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# Deficiency of all-spectrin affects endothelial cell–matrix contact and migration leading to impairment of angiogenesis in vitro



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## Abstract

**Background:** Precise coordination of cytoskeletal components and dynamic control of cell adhesion and migration are required for crucial cell processes such as differentiation and morphogenesis. We investigated the potential involvement of all-spectrin, a ubiquitous scaffolding element of the membrane skeleton, in the adhesion and angiogenesis mechanism.

**Methods:** The cell models were primary human umbilical vein endothelial cells (HUVECs) and a human dermal microvascular endothelial cell line (HMEC-1). After siRNA- and shRNA-mediated knockdown of all-spectrin, we assessed its expression and that of its partners and adhesion proteins using western blotting. The phenotypes of the control and spectrin-depleted cells were examined using immunofluorescence and video microscopy. Capillary tube formation was assessed using the thick gel Matrigel matrix-based method and a microscope equipped with a thermostatic chamber and a Nikon Biostation System camera.

**Results:** Knockdown of all-spectrin leads to: modified cell shape; actin cytoskeleton organization with the presence of peripheral actin patches; and decreased formation of stress fibers. Spectrin deficiency affects cell adhesion on laminin and fibronectin and cell motility. This included modification of the localization of adhesion molecules, such as  $\alpha V\beta 3$ - and  $\alpha 5$ -integrins, and organization of adhesion structures, such as focal points. Deficiency of all-spectrin can also affect the complex mechanism of in vitro capillary tube formation, as demonstrated in a model of angiogenesis. Live imaging revealed that impairment of capillary tube assembly was mainly associated with a significant decrease in cell projection length and stability. all-spectrin depletion is also associated with significantly decreased expression of three proteins involved in capillary tube formation and assembly: VE-cadherin, MCAM and  $\beta 3$ -integrin.

**Conclusion:** Our data confirm the role of all-spectrin in the control of cell adhesion and spreading. Moreover, our findings further support the participation of all-spectrin in capillary tube formation in vitro through control of adhesion molecules, such as integrins. This indicates a new function of all-spectrin in angiogenesis.

**Keywords:** all-spectrin, Endothelial cells, Adhesion, Migration, Actin cytoskeleton, Capillary tube assembly



## Introduction

The peripheral protein network of the red blood cell (RBC) membrane is mainly made of spectrin, actin and protein 4.1. Many studies on RBC, particularly analyses of mutations causing hereditary hemolytic anemia, have defined the importance of this network for maintaining cell shape and membrane integrity [1, 2].

The fundamental member of this network, spectrin, is thought to be present in all metazoan cells. In non-erythroid cells, it also occurs in endomembranes of the nucleus, cytoplasmic vesicles and the Golgi complexes [3]. Recent studies have shown that similar spectrin-based structures participate in both the establishment and maintenance of many highly specialized membrane domains that enable the cell to execute a variety of physiological functions, including morphogenesis [4–7].

In mammalian RBCs, spectrins mainly occur as large and flexible heterotetramers made of a set of two  $\alpha$ I and two  $\beta$ I subunits. These tetramers, as basic filaments of the network, cross-link short actin filaments via the actin-binding domain present in  $\beta$ -spectrins. In nucleated cells, there are a large number of possible combinations of spectrin isoforms. They are expressed from two genes that code  $\alpha$ -spectrins ( $\alpha$ I and  $\alpha$ II subunits) and five that code  $\beta$ -spectrins ( $\beta$ I through  $\beta$ V subunits) [8].

The occurrence of the spectrin-based skeleton in various cellular environments and its interaction with multiple proteins indicate that spectrin plays a role in many different physiological pathways involved in cell proliferation and differentiation. Some studies show that spectrin is essential for normal embryogenesis and organ development [9]. For example,  $\alpha$ II-spectrin appears to be a key component in cell spreading, tissue control and organ development in vertebrates [10]. Furthermore, in *Drosophila*, cortical  $\beta$ (H)-spectrin is directly involved in the function of *sosie*, which is an essential gene for oogenesis. Dysfunction of *sosie* in the germ and soma cell lines of *Drosophila* leads to defective organization of cytoplasmic actin networks [11].

Our recent studies also confirmed the contribution of  $\alpha$ II-spectrin in cell adhesion processes and in organization of the actin cytoskeleton in various cell models. siRNA-mediated depletion of  $\alpha$ II-spectrin in a melanoma cell line revealed defects in cell adhesion, such as changes in actin stress fibers, modification of focal adhesion and altered levels of some integrins [12]. Such alterations have also been observed in embryonic fibroblasts from  $\alpha$ II-spectrin<sup>-/-</sup> mice [10]. In human neuroblastoma cells, depletion of  $\alpha$ II-spectrin causes loss of adhesive properties in cell bodies and neurites [13]. Moreover, spectrin may also regulate the function and development of actin-rich, dynamic invadosomes by controlling the mobility of the integrins in the membrane [14]. Furthermore, the regulatory role of spectrin in cell–cell contact and adhesion processes in the first stages of immunological synapse (IS) formation was recently demonstrated. Loss of  $\alpha$ II-spectrin was associated with loss of actin-rich lamellipodia in activated T lymphocytes [15].

In this study, we used different endothelial cell (EC) models to investigate the involvement of  $\alpha$ II-spectrin in: cell adhesion to the extracellular matrix; cell motility; and actin cytoskeleton dynamics. We further analyzed the impact of  $\alpha$ II-spectrin depletion on the assembly of capillary tubes in vitro to ascertain its role in modulating endothelial migration during angiogenesis.

## Experimental procedure

### Cell culture

The human microvascular endothelial cell line HMEC-1 (ATCC, CRL-10636) was grown in MCDB131 (Gibco) supplemented with 15% FCS (FCS PAN Biotech), 2 mM L-glutamine, 1  $\mu$ g of dexamethasone (D8893, Sigma) and 100 ng of EGF (Invitrogen). The human umbilical vein endothelial cells (HUVECs) were cultured in M199 containing 20% FCS, 1% L-glutamine and 10 mM HEPES. The culture media contained 1% penicillin ( $10^3$  units/ml) and streptomycin ( $10^3$   $\mu$ g/ml) (Invitrogen). Cells were plated on plastic coated with gelatin 0.2% (Sigma) and incubated at 37 °C in a water-saturated atmosphere with 5% CO<sub>2</sub>. The HUVECs used for the experiments were from passages 1 to 5 and the HMEC-1 cells from passages 10 to 25.

### Transfection

HUVECs and HMEC-1 cells were transfected with either siRNA heteroduplexes or GFP-shRNA (Sp shRNA) plasmids using the HUVEC Nuclofactor Kit (Amaxa Biosystem) and the general JET PEI transfection reagent (Polyplus), respectively, according to the manufacturer's instructions. The culture medium was changed 24 h after transfection to remove transfection products.

Transfection efficiency was estimated via flow cytometry using a FACSCalibur flow cytometer (BD Biosciences) either with control siRNAs (non-relevant siRNA, Nr siRNA) labelled with Alexa Fluor 488 or 568 (Qiagen) for HUVEC and HMEC-1 cells, or with control plasmids expressing both non-relevant shRNA (Nr shRNA) and GFP for HMEC-1 cells. Cell viability was also determined using flow cytometry 24 h after transfection in the presence of 5  $\mu$ g/ml propidium iodide (PI).

The siRNA duplexes targeting human  $\alpha$ II-spectrin (Sp siRNA) were Dharmacon Individual siGENOME duplex D-009933-01, D-009933-02, D-009933-03 and D-009933-04, and Ambion Silencer Pre-designed siRNAs 12,798 and 142,727. The negative silencer control siRNAs (Nr siRNA) were Dharmacon siCONTROL Non-Targeting siRNA Pool. The shRNA plasmids targeting human  $\alpha$ II-spectrin (Sp shRNA) were SABiosciences KH18852G clone 1, 2, 3 and 4. We validated the efficiency of Sp siRNA 1 and 17 and Sp shRNA 3 and 4 in our experiments.

### Antibodies

Monoclonal antibodies directed against vinculin were obtained from Sigma-Aldrich;  $\alpha$ V $\beta$ 3-integrin from Chemicon International;  $\alpha$ 5-integrin from BD Biosciences; and  $\alpha$ II-spectrin clone AA6 from Biohit and Millipore. Polyclonal antibodies against lamin A/C were purchased from Santa Cruz Biotechnology; VASP and ABI-1 from Sigma-Aldrich; and VE-cadherin from Abcam. Polyclonal antibodies directed against  $\alpha$ II-spectrin were obtained after the immunization of guinea pig (Eurogentec) using the recombinant peptide SH3- $\alpha$ 8- $\alpha$ 11-His<sub>6</sub>  $\alpha$ II-spectrin repeats.

### Western blot analysis

After two washes with pre-warmed Dulbecco's PBS (Gibco), the cells were directly lysed on plates in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>) containing 1% SDS, anti-protease cocktail (Sigma) and OmniCleave

endonuclease (Epicentre). Protein concentrations were estimated in a colorimetric assay using the BCA method (microAssay Uptima) with BSA as the standard protein. Aliquots of cell lysates (between 20 and 40  $\mu\text{g}$ ) were resolved on SDS poly-acrylamide gels and transferred onto Protan nitrocellulose membrane (0.45  $\mu\text{m}$ ; Schleicher & Schuell) using a Tris-glycine buffer. After saturation in 5% non-fat-milk, 0.05% Tween 20, PBS buffer (pH 7.5), the membranes were probed overnight at 4 °C with the indicated primary antibodies. After extensive washing, blots were incubated for 1 h at room temperature with secondary antibodies conjugated with horseradish peroxidase (Nordic Immunological laboratories). Immune complexes were detected using the Supersignal West Pico chemiluminescence substrate (Pierce). The chemiluminescence was quantified using the Quantity One 1-D Analysis software (Bio-Rad) after acquisition with Molecular Imager Gel Doc (Bio-Rad).

### **Immunofluorescence studies**

Cells grown on CC2 or permanox slides (Nunc) were washed in pre-warmed Dulbecco's PBS, fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and saturated for 30 min either with buffered saline solution containing 0.1% BSA or with Image-iT Signal Enhancer (Molecular Probes). Primary and secondary antibody dilutions were made in background-reducing buffer (DakoCytomation, Invitrogen). In a final step, immunolabelled cells were mounted in ProLong Antifade Gold solution. Secondary labelled anti-IgG antibodies were purchased from Molecular Probes (Alexa Fluor 488 and 568). F-actin was labelled with Alexa Fluor 568 or 488 phalloidin (Molecular Probes). The fluorescence was observed via confocal microscopy using either a Zeiss LSM 510 META or a Nikon Eclipse TE300 microscope upgraded with a D-Eclipse C1 confocal system.

### **Static cell adhesion assays**

Adhesion assays were performed 48 h after transfection on culture dishes coated with either fibronectin or laminin 510/511. Control and transfected cells were respectively stained with the vital dyes Hoechst 33342 (1  $\mu\text{g}/\text{ml}$ ) and calcein (10  $\mu\text{g}/\text{ml}$ ) AM (Molecular Probes). After washing in D-PBS, the cells were detached using trypsin-EDTA and washed in complete culture medium, then both labelled cell populations were mixed in a 1/1 ratio. These mixtures were plated in triplicate on 12-well plates ( $2 \times 10^6$  cells per well) and incubated for 2 h at 37 °C in 5%  $\text{CO}_2$ . After two washes with complete warmed culture medium, the remaining adherent cells were visualized via fluorescence using an Evolution VF camera (Media Cybernetics). Ten images were acquired for each sample of mixed cells. Adherent cells were counted using Image-Pro Plus software. The results are expressed as the mean percentages of adherent transfected cells versus adherent control cells (100%). Spread cells were discriminated from round cells based on the fluorescence intensity of Calcein and Hoechst: the round cells showed a higher intensity.

### **Study of all-spectrin distribution during the adhesion process**

HMEC-1 cells were plated on CC2 slides coated with either fibronectin or laminin and incubated for 5, 10 and 30 min at 37 °C in 5%  $\text{CO}_2$ . Cells were gently washed once with

complete culture medium before fixation with 4% paraformaldehyde. The remaining adherent cells were detected using immunofluorescence after actin and  $\alpha$ II-spectrin labelling.

#### **Study of capillary tube formation via video microscopy**

Capillary tube formation was assessed using the thick gel Matrigel matrix-based method. Matrigel Basement Membrane Matrix Phenol Red Free (BD Biosciences) was first thawed at 4 °C for one night then mixed using cooled pipettes before being used to coat permanox coverslips on ice. Incubation was at 37 °C for 30 min to allow gel polymerization.

Dynamic studies of capillary tube formation were performed in HMEC-1 cells transfected with plasmids expressing GFP and either non-relevant shRNA (Nr shRNA-GFP) or shRNA targeted  $\alpha$ II-spectrin (Sp shRNA-GFP). Cells were plated on permanox slides coated with Matrigel at a density of  $10^5$  in a 2 ml final volume and then incubated for 12 h at 37 °C in 5% CO<sub>2</sub> in the Biostation system. Capillary tube formation was followed using a microscope equipped with a thermostatic chamber and a camera (Biostation system, Nikon). Images were registered every 2 min. Three principal characteristics were measured: the mean number of filopodia per cell, their maximal size and their stability. From three different experiments (analysis of 10 images per experiment), the number of filopodia was calculated by counting the maximal number of filopodia or other extensions formed by cells. The size of the filopodia was measured when filopodia were at their maximum length in the same images. Their stability was assessed by measuring the time interval between the beginning of filopodia formation and their retraction.

#### **Statistical analysis**

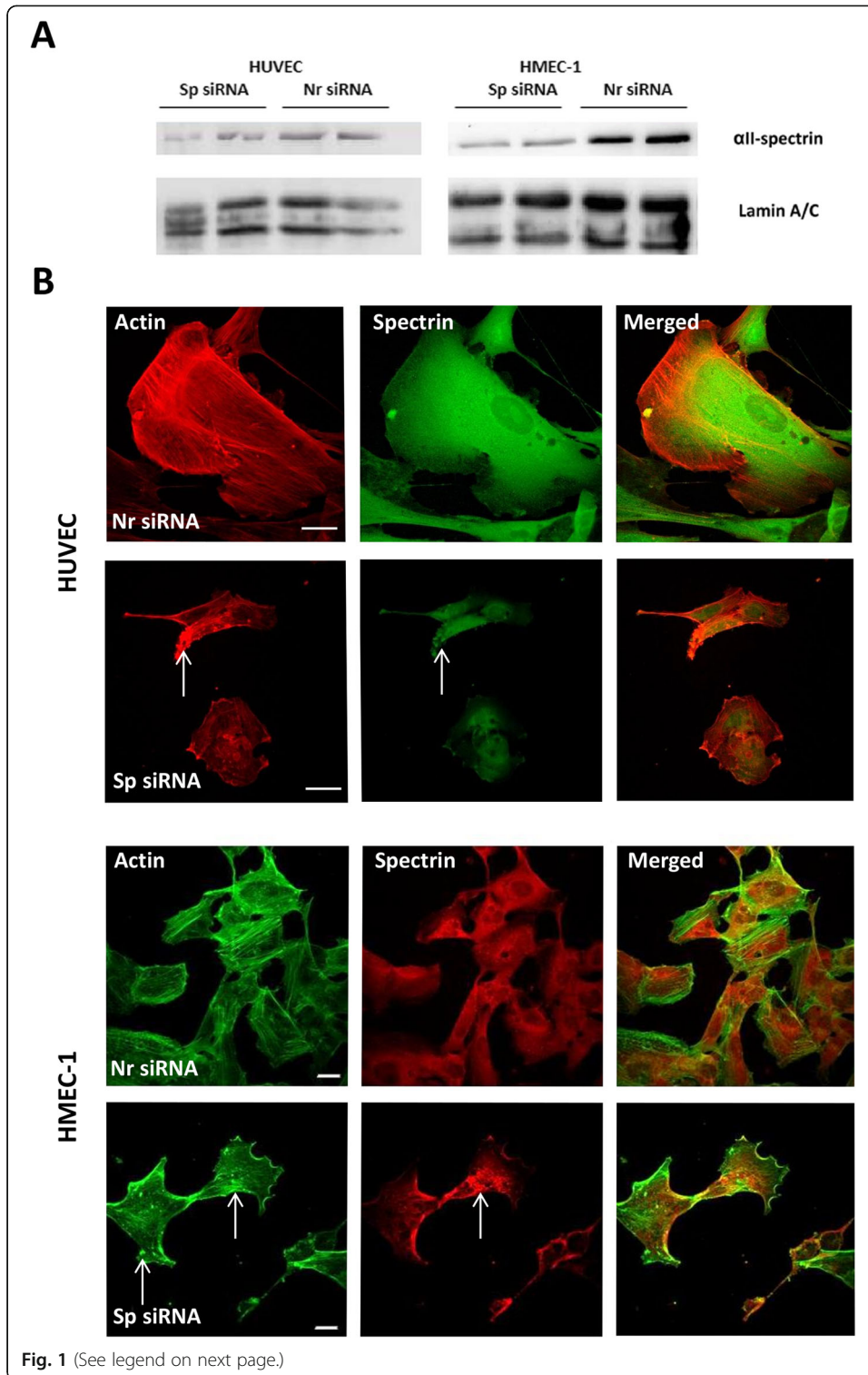
The statistical significance was mainly calculated using Student's test. Capillary tube experiments were analyzed using two tests: the Bartlett test for comparison of variance and the Mann-Whitney test.

## **Results**

### **Spectrin depletion modifies the actin cytoskeleton in endothelial cells**

We previously found that spectrin deficiency in a human melanoma cell line [12] and in T cells [15] is associated with modifications of the actin cytoskeleton and with defects in cell adhesion, spreading and cell–cell contact. To test whether these events, which are associated with spectrin loss, are common features in any cellular context, we here investigated the effects of spectrin knockdown using a RNAi strategy in two endothelial cell lines: HMEC-1 and HUVECs. Using at least two different siRNA or siRNA pools, a roughly 70% decrease in spectrin expression was obtained in both cell types as evaluated via western blot (Fig. 1a, Additional file 5: Figure S4).

Immunofluorescence showed that HUVEC and HMEC-1 cells treated with non-relevant siRNA (Fig. 1b) exhibit phenotype control cells (non-transfected cells, Additional file 1: Figure S1): the cell shape is not modified and the cells are well spread. Alpha II-spectrin labelled with different antibodies (mono- and polyclonal) is mainly present in the cytoplasm and around the nucleus, with weak labelling in the nucleus.



(See figure on previous page.)

**Fig. 1** all-spectrin depletion in endothelial cells modifies their shape and actin cytoskeleton. **a** Western blotting of all-spectrin in HMEC-1 cells and HUVECs. Lysates (20  $\mu$ g) of cells transfected with either siRNAs targeting all-spectrin (Sp siRNA) or non-relevant siRNA (Nr siRNA) were analyzed 72 h after transfection. all-spectrin and lamin A/C (used as a loading control) levels were checked using polyclonal antibodies. Knockdown of all-spectrin was efficient with two siRNAs (see the Experimental procedure section). Residual expression of all-spectrin in Sp siRNA-transfected cells was about 20 to 30%. The transfection efficiency in the cells was about 95% (as evaluated by flow cytometry, data not shown). **b** Analysis of cell morphology and actin cytoskeleton. HMEC-1 cells and HUVECs were transfected with siRNAs targeting either all-spectrin (Sp siRNA) or non-relevant siRNA (Nr siRNA). Cells were labelled 72 h after transfection with polyclonal antibodies directed against all-spectrin (labelled green in HUVECs and red in HMEC-1 cells) and phalloidin toxin detecting actin (red in HUVECs and green in HMEC-1 cells). Sp siRNA transfection induces a decreased labelling of all-spectrin that accumulates into some aggregates (arrows). Spectrin depletion also modifies the actin architecture in endothelial cells: depleted HUVECs and HMEC-1 cells present a marked phenotype with disorganization of stress fibers, patches and aggregates (arrows). Scale bar = 20  $\mu$ m

Spectrin is also present at the cell membrane and at the leading edge, where it can accumulate as some patches (Additional file 1: Figure S1). In most cells, phalloidin labelled actin is present mainly as stress fibers.

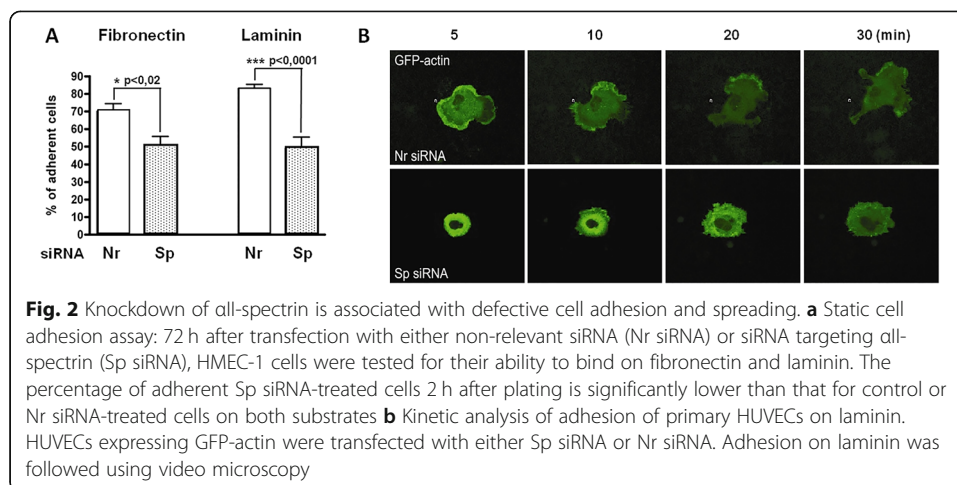
In cells treated with siRNA targeting  $\alpha$ II-spectrin, spectrin labelling is reduced, indicating efficient depletion (Fig. 1b). Decreased spectrin expression is associated with important modifications in cell shape, as manifested in the reduction in the size and number of adherent cells. In  $\alpha$ II-spectrin-depleted cells, the remaining spectrin accumulates as aggregates in the cytoplasm. Phalloidin labelling showed disorganization of the actin network in the form of a reduction in the basal stress fibers and the presence of actin patches or aggregates.

Video microscopy analysis of cells transfected with both GFP-actin and siRNAs (Nr and Sp) confirmed the observations obtained on fixed cells (Additional file 2: Figure S2): spectrin-depleted cells had reduced size with disorganization of the actin network and loss of stress fibers. In cells transfected with a non-relevant siRNA, GFP-actin produces very dynamic stress fibers and accumulates at the leading edge. By contrast, in spectrin-depleted cells stress fibers are not formed and GFP-actin accumulates in dynamic patches. These modifications within the actin skeleton, mainly the disappearance of stress fibers, indicate the links between spectrin and the actin-based cytoskeleton.

#### Cell adhesion and spreading are impaired in spectrin-deficient cells

The smaller size of spectrin-depleted cells correlates with impaired spreading. This raises the question of potential associated defects in cell adhesion and spreading. For endothelial HMEC-1 cells, these processes were assessed based on two cell matrix components: fibronectin and laminin 510/511.

Static adhesion assays performed 72 h after siRNA transfection showed a statistically significant decrease in the number of adherent cells treated with siRNA targeting spectrin compared to the number of adherent cells treated with non-relevant siRNA (as described in the Experimental procedure section). Compared to control cells, the percentage of remaining adherent cells treated with Sp siRNAs (obtained in four independent experiments) was around 50% on both matrices (Fig. 2a), while the number of adherent cells treated with Nr siRNA was reduced by 10 to 30% based on the matrix component.



The analysis of HMEC-1 cells 2 h after seeding on fibronectin, revealed a defective spreading for cells treated with siRNAs targeting spectrin. The ratio of spread versus non-spread cells in non-relevant siRNA-treated samples was 2.0, whereas it was three times less (0.7) in spectrin-depleted cells.

The beginning of the spreading process was followed in living cells transfected with both GFP-actin and siRNAs. At the beginning of the adhesion process (Fig. 2b, 5 min) and during spreading (Fig. 2b, 10 to 20 min), Nr siRNA-treated cells spread well, were motile and presented highly dynamic actin-rich lamellipodia (Fig. 2b and Additional file 3: Figure S3A and Additional file 4: Figure S3B). In spectrin-depleted cells, lamellipodia were less dynamic and actin was present in patches, confirming previous observations of fixed cells. The cells were smaller and more rounded. They also exhibited a lower mobility. All the Nr siRNA-treated cells were completely spread at 30 min after plating, whereas the spectrin-depleted cells remained rounded (Fig. 2b, 30 min). Therefore, spectrin depletion induces a defect in adhesion and spreading in both endothelial cell types.

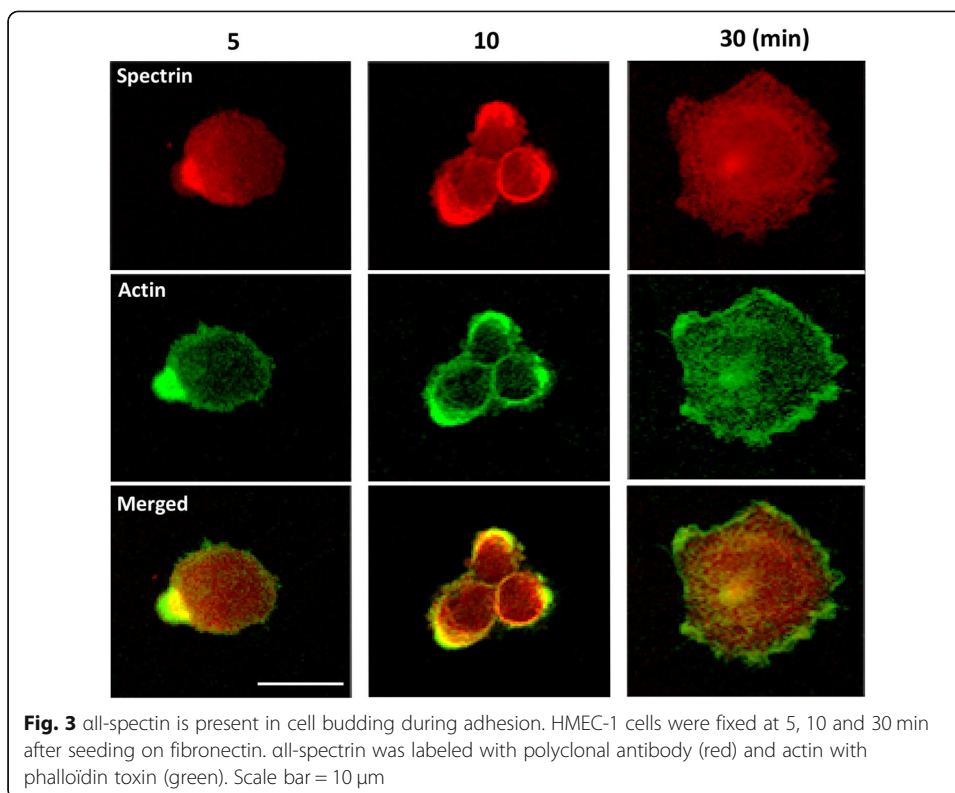
#### Spectrin is recruited at the leading edge during adhesion and spreading

As  $\alpha$ II-spectrin loss in endothelial cells causes a significant defect in cell adhesion and spreading, the localization of  $\alpha$ II-spectrin during these processes was investigated via immunofluorescence. HMEC-1 cells were plated on fibronectin-coated slides and fixed at different times. At the beginning of the adhesion process (5 and 10 min),  $\alpha$ II-spectrin accumulates in the cell budding and is partially colocalized with actin at their bases, with the actin more at the front of the membrane (Fig. 3). This particular accumulation of spectrin was observed not only in human endothelial cells, but also in human WM266 and Jurkat cells and mouse BF16 melanoma cells (data not shown). At 30 min after plating, the cells are well spread, and actin is mainly present at the front of the membrane. Spectrin labelling is mainly found in the cytoplasm with weak labelling at the membrane.

#### Adhesion structures such as focal points are modified in spectrin-depleted cells

As spectrin-depleted cells exhibit adhesion and spreading defects, the adhesion structures such as focal points were analyzed using antibodies directed against vinculin, a





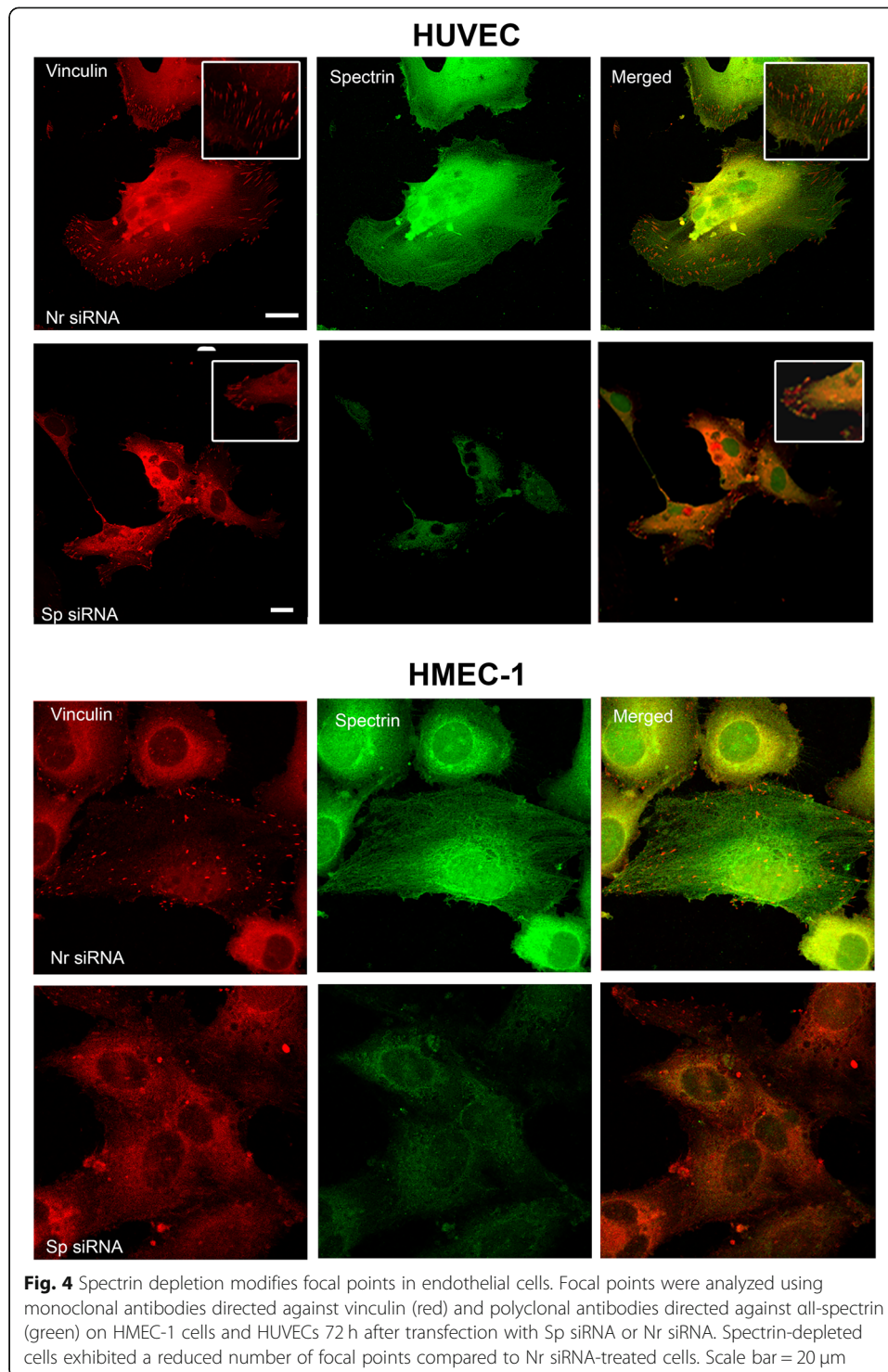
component of these structures (Fig. 4). Spectrin-depleted HMEC-1 cells and HUVECs presented fewer focal points.

Concerning adhesion molecules, we observed a modified location for  $\alpha$ 5-integrin and  $\alpha$ V $\beta$ 3-integrin. Depletion of spectrin is associated with aggregates of  $\alpha$ 5-integrin. We also observed the absence of  $\alpha$ V $\beta$ 3-integrin accumulation at the focal points. Instead, it formed aggregates in the cytoplasm, most of them without actin labelling (Fig. 5). As shown above, spectrin-depleted cells present a reduced size. Therefore, knockdown of  $\alpha$ II-spectrin is associated with a modification of adhesion structures and an abnormal distribution of the main adhesion proteins.

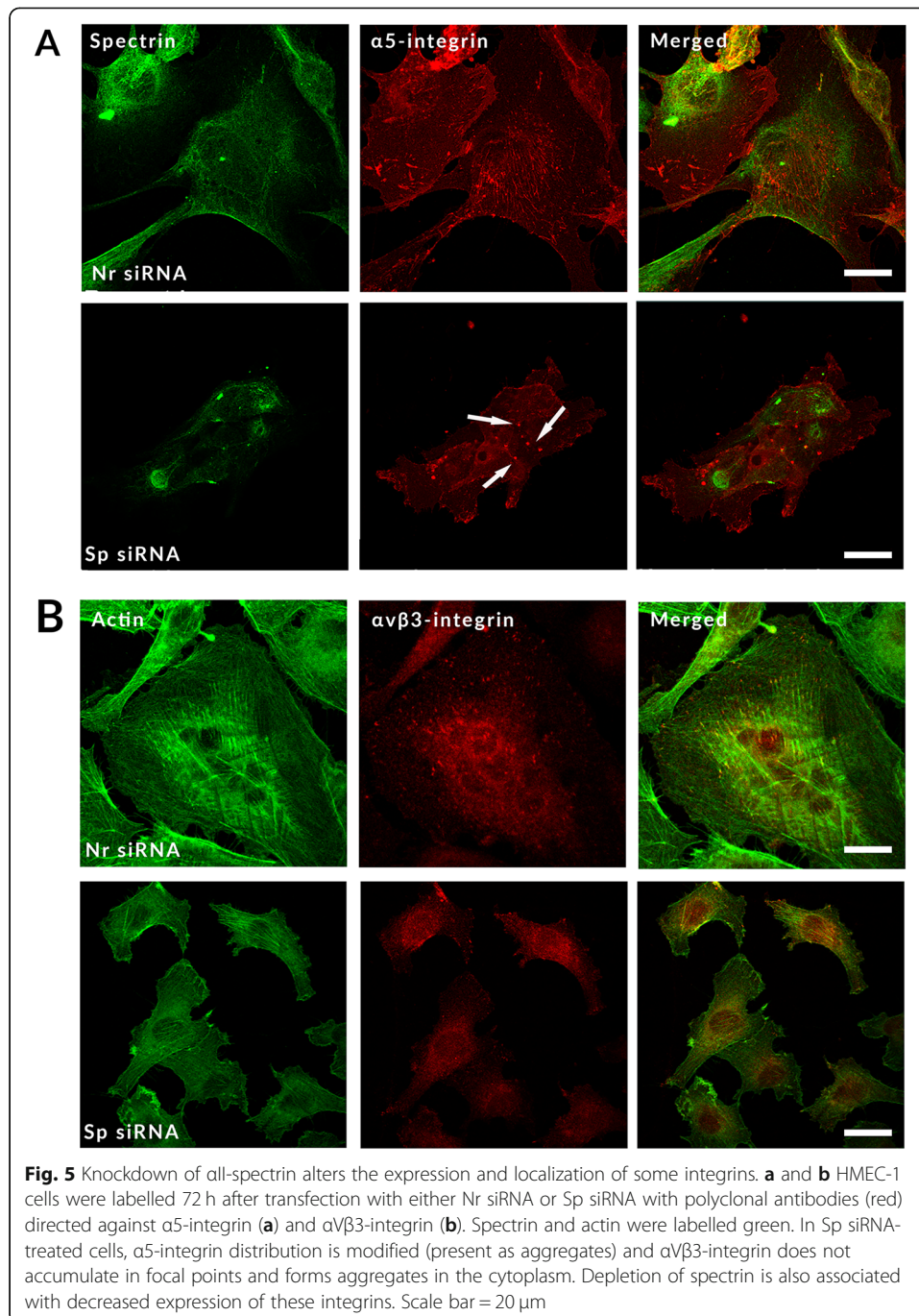
#### Spectrin depletion affects the formation of capillary tube structures on Matrigel

Since spectrin loss alters endothelial cell adhesion and spreading, we investigated the involvement of  $\alpha$ II-spectrin in the physiological mechanism of angiogenesis based on these two processes. Endothelial cells plated on Matrigel reorganize and form characteristic structures called capillary tube structures in a process that mimics angiogenesis. The ability of spectrin-depleted cells to participate in the formation of capillary tube structures on Matrigel was tested.

These experiments were performed on HMEC-1 cells transfected with a plasmid coding for both shRNAs and GFP. 72 h after transfection, depletion of  $\alpha$ II-spectrin was analyzed via western blot and found to be efficient: its residual expression was around 35%. Cells transfected with non-relevant shRNA (Nr shRNA-GFP) show similar capillary tube formation to non-transfected cells (Fig. 6a), with most of the transfected cells participating in capillary tube formation. By contrast, the presence of spectrin-depleted

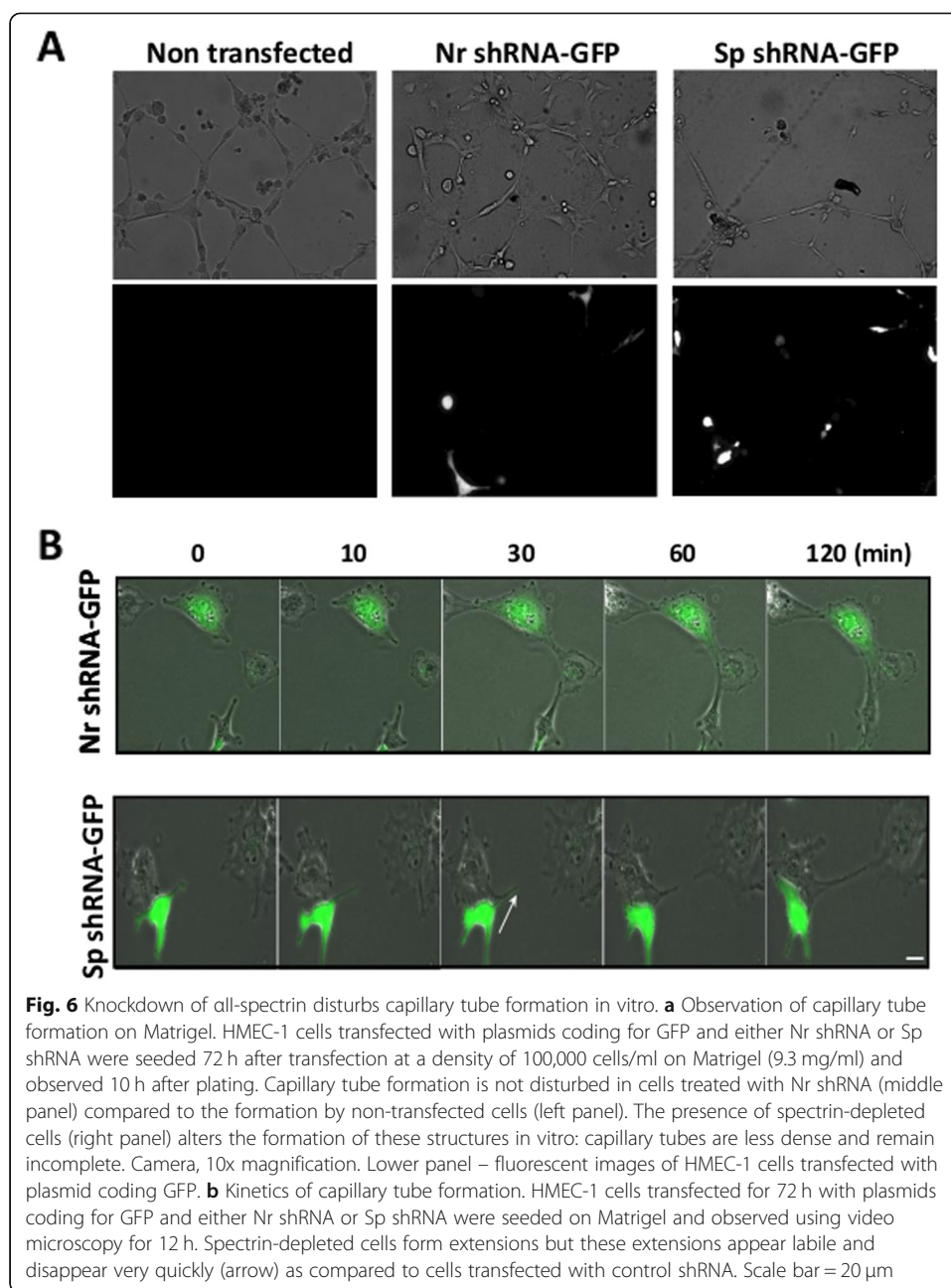


cells (Sp shRNA-GFP) disturbs the capillary tube formation and it appears that most of these cells do not participate in the formation of the network. The Sp shRNA-GFP capillary tube network is less dense and remained incomplete at the end of the process (Fig. 6a). Spectrin-depleted cells were not completely spread as compared to non-

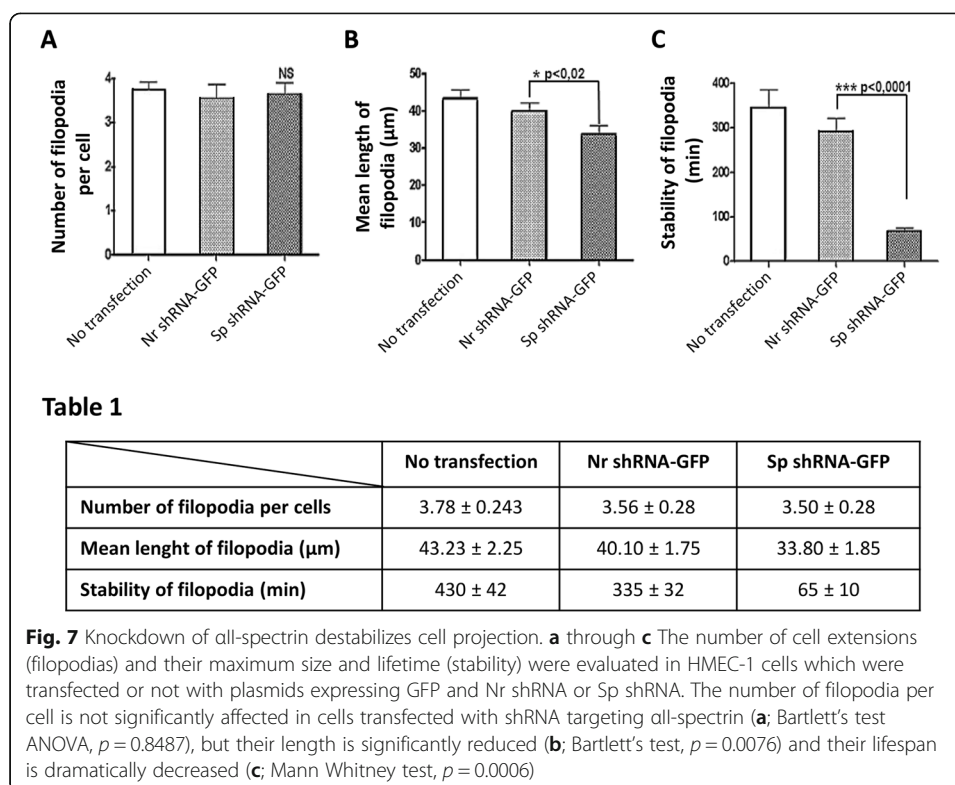


transfected cells and cells transfected with a non-relevant shRNA that provides long extensions (filopodia).

Video microscopy analysis during capillary tube formation revealed that spectrin-depleted cells form extensions and initiate contact. However, these extensions and contacts do not seem stable as the filopodia retract. As a result, spectrin-depleted cells are excluded from the capillary tube (Fig. 6b, arrows). The number of filopodia per cell was similar to that observed in Nr shRNA-GFP treated cells and was around 3.5 vs 3.7 in non-transfected cells (Fig. 7a). However, the spectrin-depleted cell filopodia are shorter



than those of control cells or non-relevant transfected shRNA cells ( $43.23 \pm 2.25$  in non-transfected cells,  $40.10 \pm 1.75$  in Nr shRNA-GFP-transfected cells and  $33.8 \pm 1.85$  in Sp shRNA-GFP-transfected cells; Fig. 7b). The most relevant feature is the shorter stability of extensions in spectrin-depleted cells compared to the control cells and Nr shRNA-GFP-transfected cells (stable for  $430 \pm 42$  min in control cells,  $335 \pm 32$  min in Nr shRNA-GFP-transfected cells and  $85 \pm 10$  min in Sp shRNA-GFP-transfected cells; Fig. 7c). Spectrin-depleted cells could emit extensions, but the lifespan of these extensions is around six times shorter than those observed in control and non-relevant shRNA transfected cells. Depletion of  $\alpha$ II-spectrin alters the stability of cellular extensions, which could participate in the defective formation of the capillary tube.

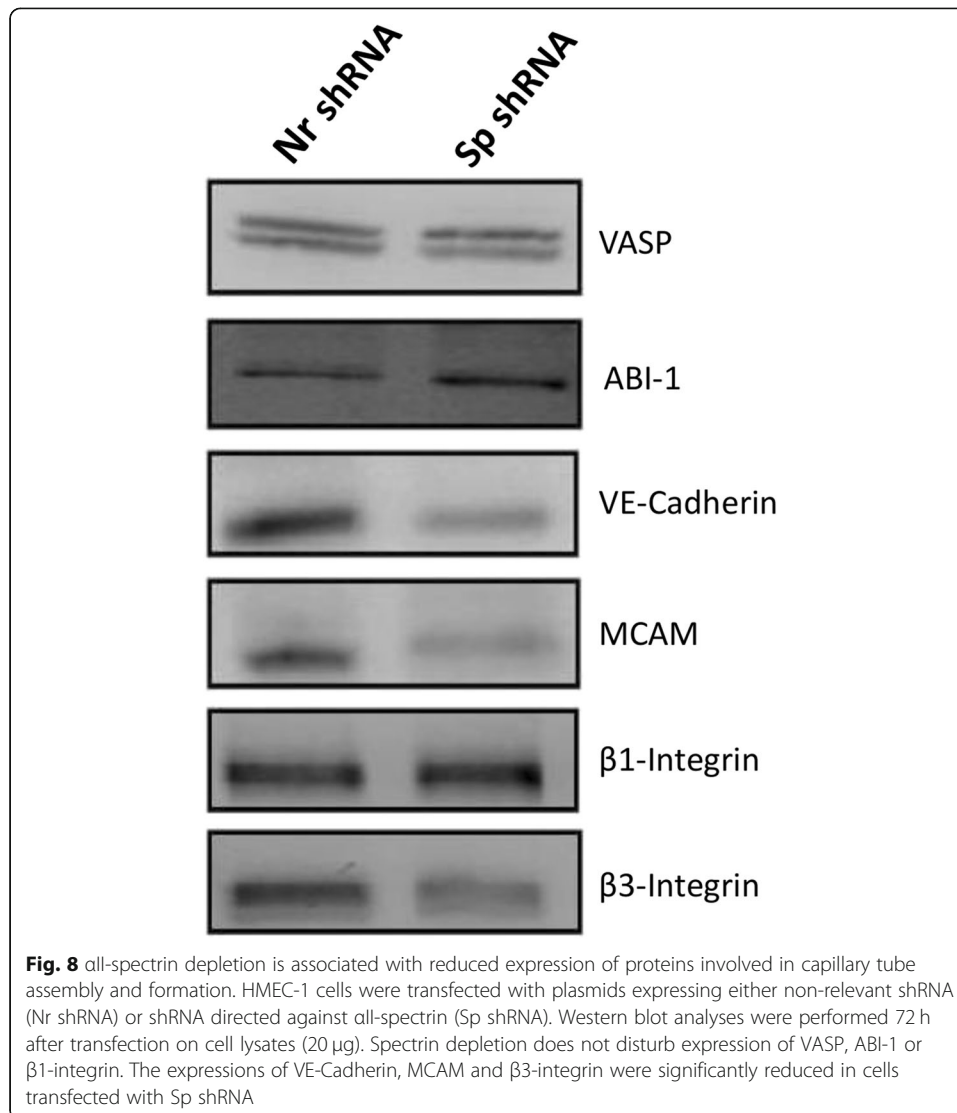


Recent findings show that  $\alpha$ II-spectrin is a partner of two proteins, VASP and Abi1, which are involved in cell–cell contacts [16, 17]. In particular, spectrin participates in the recruitment of VASP at the cell membrane and  $\alpha$ II-spectrin–VASP complexes regulate the cytoskeleton assembly at endothelial cell–cell contacts. Therefore, we assessed VASP and ABI-1 expression. Western blot did not reveal any differences in the expression of these proteins in spectrin-depleted cells. We also analyzed the expressions of adhesion proteins involved in the formation and/or assembly of the capillary tube: VE-cadherin, MCAM, and  $\beta$ 1- and  $\beta$ 3-integrins. Western blot revealed a considerable decrease in the expression of VE-cadherin (80%), MCAM (75%) and  $\beta$ 3-integrin (70%), but  $\beta$ 1-integrin expression was not changed (Fig. 8).

## Discussion

Many recent reports have highlighted the importance of spectrin in maintaining cell shape, physical properties and membrane integrity and controlling the localization and stability of many surface proteins. The absence of homologous  $\alpha$ II-spectrin in *D. melanogaster*, *C. elegans* and mice is associated with abnormal development and lethality [10, 18–20].

Organ development and size can be stimulated by various factors. Biophysical mediators can induce changes in the actin dynamic to elicit gene-specific transcription [21]. Recent findings strongly indicate that the spectrin network is essential for cell spreading, tissue differentiation and growth of organs, but little is known about its development-related roles in vertebrates. In this study, we focused on the functions of spectrin in endothelial cells and its role in the control of morphogenesis.



### Spectrin regulates the organization of actin skeleton

We previously reported that  $\alpha$ II-spectrin depletion is associated with modifications of the actin skeleton, mainly characterized by the disappearance of stress fibers in a melanoma cell line [12] and the actin-rich lamellipodia in T cells [15]. These observations were validated in embryonic fibroblasts from an *spna2<sup>-/-</sup>* mouse model that also presented the loss of stress fibers and the absence of cortical actin in lamellipodia [10]. Moreover, reducing the expression of  $\alpha$ II-spectrin in endothelial cells affects the organization of actin-rich invadosomes and reduces their ability to invade [14]. In neurites with reduced expression of  $\alpha$ II-spectrin, cortical F-actin loss and changes in shape are observed [13]. In endothelial cells, we confirmed the disturbance of actin organization, associated again with a loss of actin stress fibers. Epithelial cells overexpressing  $\beta$ -spectrin had a changed morphology/changed their morphology and were degraded [22]. However, the SH3 domain of  $\alpha$ II-spectrin has been revealed to bind proteins involved in actin dynamics, such as Abi1, EVL, VASP and Tes [16, 17, 23, 24]. Abi1 (also called e3b1), which is part of the WAVE protein complex, is involved in

reorganization of the actin cytoskeleton: the direct interaction of Abi1 with WAVE2 increases WAVE2 activity for actin polymerization [25]. Two members of the Mena/VASP family, EVL and VASP, are situated in focal adhesions and filopodias [16]. Moreover,  $\alpha$ II-spectrin–VASP complexes were reported to regulate cytoskeleton assembly at endothelial cell–cell contacts [23]. Rotter et al. [24] highlight a direct interaction between  $\alpha$ II-spectrin and Tes, which is a tumor suppressor found along the actin stress fibers and at focal adhesion points. Tes interacts with vinculin, zyxin, talin or EVL, Mena and VASP, which are various proteins of cytoskeleton focal adhesion [26, 27]. The depletion of Tes in cells leads to damage of the actin stress fibers [27]. Furthermore, it was reported that  $\alpha$ II-spectrin participates in the Rac activation for actin filament formation and spreading through its SH3 domain [28]. We also hypothesized that spectrin, through direct interaction with VASP, indirectly controls the activation of talin, thus participating in the regulation of LFA1 integrin clustering in the immunological synapse region [15]. The possible integration between the spectrin-based skeleton and the proteins involved in actin dynamics suggests a new way of linking the spectrin-based skeleton with actin reorganization.

#### **Spectrins play a role in the control of cell adhesion and migration**

Using siRNA approaches, we previously found that  $\alpha$ II-spectrin, which is constitutively expressed in all nucleated cells, is also an main actor for nucleated cell shape and cell–matrix adhesion [12–15]. As this study showed, depletion of  $\alpha$ II-spectrin in endothelial cell lines is associated with defective adhesion, loss of cell spreading, modification of cell shape, and fewer, less dense and less regular focal adhesion points. Similarly, siRNA-mediated depletion of  $\beta$ II-spectrin in epithelial cells modifies the cell shape with a loss of the lateral membrane [29]. Other studies revealed that other elements of the spectrin-based skeleton, such as ankyrin-G, are involved in interactions with  $\beta$ II-spectrin related to epithelial cell polarity and the formation of the lateral membrane [30]. As shown recently, protein 4.1R is also implicated in adhesion, spreading, migration and motility of mouse keratinocytes [31, 32].

In a melanoma cell line [12], we observed that depletion of  $\alpha$ II-spectrin in endothelial cells alters cell adhesion and *a fortiori* spreading. The defect is more pronounced on laminin (the  $\beta$ 3-integrin ligand) than on fibronectin (the  $\beta$ 1-integrin ligand). These changes are associated with decreased expression of  $\beta$ 1-integrin, which remains normally distributed, and with accumulation aggregates of  $\alpha$ V $\beta$ 3-integrin which remains normally expressed. Furthermore,  $\alpha$ II-spectrin depletion induces a decreased number of focal adhesions points associated with modification of their architecture. Therefore, decreased expression of  $\alpha$ II-spectrin may lead to inefficient accumulation of integrins in the membrane and disorganization of focal adhesion points.

$\alpha$ II-spectrin accumulates in specialized integrin clusters that initiate cell adhesion [28]. The SH3 domain of  $\alpha$ II-spectrin colocalizes with  $\beta$ 3-integrin at adhesion sites and disappears at a later stage of cell spreading. On the other hand, recent data indicates extensive regulation of integrins by Abi1 [33]. Spectrin downregulation, which affects cell–matrix adhesion and impairs spreading, can be associated with Abi1, which is directly bound to the spectrin SH3 domain [17]. This connection may be regulated by phosphorylation of Abi1, not only by the levels of this protein.

Our results highlight an essential role of non-erythroid spectrin in cell adhesion, regardless of the cell context. They also confirm the link between spectrin and adhesion molecules such as  $\beta 3$ -integrin. Spectrin, in both RBCs and non-erythroid cells, is found in adhesion complexes involved in cell adhesion to the extracellular matrix and in regulation of cell–cell contact. Details of these specific interactions are presented in a new review [9]. Numerous studies have shown that immunoglobulin superfamily cell adhesion molecules (CAMs) can regulate the cytoskeleton and that the cytoskeleton directly controls the function and level of CAMs, for example in neurons [34].

We also observed decreased expression of the two endothelial adhesion molecules, VE-cadherin and MCAM, in spectrin-depleted cells. This decreased expression of adhesion molecules may be the reason for the observed defect in adhesion with the extracellular matrix. As yet, there are no data confirming a link between  $\alpha$ II-spectrin and these molecules.

### Spectrin is engaged in angiogenesis

Angiogenesis is a physiological mechanism based on cell adhesion and spreading. We revealed for the first time that spectrin may be involved in this process. We observed that  $\alpha$ II-spectrin depletion impairs capillary tube formation in vitro. Spectrin depletion (knockdown of  $\alpha$ II-spectrin) disturbs the stability of cell projections and cell–cell contacts, but also correlates with a decreased level of VE-cadherin, MCAM and  $\beta 3$ -integrins, three proteins involved in angiogenesis [35]. MCAM shows pro-angiogenic potential: it is a membrane signal receptor in tumor-induced angiogenesis [36] and has been identified as a novel target for anti-angiogenic agents in anticancer therapy [37]. Moreover,  $\alpha v \beta 3$  integrin, which is upregulated in tumor-associated blood vessels, has received much attention because of its anti-angiogenic potential [38].

In conclusion,  $\alpha$ II-spectrin seems to be involved in the expression of proteins strongly engaged in angiogenesis under both physiological and pathological conditions. We conclude that non-erythroid spectrin may play a crucial role in the control of endothelial cell–matrix contact and migration, and that its depletion leads to impairment of angiogenesis in vivo.

### Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s11658-020-0200-y>.

**Additional file 1: Figure S1.** The distribution of all-spectrin in endothelial cells: HMEC-1 cells and HUVECs were labeled with polyclonal antibodies directed against all-spectrin. Scale bar = 20  $\mu$ m.

**Additional file 2: Figure S2.** all-spectrin depletion modified actin dynamics in living HUVECs. HUVECs expressing GFP-actin and transfected with siRNAs (Sp and Nr siRNAs) were analyzed using video microscopy 72 h after transfection. Cells treated with Sp siRNAs exhibit a reduced size and global disorganization of the actin network. Actin mainly accumulates in dynamic patches when in cells treated with Nr siRNAs. Actin formed very dynamic stress fibers and accumulated at the leading edge of the cells.

**Additional file 3: Figure S3A.** Video illustrating the Nr siRNA case for Fig. 2c.

**Additional file 4: Figure S3B.** Video illustrating the Sp siRNA case for Fig. 2c.

**Additional file 5: Figure S4.** Raw image file for western blots shown in Fig. 1a.

### Abbreviations

Abi1: Abelson interactor 1; CAMs: Cell adhesion molecules; EC: Endothelial cell; EVL: Ena/VASP-like protein; GFP: Green fluorescence protein; HMEC-1: Human microvascular endothelial cell; HUVECs: Human umbilical vein endothelial cells; IS: Immunological synapse; LFA1: Lymphocyte function-associated antigen 1; MCAM: Melanoma cell adhesion molecule; RBC: Red blood cell; SH3 domain: SRC Homology 3 Domain; shRNA: Short hairpin RNA; siRNA: Small interfering RNA; VASP: Vasodilator-stimulated phosphoprotein; VE-cadherin: Vascular endothelial cadherin



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**Authors' contributions**

This research was performed by BM and AP. Both authors wrote the first draft and edited the manuscript. MCL supervised the study and reviewed the manuscript. JP was the engineer of the imaging platform. YC participated in supervising the study and reviewed the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during this study are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

All the authors listed have approved the manuscript for publication.

**Competing interests**

The authors declare that they have no competing interests.

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