time points, total RNA was extracted and *ezrin*/*merlin* mRNA expression was detected by RT–PCR. *GAPDH* was used a reference. (A, B) The expression of *ezrin* mRNA after induction by oHA and nHA in HUVECs. (C, D) The expression of *merlin* mRNA after induction by oHA and nHA in HUVEC. Representative image of three independent experiments was shown. *$P < 0.05$ vs. PBS group.

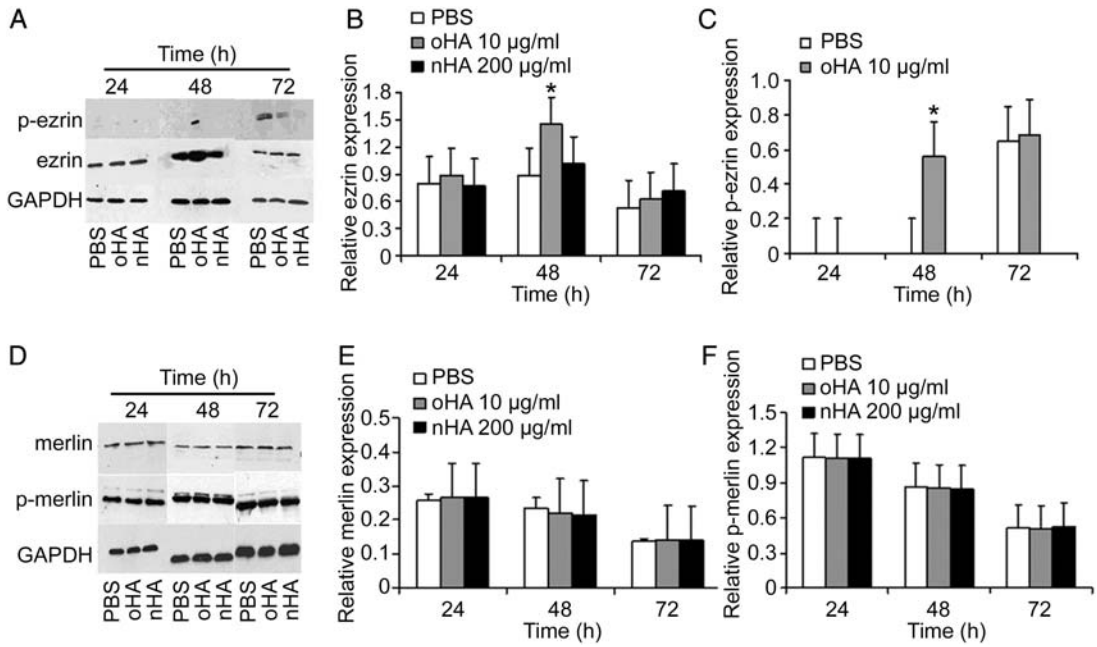

Figure 4 The effects of oHA and nHA on ezrin/merlin protein expression and their phosphorylation in HUVECs HUVECs were treated with 10 μg/ml oHA or 200 μg/ml nHA for different time points, and total cell lysates were extracted. Protein expression and phosphorylation of ezrin and merlin were examined by western blot using specific antibodies. (A, B, C) The expression of ezrin protein and p-ezrin (Thr567) of HUVECs stimulated by oHA and nHA for different time points. (D, E, F) The expression of merlin protein and p-merlin (Ser518) of HUVECs after induction by oHA and nHA for different time points. Representative image of three independent experiments was shown. GAPDH was used as a reference. *$P < 0.05$ vs. PBS group.

48 h after 10 μg/ml oHA treatment and predominant phosphorylation at Thr567 occurred. Further, the ezrin and p-ezrin protein expression in HUVECs treated with 200 μg/ml nHA showed no obvious change [**Fig 4(A,B,C)**]. There was no significant difference in merlin protein expression at any time point in HUVECs treated with 10 μg/ml oHA. Additionally, merlin and p-merlin protein expression in HUVECs incubated with 200 μg/ml nHA showed no obvious differences [**Fig. 4(D,E,F)**].

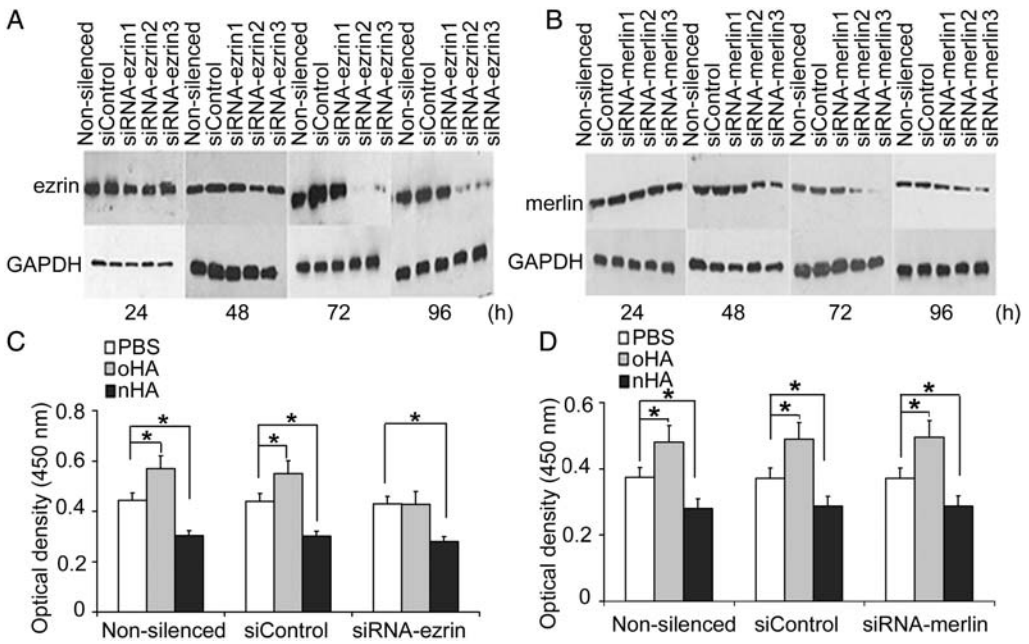

Figure 5 Effects of oHA and nHA on HUVECs proliferation after silencing of ezrin/merlin After HUVECs were transfected with siControl, three siRNA-ezrin or siRNA-merlin sequences for different time points, protein expression of ezrin (A) and merlin (B) was detected by western blot analysis. GAPDH was used as a reference. After silencing of ezrin (C) or merlin (D), HUVECs were stimulated with PBS, 10 μg/ml oHA and 200 μg/ml nHA for 72 h. The number of viable cells was determined using BrDu ELISA method. PBS and siControl are used as controls, respectively. Data were expressed as mean ± SD from three different experiments.

siRNA-mediated knockdown of ezrin and merlin expression

By western-blot analysis, we found that delivery of ezrin and merlin siRNA into HUVECs by electroporation led to a time-dependent decrease of ezrin and merlin expression and more than 70% reduction of ezrin/merlin protein level was achieved 72 h after knockdown by siRNA-ezrin2 or siRNA-merlin3 [**Fig. 5(A,B)**], demonstrating the efficacy and specificity of the ezrin/merlin siRNA used. Then, 10 μg/ml oHA and 200 μg/ml nHA were used to treat siRNA-HUVECs for 24, 48, 72, and 96 h, and the proliferation data were recorded to calculate the relative proliferation rate. The time–proliferation rate curve is shown in **Supplementary Fig. S2**. At 72 h, the best efficiency was reached.

Silencing of ezrin/merlin influences oHA/nHA-stimulated HUVEC proliferation

As illustrated in **Fig. 5(C)**, when ezrin was silenced, as compared with PBS control, 10 μg/ml oHA treatment for 72 h did not obviously change the proliferation of HUVECs, showing entire difference from its effects on non-silenced and siControl-treated HUVECs. That is to say, the effect of oHA on HUVEC proliferation following ezrin knocking-down by siRNA was remarkably decreased ($P < 0.05$). Meanwhile, 200 μg/ml nHA treatment still significantly inhibited HUVEC proliferation, just like its effects on non-silenced and siControl-treated HUVECs, suggesting that the role of nHA in HUVEC proliferation was not influenced by ezrin silencing.

When merlin was silenced, as compared with PBS control, 10 μg/ml oHA treatment caused an increased proliferation rate of HUVECs ($P < 0.01$) and 200 μg/ml nHA treatment caused a decrease of proliferation of HUVECs, in the same way as to the non-silenced and siControl HUVECs. In other words, HUVECs with merlin knocking-down by siRNA showed no obvious change in proliferation ability [**Fig. 5(D)**] either in the presence of PBS, oHA, or nHA.

The influence of oHA and nHA to mRNA expression of HUVECs after ezrin/merlin were silenced

After incubation with 10 μg/ml oHA, expression of the *merlin* gene in HUVECs with ezrin silencing showed no marked change [**Fig. 6(A,B)**], compared with the PBS treatment control. Treatment of 200 μg/ml nHA for 24 h induced an obvious increase in *merlin* mRNA expression in HUVECs with ezrin silencing [**Fig. 6(A,B)**].

Compared with the PBS treatment control, the expression of *ezrin* mRNA showed a significant increase in HUVECs with merlin silencing after 24 h treatment with 10 μg/ml oHA [**Fig. 6(C,D)**]. However, no significant change in the expression of *ezrin* mRNA in HUVECs with merlin silencing after incubation with 200 μg/ml nHA [**Fig. 6(C,D)**].

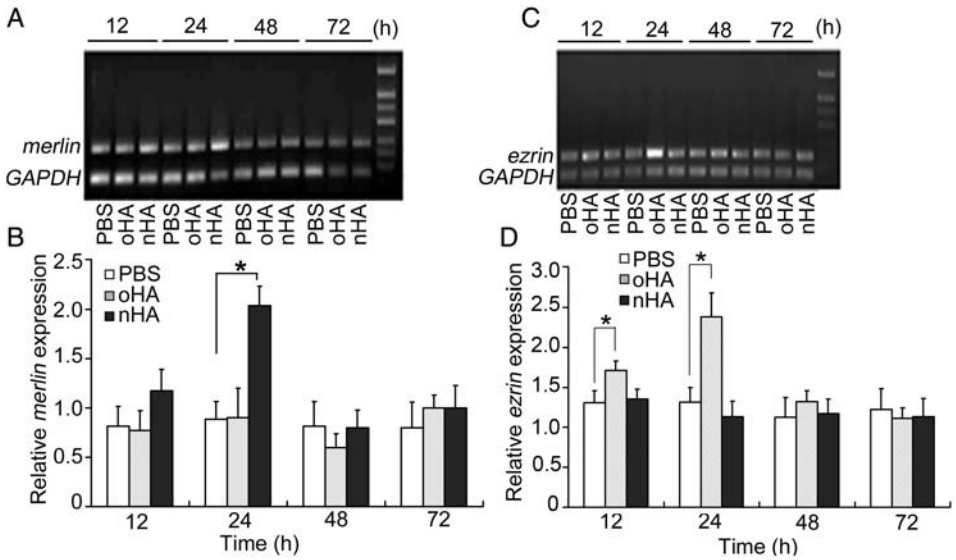

Figure 6 The effects of oHA and nHA on *ezrin*/merlin mRNA expression after silencing of ezrin/merlin After ezrin- or merlin-targeting siRNA was transfected into HUVECs, 10 μg/ml oHA or 200 μg/ml nHA was added. After incubation with oHA/nHA for different time points, total RNA was extracted and ezrin/merlin mRNA expression was determined by RT–PCR. (A, B) The effects of oHA and nHA on *merlin* mRNA expression of HUVECs after transfection of ezrin-targeting siRNA. (C, D) The effects of oHA and nHA on *ezrin* mRNA expression of HUVECs after transfection of merlin-targeting siRNA. GADPH is used as a reference.

The influence of oHA and nHA on protein expression in HUVECs with ezrin/merlin-silencing

Compared with the PBS treatment control, no marked change was seen in the expression of merlin and phosphorylated merlin at Ser518 in HUVECs with ezrin silencing after 72 h induction with 10 μg/ml oHA. However, an obvious increase was observed in expression of merlin and phosphorylated merlin at Ser518 in HUVECs with ezrin silencing after 200 μg/ml nHA induction [**Fig. 7(A,B)**].

After merlin silencing, HUVECs were treated with 10 μg/ml oHA for 72 h and then the expression of ezrin and phosphorylated ezrin at Thr567 were detected. The results showed a remarkable increase in the expression of ezrin and p-ezrin was observed compared with the PBS treatment control. In contrast, no marked change in the expression of ezrin and phosphorylated ezrin at Thr567 in HUVECs with merlin silencing was found after 200 μg/ml nHA treatment for 72 h [**Fig. 7(C,D)**].

Discussion

Since first described in 1934 [19], HA has been used across a wide variety of medical fields as diverse as neurosurgery and cutaneous wound healing. HA is a high-molecular-weight, non-sulfated linear GAG comprised of $<$10,000 repeating units of (β,1→4)-D-glucuronic acid-(β, 1→3)-*N*-acetyl-D-glucosamine [20], the simplest aminoglycan. Of the biological activities of HA, the most interesting may be that its degradation fragments are able to stimulate angiogenesis and become extraordinarily active in pathological events. However, as in tumor metastasis, wound repair, and inflammatory events, little is known about the role of such HA oligosaccharides in regulating angiogenesis and as a target for clinical treatments. Although the mechanism is uncertain, it might be initiated through cell surface receptors or HA-binding proteins, resulting in signal transduction activation and ultimately mitogenesis [5]. Moreover, oHA, the usual degradation product from nHA under pathological conditions, displayed a different, opposite function from that of nHA. Although hundreds of papers have been published on the biological activity of oHA and nHA, the detailed mechanism is unclear. In our study, we observed the regulatory effect of oHA or nHA on the growth of HUVEC, demonstrating the inhibition effect of nHA and the stimulation effect of oHA on HUVEC proliferation (**Fig. 1**, **Supplementary Fig. S1**), consistent with the previous reports [2,3]. Moreover, the mechanisms for the different influences of nHA and oHA on HUVEC function have not been explained definitely yet and are still a hotspot in the related fields. Based on the siRNA data, we propose in our study that nHA and oHA probably function through merlin and ezrin, respectively, to regulate HUVEC proliferation. However, additional work is ongoing to provide more direct evidence for this proposal. In addition, our study may provide support for establishing the relationship of HA with angiogenesis, furthering its clinical use.

Some studies suggested that nHA/oHA acted via modulating the activity of related downstream signal molecules to influence cell functions [7,21]. Most studies concerned the activation of cellular signal molecules and the function of kinases [10], suggesting the probable existence of a

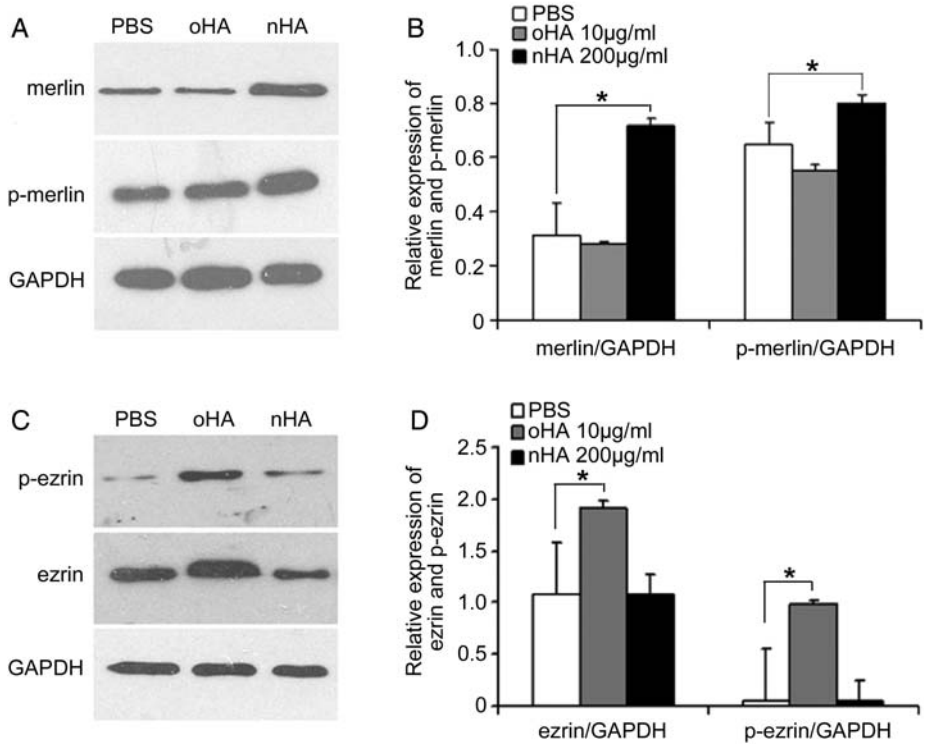

Figure 7 The effects of oHA and nHA on ezrin/merlin protein expression and their phosphorylation after silencing of ezrin/merlin After transfection with siRNA-ezrin or siRNA-merlin, HUVECs were treated with 10 μg/ml oHA or 200 μg/ml nHA for 72 h. Then, total cell lysates were extracted and ezrin/merlin protein expression and their phosphorylation levels were detected by western blot analysis. (A, B) The effects of oHA and nHA on merlin protein expression and phosphorylation of HUVECs after transfection with siRNA-ezrin before and 72 h after adding ezrin siRNA. (C, D) The effect of oHA and nHA on ezrin protein expression and phosphorylation of HUVECs after transfection with siRNA-ezrin. GADPH is used as a reference.

related molecule that can bind to HA receptor directly and participate the regulatory process of HA on cell proliferation. As the dominant receptor of HA, CD44 can bind to an ezrin protein subfamily, related to proliferation, via the cellular C-terminus [14]. Typical members of this subfamily include ezrin and merlin, which are the important proteins responsible for proliferation and inhibition and can bind to CD44; moreover, merlin probably competed with ezrin to bind to CD44 [15]. Therefore, we speculate that the mechanism of different molecular weight HA regulating cell proliferation is probably associated with the different members (ezrin and merlin) of the ezrin subfamily. Although previous study has proposed similar theories [18], up to date, there is no definitive evidence to support this speculation.

In our experiment, we observed that ezrin and merlin were widely expressed in the isolated HUVECs (**Fig. 2**). To find whether or not the members of ezrin family have a role in the regulatory effect of HA on EC proliferation, we also detected the expression of ezrin and merlin in the HUVECs after treatment with different molecular weight HA products. It showed that oHA could induce an increased expression of the ezrin mRNA and protein as well as significantly activate Thr567 phosphorylation on ezrin, which is related to proliferation (**Figs. 3** and **4**). This finding suggests that the regulation of oHA on EC proliferation probably acts via ezrin. However, the expression of *ezrin* and *merlin* in HUVECs after nHA treatment showed no significant difference, suggesting that merlin is probably not the main regulator for nHA inhibition of EC proliferation. The ERM (ezrin, radixin, moesin)-merlin dependence study revealed that both ezrin and merlin probably compete with each other in binding to the CD44 molecule [15], especially during the log phase of cell growth. Ezrin binds to the end of CD44 and the conformation of merlin molecule changes to a cyclic structure that binds to ezrin upon inactivation by phosphorylation, resulting in the absence of the characteristic inhibition of cell growth [15,22]. To avoid the interference of ezrin on the function of merlin, we designed an ezrin siRNA sequence, that is, when the ezrin gene was silenced, ECs were treated with nHA or oHA and the change in expression and activation of merlin can be observed. Also, the change in expression and activation of ezrin was studied in HUVECs treated with nHA or oHA after merlin silencing. As a result, we can elucidate the effects of HA molecular weight variants on cell proliferation via the two regulators: ezrin and merlin.

First, we silenced the ezrin gene (**Supplementary Fig. S3**). Forty-eight hours later, the expression of the merlin mRNA and protein was tested. The data showed that there was no change for merlin at the transcriptional level, but protein expression was increased. However, there was no change in the level of phosphorylation at Ser518 (**Supplementary Fig. S3**). This implies that the expression of merlin is probably competitively inhibited by the expression of ezrin, though its activation was not influenced. The fact that the proliferation ability of HUVECs with siRNA-ezrin was weaker than those without the siRNA supports ezrin as an important proliferation-promoting molecule [**Fig. 5(C)**]. After induction by 200 μg/ml nHA, the mRNA and protein expression levels of merlin and level of phosphorylation at Ser518 were significantly increased [**Fig. 6(A,B)** and **7(A,B)**]. This reveals that in the absence of ezrin, nHA can increase merlin expression, suggesting that nHA inhibits EC proliferation probably via a nHA-merlin pathway. In contrast, after ezrin was silenced, in HUVECs treated with 10 μg/ml oHA there was no significant change in expression of the merlin mRNA, protein, and phosphorylation at Ser518, implying that proliferation promotion of EC by oHA probably did not function via the oHA-merlin pathway. Taken together, we speculate that nHA inhibits EC proliferation possibly through a nHA-merlin cascade reaction. However, it was not the only pathway in this study and was influenced by many factors. Thus, a detailed mechanism requires further study. Based on the combination of the above reports [23–25] and our data, we propose that when the concentration of intercellular nHA increases, high-molecular-weight HA can cross-link with several consecutive receptors and promote cell aggregation, raising the cell density. Afterwards, a large amount of intercellular high-molecular-weight HA form the whole network structure and merlin protein can be released from the ERM complex and activated through dephosphorylation to function as an anti-proliferation agent. As a result, the growth status of cells is inhibited. In contrast, low-molecular-weight HA cannot cross-link with cells, thus, it cannot promote cell aggregation; therefore, phosphorylated merlin probably binds to CD44 with the ezrin- and moesin-forming complex, promoting cell proliferation and stimulating cell growth.

Second, we established the merlin siRNA interference model (**Supplementary Fig. S4)**, wherein the *merlin* gene was silenced, and there was no change in *ezrin* expression (**Supplementary Fig. S4**). In addition, there was no obvious change in the EC proliferation ability [**Fig. 5(D)** and **Supplementary Fig. S2**]. Next, we treated merlin-silenced HUVECs using oHA and nHA. The results showed the ezrin mRNA and protein expression as well as phosphorylation (Thr567) significantly increased in the oHA-stimulated group compared with the control group, indicating that the oHA-ezrin is the pathway for oHA promotion of EC proliferation and activation [**Fig. 6(C,D)** and **7(C,D)**]. However, the insignificant differences in ezrin mRNA and protein expression between the nHA-stimulated group and the PBS treatment control demonstrated that nHA cannot cause a change in ezrin expression, that is, the nHA-ezrin pathway was not detected [**Fig. 6(C,D)** and **7(C,D)**].

Combining the oHA induction data obtained before and after the two genes were silenced, we supposed that oHA can trigger the oHA-ezrin pathway and nHA can activate merlin (which inhibits proliferation). nHA-merlin and oHA-ezrin may function as the important pathway for HA regulation of HUVEC proliferation. As reported previously, ezrin and merlin can interact with the cellular C-terminus of CD44 [15,24]. We are carrying out experiments now to verify the role of CD44 in HA/ERM cellular activating pathway. Our study provides a theoretical basis for further mechanism exploration of oHA and nHA in regulating neovascularization, and further elucidation of these mechanisms will, undoubtedly, lead to a better understanding of HA function and may suggest therapeutic strategies for angiogenesis-related diseases.

Supplementary Data

Supplementary data are available at *ABBS* online.

Funding

This work was supported by grants from the Science and Technology Commission of Shanghai Municipality (Key Technology Support Programme, 08411952700), the National Natural Science Foundation of China (81071814), and the Program of Shanghai Subject Chief Scientist (11XD1404000).

References

1 Toole BP. Hyaluronan: from extracellular glue to pericellular cue. Nat Rev Cancer 2004, 4: 528–539.
2 Nissen NN, Shankar R, Gamelli RL, Singh A and DiPietro LA. Heparin and heparan sulphate protect basic fibroblast growth factor from non-enzymic glycosylation. Biochem J 1999, 338: 637–642.
3 Terramani TT, Eton D, Bui PA, Wang Y, Weaver FA and Yu H. Human macrovascular endothelial cells: optimization of culture conditions. In Vitro Cell Dev Biol Anim 2000, 36: 125–132.
4 Noble PW. Hyaluronan and its catabolic products in tissue injury and repair. Matrix Biol 2002, 21: 25–29.
5 Slevin M, Kumar S and Gaffney J. Angiogenic oligosaccharides of hyaluronan induce multiple signaling pathways affecting vascular endothelial cell mitogenic and wound healing responses. J Biol Chem 2002, 277: 41046–41059.
6 West DC, Hampson IN, Arnold F and Kumar S. Angiogenesis induced by degradation products of hyaluronic acid. Science 1985, 228: 1324–1326.

7 Gao F, Cao M, Yang C, He Y and Liu Y. Preparation and characterization of hyaluronan oligosaccharides for angiogenesis study. J Biomed Mater Res B Appl Biomater 2006, 78: 385–392.

8 Yang CX, Liu YW, He YQ and Gao F. Src kinase-MAPK signal pathway plays a role in proliferation of endothelial cells induced by o-HA. Fen Zi Xi Bao Sheng Wu Xue Bao 2006, 39: 495–501.

9 Bourguignon LY, Singleton PA, Zhu H and Zhou B. Hyaluronan promotes signaling interaction between CD44 and the transforming growth factor beta receptor I in metastatic breast tumor cells. J Biol Chem 2002, 277: 39703–39712.

10 Bourguignon LY, Gilad E, Brightman A, Diedrich F and Singleton P. Hyaluronan-CD44 interaction with leukemia-associated RhoGEF and epidermal growth factor receptor promotes Rho/Ras co-activation, phospholipase C epsilon-Ca2+ signaling, and cytoskeleton modification in head and neck squamous cell carcinoma cells. J Biol Chem 2006, 281: 14026–14040.

11 Turley EA, Noble PW and Bourguignon LY. Signaling properties of hyaluronan receptors. J Biol Chem 2002, 277: 4589–4592.

12 Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U and Bell JI. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc Natl Acad Sci USA 1992, 89: 12160–12164.

13 Zohar R, Suzuki N, Suzuki K, Arora P, Glogauer M, McCulloch CA and Sodek J. Intracellular osteopontin is an integral component of the CD44-ERM complex involved in cell migration. J Cell Physiol 2000, 184: 118–130.

14 Martin TA, Harrison G, Mansel RE and Jiang WG. The role of the CD44/ezrin complex in cancer metastasis. Crit Rev Oncol Hematol 2003, 46: 165–186.

15 Morrison H, Sherman LS, Legg J, Banine F, Isacke C, Haipek CA and Gutmann DH, *et al*. The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. Genes Dev 2001, 15: 968–980.

16 Bai Y, Liu YJ, Wang H, Xu Y, Stamenkovic I and Yu Q. Inhibition of the hyaluronan-CD44 interaction by merlin contributes to the tumor-suppressor activity of merlin. Oncogene 2007, 26: 836–850.

17 Brown KL, Birkenhead D, Lai JC, Li L, Li R and Johnson P. Regulation of hyaluronan binding by F-actin and colocalization of CD44 and phosphorylated ezrin/radixin/moesin (ERM) proteins in myeloid cells. Exp Cell Res 2005, 303: 400–414.

18 Ponta H, Sherman L and Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 2003, 4: 33–45.

19 Weigel PH, Frost SJ, LeBoeuf RD and McGary CT. The specific interaction between fibrin(ogen) and hyaluronan: possible consequences in haemostasis, inflammation and wound healing. Ciba Found Symp 1989, 143: 248–261; discussion 261–264, 281–285.

20 Jaffe EA, Nachman RL, Becker CG and Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973, 52: 2745–2756.

21 Gao F, Yang CX, Mo W, Liu YW and He YQ. Hyaluronan oligosaccharides are potential stimulators to angiogenesis via RHAMM mediated signal pathway in wound healing. Clin Invest Med 2008, 31: E106–E116.

22 Shaw RJ, McClatchey AI and Jacks T. Regulation of the neurofibromatosis type 2 tumor suppressor protein, merlin, by adhesion and growth arrest stimuli. J Biol Chem 1998, 273: 7757–7764.

23 Kissil JL, Johnson KC, Eckman MS and Jacks T. Merlin phosphorylation by p21-activated kinase 2 and effects of phosphorylation on merlin localization. J Biol Chem 2002, 277: 10394–10399.

24 Gao S, Dai Y, Yin M, Ye J, Li G and Yu J. Potential transcriptional regulatory regions exist upstream of the human ezrin gene promoter in esophageal carcinoma cells. Acta Biochim Biophys Sin 2011, 43: 455–464.

25 Guo LX, Zou K, Ju JH and Xie H. Hyaluronan promotes tumor lymphangiogenesis and intralymphantic tumor growth in xenografts. Acta Biochim Biophys Sin 2005, 37: 601–606.