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Papilloma protein E6 abrogates shear stress-dependent survival in human endothelial cells: Evidence for specialized functions of paxillin

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Abstract

Background: To investigate how endothelial cells transduce intracellular signals in response to laminar shear stress (SS), we made use of the papilloma virus oncoprotein E6 which interacts with and induces degradation of numerous cellular proteins including p53 and members of the PDZ-domain family. E6 also recognizes paxillin (PXN), a fundamental component of focal adhesions, interfering with its association to focal adhesion kinase (FAK).

Methods and results: Human umbilical vein endothelial cells, expressing E6 or its mutated variant $\Delta E6^{105-110}$ (ΔE6) which does not inactivate p53, were cultured under static conditions or exposed to a laminar SS of 12 dyn/cm^2 for 16 h. In response to SS, cells expressing E6 or ΔE6 failed to synthesise nitric oxide and directionally remodel their cytoskeleton, as indicated by morphology and phalloidin staining of actin microfilaments. Under these conditions, PXN association with FAK, its localization to the plasma membrane, and its phosphorylation on tyrosine-31, which partially encompasses the PXN/FAK docking site, were severely compromised. These alterations were paralleled by the impairment of important SS-dependent endothelial functions, including nitric oxide production and survival upon serum deprivation. The direct targeting of PXN expression by RNA interference partially reproduced the E6 phenotype, impairing flow-dependent cell orientation and survival but not nitric oxide production.

Conclusions: These results provide evidence that papilloma virus E6 protein interferes with the function of the SS-mechanosensor and suggests a potential a role for PXN in this process.

Keywords: Flow; Signal transduction; Apoptosis; Endothelial; Integrin

1. Introduction

Laminar shear stress (SS) is an important source of biomechanical stimuli modulating cardiac morphogenesis [1] and endothelial cell (EC) function by a signal transduction cascade that leads to chromatin remodeling [2], survival [3,4], and the regulation of panels of specific transcription units [5]. The proposed nature of the SS mechanoreceptor is multifaceted and includes stretch-sensitive ion channels, protein kinases associated with the cytoskeleton, integrin–cytoskeletal interactions, cytoskele-

Abbreviations: BAEC, bovine aortic endothelial cells; ERK, extracellular signals regulated kinase; HUVEC, human umbilical endothelial cell; IP, immunoprecipitation; LD, leucine-aspartate region; LIM domains, lin-11, isl-1 mec-3 domain; MAPK, mitogen-activated protein kinase; PDZ, protein domain Z; PI3K, phosphoinositide-3-kinase; PXN, paxillin; shPXN, short hairpin of paxillin RNA; SS, shear stress; ST, static

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tal–nuclear interactions, and oxidase systems capable of generating reactive oxygen species. The molecular identity of the mechanosensor, however, is only partially known; it is neither clear whether multiple sensing mechanisms exist and/or may be simultaneously activated [5]. Notably, integrin complexes, such as $\alpha_v\beta_1$, β_3 and β_5 [6] and their interacting molecules, play an important role in the early steps of this process [7]. In response to SS, in fact, focal adhesions undergo directional remodeling [8] and the integrin-associated protein kinases src and focal adhesion kinase (FAK) become activated and interact with the docking proteins grb-2, shc, and paxillin (PXN) [9], stimulating the downstream MAP kinase (MAPK) signalling pathway [10] leading to the transcriptional induction of immediate–early genes [2,11].

The papilloma virus protein E6 represents a valuable tool for a simultaneous interference along multiple intracellular pathways [12]. It interacts with several intracellular targets including the tumour-suppressor protein p53, which is rapidly ubiquitinylated and degraded via the proteasome pathway, and members of the PDZ domain protein family Disc Large, Scribble and MUPP1 [13,14]. E6 recognizes also the LD repeats of PXN and blocks its interaction with FAK and vinculin, disrupting the structure of the cytoskeleton, which plays an important role in the directional remodeling induced by laminar flow.

This work establishes that papillomavirus E6 protein compromises endothelial cells response to laminar SS and provides the evidence that PXN may play an important role in this process.

2. Materials and methods

2.1. Cell culture

$HUVEC^{pBABE}$, $HUVEC^{E6}$ and $HUVEC^{\Delta E6}$ cells were cultured in endothelial cell basal medium (EBM-2, Clonetics) supplemented with an endothelial cell Bullet Kit (Clonetics) (2% FCS, hEGF-2, hFGF-2, heft, R3-IGF-1, ascorbic acid, hydrocortisone, heparin, gentamycin, amphotericin-B, Bio-Whittaker). Cells were used between passage 4 and 6. Bovine aortic endothelial cells (BAECs) were isolated and cultured in DMEM with 10% FCS as previously described [15]. For all experiments, cells were used between passages 3 and 8.

2.2. Retroviral infection

Phoenix-ampho cells (American Type Culture Collection) were cultivated and transfected with pBABE-puro [16], pBABE-puro E6 and pBABE-puroΔE6 as described by Pear et al. [17]. Briefly, Phoenix-ampho packaging cells were transfected with 10 μg/p100 dish of each retroviral vector using Fugene6 reagent (Roche) according to manufacturer instructions. Medium containing the emerging retrovirus was harvested 36 h after transfection, filtered and incubated for 5 h with HUVEC in the presence of 4 μg/ml of polybrene. Twelve hours later, the retroviral supernatant was harvested for a second time and HUVEC infected again. Forty-eight hours after the second round of infection, HUVEC were selected in puromycin-containing medium (0.5 μg/ml, Sigma), obtaining $HUVEC^{pBABE}$, $HUVEC^{E6}$ and $HUVEC^{\Delta E6}$ cell lines. pBABE-puro E6 and pBABE-puro ΔE6 plasmids were generated from pGST-E6 and pGST-ΔE6, respectively, using standard techniques [18].

2.3. Short-hairpin RNA interference

Short interfering RNA constructs (shRNAi) were made in pSuper.retro expression vector (Oligoengine). To suppress endogenous paxillin expression, sense and anti-sense oligonucleotides GATCCCGGCAAGGACTACTTCGACATGT corresponding to nucleotides 1292–1312 of the human paxillin cDNA sequence were cloned into pSUPER (pSuper-shPXN). Phoenix cells were transfected with pSuper-shPXN construct using Fugene (Roche) to produce retroviral particles as described above. The cells were infected and selected in puromycin containing medium obtaining $HUVEC^{pSuper}$ and $HUVEC^{shPXN}$ cell lines.

2.4. Transfection and immunofluorescence

The pGFP-paxillin was constructed by excising human paxillin cDNA from pCMV/IL2R-paxillin (kindly provided by Dr. K. Nakata, NIDCR, NIH) at *Hin*dIII and *Xba*I sites and inserting it into the *Hin*dIII and *Xba*I sites of a pRK5-based GFP tagged plasmid, pGZ21XdZ (gift from Dr. Shinichi Aota, NIDCR, NIH). The single AA point mutant paxillin Y31A was constructed using a QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. The mutagenesis was performed by using the following primers and pGFP-paxillin as template: forward primer 5′-CTTGTCGGAGGAGACCCCC***GC***CTCATACCCAACTGGAAACCACAC-3′; reverse primer 5′-GTGTGGTTTCCAGTTGGGTATGAG***GC***GGGGGTCTCCTCCGACAAG-3′. The mutations were verified by sequencing the entire paxillin cDNA. Expression vectors for Bovine papilloma virus E6 (BPE6) and an E6 mutant Δ134–137 with reduced affinity for paxillin ($BPE6^{mut}$) were kindly provided by Howley PM [19]. For transient transfection experiments all vectors were transfected into BAEC cells according to a prior published protocol [20]. After transfection, the same cell population was divided to 1/2 in 150 mm^2 cell culture plate. Near-confluent cells were exposed to a laminar SS of 12 dyn/cm^2, for 12 to 18 h by using a cone-and-plate apparatus [21]. Control cells were kept under static culture condition. At the end of each experiment cells were fixed with 3.7% paraformaldehyde (Sigma) in PBS for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS

for 2 min, soaked in PBS containing 2% bovine serum albumin (blocking solution) for 1 h at room temperature. Actin filaments were visualized by incubation for 1 h with FITC-conjugated phalloidin (Molecular Probes, Eugene, OR) at 1:2000 dilution. After washing, the samples were mounted in 50% glycerol/phosphate-buffered saline, and images were acquired using standard fluorescence microscopy (Axioplan 2; Carl Zeiss, Inc.).

2.5. Western blot and immunoprecipitation analyses

For Western blot analysis, cells were rinsed with ice-cold phosphate-buffered saline (PBS), scraped, and lysed for 15 min as previously described [4]. For immunoprecipitation experiments, 150 μg of whole cell extracts were incubated with the indicated antibody under gentle rocking at 4 °C overnight. After two pre-clearing washes, the protein A-agarose (Santa Cruz) was incubate for 2 h at 4 °C with the lysate. Immunoprecipitates were resuspended in 20 μl of 4× SDS Laemli buffer and resolved by SDS–PAGE before electro-transfer to nitrocellulose (Amersham, Uppsala, Sweden). The following antibodies were used to detect the proteins of interest: phospho-FAK (Y397) (BD Transduction Laboratories), FAK (C-903) (Santa Cruz Biotechnologies), phospho-AKT (Biosource International), AKT 1-2 (Santa Cruz Biotechnologies), paxillin (5H11) (Transduction Laboratories), phospho-paxillin pY118 (Biosource International), phospho-paxillin pY181 (Biosource International), phospho-paxillin pY31 (Biosource International), tubulin (Santa Cruz Biotechnologies), vinculin (Santa Cruz Biotechnologies), phospho-ERK 1–2 (Santa Cruz Biotechnologies), ERK 1–2 (Santa Cruz Biotechnologies), paxillin (H-114) (Santa Cruz Biotechnologies), phospho-eNOS (Cell Signalling), e-NOS (Transduction Laboratories), p53 (Ab-1, Oncogene) and anti-c-*fos* antibody (Santa Cruz); they were used according to the manufacturer's instruction. Normalization of protein loading was obtained using red-Ponceau staining of the membranes and/or an anti-histone H1 antibody (Upstate Biotechnology).

2.6. Determination of nitric oxide production

Nitric oxide production was evaluated by the 4,5-diaminofluorescein (DAF-2 DA) (Alexis) added to the complete medium. Cells were cultured under SS for 18 h and, at the end of the experiment, washed with PBS, trypsinized, centrifuged, and analysed by flow cytometry to detect intracellular NO production.

2.7. Evaluation of cell death

Cell death was induced in 50–70% confluent cultures by treatment with 100 μM $CoCl_2$ (Sigma-Aldrich) in EBM-2 serum-free medium (Clonetics) as previously described [4]. The evaluation of the sub-G_1 DNA content was performed after incorporation of propidium iodide by FACS analysis (FACSCalibur, Becton & Dickinson).

2.8. Statistical analysis

Variables were analysed by Student's *t* test. A value of $P \leq 0.05$ was deemed statistically significant.

3. Results

3.1. Papilloma virus E6 protein impairs endothelial cell responses to laminar flow

A series of experiments were performed in human endothelial cells expressing papilloma virus proteins E6 ($HUVEC^{E6}$) or its mutant ΔE6 ($HUVEC^{\Delta E6}$) which retains the capacity of PXN binding but does not stimulate p53 degradation (see inset of Fig. 1A). Fig. 1A shows that $HUVEC^{E6}$ and $HUVEC^{\Delta E6}$ exposed to SS lost their capacity to align along the SS direction as indicated by phalloidin staining of actin filaments, putting in evidence that E6 and ΔE6 proteins act through a common p53-independent mechanism possibly due to PXN binding.

One of the most relevant biological effect of SS is its property to protect ECs from apoptosis. In our prior work [4], we reported that growth factor deprivation (GDF) and chemical hypoxia generated by using $CoCl_2$ induced apoptosis in HUVEC cells kept in static culture while, in this condition, SS treatment efficiently protected cells from death. In this work we investigated how $HUVEC^{E6}$ and $HUVEC^{\Delta E6}$, exposed to GDF and $CoCl_2$, responded to SS.

$HUVEC^{E6}$ cells, expressing E6, presented a very low level of p53 expression (see inset in Fig. 1A) and were resistant to cell death both in ST as SS condition. While in the presence of ΔE6, which binds PXN but does not induces p53 degradation (see inset in Fig. 1A), laminar flow failed to protect cells from cell death revealing a marked impairment in the SS signalling aimed at protecting ECs from cell death (Fig. 1B). Therefore, the absence of p53, determined by E6 expression, prevented endothelial cells from undergoing death; however, cell viability was markedly decreased in cells expressing ΔE6, which preserves p53 expression, indicating that p53 is downstream of the SS mechanosensor. Further, these observations suggest that PXN, acting upstream of p53, could be potentially involved in the antiapoptotic function of SS.

In the same experimental condition, after SS treatment, both cell lines showed a reduction in NO production (Fig. 1C, upper panel). Noteworthy, in this experiment, the E6 mutant ΔE6 appears more efficient in inhibiting NO production compare to the wild type protein E6 (Fig. 1C, upper panel). In order to ascertain the role of NO in this system, E6 and ΔE6 cells were cultured in presence or absence of a sodium-nitroprusside, a well known NO donor,

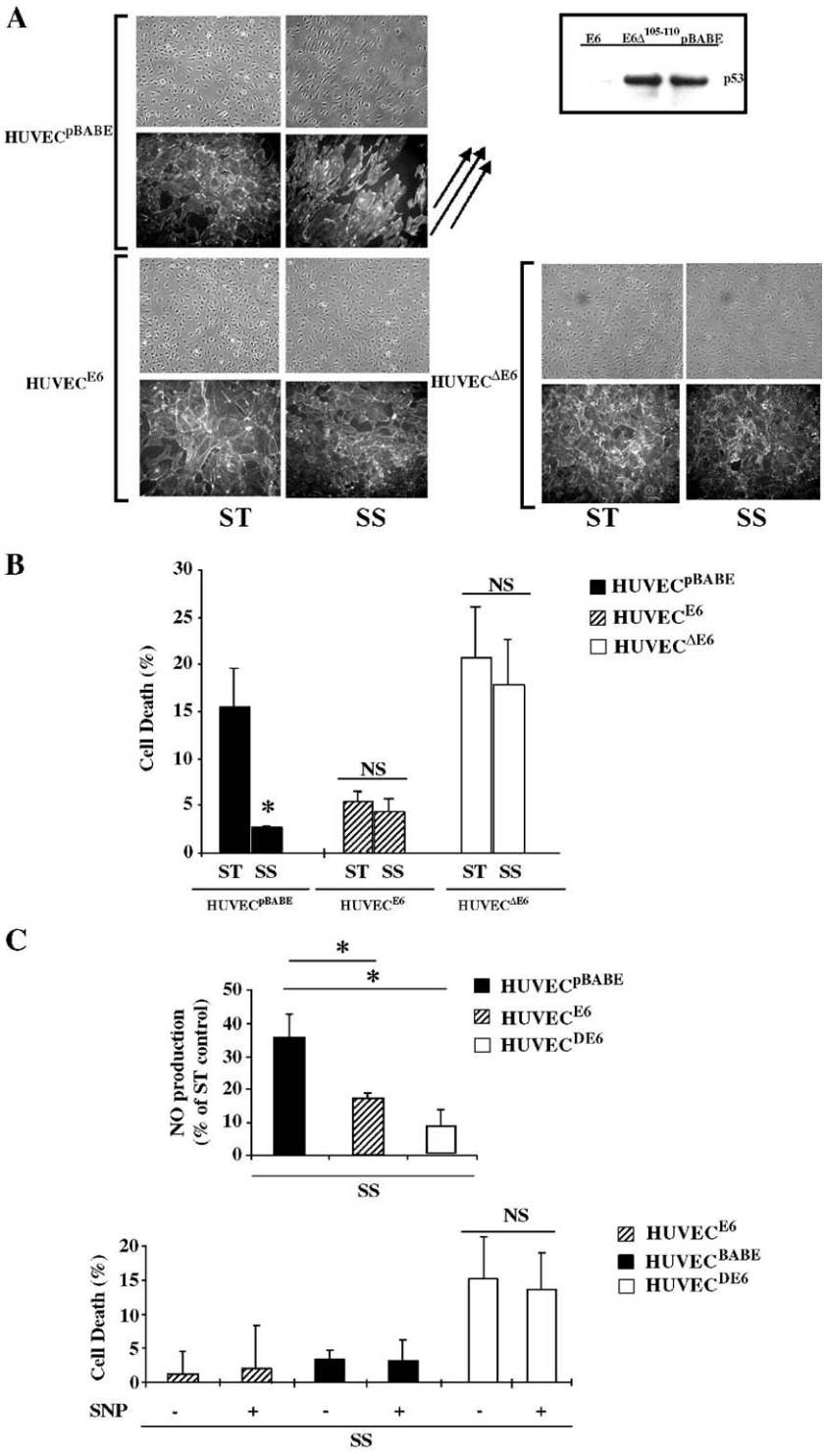

Fig. 1. (A) Papilloma virus protein E6 abrogates orientation remodelling in presence of laminar SS. The picture shows representative images of phase contrast and fluorescent phalloidin staining of $HUVEC^{pBABE}$ (upper), $HUVEC^{E6}$ (lower left) and $HUVEC^{\Delta E6}$ (lower right) exposed to a laminar SS of 12 dyne/cm^2 for 16 h ($n=4$). Arrows indicate the direction of flow. The inset shows the Western blotting analysis of p53 protein level in control cells and in cells expressing E6 and ΔE6 papilloma virus proteins, showing the differences between the three cell lines. (B) SS antiapoptotic effects are compromised in HUVEC expressing papilloma virus proteins E6 and $E6^{\Delta 105-110}$ (ΔE6). The graph represents the results of three independent experiments performed in duplicate in which laminar SS effects on cell viability were evaluated by trypan blue exclusion and cell count ($p>0.05$). (C) NO production is reduced in the presence of E6 and does not protect ECs from apoptosis. The upper panel shows the results of three independent experiments performed in duplicate in which NO production was determined by DAF incorporation and FACS analysis of $HUVEC^{pBABE}$, $HUVEC^{E6}$ and $HUVEC^{\Delta E6}$ exposed to a laminar SS of 12 dyne/cm^2 or kept in static for 16 h ($p<0.05$). Data are displayed as the percent increase of NO production compared to static controls. The lower panel shows that 12 dyne/cm^2 of laminar SS and 100 μM of the nitric oxide donor sodium-nitroprusside (SNP) fail to rescue $HUVEC^{\Delta E6}$ from cell death. The graph represents the mean ± S.D. of three independent experiments performed in duplicate.

treated with $CoCl_2$ and serum deprivation and exposed to laminar flow. Fig. 1C, lower panel shows that, as expected, pBABE and E6 cells were protected from cell death while, although in the presence of the NO donor, papilloma virus ΔE6 cells still underwent cell death. This result indicates that, in the presence of papilloma ΔE6 protein which does not stimulate p53 degradation, retains PXN binding and prevents endothelial cells from aligning along the direction of flow, addition of NO is not sufficient to protect ECs from cell death.

3.2. E6 protein alters SS signalling in human endothelial cells

In the presence of SS the PI3K–AKT pathway is strongly activated and contributes to the production of NO [22] and to the antiapoptotic effect of flow [23]. Fig. 2A shows that in the presence of E6 or ΔE6, SS-dependent activation of AKT is delayed being detectable only after 15 to 30 min of SS exposure compared to control cells where it becomes phosphorylated at 1 min of SS treatment. This evidence is in agreement with the reduced NO production showed in Fig. 1C and suggests that the papilloma E6 protein may interfere with the SS-dependent activation of PI3K–AKT pathway which is known to regulate eNOS function [23]. In order to investigate the effect of laminar SS on the activation of endothelial nitric oxide (eNOS) in cells expressing E6 and ΔE6, phosphorylation of this protein was evaluated along a time of SS treatment from 1 to 30 min. Fig. 2B shows that eNOS phosphorylation occurs between 1 and 5 min after exposure to SS and it progressively increases during the time course. Remarkably, the presence of papilloma proteins completely abolished this process. Although further experiments are required to provide the molecular basis of this

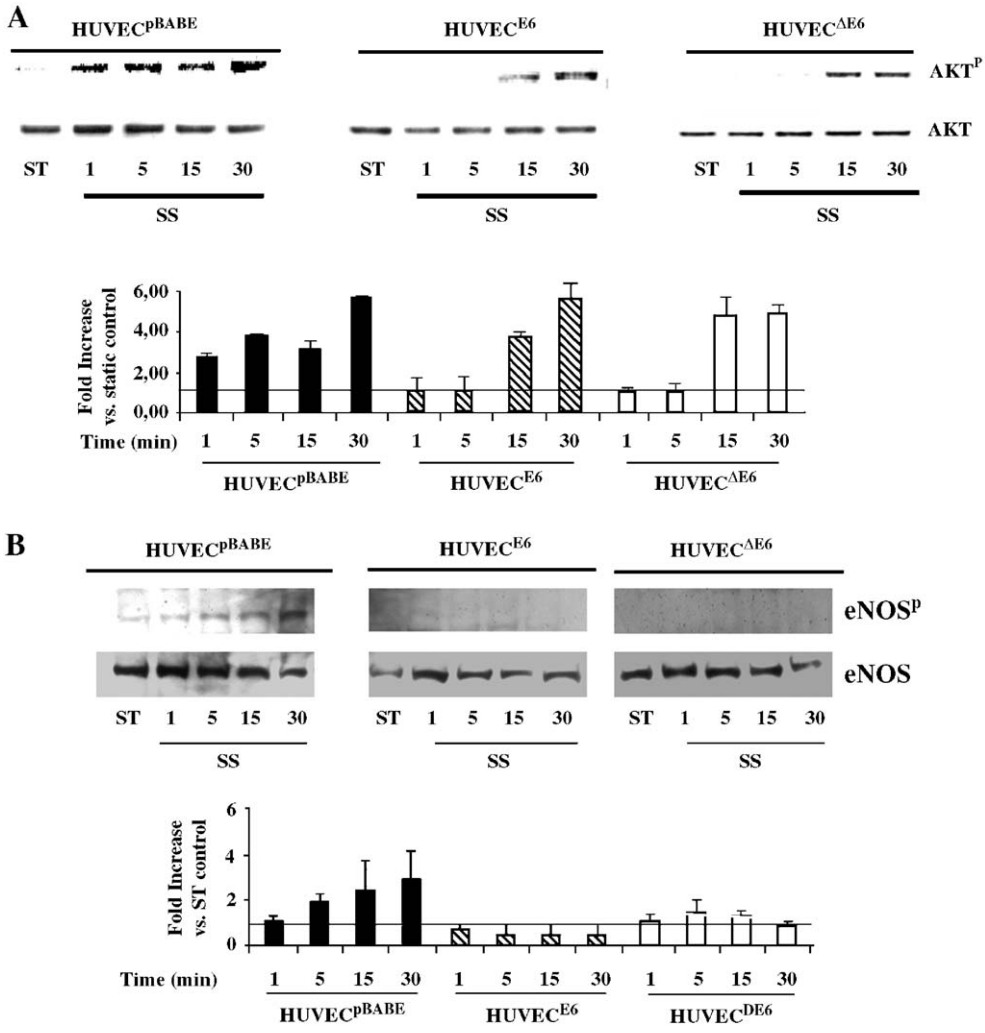

Fig. 2. (A) SS-dependent activation of AKT is delayed in HUVEC expressing papilloma E6 and ΔE6 proteins. The picture shows representative panels of Western blotting and densitometry analyses (below) revealing AKT total protein and phosphorylation levels in $HUVEC^{pBABE}$, $HUVEC^{E6}$ and $HUVEC^{\Delta E6}$ exposed to a laminar SS of 12 dyne/cm^2 from 1 to 30 min ($n=4$). (B) Endothelial nitric oxide synthase (eNOS) activation is compromised in the presence of papilloma virus proteins. The picture shows representative panels of Western blotting and densitometry analyses (below) revealing total eNOS protein and phosphorylation levels in $HUVEC^{pBABE}$, $HUVEC^{E6}$ and $HUVEC^{\Delta E6}$ exposed to a laminar SS of 12 dyne/cm^2 from 1 to 30 min ($n=3$).

alteration, taken together this result suggests that E6 and ΔE6 are able to interfere with eNOS function.

The investigation of other pathway like ERK 1/2, which has been described to be activated and involved in SS-dependent signal transduction [24], was also investigated. We found a delay ($HUVEC^{E6}$) or weaker ($HUVEC^{\Delta E6}$) activation of ERK 1/2 pathway than in control cells ($HUVEC^{pBABE}$) (not shown). To analyse whether SS-dependent gene transcription was altered in $HUVEC^{E6}$ and $HUVEC^{\Delta E6}$, cFos early gene expression was investigated [10]. In these cells, Western blot analysis highlighted a significant reduction in cFos protein level in the presence of E6 while an increase of about threefold was found in control cells (not shown). Altogether these results suggest that papilloma virus E6 interferes, at multiple level, with different SS-dependent pathways.

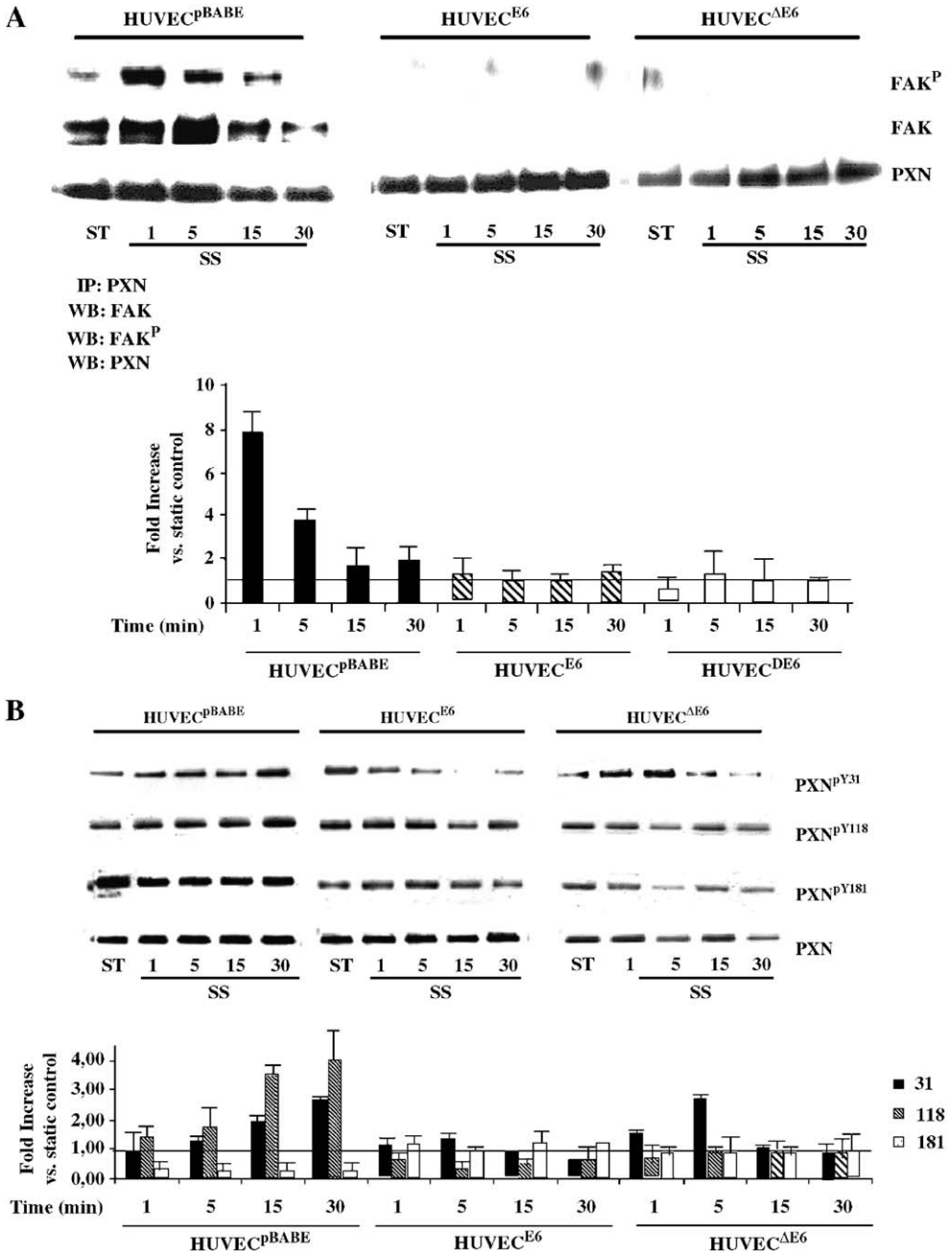

Fig. 3. (A) Co-immunoprecipitation of PXN revealing its interaction with FAK in cells expressing E6 and $E6^{\Delta 105-110}$ proteins. The figure shows representative panels from a series of PXN immunoprecipitation, Western blotting and densitometry analyses (below) revealing its association with FAK. The experiments were performed in $HUVEC^{pBABE}$, $HUVEC^{E6}$ and $HUVEC^{\Delta E6}$ exposed to a laminar SS of 12 dyne/cm^2 for 1 to 30 min ($n=4$). (B) PXN phosphorylation on tyrosine-31 (Y31) is inhibited in cells expressing E6 and $E6^{\Delta 105-110}$ proteins. The figure shows representative Western blotting and densitometry analyses (below) of phosphorylated tyrosine 31, 118 and 181 PXN residues in cell extracts obtained from $HUVEC^{pBABE}$, $HUVEC^{E6}$ and $HUVEC^{\Delta E6}$ exposed to a laminar SS of 12 dyne/cm^2 for 1 to 30 min ($n=4$).

3.3. E6 alters SS-dependent PXN/FAK association and site specific PXN phosphorylation

E6 protein interacts with PXN in proximity of the structural LD1 domain and interferes with PXN phosphorylation and FAK binding in tyrosine 31 [25]. In our experiments, human endothelial cells, expressing E6 or ΔE6, revealed a reduced level of FAK phosphorylation and PXN association while both were enhanced in control cells exposed to SS as indicated by the co-immunoprecipitation experiments (Fig. 3A).

In normal cells, upon mitogen induction, FAK phosphorylates PXN on tyrosine residues at position 31 and 118 [26] and regulates cell adhesion. In order to investigate the effect of SS on PXN phosphorylation, we examined tyrosines 31, 118 and tyrosine 181, which is not a FAK substrate [26]. Fig. 3B shows that, in control cells, tyrosine 31 and 118 phosphorylation increases from 1 to 30 min of SS treatment

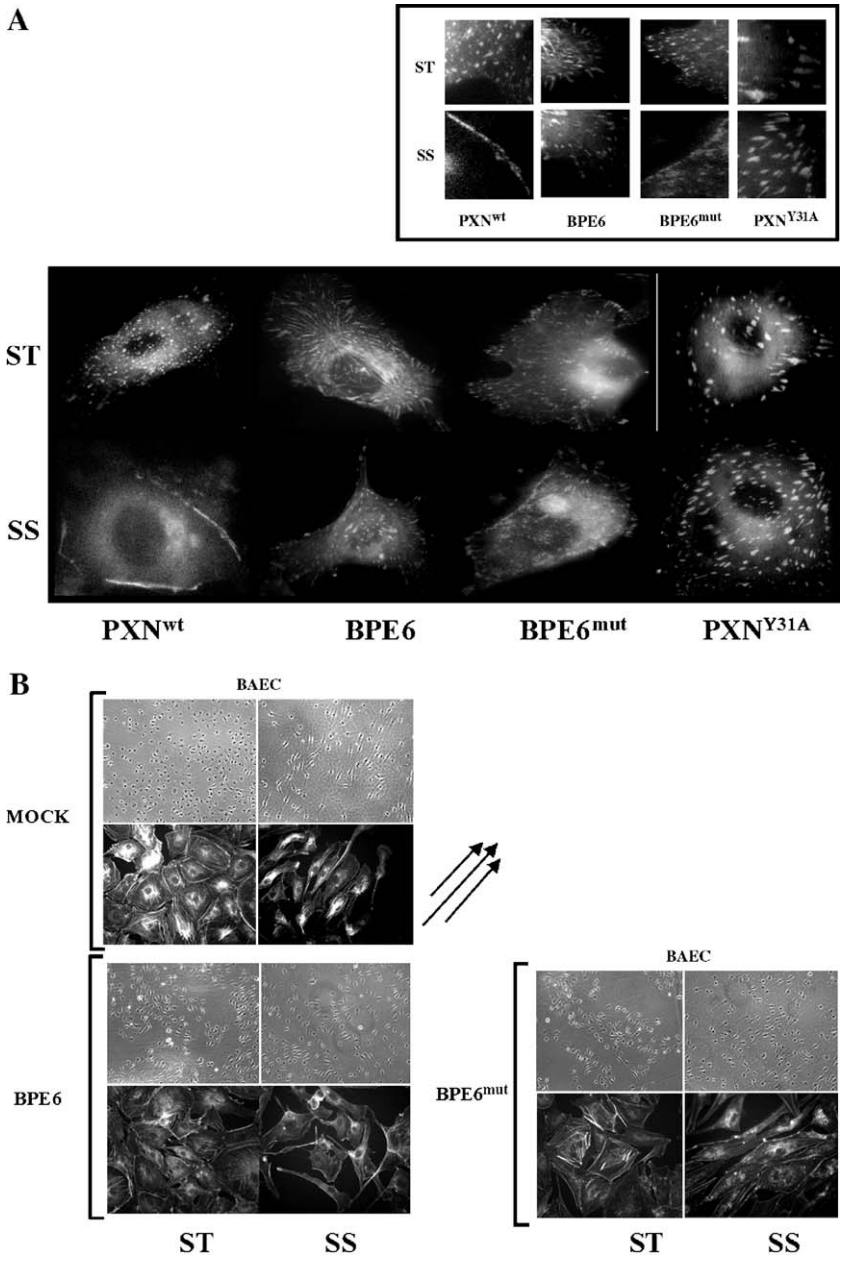

Fig. 4. (A) Papilloma E6 alters PXN intracellular localization. The picture shows representative images (40×) of BAEC co-transfected with a PXN–GFP construct and (in sequence from left to right) a Bluescript vector, bovine papilloma E6 or a PXN Y31A mutant alone. Cells were kept in static condition (upper panels) or exposed to a laminar SS of 12 dyne/cm^2 for 16 h (lower panels). The inset shows details, at larger magnification (100×), of the same cells (n=4). (B) Endothelial cells align along the direction of flow in the presence of a papilloma virus E6 mutant unable to recognize PXN. The picture shows representative images of phase contrast (magnification: 20×) and fluorescent phalloidin staining (magnification: 40×) of mock (upper), BPE6 (lower left) and BPE6^{mut} (lower right) transfected BAEC exposed to a laminar SS of 12 dyne/cm^2 for 16 h (n=3). Arrows indicate the direction of flow.

while in the presence of E6 and ΔE6, tyrosine phosphorylation at position 31 was altered decreasing progressively from 5–15 to 30 min after SS treatment. Tyrosine phosphorylation at position 118 was only partially modulated in the presence of ΔE6 and tyrosine 181 did not show significant modulations in any of the conditions tested.

3.4. Papilloma E6 alters PXN re-localization to plasma membrane

Prior studies indicate that focal adhesions re-organization occurs as an early event in response to SS [8]. Fig. 4A shows that PXN is localized at focal adhesions dispersed throughout the cytoplasm of unstimulated bovine aortic endothelial cells (BAEC), but its concentration at the edge of the cell membrane becomes higher in SS treated cells (WT). Notably, in the presence of bovine papilloma E6 protein (BPE6) PXN fails to localize into macro-aggregated structures. The expression of a BPE6 mutant, unable of PXN binding, did not significantly interfere with PXN re-localization during SS treatment. The intracellular distribution of a PXN mutant in which the tyrosine at position 31 was replaced with an alanine (Y31A) revealed that, although distributed normally in unstimulated cells, this mutant was unable to localize at the cell membrane. The inset shows enlarged details of PXN intracellular localization corresponding to the experimental conditions depicted in Fig. 4A. Remarkably, the expression in BAEC of the BPE6 Δ134–137 mutant protein [19], which is impaired in its PXN binding capacity, failed to prevent ECs alignment along the direction of flow thus indicating that an appropriate E6–PXN interaction is required to impair this process (Fig. 4B).

3.5. Targeting PXN expression partly reproduces the E6 phenotype

PXN is an important component of focal adhesions providing physical links to membrane-bound integrins, intermediate filaments and several protein kinases including FAK. E6 targets PXN within the LD1 structural domain disrupting its binding to vinculin and reducing the possibility of interaction with actin and phosphorylated FAK [27]. The evidences provided in this work suggest that PXN could be involved in the E6-dependent regulation of SS signalling. To investigate this possibility, a series of experiments were performed in normal HUVEC in which PXN

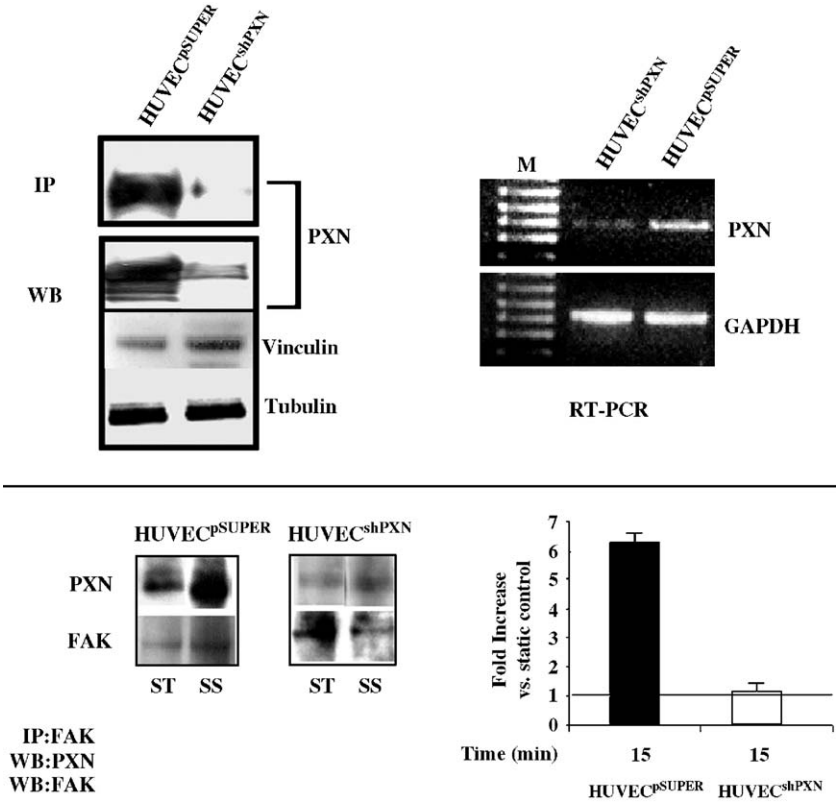

Fig. 5. (A) RNA interference reduces PXN expression at mRNA and protein level and PXN–FAK association is impaired in endothelial cells bearing PXN interference (HUVEC^{shPXN}). The upper picture shows PXN mRNA and protein levels evaluated, respectively, by RT–PCR (on the right), immunoprecipitation and Western blotting (on the left) in control endothelial cells (HUVEC^{pSuper}) and in cells bearing stable expression of a specific retroviral vector encoding for a short hairpin RNA targeted to PXN (HUVEC^{shPXN}). Tubulin has been used as loading control for westerns and Vinculin has been used as control of the capacity of the interference to target only PXN and not other cytoskeletal protein. The figure below shows a representative Western blotting analysis from a series ($n=3$) of co-immunoprecipitations indicating the reduction in PXN–FAK association in HUVEC^{shPXN} exposed to a laminar SS of 12 dyn/cm^2 for 15 min.

expression was stably knocked-down by short hairpin RNA interference (shRNAi). Specific oligos designed for the RNA interference of PXN expression (shPXN) were effective decreasing PXN mRNA and protein levels, as determined by RT–PCR, immunoprecipitation, and Western blotting analyses (Fig. 5). Further analyses revealed that in cells with reduced PXN levels (HUVEC^{shPXN}) of association with FAK was significantly reduced before and after SS treatment (Fig. 5). Remarkably, lower levels of PXN did not alter SS-dependent c-*fos* gene expression (not shown).

Fig. 6A shows that endothelial cells bearing shPXN were unable to align their cytoskeleton in the direction of flow.

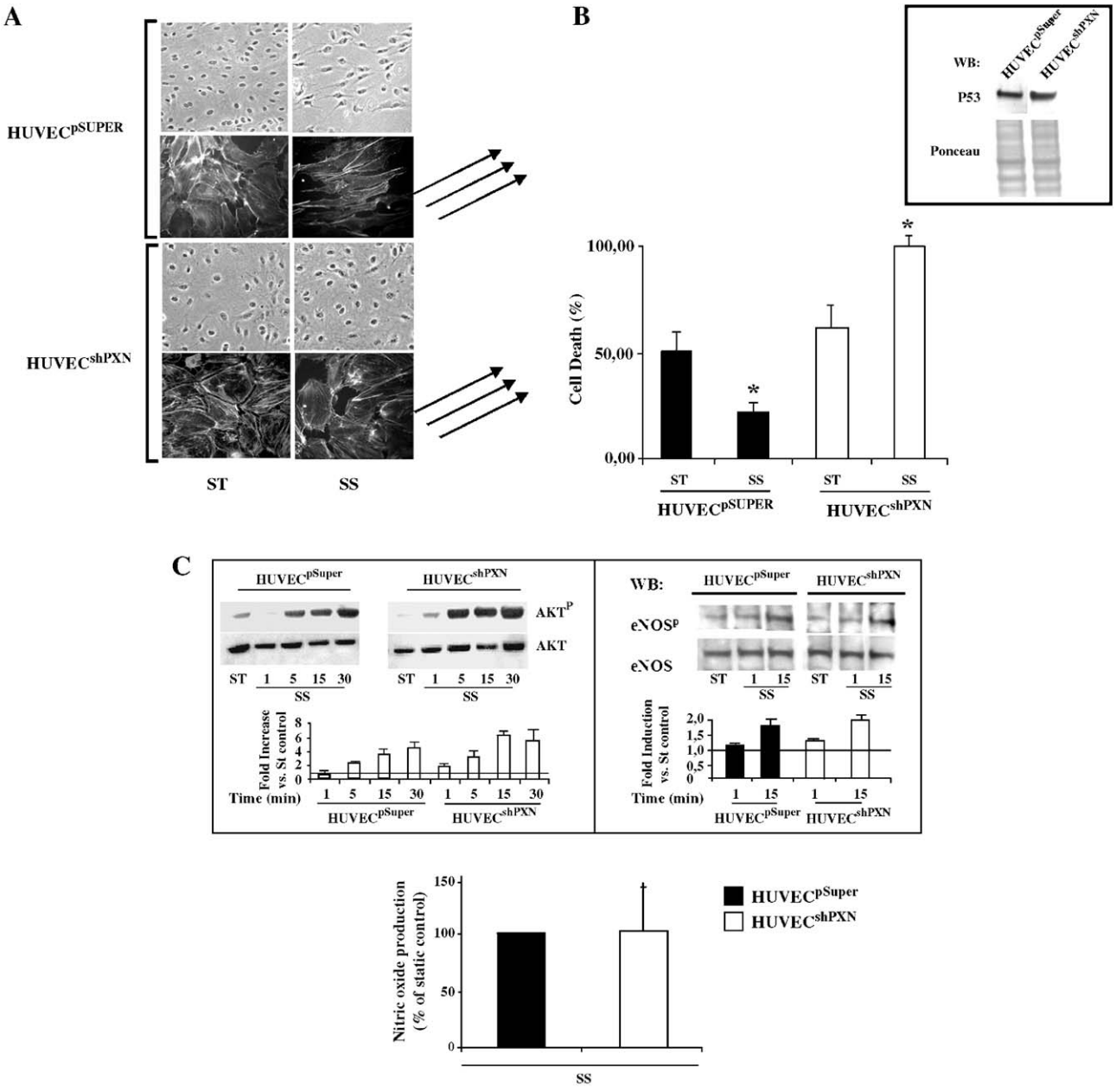

Fig. 6. (A) RNA interference of PXN abrogates orientation remodelling in presence of laminar SS. The picture shows representative micro-photograms of phase contrast and fluorescent phalloidin staining of control cells (upper) and HUVEC^{shPXN} (lower) exposed to a laminar SS of 12 dyn/cm^2 or kept in ST condition for 16 h ($n=3$). (B) SS antiapoptotic effects are altered in HUVEC^{shPXN} but RNA interference of PXN expression does not alter NO production. The graph represents the results of three independent experiments performed in duplicate in which laminar SS effects on cell death were evaluated by propidium iodide incorporation and FACS analysis. The control cells (black bars) and cells bearing PXN RNA interference (white bars) were exposed to a laminar SS of 12 dyn/cm^2 or kept in ST condition for 6 h ($p<0.05$). The inset shows that in this condition the altered levels of PXN do not influence the expression of p53. (C) AKT, eNOS and NO production are normal in HUVEC^{shPXN} exposed to laminar SS. The graph depicts the levels of NO in control cells (black bars) and HUVEC^{shPXN} (white bars) exposed to a laminar SS of 12 dyn/cm^2 or kept in ST condition for 16 h as indicated by DAF incorporation and FACS analysis. Data are represented as percent of static control. Each experiment has been repeated three times in duplicate. The inset show Western blotting analysis depicting total and phosphorylated AKT (left) and eNOS (right) levels in HUVEC^{shPXN} during a time course of laminar SS treatment.

Recent evidences indicate that PXN could play an important role in protecting cardiomyocytes from apoptosis [28] and prior studies indicate that laminar SS protects HUVEC from apoptosis induced by serum starvation and chemical hypoxia [4]. In a new series of experiments, control cells and HUVEC^{shPXN} were serum starved in the presence of $CoCl_2$ and were exposed to laminar SS or kept in ST. Fig. 6B shows that in this condition SS failed to protect HUVEC^{shPXN} from cell death compared to control. Further analyses revealed that in the presence of SS HUVEC^{shPXN} retains their capacity to synthesize NO as indicated by DAF incorporation and FACS analysis (Fig. 6C). In agreement, the activation of AKT and eNOS phosphorylation in the presence of SS were comparable to that of control cells (see inset in Fig. 6C) indicating that PXN may function downstream of the PI3K–AKT–eNOS pathway. Altogether these results indicate that E6 actively interferes with NO production in presence of SS while PXN does not affect this type of endothelial response to mechanical stress. Further, this observation suggests that E6 is able to exert a broader inhibitory effect on a variety of SS functions while the role of PXN may be limited to the cytoskeletal adaptation to flow and pro-survival effects.

4. Discussion

Laminar flow provides important physiologic stimuli to remodel chromatin structure [2], modulates gene expression, stimulates the release of NO and other biologically active agents important to ensure endothelial cell survival and function [4]. This manuscript provides the evidence that the oncoviral protein E6 from the type 16 human papillomavirus interferes with SS responses in endothelial cells. E6 is known to bind and/or interact with multiple cellular factors. E6-binding partners, including PXN and FAK, contain a structural domain of seven amino acids which form part of an α-helix that is necessary for the association with E6 [29]. Specifically, PXN participates in the formation of links between the cytoskeleton and integrin proteins that in turn mediate tension transmission between the contractile apparatus and the extracellular matrix and it may be required for the transmission of SS signals [5].

Our findings indicate that in human endothelial cells, in the presence of E6, the whole response to SS is altered. E6, in fact, reduces PXN phosphorylation on tyrosine 31 (Fig. 3B), alters its interaction with FAK (Fig. 3A), interferes with its redistribution capacity to the cell membrane in response to SS and impairs NO production (Fig. 1A and B). In endothelial cells, NO plays, among others, an important antiapoptotic role; however, in cells expressing papilloma virus protein E6 and unable to redirect their cytoskeleton along the direction of flow, NO did not prevent cell death. Intriguingly, this observation is in agreement with the evidence that ECs with reduced PXN expression fails to align their cytoskeleton in the direction of flow underwent to a cell death program although normal amounts of NO were synthesised. In fact, the reduction of the intracellular levels of PXN, only partially reproducing the E6-dependent phenotype, suggests that, in our experimental setting, nitric oxide production may be dispensable for cell survival and that PXN may have a crucial role in the flow adaptive response of human endothelial cells. Specifically, our experiments indicate PXN as an important mediator in the SS-dependent cytoskeleton remodelling and endothelial survival. A large body of literature has shown that SS prevents apoptosis triggered by a variety of stimuli including growth factors deprivation [3], TNF-α [22], oxidative stress [30], oxidated LDL [22], as well as ECs death occurring in the absence of flow when ECs are cultured in the presence of serum and growth factors [31]. SS induces a CD-31- and VEGFR-2-dependent activation of the PI3K–AKT pathway [7] promoting the activation of an integrin-dependent signalling cascade [5], the production of NO, and the inhibition of caspase-3 activity and cell death [32]. Our work reports that in the presence of reduced levels of PXN, SS fails to protect cells from cell death induced by serum starvation and chemical hypoxia leaving unaltered the production of NO. This result suggests that PXN-independent signals are involved in controlling NO production while PXN, acting possibly downstream of NO, may be required for the antiapoptotic effects of flow [28]. In this context, the role of endothelial cells re-orientation which occurs in presence of laminar flow emerges as a potential important regulatory component of the flow-dependent pro-survival function. Further characterizations are required to elucidate the role of the cytoskeleton determining the prosurvival effect of SS and NO in endothelial cells.

In conclusion, this work provides the evidence that papilloma E6 virus largely compromises the property of endothelial cells to sense and adapt to SS. Further, the experimental evidences strongly indicate that PXN, whose inactivation partially reproduces the E6 phenotype, is an important mediator of cytoskeleton remodelling and cell survival in response to laminar flow signalling. Although further experiments are required to investigate whether PXN may play a role during endothelial cells differentiation and angiogenesis, the identification of this molecule involved in important flow-dependent adaptive responses may be relevant to design preventive interventions aimed at reducing endothelial cell damage and dysfunction.

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References

[1] Hove JR, Koster RW, Forouhar AS, Acevedo-Bolton G, Fraser SE, Gharib M. Intracardiac fluid forces are an essential epigenetic factor for embryonic cardiogenesis. Nature 2003;421:172–7.

[2] Illi B, Nanni S, Scopece A, Farsetti A, Biglioli P, Capogrossi MC, et al. Shear stress-mediated chromatin remodeling provides molecular basis for flow-dependent regulation of gene expression. Circ Res 2003;93:155–61.

[3] Dimmeler S, Haendeler J, Rippmann V, Nehls M, Zeiher AM. Shear stress inhibits apoptosis of human endothelial cells. FEBS Lett 1996;399:71–4.

[4] Mattiussi S, Turrini P, Testolin L, Martelli F, Zaccagnini G, Mangoni A, et al. p21(Waf1/Cip1/Sdi1) mediates shear stress-dependent antiapoptotic function. Cardiovasc Res 2004;61:693–704.

[5] Alenghat FJ, Ingber DE. Mechanotransduction: all signals point to cytoskeleton, matrix, and integrins. Sci STKE 2002;2002:E6.

[6] Tzima E, del Pozo MA, Shattil SJ, Chien S, Schwartz MA. Activation of integrins in endothelial cells by fluid shear stress mediates Rho-dependent cytoskeletal alignment. EMBO J 2001;20:4639–47.

[7] Tzima E, Irani-Tehrani M, Kiosses WB, Dejana E, Schultz DA, Engelhardt B, et al. A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. Nature 2005;437:426–31.

[8] Davies PF, Robotewskyj A, Griem ML. Quantitative studies of endothelial cell adhesion. Directional remodeling of focal adhesion sites in response to flow forces. J Clin Invest 1994;93:2031–8.

[9] Kano Y, Katoh K, Masuda M, Fujiwara K. Macromolecular composition of stress fiber-plasma membrane attachment sites in endothelial cells in situ. Circ Res 1996;79:1000–6.

[10] Jalali S, Li YS, Sotoudeh M, Yuan S, Li S, Chien S, et al. Shear stress activates p60src–Ras–MAPK signaling pathways in vascular endothelial cells. Arterioscler Thromb Vasc Biol 1998;18:227–34.

[11] Chen KD, Li YS, Kim M, Li S, Yuan S, Chien S, et al. Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins, and Shc. J Biol Chem 1999;274:18393–400.

[12] Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, et al. Mechanisms of human papillomavirus-induced oncogenesis. J Virol 2004;78:11451–60.

[13] Massimi P, Gammoh N, Thomas M, Banks L. HPV E6 specifically targets different cellular pools of its PDZ domain-containing tumour suppressor substrates for proteasome-mediated degradation. Oncogene 2004;23:8033–9.

[14] van Ham M, Hendriks W. PDZ domains–glue and guide. Mol Biol Rep 2003;30:69–82.

[15] Sterpetti AV, Cucina A, Morena AR, Di Donna S, D'Angelo LS, Cavalarro A, et al. Shear stress increases the release of interleukin-1 and interleukin-6 by aortic endothelial cells. Surgery 1993;114:911–4.

[16] Morgenstern JP, Land H. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res 1990;18:3587–96.

[17] Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. Proc Natl Acad Sci U S A 1993;90:8392–6.

[18] Lechner MS, Laimins LA. Inhibition of p53 DNA binding by human papillomavirus E6 proteins. J Virol 1994;68:4262–73.

[19] Tong X, Howley PM. The bovine papillomavirus E6 oncoprotein interacts with paxillin and disrupts the actin cytoskeleton. Proc Natl Acad Sci U S A 1997;94:4412–7.

[20] Giampietri C, Levrero M, Felici A, D'Alessio A, Capogrossi MC, Gaetano C. E1A stimulates FGF-2 release promoting differentiation of primary endothelial cells. Cell Death Differ 2000;7:292–301.

[21] Davies PF, Remuzzi A, Gordon EJ, Dewey Jr CF, Gimbrone Jr MA. Turbulent fluid shear stress induces vascular endothelial cell turnover in vitro. Proc Natl Acad Sci U S A 1986;83:2114–7.

[22] Dimmeler S, Hermann C, Galle J, Zeiher AM. Upregulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells. Arterioscler Thromb Vasc Biol 1999;19:656–64.

[23] Dimmeler S, Assmus B, Hermann C, Haendeler J, Zeiher AM. Fluid shear stress stimulates phosphorylation of Akt in human endothelial cells: involvement in suppression of apoptosis. Circ Res 1998;83:334–41.

[24] Traub O, Berk BC. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. Arterioscler Thromb Vasc Biol 1998;18:677–85.

[25] Tong X, Salgia R, Li JL, Griffin JD, Howley PM. The bovine papillomavirus E6 protein binds to the LD motif repeats of paxillin and blocks its interaction with vinculin and the focal adhesion kinase. J Biol Chem 1997;272:33373–6.

[26] Schaller MD, Parsons JT. pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. Mol Cell Biol 1995;15:2635–45.

[27] Tumbarello DA, Brown MC, Turner CE. The paxillin LD motifs. FEBS Lett 2002;513:114–8.

[28] Melendez J, Turner C, Avraham H, Steinberg SF, Schaefer E, Sussman MA. Cardiomyocyte apoptosis triggered by RAFTK/pyk2 via Src kinase is antagonized by paxillin. J Biol Chem 2004;279:53516–23.

[29] Be X, Hong Y, Wei J, Androphy EJ, Chen JJ, Baleja JD. Solution structure determination and mutational analysis of the papillomavirus E6 interacting peptide of E6AP. Biochemistry 2001;40:1293–9.

[30] Dimmeler S, Zeiher AM. Endothelial cell apoptosis in angiogenesis and vessel regression. Circ Res 2000;87:434–9.

[31] Kaiser D, Freyberg MA, Friedl P. Lack of hemodynamic forces triggers apoptosis in vascular endothelial cells. Biochem Biophys Res Commun 1997;231:586–90.

[32] Rossig L, Fichtlscherer B, Breitschopf K, Haendeler J, Zeiher AM, Mulsch A, et al. Nitric oxide inhibits caspase-3 by *S*-nitrosation in vivo. J Biol Chem 1999;274:6823–6.