

# Acquisition of anoikis resistance promotes alterations in the Ras/ERK and PI3K/Akt signaling pathways and matrix remodeling in endothelial cells

Ana Paula de Sousa Mesquita<sup>1,2</sup> · Silvana de Araújo Lopes<sup>1,2</sup> · Paulo Castanho A. Pernambuco Filho<sup>1,2</sup> · Helena B. Nader<sup>2</sup> · Carla Cristina Lopes<sup>1,2</sup>

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**Abstract** Anoikis is a programmed cell death induced upon cell detachment from extracellular matrix. Anoikis resistance is a critical mechanism in tumor metastasis. Cancer cells deregulate and adapt their metabolism to survive in the absence of adhesion, spreading metastases to distant organs. These adaptations include abnormal regulation of growth factor receptors activating prosurvival signaling pathways, such as the Ras/ERK and PI3K/Akt pathways, and extracellular matrix remodeling, leading to metastasis by an increase of invasiveness and inhibiting anoikis. This study investigates the possible involvement of ECM components and signaling pathways in the regulation of resistance to anoikis in endothelial cells (EC). Endothelial cells submitted to stressful conditions by blocking adhesion to substrate (anoikis resistance) display an up-regulation of Ras/ERK and PI3k/Akt pathways by high expression of Ras, ERK, PI3K (p110 $\alpha$ ) and Akt (Thr 308). After ERK and PI3K inhibiting, all EC-derived cell lines studied showed lower growth, a decrease in invasive potential and a higher rate of apoptosis. Furthermore, anoikis-resistant cell lines display a decrease in the expression of fibronectin, collagen IV and hyaluronic acid and an increase in the expression of laminin, perlecan,  $\alpha$ v,  $\beta$ 3,  $\alpha$ 5 and  $\beta$ 1 integrins subunits, hyaluronidases 1, 2 and 3 and metalloproteinases

2 and 9. These results indicate that the acquisition of anoikis resistance induced remodeling of the extracellular matrix and overexpression of the PI3K/Akt and Ras/ERK pathway components. Acquisition of resistance to anoikis is a potentially crucial step in endothelial cell transformation.

**Keywords** Anoikis · Ras/ERK and PI3K/Akt pathways · Extracellular matrix remodeling · Endothelial cells

## Introduction

Attachment of a cell to the extracellular matrix (ECM) is vital for the proliferation and survival functions to be carried out efficiently. If a cell gets detached, apoptosis may be induced. Cell death triggered by lack of attachment to the ECM is defined as anoikis [1]. Anoikis acts as an important defense for the organism by preventing re-adhesion of detached cells to new matrices in incorrect locations and their dysplastic growth [2].

Dysregulation of anoikis, such as anoikis resistance, is a critical mechanism in tumor metastasis. Cancer cells rapidly develop several mechanisms to resist anoikis, progress towards malignancy and spread metastases to distant organs [3]. First, the cancer cells acquire a migratory and invasive phenotype, which allows them to degrade the ECM, invade through the basement membrane and enter into either the circulatory or lymphatic system. Later, these cells deregulate and adapt their metabolism to survive in the absence of adhesion to the ECM (anoikis resistance) [4].

Tumors produce several molecules that facilitate their proliferation, invasion and maintenance, especially proteoglycans. The syndecan-4, a heparan sulfate proteoglycan, can act as a co-receptor of growth factors and proteins of the extracellular matrix by increasing the

Ana Paula de Sousa Mesquita and Silvana de Araújo Lopes have contributed equally to this work.

✉ Carla Cristina Lopes  
lopesazevedo.bioq@epm.br; cclazevedo@gmail.com

<sup>1</sup> Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, Diadema, SP, Brazil

<sup>2</sup> Disciplina de Biologia Molecular, Departamento de Bioquímica, Universidade Federal de São Paulo, São Paulo, SP, Brazil

affinity of adhesion molecules to their specific receptors [4, 5]. Changes in the expression of syndecan-4 have been observed in tumor cells, indicating its involvement in cancer. Acquisition of anoikis resistance induced syndecan-4 up-regulation in endothelial cells [6].

In addition to the expression of syndecan-4, anoikis resistance involves unregulated expression of growth factor receptors or components of their signaling pathways that inhibit cell death pathways and active cell survival pathways [5]. Abnormal regulation of growth factor receptors activates prosurvival signaling pathways, such as the Ras/ERK, PI3K/Akt, NF- $\kappa$ B, and Rho-GTPase pathways, leading to metastasis by inhibiting anoikis [5, 7, 8].

The Ras/extracellular signal-regulated kinase (Ras/ERK) and phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signaling pathways are the chief mechanisms for controlling cell survival, differentiation, proliferation, metabolism, and motility in response to extracellular cues. These pathways can intersect to regulate each other and co-regulate downstream functions. The extent of this cross-talk and its significance in cancer therapeutics are targets of many studies [9].

Ras/ERK pathway is activated by growth factors, polypeptide hormones, neurotransmitters, chemokines, and phorbol esters, which signal through their cognate receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs), or by direct activation of protein kinase C (PKC) [9–11].

Growth factors also activate the lipid kinase PI3K through either direct PI3K recruitment to the growth factor receptors or indirect recruitment involving the docking proteins insulin receptor substrate (IRS) or GRB2-associated binder (GAB). PI3K generates phosphatidylinositol 3, 4, 5 triphosphate (PIP3), which recruits the protein kinase Akt to the plasma membrane where it is activated by 3-phosphoinositide-dependent kinase 1 (PDK1) and the second mTOR complex, mTORC2. Akt phosphorylates many survival, proliferation and motility factors [9, 12].

These pathways are important on the tumorigenesis, maintenance of phenotype and protection from apoptosis, and are further regulated by cell–ECM interaction. Binding of integrins and ECM molecules transmit signals from the extracellular environment to the intracellular network, as well as signals from the intracellular network to extracellular environment. Alterations in integrins expression or in ECM composition can change the transmission mediated by integrin-activated signaling molecules, such as focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3K), and members of the extracellular signal-regulated kinase 1 and 2/mitogen activated protein (ERK1 and 2/MAP) kinase family, also altering cell proliferation, migration, and apoptosis [3, 13, 14].

The ECM is the first barrier to developing metastasis. Conversely, extracellular molecules also contribute in a critical way to the progress of cancer cell invasion. These molecules can be: (a) part of the ECM, (b) secreted in the ECM, (c) secreted but also attached on the cell surface, (d) cell membrane proteins such as receptors [15].

ECM consists of several proteins and glycoproteins (including collagen, laminin, fibronectin, vitronectin), in addition to glycosaminoglycans (GAGs) such as hyaluronan (HA) and proteoglycans such as perlecan, with different physical and biochemical properties that regulate cell growth, survival, motility, differentiation and apoptosis by ligating specific receptors such as integrin receptors [16–20]. Integrins are mediators of cell–ECM and cell–cell interaction that can transduce biochemical signals both into and out of cells [21].

Matrix degradation is central to tumor development. Enzymes that degrade extracellular matrix are abundant in tumors. The most significant enzymes in ECM remodeling are metalloproteinases (MMPs) [22]. The MMP family are best known for their ability to cleave components of the ECM but they also can cleave other proteinases, proteinase inhibitors, latent growth factors, chemotactic molecules, growth factor binding proteins, cell surface receptors and cell–cell adhesion molecules [23, 24]. ECM proteoglycans and glycoproteins are also targeted by MMPs and ADAMTS, a disintegrin and metalloproteinase with thrombospondin domains. Additionally, the various GAG polysaccharide chains can be modified or removed by enzymes that specifically target them, changing ECM function. For example, hyaluronidases (HYALs), a class of enzymes that predominantly degrade HA and its production can be increased or suppressed in some malignant conditions compared with normal tissues [22, 25].

These and other results led us to investigate the possible involvement of ECM components and signaling pathways in the regulation of resistance to anoikis in endothelial cells. We now report that anoikis-resistant endothelial cells display remodeling of the extracellular matrix and overexpression of the PI3K/Akt and Ras/ERK pathways components.

## Methodology

### Reagents and antibodies

Rabbit polyclonal antibody fibronectin, rabbit polyclonal antibody laminin, rabbit polyclonal antibody collagen IV and goat anti-Rat IgG H&L (Alexa Fluor<sup>®</sup> 594) secondary antibody were purchased from Abcam (Cambridge, MA, USA). Rat monoclonal antibody heparan sulfate proteoglycan (perlecan) clone A7L6, rabbit monoclonal antibody

Phospho-Akt (Thr 308), rabbit monoclonal antibody PI3K p110 $\alpha$  and rabbit monoclonal antibody Phospho-ERK 1/2 (Thr202/Tyr204, Thr185/Tyr187) were acquired from Merck Millipore (Danvers, MA, USA). Mouse monoclonal antibody  $\beta$ -Actin, rabbit monoclonal antibody MMP2, rabbit monoclonal antibody MMP9, rabbit monoclonal antibodies integrins  $\alpha$ v,  $\beta$ 3,  $\alpha$ 5,  $\beta$ 1 and HRP-linked goat anti-rat IgG secondary antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse monoclonal antibody HYAL-1, rabbit polyclonal antibody HYAL-2, rabbit polyclonal antibody HYAL-3, mouse monoclonal antibody GAPDH and rabbit polyclonal antibody H-Ras were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-linked goat anti-mouse IgG and HRP-linked goat anti rabbit IgG secondary antibodies were obtained from Sigma-Aldrich (St Louis, MO, USA). Goat anti-rabbit IgG (H+L) highly cross-adsorbed labeled with Hilyte Fluor 594 secondary antibody was obtained from Anaspec (Fremont, CA, USA). Specific inhibitor of PI3K, LY294002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4) was purchased from Merck Millipore (Danvers, MA, USA).

### Cell culture

Endothelial cell line derived from rabbit aorta (EC) [26], EC transfected with EJ-ras oncogene (EJ-ras EC) [27] and EC resistant to anoikis (Adh1-EC and Adh2-EC) [6] were maintained in F12 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal calf serum (FCS) (Athena—Campinas, Brazil) at 37 °C, 2.5% CO<sub>2</sub>.

### RT-PCR

For RT-PCR, total RNA was isolated from the endothelial cells with the Trizol-reagent from Invitrogen (Carlsbad, USA) and quantitated by spectrophotometer. RT-PCR was prepared using TaqMan<sup>®</sup> Reverse Transcription Reagents kit (Applied Biosystems, Foster, USA), according to the manufacturer's instructions.

### Quantitative real-time PCR (qPCR)

For quantitative PCR (qPCR), 2  $\mu$ g of total RNA per sample was reverse transcribed to cDNA with TaqMan<sup>®</sup> Reverse Transcription Reagents kit (Applied Biosystems, Foster, USA) and then 500 ng of cDNA was analyzed using the Maxima SYBR Green qPCR Master Mix Kit (Thermo Fisher Scientific Inc., Rockford, USA) with a ABI 7500 real-time PCR instrument (Applied Biosystems, Foster, USA), according to the manufacturer's instruction, respectively. The primers are used as follow: Fibronectin Fwd (5'-CCTATGGAGAAACAGGAGGAAATAG-3')

and Fibronectin Rev (5'-CAGCGTACACAGTGATGG TATAG-3'); Collagen IV Fwd (5' GGTGATGCTGCCAGT GAGTT-3') and Collagen IV Rev (5'- CTACCTGGCTCA CCCTTGG-3'); Perlecan Fwd (5'-GCCTCGAGCACC TCTCGGAACT 3') and Perlecan Rev (5'-CTCAGGAGA CGA CCTGGGCAGT-3'); Integrin  $\beta$ 1 Fwd (5'- ACGGGA AACTTGGTGGTATT-3') and Integrin  $\beta$ 1 Rev (5'- GTG GGC AAT AGA AGG GTA GTC-3'); PI3K Fwd (5'- AGC AAGCGCCCCAACTGGAA3') and PI3K Rev (5'-TGC AGGCCACTGGTGAAGCAA-3'); Akt Fwd (5'-CAC ATAGCAGGGGCACCTTCCG-3') and Akt Rev (5'-AGG GTGAAAGGTGGCGAGGGG-3'); ERK Fwd (5'-AGT TCTTGACCCCTGGTCCT3-') and ERK Rev (5'-AAT GGGTGACACACACAGGG-3'); Ras Fwd (5'-ACAGTG CAATGAGGGACCAGTA 3-') and Ras Rev (5'-GTATAG AAGGCATCAACACC-3'); GAPDH Fwd (5'-CGCTTC GCTCTCTGCTCCTCC-3') and GAPDH Rev (5'-TGG TGACCAGGCGCCCAATAC-3'). PCR was performed at 95 °C for 15 s and 60 °C for 60 s for 40 cycles. GAPDH was used as an internal standard to normalize mRNA levels for differences in sample concentration and loading. Fold changes in the expression of each target mRNA relative to GAPDH was calculated based on the threshold cycle (Ct) as  $2^{-\Delta(\Delta Ct)}$ , where  $\Delta Ct = Ct_{\text{target}} - Ct_{\text{GAPDH}}$  and  $\Delta(\Delta Ct) = \Delta Ct_{\text{Adh-EC}} - \Delta Ct_{\text{EC}}$ . The post-amplification melting curve analysis was performed to confirm whether the nonspecific amplification was generated from primer-dimers. Quantitative PCR reactions were performed in triplicate and repeated three times.

### Western blotting

Endothelial cells treated and untreated with LY294002 (Merck Millipore) 3  $\mu$ M for 1 h were lysed in buffer [50 mM Tris-HCl, pH 8, 1% Triton, 150 mM NaCl, 0.5% Deoxycholate and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc.—Rockford, USA)] for 2 h at 4 °C. The homogenates were spun at 14,000 rpm for 15 min and the supernatant was collected. Proteins were quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific Inc.—Rockford, USA). Equal amounts of protein extracts (40  $\mu$ g) were subjected to 10% SDS-PAGE and blotted onto a PVDF membrane (Thermo Fisher Scientific Inc.—Rockford, USA). After blocking, the membrane was incubated with the primary antibody (rabbit anti-fibronectin 1: 1000; rabbit anti-integrin  $\alpha$ v,  $\alpha$ 5,  $\beta$ 1 and  $\beta$ 3 1: 1000; rabbit anti-laminin 1: 1000; rabbit anti-collagen IV 1:1000; rat anti-perlecan 1:1000; rabbit anti-MMP2 and MMP9 1: 1000; mouse-anti HYAL-1; rabbit anti-HYAL-2 1:1000; goat anti-HYAL-3 1:1000; rabbit anti-PI3K (p110 $\alpha$ ) 1:500; rabbit anti-phospho-Akt (Thr 308) 1:1000; rabbit anti-phospho-ERK 1/2 (Thr202/Tyr204, Thr185/Tyr187) 1:1000; rabbit anti-H-Ras 1:5000; mouse anti-GAPDH 1:500 and

mouse anti-beta actin 1:1000) diluted in blocking buffer overnight at 4 °C and followed by incubation with secondary antibodies (1:10,000) for 1 h at room temperature. The samples were detected by enhanced chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA). An Alliance mini photodocumentation system from UVITEC (Cambridge, UK) was used to scan the films and UVIBAND MAX v1503b software (UVITEC—Cambridge, UK) was used to measure the amount of protein detected by each antibody. The experiments were performed in duplicate and repeated twice.

### Immunocytochemistry

Cells were cultured on 13.0 mm diameter glass coverslips ( $5 \times 10^3$  cells/coverslip) in 24-well plates for 2 days followed by fixation with 4% formaldehyde in PBS (pH 7.4) for 20 min at room temperature. The cells were washed four times with PBS and once with PBS containing 0.1 M glycine. They were then permeabilized with PBS containing 1% BSA and 0.01% saponin for 15 min at room temperature. After this step, the cells were washed with PBS and incubated with rabbit anti-fibronectin (1:200), rabbit anti-laminin (1:200), rat anti-perlecan (1:200), rabbit anti-collagen IV (1:200), rabbit anti-PI3K (p110 $\alpha$ ) (1:200), rabbit anti-phospho-Akt (Thr 308) (1:200), rabbit anti-phospho-ERK 1/2 (Thr202/Tyr204, Thr185/Tyr187) (1:200) and rabbit anti-H-Ras (1:200) diluted in PBS or PBS containing 0.01% saponin for 1 h at room temperature. After washing, the cells were incubated with goat anti-rabbit labeled with Hilyte Fluor 594 (1:300) or goat anti-rat labeled with Alexa Fluor® 594 (1:300) and DAPI (1:10,000) in PBS containing 0.01% saponin for 20 min at room temperature. The cells were washed and mounted in Fluoromount-G and examined using an inverted confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystems, Wetzlar, DE). Each figure shown in the “Results” corresponds to the best of two experiments.

### Cell growth analysis

Equal numbers ( $5 \times 10^4$ ) of EC, EJ-ras EC, Adh1-EC and Adh2-EC cells, treated and untreated with U0126 (Merck Millipore) 10  $\mu$ M for 2 h or LY294002 (Merck Millipore) 3  $\mu$ M for 1 h, were added into the different cell culture dishes and maintained in F12 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal calf serum (FCS) (Athena, Campinas, Brazil) at 37 °C, 2.5% CO<sub>2</sub>. Every two days, cells were removed from the dishes, washed with PBS and counted using a Neubauer chamber. Triplicate dishes were used for each time point (0, 2, 4, 6 and 8 days). Each experiment was repeated three times.

### Annexin V-FITC/PI staining assay

Apoptosis was assessed by the binding of annexin V conjugated with fluorescein isothiocyanate (FITC) to phosphatidylserine, which is externalized to the outer leaflet of the plasma membrane early during induction of apoptosis. Briefly, EC cells and its derived clones ( $1 \times 10^6$ ), treated and untreated with U0126 (Merck Millipore) 10  $\mu$ M for 2 h or LY294002 (Merck Millipore) 3  $\mu$ M for 1 h, were resuspended in the binding buffer provided in the annexin V-FITC apoptosis detection kit II (BD Pharmingen, San Diego, CA, USA) and reacted with 5  $\mu$ L of annexin V-FITC reagent and 5  $\mu$ L of propidium iodide (PI) for 30 min at room temperature in the dark. Stained cells were analyzed by flow cytometry (FACSCalibur—Becton, Dickinson and Co, Franklin Lakes, NJ, USA) using the FlowJo program (Tree Star Inc., Ashland, OR, USA). Each experiment was repeated three times.

### Invasion assay

The invasion assays were performed on 24-well plates using Polycarbonate Transwell of 8  $\mu$ m pore size (Corning Inc. Lowell, MA, USA) coated with ECL cell attachment matrix (entactin, collagen IV and laminin) (1 mg/mL) (Merck Millipore, Danvers, MA, USA). Briefly, the coated inserts were hydrated with warm F12 without serum in 2.5% CO<sub>2</sub> at 37 °C for 2 h. A total of  $5 \times 10^4$  endothelial cells, treated and untreated with U0126 (Merck Millipore) 10  $\mu$ M for 2 h or LY294002 (Merck Millipore) 3  $\mu$ M for 1 h were seeded into the upper chamber containing F12 without serum and the lower chamber contained F12 with 10% FCS. The cells were incubated at 37 °C in 2.5% CO<sub>2</sub> for 48 h. Non-invading cells were removed from the upper membrane by scrubbing with a cotton swab. Invading cells were fixed with 4% formaldehyde at room temperature for 5 min and methanol (p.a. 99.8%) for 20 min, stained with DAPI (1:10,000) dissolved in PBS for 20 min in darkness and visualized under an inverted microscope Axio Observer Z1 (Zeiss, Jena, Germany). The results were measured by counting the total number of nuclei stained with DAPI. ImageJ software (Bethesda, MA, USA) was used in the quantification and analysis of images. Each experiment was repeated three times.

### Extraction and quantification of hyaluronic acid (HA)

Determination of HA was performed using a highly specific, highly sensitive “ELISA-like” fluorimetric method that employed a biotinylated HA-binding protein and europium-labeled streptavidin. The detection limit of this method is ca. 0.2–500 ng/mL of HA [28]. The samples were assayed in triplicate and repeated three times.



The plates were read in a Victor 2-Wallac Ou fluorimeter (Turku, Finland).

### Statistical analysis

All data are expressed as the mean  $\pm$  standard error of the mean. Statistical significance was assessed using analysis of ANOVA. *P* values  $< 0.05$  were considered statistically significant.

## Results

### Analysis of Ras/ERK signaling pathway in anoikis-resistant endothelial cells

To investigate the gene and protein expression levels of H-Ras and ERK by parental, Adh-EC and EJ-ras EC cells, we used qPCR and Western Blotting analyses, respectively.

Figure 1a shows an increase in the expression of H-Ras gene in Adh-EC and EJ-ras EC cells when compared with EC cells. In addition, Fig. 1b shows an increase in the proteic expression of H-Ras in Adh-EC and EJ-ras EC cells when compared to the parental line. Similar data were observed using immunofluorescence assay (Fig. 1c).

The ERK gene expression analysis by qPCR revealed an increase of ERK mRNA levels in the Adh-EC and EJ-ras EC cells, when compared to EC, as shown in Fig. 2a. The level of phosphorylated ERK 1/2 (Thr202/Tyr204, Thr185/Tyr187) is significantly higher in Adh-EC and EJ-ras EC cells compared to parental lines (Fig. 2b). Similar data were observed using immunofluorescence assay (Fig. 2c).

### Analysis of PI3K/Akt signaling pathway in anoikis-resistant endothelial cells

In order to also investigate the gene and protein expression levels of PI3K and Akt by parental, Adh-EC and EJ-ras EC cells, we used qPCR and Western Blotting analyses, respectively.

Figure 3a shows an increase in the expression of PI3K (p110 $\alpha$ ) gene in endothelial cells resistant to anoikis and EJ-ras transfected cells in relation to parental cells. Consistent with these findings, western blot (Fig. 3b) and immunofluorescence (Fig. 3c) analysis shows an increase in the PI3K (p110 $\alpha$ ) protein levels.

Furthermore, we analysed the Akt expression in endothelial cells resistant to anoikis. qPCR analysis showed that Akt gene expression levels were similar in Adh-EC and EC cells (Fig. 4a). For protein expression, we observed an increase in the Akt (phosphorylated at Thr 308) expression in anoikis-resistant endothelial cells and EJ-ras EC in relation to EC cells (Fig. 4b, c).

### ERK and PI3K inhibition affects growth, apoptosis and invasiveness in anoikis-resistant endothelial cells

To test the role of the Ras/ERK and PI3K/Akt pathways in cell growth, apoptosis and invasiveness of endothelial cells resistant to anoikis, selective inhibitors of MEK 1/2, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) and PI3K, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4 (LY294002) were used [29, 30].

To analyze the cell growth, EC, EJ-ras EC, Adh1-EC and Adh2-EC cells were treated with U0126 10  $\mu$ M for 2 h or LY294002 3  $\mu$ M for 1 h and added into the different cell culture dishes and maintained in F12 medium supplemented with 10% FCS. Every 2 days, cells were removed from the dishes and counted using a Neubauer chamber. As shown in Figs. 5a and 6a, respectively, the cell growth rate decreased in all cell lines after treatment with U0126 or LY294002, especially in cells resistant to anoikis.

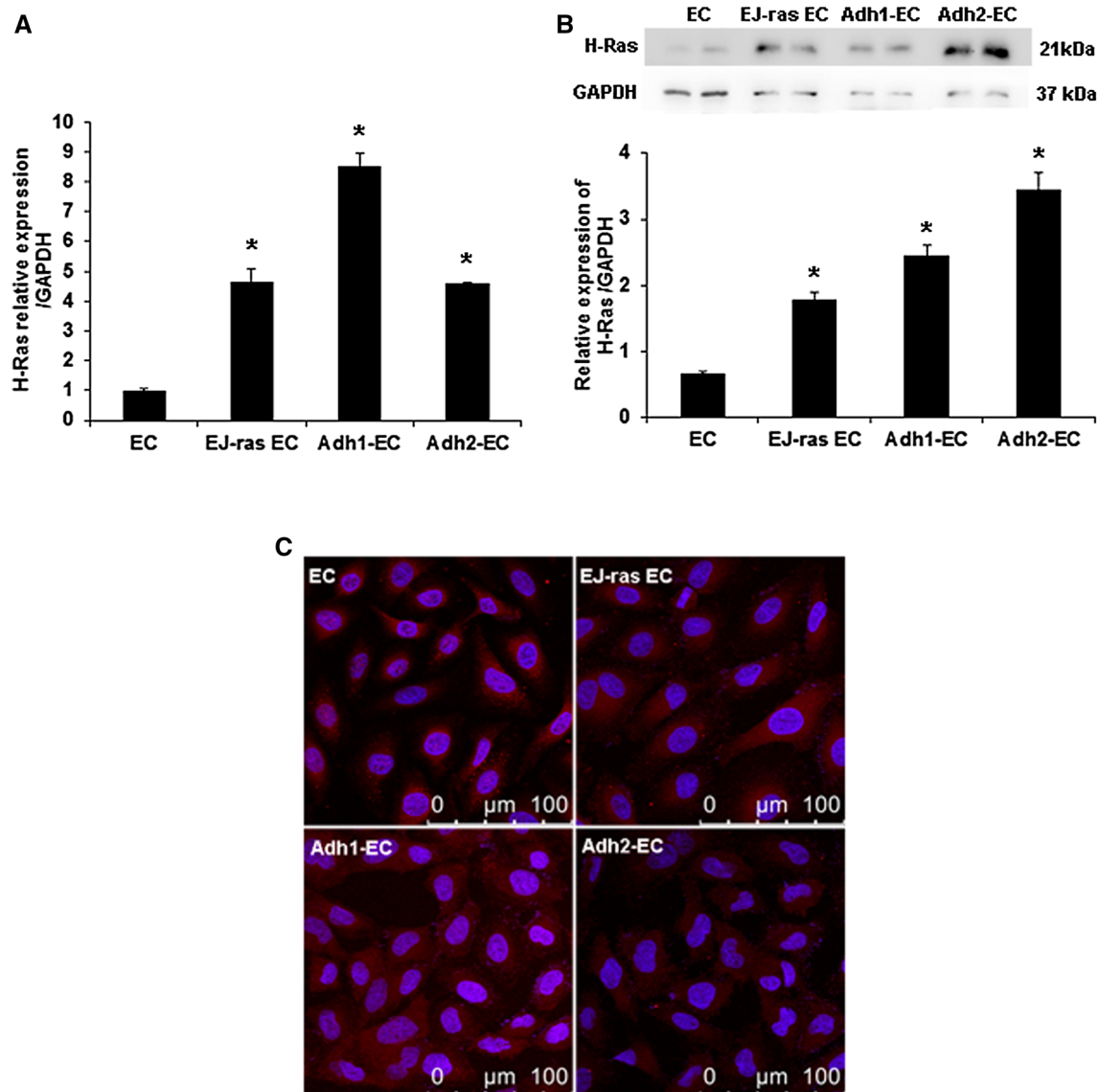
To determine the percentage of apoptotic cells, EC, EJ-ras EC, Adh1-EC and Adh2-EC cells were treated with U0126 10  $\mu$ M for 2 h or LY294002 3  $\mu$ M for 1 h, harvested and stained with Annexin V-FITC and propidium iodide (PI). Stained cells were analyzed by flow cytometry. The results obtained with flow cytometry revealed a significant increase in apoptosis rate in all EC-derived cell lines treated with U0126 or LY294002 inhibitors (Figs. 5b, 6b, respectively).

To analyze the invasiveness, EC, EJ-ras EC, Adh1-EC and Adh2-EC cells were treated with U0126 10  $\mu$ M for 2 h or LY294002 and added into the plates using polycarbonate transwell coated with ECL cell attachment matrix (Merck Millipore). As shown in Figs. 5c and 6c, respectively, the invasiveness decreased in all cell lines after treatment with U0126 or LY294002, mainly in Adh1-EC, Adh2-EC and EJ-ras EC cells.

### Expression of ECM molecules and receptors in anoikis-resistant endothelial cells

In order to investigate the role of ECM in anoikis resistance, we analyzed the expression of some molecules present in the extracellular matrix, such as: fibronectin, collagen IV, laminin, vitronectin, perlecan, hyaluronic acid, hyaluronidases 1, 2 and 3, MMPs 2 and 9,  $\alpha$ v $\beta$ 3 and  $\alpha$ 5 $\beta$ 1 integrins. The gene and protein expression levels were determined using qPCR and Western blot analysis, respectively. The hyaluronic acid synthesis was determined by “ELISA-like” fluorimetric method [28].

Figure 7a shows a decrease in the expression of fibronectin gene in Adh-EC and EJ-ras EC cells when compared with EC cells. In addition, Fig. 7b shows a decrease in the proteic expression of fibronectin in Adh-EC and EJ-ras EC



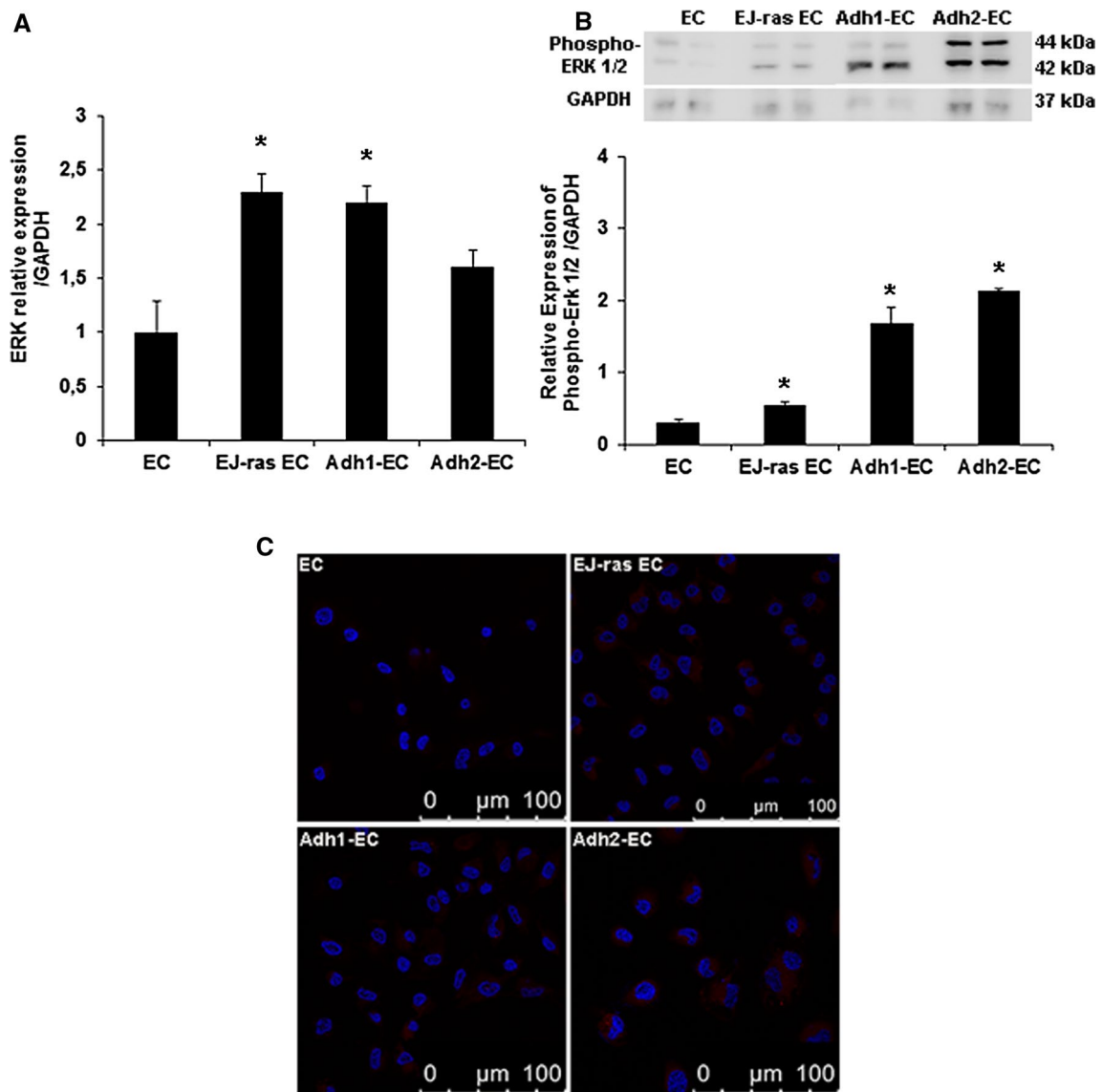
**Fig. 1** Gene and protein expression of H-ras in EC-derived cell lines. **a** Expression of H-Ras detected by qPCR. GAPDH was used as a loading control. **b** Protein expression of H-Ras by western blot. GAPDH was used as a loading control. Histogram depicting H-Ras protein levels normalized to GAPDH. **c** Immunofluorescent analysis of H-Ras. H-Ras was stained with anti-H-Ras antibody followed by secondary Hilyte Fluor™-594-labelled antibody (red). Nucleus stained with DAPI (blue). The images of immunofluorescence were

obtained using an inverted confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystems) and analyzed by Leica Application Suite Advanced Fluorescence software (100 μm Scale bar). **a** Experiment was performed in triplicate and **b** and **c** experiments were performed in duplicate. EC parental endothelial cells, EJ-ras EC EJ-ras transfected endothelial cells, Adh1-EC and Adh2-EC anoikis-resistant endothelial cells. The bars represent the standard error. \* $P < 0.05$ . (Color figure online)

cells when compared to the parental line. Similar data were observed using immunofluorescence assay (Fig. 7c).

Figure 8a shows an increase in the expression of collagen IV gene in Adh-EC and EJ-ras EC cells when compared with EC cells. Furthermore, Fig. 8b shows a decrease in the proteic expression of collagen IV in Adh-EC and EJ-ras EC cells when compared to the parental line. Similar data were observed using immunofluorescence assay (Fig. 8c).

The perlecan gene expression analysis by qPCR revealed an increase of perlecan mRNA levels in Adh-EC and EJ-ras EC cells when compared to EC, as shown in Fig. 10a. In addition, western blot analysis showed that protein expression levels of laminin and perlecan are increased in endothelial cells resistant to anoikis and EJ-ras transfected cells in relation to parental cells (Figs. 9a, 10b, respectively). Similar data were observed using immunofluorescence assay (Figs. 9b, 10c).



**Fig. 2** Gene and protein expression of ERK in EC-derived cell lines. **a** Expression of ERK detected by qPCR. GAPDH was used as a loading control. **b** Protein expression of phospho-ERK 1/2 (Thr202/Tyr204, Thr185/Tyr187) by western blot. GAPDH was used as a loading control. Histogram depicting ERK protein levels normalized to GAPDH. **c** Immunofluorescent analysis of phospho-ERK 1/2 (Thr202/Tyr204, Thr185/Tyr187) ERK was stained with anti-Phospho-ERK antibody followed by secondary Hilyte Fluor™-594-labelled antibody (red). Nucleus stained with DAPI (blue). The

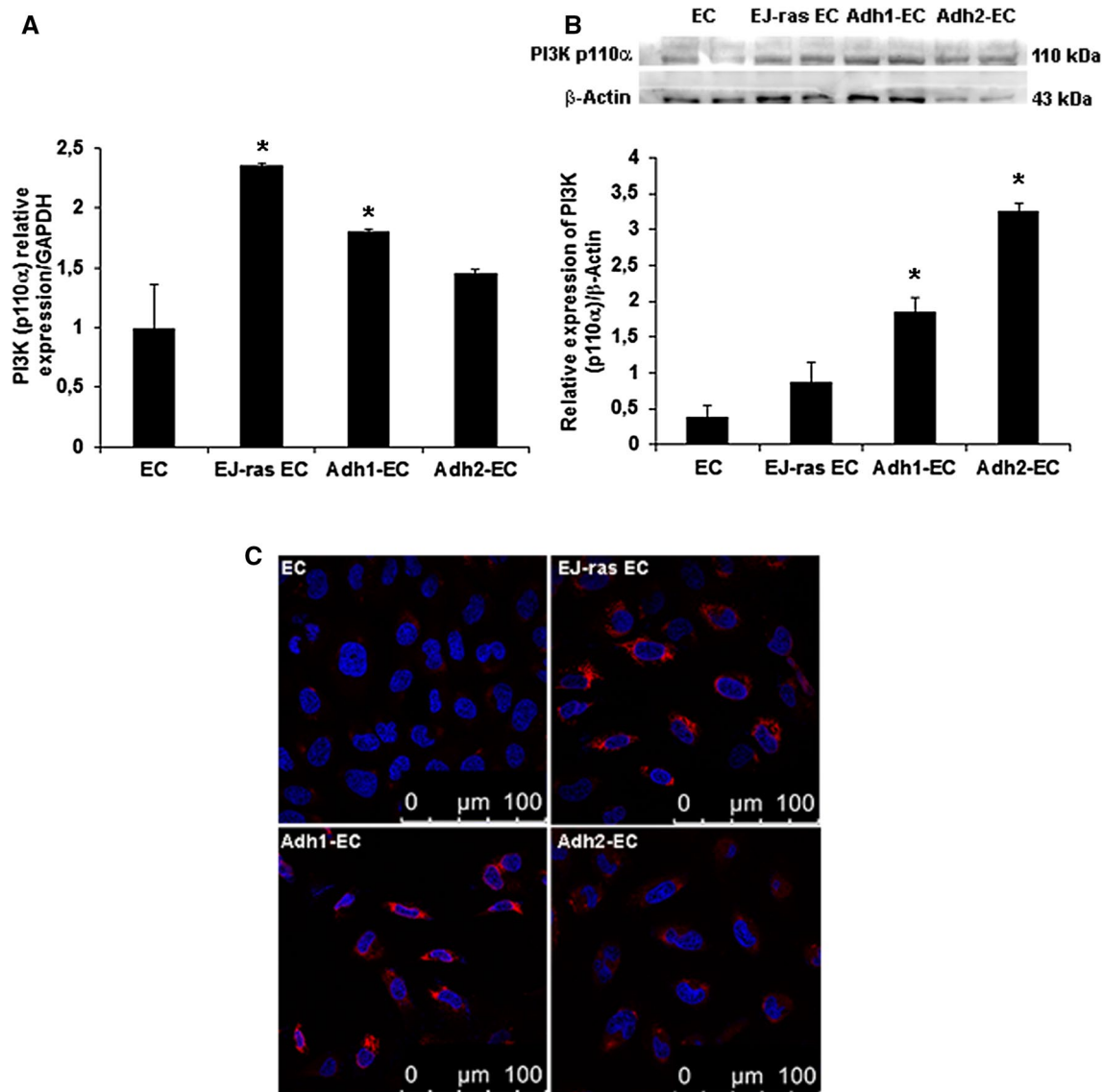
images of immunofluorescence were obtained using an inverted confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystems) and analyzed by Leica Application Suite Advanced Fluorescence software (100 μm Scale bar). **a** Experiment was performed in triplicate and **b** and **c** experiments were performed in duplicate. EC parental endothelial cells, EJ-ras EC: EJ-ras transfected endothelial cells, Adh1-EC and Adh2-EC anoikis-resistant endothelial cells. The bars represent the standard error. \* $P < 0.05$ . (Color figure online)

Figure 9c shows that the protein expression levels of vitronectin were similar in all cell lines.

The hyaluronic acid synthesis was determined by fluorimetric assay [28]. In Fig. 11a, it is possible to observe a decrease in the synthesis of this glycosaminoglycan in cells resistant to anoikis (Adh1-EC and Adh2-EC) and cells transfected with EJ-ras oncogene when compared to parental cells.

As the hyaluronic acid synthesis is reduced, we decided to analyze the expression of enzymes that degrade this molecule, the hyaluronidases (HYALs), in anoikis-resistant endothelial cells. The protein expression levels of HYALs 1, 2 and 3 are significantly higher in Adh-EC and EJ-ras EC cells compared to parental cells (Fig. 11b–d, respectively).

As changes were observed in the expression of syndecan-4 [6], fibronectin and collagen IV in anoikis-resistant



**Fig. 3** Gene and protein expression of PI3K (p110 $\alpha$ ) in EC-derived cell lines. **a** Expression of PI3K (p110 $\alpha$ ) detected by qPCR. GAPDH was used as a loading control. **b** Protein expression of PI3K (p110 $\alpha$ ) by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting PI3K protein levels normalized to  $\beta$ -Actin. **c** Immunofluorescent analysis of PI3K (p110 $\alpha$ ). PI3K was stained with anti-PI3K (p110 $\alpha$ ) antibody followed by secondary Hilyte Fluor<sup>TM</sup>-594-labelled antibody (red). Nucleus stained with DAPI (blue). The images of

immunofluorescence were obtained using an inverted confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystems) and analyzed by Leica Application Suite Advanced Fluorescence software (100  $\mu$ m Scale bar). **a** Experiment was performed in triplicate and **b** and **c** experiments were performed in duplicate. *EC* parental endothelial cells, *EJ-ras EC* EJ-ras transfected endothelial cells, *Adh1-EC* and *Adh2-EC* anoikis-resistant endothelial cells. The bars represent the standard error. \* $P < 0.05$ . (Color figure online)

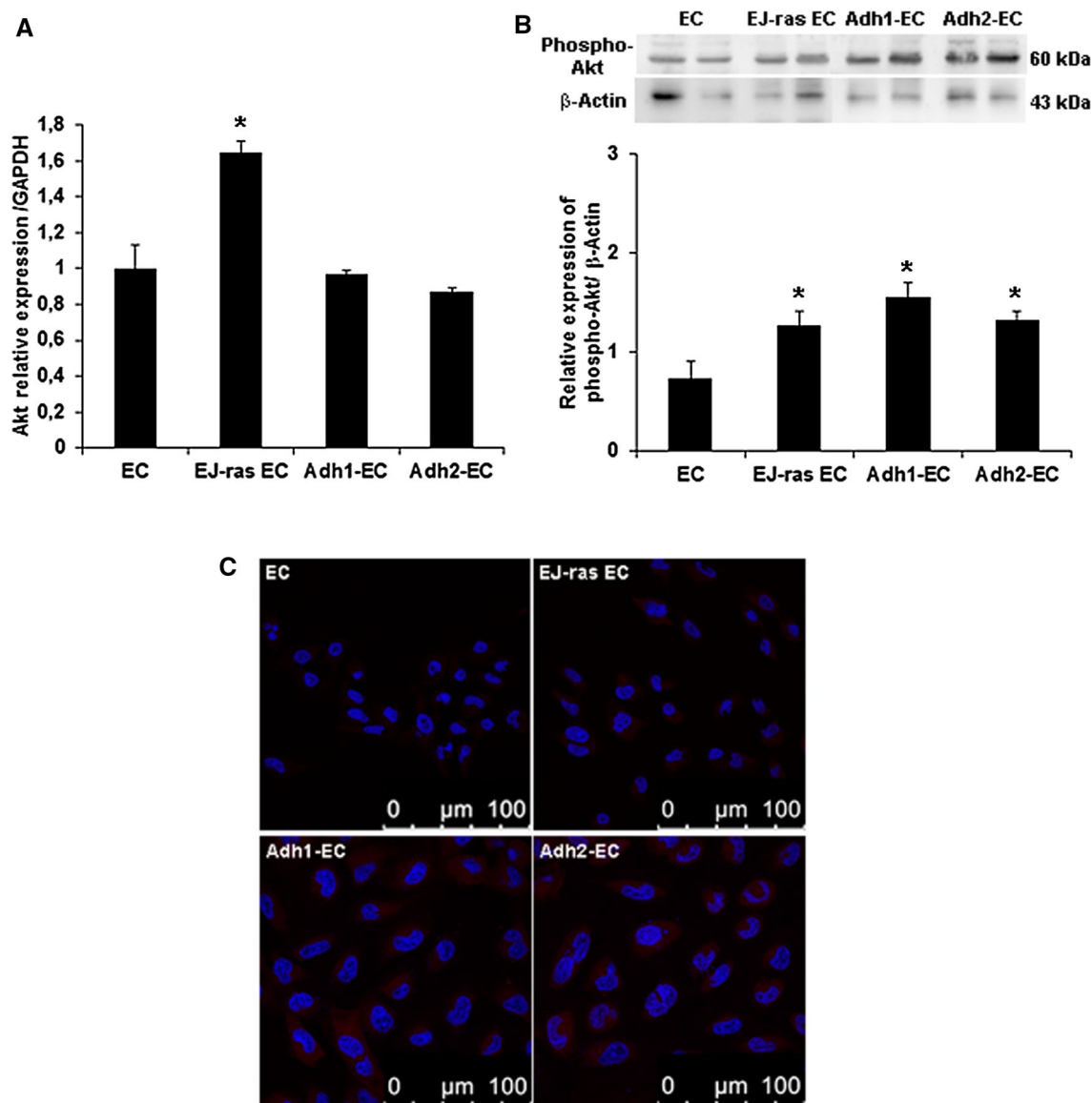
endothelial cells, we decided to investigate the expression of enzymes involved in the degradation of these molecules, the metalloproteinases (MMPs) 2 and 9, in these cells. Figure 12a, b show an increase in the proteic expression of MMP 2 and 9, respectively, in Adh-EC and EJ-ras EC cells when compared to the parental cells.

Based on these results, we decided to evaluate the expression levels of  $\alpha$ v,  $\beta$ 3,  $\alpha$ 5 and  $\beta$ 1 integrins subunits in all cell lines using western blot analysis. Figure 13a–d

show an increase in the expression levels of all subunits in endothelial cells resistant to anoikis and EJ-ras transfected cells in relation to parental cells.

Also, as changes were observed in the expression of ECM molecules and H-Ras in cells resistant to anoikis, we decided to investigate the expression these molecules after treatment with PI3K inhibitor. Figure 14 shows that inhibition of the PI3K pathway leads to reversal of protein expression changes of perlecan, collagen IV and laminin in





**Fig. 4** Gene and protein expression of Akt in EC-derived cell lines. **a** Expression of Akt detected by qPCR. GAPDH was used as a loading control. **b** Protein expression of phospho-Akt (Thr308) by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting Akt protein levels normalized to  $\beta$ -Actin. **c** Immunofluorescent analysis of phospho-Akt (Thr308). Akt was stained with anti-phospho-Akt (Thr308) antibody followed by secondary Hilyte Fluor<sup>TM</sup>-594-labelled antibody (red). Nucleus stained with DAPI (blue). The

images of immunofluorescence were obtained using an inverted confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystems) and analyzed by Leica Application Suite Advanced Fluorescence software (100  $\mu$ m Scale bar). **a** Experiment was performed in triplicate and **b** and **c** experiments were performed in duplicate. EC parental endothelial cells, EJ-ras EC EJ-ras transfected endothelial cells, Adh1-EC and Adh2-EC anoikis-resistant endothelial cells. The bars represent the standard error. \* $P < 0.05$ . (Color figure online)

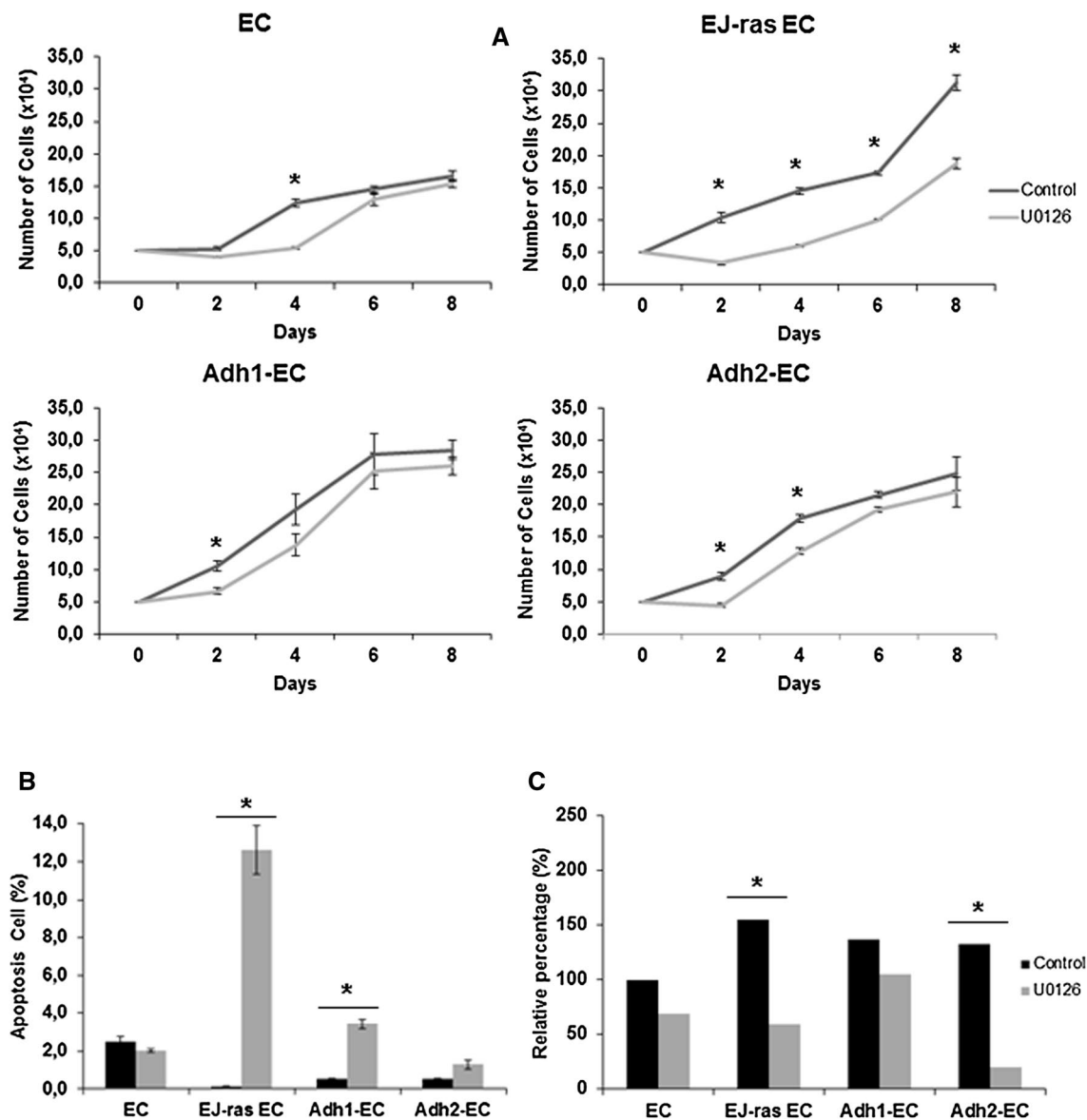
anoikis-resistant endothelial cells. In contrast, vitronectin and H-Ras expression levels were not significantly altered.

## Discussion

Recently, we reported that anoikis-resistant endothelial cells display morphological alterations, high rate of proliferation, poor adhesion to fibronectin, laminin and collagen

IV and deregulation of the cell cycle, becoming less serum-dependent. Furthermore, anoikis-resistant cell lines display a high invasive potential and a low rate of apoptosis. This is accompanied by an increase in the levels of heparan sulfate and chondroitin sulfate as well as by changes in the expression of syndecan-4 and heparanase [6].

Anoikis resistance is a vital step during cancer progression and metastatic colonization [3]. Unregulated expression of growth factor receptors or components of their

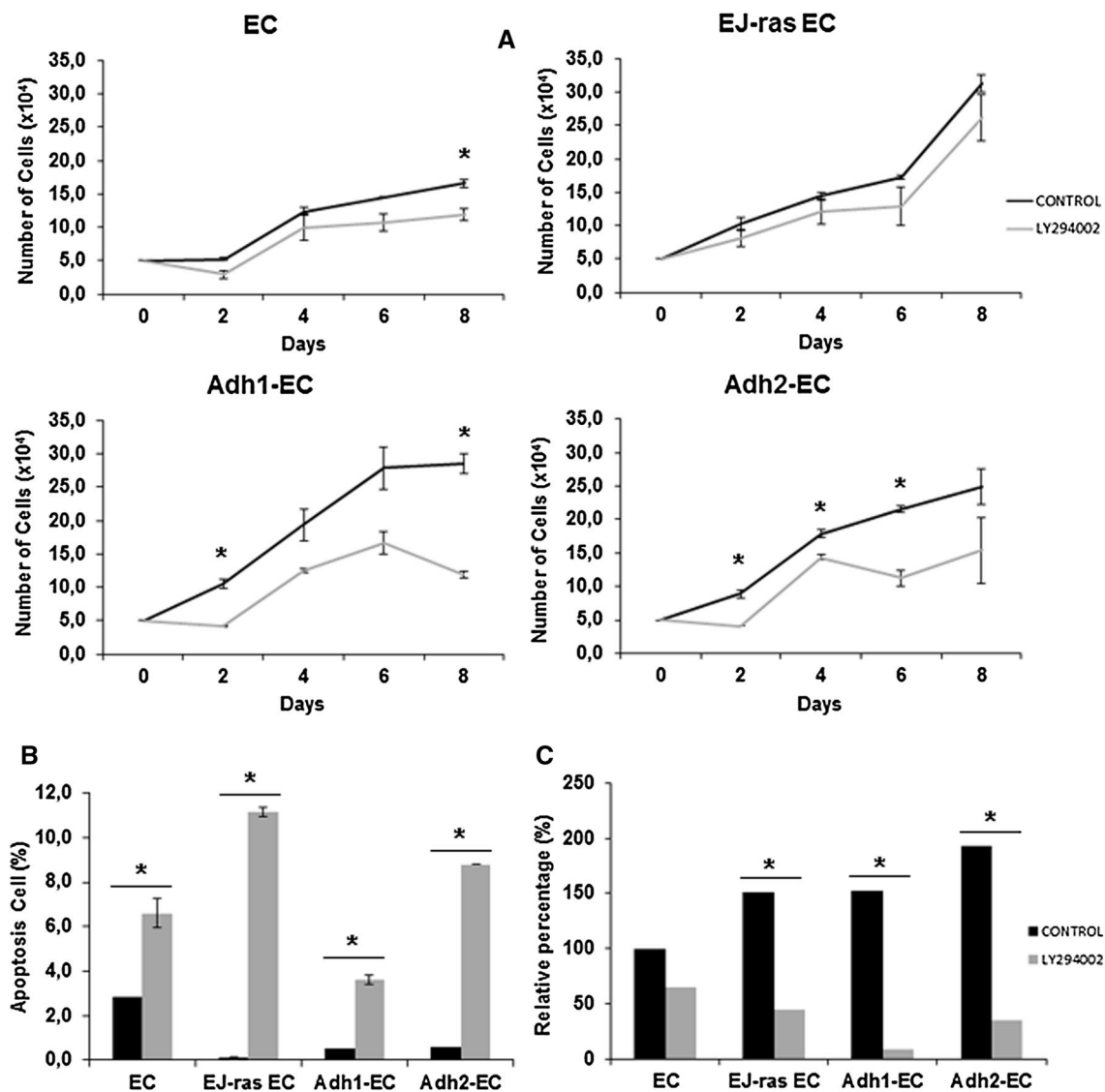


**Fig. 5** Cell growth analysis, percentage of apoptosis and invasion capacity of EC-derived cell lines after ERK inhibition. **a** Growth curves of EC, EJ-ras transfected cells (EJ-ras EC) and EC anoikis resistant (Adh1-EC and Adh2-EC) after U0126 treatment (10 μM). **b** Percentage of apoptotic cells detected by Annexin V-FITC/PI double staining method in EC, EJ-ras transfected cells (EJ-ras EC) and EC anoikis resistant (Adh1-EC and Adh2-EC) cells after U0126 treat-

ment (10 μM). **c** Invasion rate in EC, EJ-ras EC, Adh1-EC and Adh2-EC cells after U0126 treatment (10 μM). Invasion activity of EC-derived cell lines was analyzed by using a Transwell coated with ECL cell attachment matrix as described in “Methodology”. All experiments were performed in triplicate and repeated three times. The bars represent the standard error. \**P* < 0.05

signaling pathways are associated with tumor malignancy due to their inhibition of cell death pathways and activation of cell survival pathways [5, 8]. Numerous kinase/phosphatase signaling molecules have been implicated in anoikis as central regulators. Because ras activation prevents anoikis and integrins can stimulate various aspects of the ras pathway, anoikis research has focused on the two major ras effectors, the kinases PI3K (phosphoinositide-3 kinase) and raf [31].

Our findings show an increase in the levels of H-Ras and Phospho-ERK (Thr202/Tyr204, Thr185/Tyr187) in anoikis resistant endothelial cells when compared with parental cells. After treatment with U0126, a selective inhibitor of MEK1/2, all EC-derived cell lines displayed lower growth, a decrease in invasive potential and a higher rate of apoptosis. Lopes et al. [27] showed that endothelial cells transfected with the EJ-ras oncogene display morphological alterations and deregulation of the cell cycle, becoming less



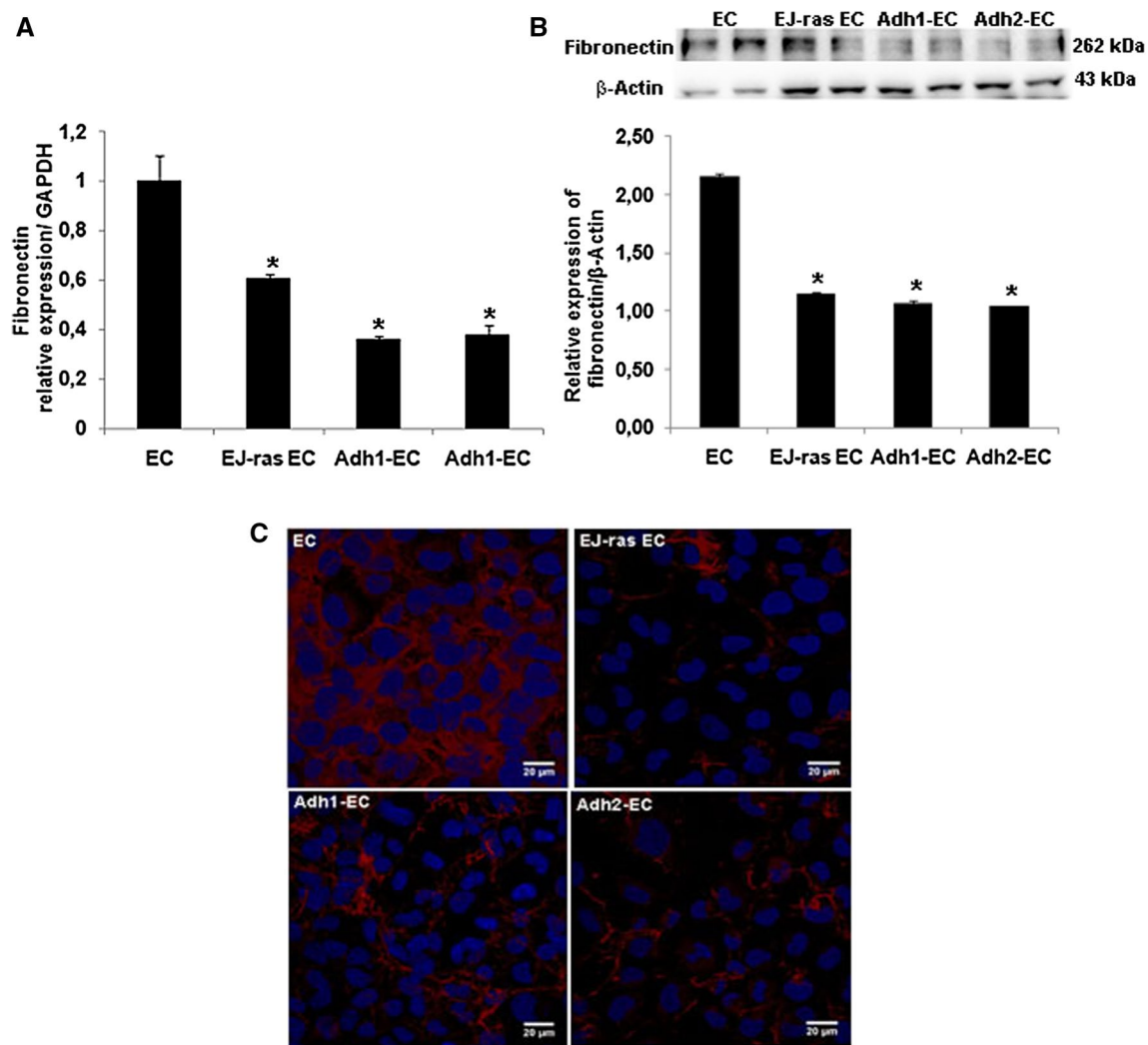
**Fig. 6** Cell growth analysis, percentage of apoptosis and invasion capacity of EC-derived cell lines after PI3K inhibition. **a** Growth curves of EC, EJ-ras transfected cells (EJ-ras EC) and EC anoikis resistant (Adh1-EC and Adh2-EC) after LY294002 treatment (3  $\mu$ M). **b** Percentage of apoptotic cells detected by Annexin V-FITC/PI double staining method in EC, EJ-ras transfected cells (EJ-ras EC) and EC anoikis resistant (Adh1-EC and Adh2-EC) cells after LY294002

treatment (3  $\mu$ M). **c** Invasion rate in EC, EJ-ras EC, Adh1-EC and Adh2-EC cells after LY294002 treatment (3  $\mu$ M). Invasion activity of EC-derived cell lines was analyzed by using a Transwell coated with ECL cell attachment matrix as described in “Methodology”. All experiments were performed in triplicate and repeated three times. The bars represent the standard error. \* $P < 0.05$

serum-dependent, in contrast with the parental endothelial cells. Activation of the Ras/ERK signaling pathway can inhibit apoptosis in response to growth factor withdrawal, death receptor activation, and, as recently described for fibroblasts and MDCK cells, detachment from extracellular matrix [32, 33]. Activation of the Raf-1/MAP kinase pathway provides a potent antiapoptotic signal in the absence of extracellular matrix support [33]. Treatment of Ras-transformed cells with the U0126 ERK inhibitor caused partial reversion to an anoikis-sensitive state, indicating that

extracellular signal-regulated kinase (ERK) activation contributes to inhibition of anoikis [34]. U0126 and PD98059, ERK inhibitors, also selectively repressed anchorage-independent growth of human breast cancer cell line MDA-MB231 [35].

ERK is a mitogen-activated protein kinase (MAPK) that functions as the major effector of the Ras oncoprotein [9]. Several reports have pointed that Raf-ERK activation providing one route that bypasses adhesion signals and elicits resistance to anoikis [36]. Enhanced ERK activity, via



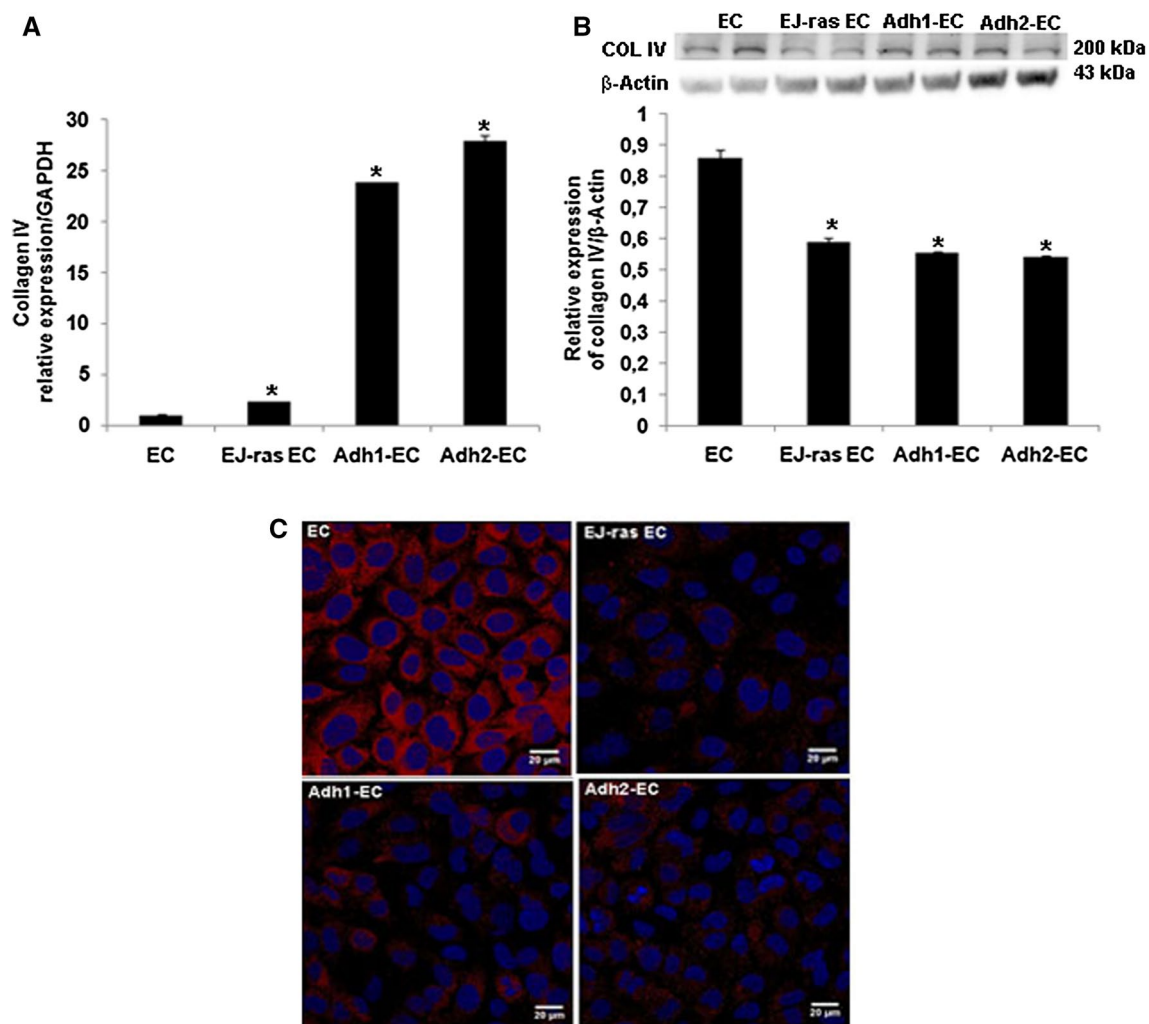
**Fig. 7** Gene and protein expression of fibronectin in EC-derived cell lines. **a** Expression of fibronectin detected by qPCR. GAPDH was used as a loading control. **b** Protein expression of fibronectin by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting fibronectin protein levels normalized to  $\beta$ -Actin. **c** Immunofluorescent analysis of fibronectin. Fibronectin was stained with anti-fibronectin antibody followed by secondary Hilyte Fluor<sup>TM</sup>-594-labelled antibody (red). Nucleus stained with DAPI (blue). The images of immuno-

fluorescence were obtained using an inverted confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystems) and analyzed by Leica Application Suite Advanced Fluorescence software (20  $\mu$ m Scale bar). **a** Experiment was performed in triplicate and **b** and **c** experiments were performed in duplicate. *EC* parental endothelial cells, *EJ-ras EC* EJ-ras transfected endothelial cells, *Adh1-EC* and *Adh2-EC* anoikis-resistant endothelial cells. The bars represent the standard error. \* $P < 0.05$ . (Color figure online)

activation of the EGF receptor, in suspended keratinocytes provides protection against anoikis [37]. Further studies showed that reactive oxygen species (ROS) leads to the SRC-mediated phosphorylation of EGF receptor at Tyr845, a site that is well known to promote the activation of EGF receptor [38]. Indeed, this ROS-mediated activation of SRC (and EGFR) was shown to be necessary for anoikis inhibition through ERK-mediated modulation of pro-apoptotic protein BIM-EL [39–41]. Another study shows that raf-1 can in turn be activated by EGFRs, this result implicates a positive feedback loop that promotes cell survival involving

raf and autocrine growth stimulation. It has been found that hepatocyte growth factor (HGF) inhibited anoikis of human head and neck squamous cell carcinoma (HNSCC) cells. The inhibition of anoikis mediated by HGF was dependent on activator protein-1 activity by activating the ERK-signalling pathway [42].

PI3K/Akt pathway also plays a major role in various cellular functions such as proliferation, invasion, and angiogenesis [43]. The role of PI3K in cancer was highlighted by the observation that the gene coding the catalytic  $\alpha$  subunit of p110 is frequently mutated in the most common human



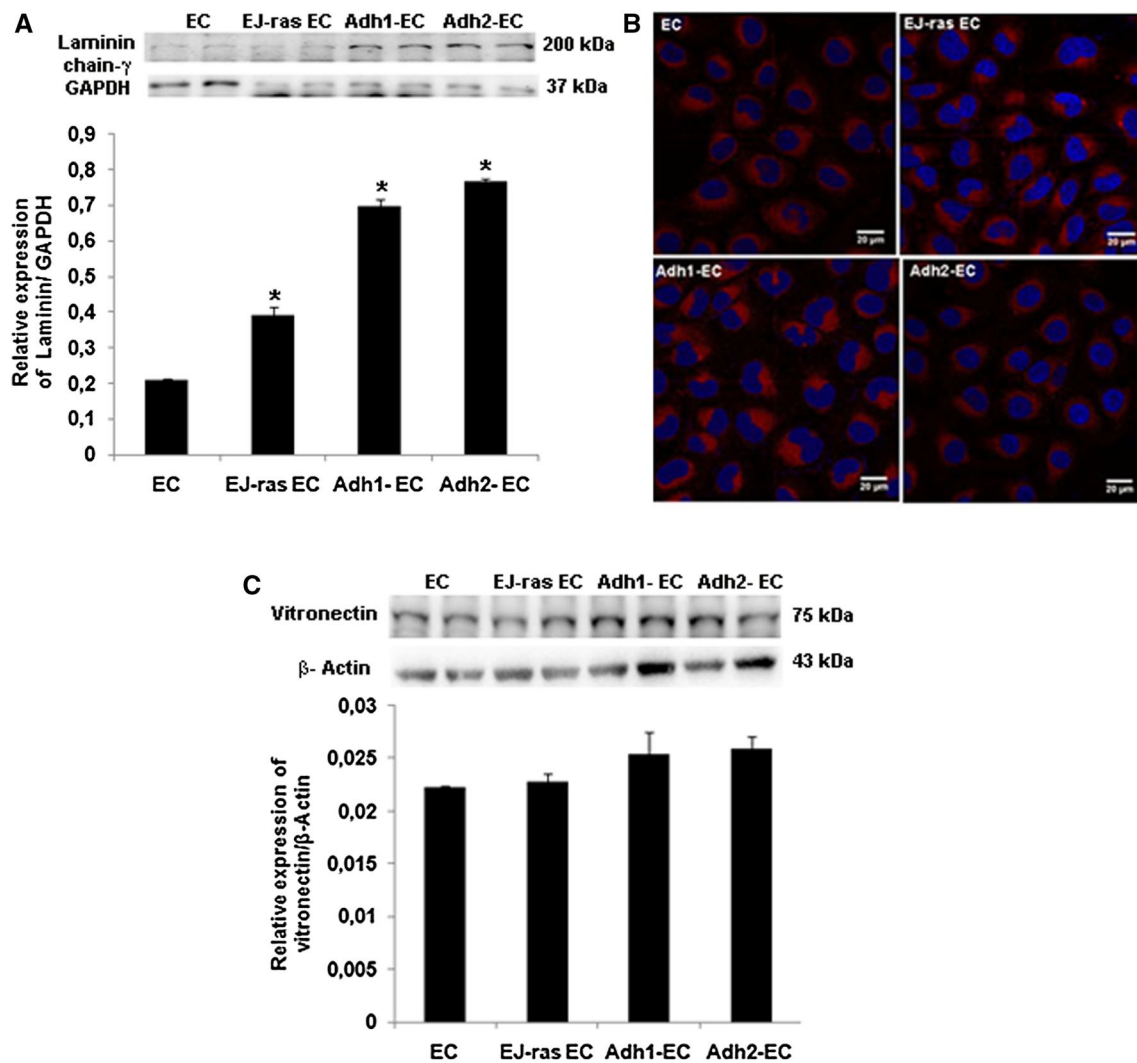
**Fig. 8** Gene and protein expression of collagen IV in EC-derived cell lines. **a** Expression of collagen IV detected by qPCR. GAPDH was used as a loading control. **b** Protein expression of collagen IV by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting collagen IV protein levels normalized to  $\beta$ -Actin. **c** Immunofluorescent analysis of collagen IV. Collagen IV was stained with anti-collagen IV antibody followed by secondary Hilyte Fluor™-594-labelled antibody (red). Nucleus stained with DAPI (blue). The

images of immunofluorescence were obtained using an inverted confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystems) and analyzed by Leica Application Suite Advanced Fluorescence software (20  $\mu$ m Scale bar). **a** Experiment was performed in triplicate and **b** and **c** experiments were performed in duplicate. EC parental endothelial cells, EJ-ras EC EJ-ras transfected endothelial cells, Adh1-EC and Adh2-EC anoikis-resistant endothelial cells. The bars represent the standard error. \* $P < 0.05$ . (Color figure online)

cancers [44, 45]. Akt is viewed as a major downstream effector of PI3K [45], as this protein kinase is recruited to the plasma membrane and is activated by phosphorylation at residues T308 and S473 by PDK1 [46]. Activated Akt is a powerful promoter of cell survival, as it antagonizes apoptosis by phosphorylating and inactivating various components of the apoptotic machinery such as Bad [47], caspase-9 [48], and forkhead transcription factor family members [49, 50]. Here we show an up-regulation of PI3K/Akt pathway by high expression of PI3K (p110 $\alpha$ ) and the high activation of Akt by phosphorylation of Thr 308

in anoikis-resistant endothelial cells. After treatment with LY294002, a selective inhibitor of PI3K, all cell lineages displayed lower growth, a decrease in invasive potential and a higher rate of apoptosis. In contrast, PI3K inhibition does not alter the H-Ras protein expression. Inhibition of PI3K-AKT pathway by LY294002 led to a decrease in growth and increase in apoptosis rates in medullary thyroid cancer cell line (TT), suggesting that PI3K-AKT pathway may play a critical role in the survival of medullary thyroid cancer cells by protecting cells from apoptosis [51]. Another study showed that the introduction of activated





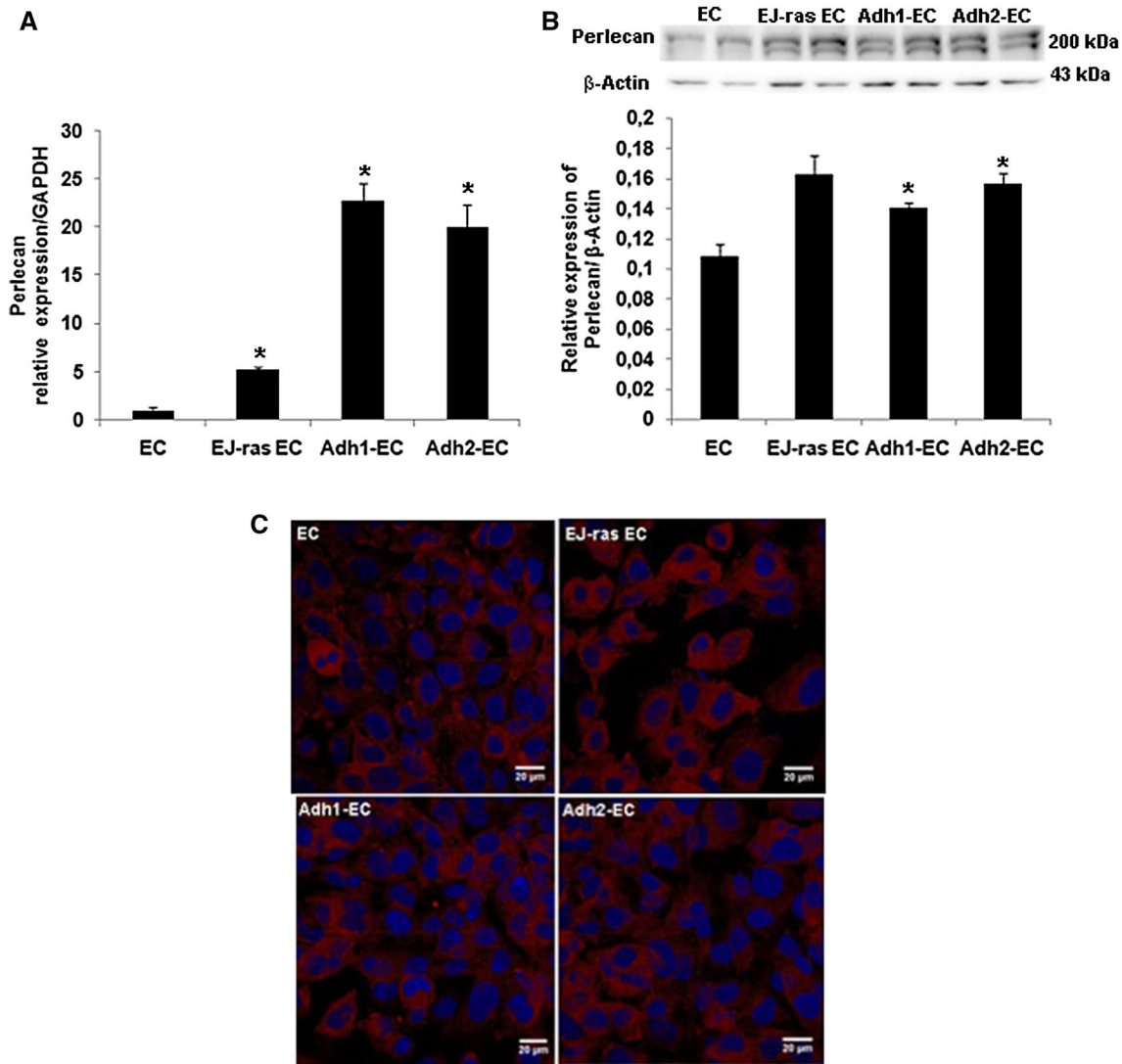
**Fig. 9** Protein expression of laminin and vitronectin in EC-derived cell lines. **a** Protein expression of laminin by western blot. GAPDH was used as a loading control. Histogram depicting laminin protein levels normalized to GAPDH. **b** Immunofluorescent analysis of laminin. Laminin was stained with anti-laminin antibody followed by secondary Hilyte Fluor™-594-labelled antibody (red). Nucleus stained with DAPI (blue). The images of immunofluorescence were obtained using an inverted confocal laser-scanning microscope (Leica

TCS SP8, Leica Microsystems) and analyzed by Leica Application Suite Advanced Fluorescence software (20  $\mu$ m Scale bar). **c** Protein expression of vitronectin by western blot.  $\beta$ -actin was used as a loading control. Histogram depicting vitronectin protein levels normalized to  $\beta$ -Actin. All experiments were performed in duplicate. EC parental endothelial cells, EJ-ras EC EJ-ras transfected endothelial cells, Adh1-EC and Adh2-EC anoikis-resistant endothelial cells. The bars represent the standard error. \* $P < 0.05$ . (Color figure online)

forms of the PI3K p110 subunit, or of Akt, rescued the MDCK cells from anoikis. Attachment-mediated activation of PI3K and protein kinase B/Akt protects cells from anoikis. In contrast, inhibition of PI3K and protein kinase B/Akt results in an induction of anoikis [52].

Ras/ERK and PI3K/Akt were originally modeled as linear signaling conduits activated by different stimuli, yet even early experiments hinted that they might intersect to regulate each other and co-regulate downstream functions. During recent years, many mechanisms and modes

of cross-talk have been uncovered. These include cross-inhibition, crossactivation, and pathway convergence on substrates [9]. The Ras/ERK pathway cross-activates PI3K/Akt signaling. Ras-GTP can directly bind and allosterically activate PI3K, similarly, components of the Ras-ERK pathway (Ras, Raf, ERK, and RSK) also positively regulate the PI3K/mTORC1 pathway [9, 53–55]. Once activated, ERK and Akt often act on the same substrate, sometimes in concert, to promote cell survival, proliferation, metabolism, and motility. Examples include FOXO (forkhead box



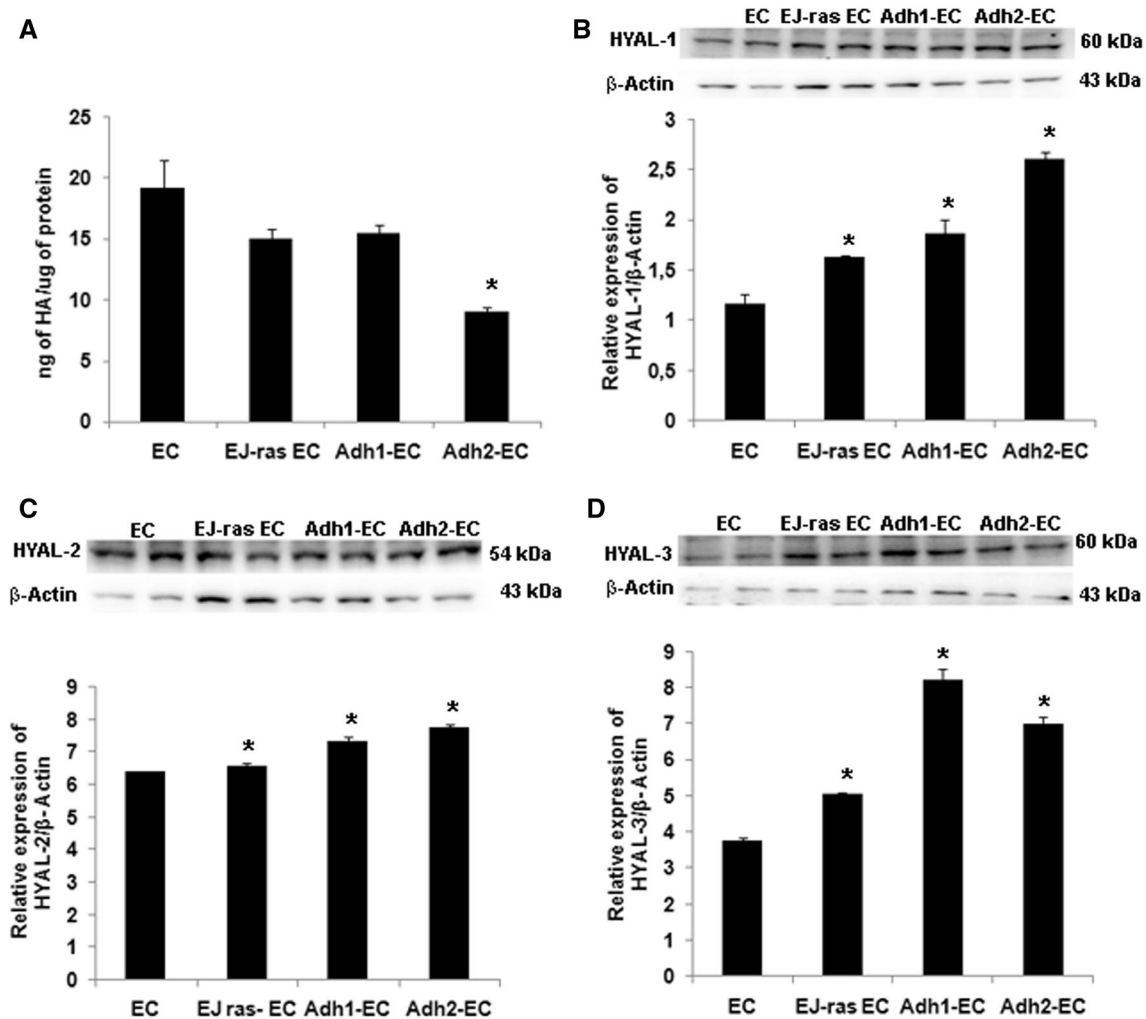
**Fig. 10** Gene and protein expression of perlecan in EC-derived cell lines. **a** Expression of perlecan detected by qPCR. GAPDH was used as a loading control. **b** Protein expression of perlecan by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting perlecan protein levels normalized to  $\beta$ -Actin. **c** Immunofluorescent analysis of perlecan. Perlecan was stained with anti-perlecan antibody followed by secondary Hilyte Fluor™-594-labelled antibody (red). Nucleus stained with DAPI (blue). The images of immunofluorescence were

obtained using an inverted confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystems) and analyzed by Leica Application Suite Advanced Fluorescence software (20  $\mu$ m Scale bar). **a** Experiment was performed in triplicate and **b** and **c** experiments were performed in duplicate. EC parental endothelial cells, EJ-ras EC EJ-ras transfected endothelial cells, Adh1-EC and Adh2-EC anoikis-resistant endothelial cells. The bars represent the standard error. \* $P < 0.05$ . (Color figure online)

O) and GSK3 (glycogen synthase kinase 3), which can be inhibited by ERK and Akt [9, 56, 57]. FOXOs regulates the expression of apoptotic proteins and cell cycle regulators to suppress cell survival and proliferation. GSK3 inhibits pro-survival, proliferation and motility proteins, such as adhesion proteins and the b-catenin transcription factor that drives the expression of cyclin D and MYC [9]. This leads us to believe that pharmacological inhibition of PI3K activity with LY294002 in endothelial cells resistant to anoikis

attenuates the negative regulation of FOXOs and GSK3 by Akt, consequently inhibits the cell proliferation and increases the apoptosis rate.

The signaling pathways that drive cell survival, proliferation and motility also are controlled by microenvironmental cues, including cell–cell interactions and ECM-integrin engagement [58]. Tumor cells actively remodel their local extracellular microenvironment either by directly releasing ECM remodeling enzymes such as MMPs,



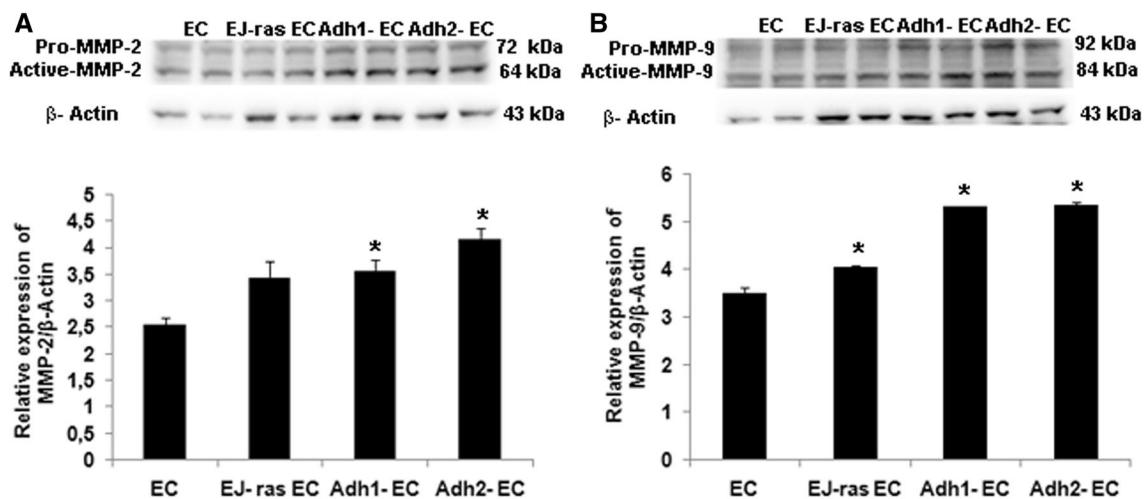
**Fig. 11** Synthesis of hyaluronic acid and protein expression of hyaluronidases 1, 2 and 3 in EC-derived cell lines. **a** Synthesis of hyaluronic acid. The samples were subjected to non-competitive fluorometric ELISA-like assay to detect HA. **b** Protein expression of hyaluronidase 1 (HYAL-1) by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting HYAL-1 protein levels normalized to  $\beta$ -Actin. **c** Protein expression of hyaluronidase 2 (HYAL-2) by western blot.  $\beta$ -Actin was used as a loading control. Histogram

depicting HYAL-2 protein levels normalized to  $\beta$ -Actin. **d** Protein expression of hyaluronidase 3 (HYAL-3) by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting HYAL-3 protein levels normalized to  $\beta$ -Actin. **a** Experiment was performed in triplicate and **b**, **c** and **d** experiments were performed in duplicate. *EC* parental endothelial cells, *EJ-ras EC* EJ-ras transfected endothelial cells, *Adh1-EC* and *Adh2-EC* anoikis-resistant endothelial cells. The bars represent the standard error. \* $P < 0.05$

serine and cysteine proteases or hyaluronidases [59–61]. Here we show that anoikis-resistant endothelial cells display a decrease in the expression of fibronectin, collagen IV and hyaluronic acid and an increase in the expression of laminin, perlecan,  $\alpha$ v,  $\beta$ 3,  $\alpha$ 5 and  $\beta$ 1 integrins subunits, HYALs 1, 2 and 3 and MMPs 2 and 9. Inhibition of the PI3K-AKT pathway by LY294002 leads to reversal of protein expression changes of perlecan, collagen IV and laminin in anoikis-resistant endothelial cells.

Different extracellular matrix components can transduce distinct survival signals through preferential

activation of subsets of multiple integrin-mediated signaling pathways. Survival signals from laminin-10/11 are mainly through the PI3 kinase/Akt pathway, whereas survival signals from fibronectin are conveyed by MEK1/ERK through FAK. Laminin-10/11 has more survival potential than fibronectin [62]. The overexpression of laminin-5  $\gamma$ 2 chain increases cell proliferation, and thereby promotes tumor growth, indicating the possibility that this molecule could be used as a marker for disease progression and malignancy [63–65]. In addition, vitronectin induces Akt activity and rescue detached HEK293



**Fig. 12** Protein expression of MMP-2 and 9 in EC-derived cell lines. **a** Protein expression of MMP-2 (active enzyme/64 kDa) by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting MMP-2 protein levels normalized to  $\beta$ -Actin. **b** Protein expression of MMP-9 (active enzyme/84 kDa) by western blot.  $\beta$ -Actin was used

as a loading control. Histogram depicting MMP-9 protein levels normalized to  $\beta$ -Actin. All experiments were performed in duplicate. *EC* parental endothelial cells, *EJ-ras EC* EJ-ras transfected endothelial cells, *Adh1-EC* and *Adh2-EC* anoikis-resistant endothelial cells. The bars represent the standard error. \* $P < 0.05$

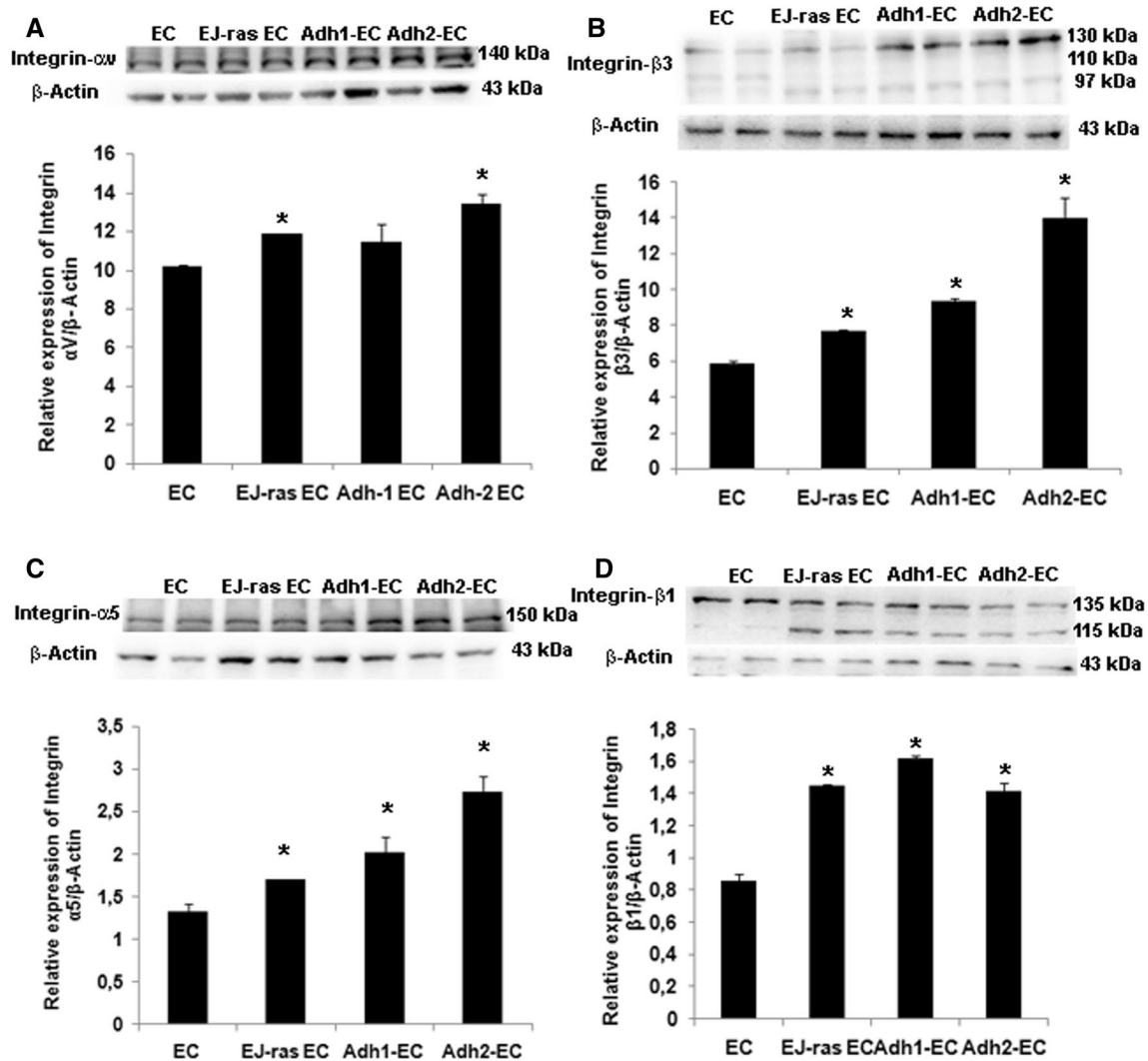
cells from anoikis [66] and loss of type IV collagen during tumor development can potentially provide a permissive microenvironment for invasiveness phenotype of cancer [67, 68]. Zeng et al. [69] showed that loss of basal membrane type IV collagen occurs during colorectal tumorigenesis and is associated with elevations in the active forms of MMP-2 and MMP-9. This consequently leads to an increase in the survival and proliferation of tumor cells.

Integrins mediate anchorage and migration of cells via recognition of variable extracellular matrix molecules. Only specific ligated integrins suppress anoikis in certain cell types. For example, serum-starved HT29 carcinoma cells readily underwent apoptosis, which was partially suppressed by transfection of the  $\alpha$  integrin subunit [70]. The ectopic expression of  $\alpha 5\beta 1$  integrin, but not  $\alpha \nu \beta 1$  integrin, suppressed anoikis of fibronectin-bound CHO cells under serum-free conditions [71].  $\alpha 5\beta 1$  integrin controls invasion of breast cancer cells by modulation of MMP-1 [72] and MMP-2 collagenase activity [73]. Fibronectin secreted by peritoneal tissue activates  $\alpha 5\beta 1$  integrin on ovarian cancer cells to stimulate their invasiveness through an increase of MMP-9 activity [74]. In addition,  $\alpha \nu \beta 3$  integrin mediates endothelial cell binding to the extracellular matrix and transduces an intracellular signal promoting survival of endothelial cells and various tumor cells [75].  $\alpha \nu \beta 3$  integrin is important for adhesion of melanoma cells to dermal collagen and the suppression of anoikis [76], most likely by altering the Bcl2/Bax ratio [77].

Other ECM molecules are also involved in anoikis resistance. Perlecan, a heparan sulfate (HS) proteoglycan, supports various biological functions, including cell adhesion, growth factor binding, and modulation of apoptosis. A C-terminal fragment released from endothelial cell-derived perlecan protects fibroblasts from apoptosis [78, 79]. Anoikis resistant melanocytes show an increase in perlecan expression [80]. Increased perlecan levels are detected in breast carcinomas and in metastatic melanomas that correlate with a more aggressive phenotype [81].

Hyaluronic acid (HA) is a nonsulfated glycosaminoglycan that interacts with cell surface receptors, including cluster determinant 44 (CD44). Binding of HA with CD44 has been implicated in a variety of physiological events including cell–cell and cell–substrate adhesion, cell migration, cell proliferation and HA uptake and degradation [82–84]. Degradation of HA is achieved through the enzymatic action of hyaluronidases (Hyal-1, Hyal-2 and Hyal-3), whose expression is increased or suppressed in some malignant conditions compared with normal tissues [85]. It is believed that the various HA fragments have distinct effects in cancer progression, angiogenesis and metastasis [61, 85] and may also activate matrix metalloproteinases (MMPs), thus enhancing tumor invasiveness [86].

During cancer progression, tumor-associated ECM is continually remodeled to create a more suitable microenvironment for malignant cellular growth, survival, adhesion and invasive migration. This remodeling



**Fig. 13** Protein expression of  $\alpha$ v,  $\beta$ 3,  $\alpha$ 5 and  $\beta$ 1 integrins subunits in EC-derived cell lines. **a** Protein expression of  $\alpha$ v integrin subunit by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting  $\alpha$ v integrin subunit protein levels normalized to  $\beta$ -Actin. **b** Protein expression of  $\beta$ 3 integrin subunit by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting  $\beta$ 3 integrin subunit protein levels normalized to  $\beta$ -Actin. **c** Protein expression of  $\alpha$ 5 integrin subunit by western blot.  $\beta$ -Actin was used as a loading control.

Histogram depicting  $\alpha$ 5 integrin subunit protein levels normalized to  $\beta$ -Actin. **d** Protein expression of  $\beta$ 1 integrin subunit by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting  $\beta$ 1 integrin subunit protein levels normalized to  $\beta$ -Actin. All experiments were performed in duplicate. *EC* parental endothelial cells, *EJ-ras EC* EJ-ras transfected endothelial cells, *Adh1-EC* and *Adh2-EC* anoikis-resistant endothelial cells. The *bars* represent the standard error. \* $P < 0.05$

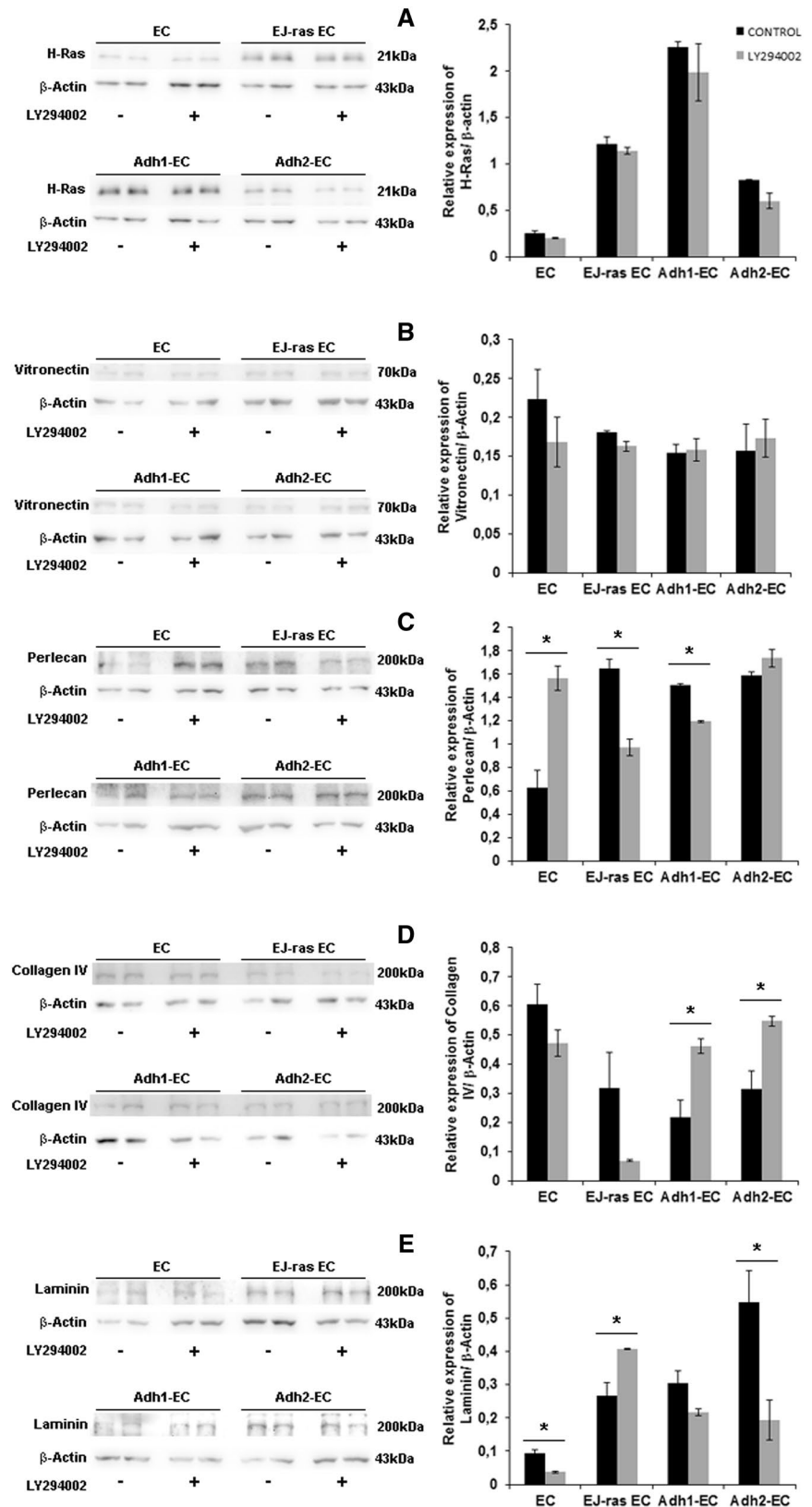
process includes the ECM degradation process mainly mediated by matrix metalloproteinases (MMPs) [87]. MMP-2 and MMP-9 have been shown to play critical roles in the “angiogenic switch” and tumor cells could synthesise and secrete large amounts of MMP-2 and MMP-9 in a paracrine and/or autocrine manner to stimulate angiogenesis and increase VEGF release [88]. Signaling pathways are involved in the regulation of MMPs. Kim et al. [89] found that both the PI3K and MAP kinase pathways are involved in the induction of MMP-9. Both pathways seem to contribute to MMP-9 production in a synergistic manner and the inhibition of these pathways

resulted in total inhibition of MMP-9 production. These data are consistent with our findings using PI3K inhibitor that decrease the invasiveness in anoikis resistant cell lines.

In conclusion, this study revealed that the acquisition of anoikis resistance induced remodeling of the extracellular matrix and overexpression of the PI3K/Akt and Ras/ERK pathways components, as this overexpression is crucial to keep the tumorigenic characteristics as higher proliferation, lower apoptosis and higher invasiveness. Acquisition of resistance to anoikis is a potentially crucial step in endothelial cell transformation.



**Fig. 14** Protein expression of H-Ras and ECM molecules in EC-derived cell lines after PI3K inhibition. **a** Protein expression of H-Ras by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting H-Ras protein levels normalized to  $\beta$ -Actin. **b** Protein expression of vitronectin by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting vitronectin protein levels normalized to  $\beta$ -Actin. **c** Protein expression of perlecan by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting perlecan protein levels normalized to  $\beta$ -Actin. **d** Protein expression of collagen IV by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting collagen IV protein levels normalized to  $\beta$ -Actin. **e** Protein expression of laminin by western blot.  $\beta$ -Actin was used as a loading control. Histogram depicting laminin protein levels normalized to  $\beta$ -Actin. All experiments were performed in duplicate. *EC* parental endothelial cells, *EJ-ras EC* EJ-ras transfected endothelial cells, *Adh1-EC* and *Adh2-EC* anoikis-resistant endothelial cells. The bars represent the standard error.  $*P < 0.05$



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