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Redundant control of migration and adhesion by ERM proteins in vascular smooth muscle cells

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ABSTRACT

Ezrin, radixin, and moesin possess a very similar structure with a C-terminal actin-binding domain and a N-terminal FERM interacting domain. They are known to be involved in cytoskeleton organization in several cell types but their function in vascular smooth muscle cells (VSMC) is still unknown. The aim of this study was to investigate the role of ERM proteins in cell migration induced by PDGF, a growth factor involved in pathophysiological processes like angiogenesis or atherosclerosis. We used primary cultured VSMC obtained from rat aorta, which express the three ERM proteins. Simultaneous depletion of the three ERM proteins with specific siRNAs abolished the effects of PDGF on cell architecture and migration and markedly increased cell adhesion and focal adhesion size, while these parameters were only slightly affected by depletion of ezrin, radixin or moesin alone. Rac1 activation, cell proliferation, and Ca^{2+} signal in response to PDGF were unaffected by ERM depletion. These results indicate that ERM proteins exert a redundant control on PDGF-induced VSMC migration by regulating focal adhesion turn-over and cell adhesion to substrate.

1. Introduction

Artery wall is constituted by a monolayer of endothelial cells surrounded by vascular smooth muscle cells (VSMC) layers separated by several collagen and elastin lattices. The major role of VSMC is to control arterial diameter and thus blood flow and tissue perfusion by contracting or relaxing in response to chemical and physical stimuli. Changes of the environmental condition associated with developmental or pathological situation promote arterial remodeling and modify vessel diameter and/or wall thickness to ensure adequate tissue perfusion [1]. This ability of VSMC to undergo remodeling in response to transient environmental cues reveals a remarkable property of these cells: their phenotype is not totally differentiated and fixed [2], leading to the concept of remodeling continuum to describe the temporal modulation of vascular structure by acute or chronic changes in cell adhesion, cytoskeleton organization or extracellular matrix (ECM) [3]. PDGF (Platelet derived growth factor) has been identified as a potent inducer of VSMC migration and proliferation after injury [4–6], tipping the balance towards a "synthetic" phenotype, in opposition to the physiological "contractile" phenotype [7].

ERM (ezrin, radixin, and moesin) are closely related proteins, characterized as actin-binding proteins potentially involved in the organization of cell cortex, especially in epithelial tissues [8]. They are generally proposed to be functionally redundant, although specific role for moesin, radixin, and ezrin is reported [9]. Their importance in the regulation of cell–cell interaction and epithelial morphogenesis has been well documented [10,11]. On the other hand, ERM proteins have also been involved in induction of cell migration and invasion [12]. However, the role of ERM proteins in the control of cell-ECM adhesions is often overlooked [12], and little is known about their function in VSMC. Moesin expression in coronary artery is increased after balloon injury and its depletion with antisense cDNA inhibits VSMC migrative and invasive potential [13] but the mechanism behind moesin involvement in the regulation of VSMC phenotype remains unknown. We observed, in a previous study [14], that moesin depletion in intact arteries potentiates the contractile response to agonist, and that, upon agonist stimulation, moesin is associated with focal adhesion proteins, in a ROCK dependent manner. As focal adhesions have long been speculated to play a critical role in the regulation of cell polarity and migration [15], we have investigated the role of ERM proteins in the regulation of VSMC adhesion to ECM, with a focus on the PDGF-induced cell migration.

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2. Materials and methods

2.1. Vascular smooth muscle cells (VSMC) culture

Aorta was isolated from male Wistar rats (150–200 g) and endothelium denuded. Pieces of aorta (2 mm^2) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and 2 mM glutamine in an incubator at 37 °C with humidified 5% CO_2. VSMC were obtained and cultured as described [16]. Before stimulation with PDGF-BB, VSMC were serum-starved in FBS-free DMEM completed with penicillin, streptomycin, and 0.2% BSA.

2.2. siRNA transfection

VSMC were cultured in DMEM supplemented with FBS 10% and without antibiotics for 24 h and then transfected with 5 nM siRNA (Stealth siRNA, Invitrogen) by using RNAiMAX Lipofectamine, following manufacturer's instructions (Invitrogen). Sequence for each siRNA is reported in the supplemental file.

Cells were cultured for 96 h to allow a maximal inhibition of protein expression. Knock-down efficiency was assessed by Western-blotting [16] with anti-ezrin (1/2000, Cell Signaling), anti-radixin (1/2000, Cell Signaling), anti-moesin (1/2000, Cell Signaling), anti-ERM proteins (1/2000, Cell signaling) antibodies, and anti-actin (1/2000, Santa Cruz) antibody as loading control. IRDye conjugated fluorescent secondary antibodies (680 and 800 nm) were used to detect primary antibodies. Bands were detected and quantified with an Odyssey infrared imaging system (Li-Cor).

2.3. Microscopy

Cells grown on glass coverslips were incubated in the presence of PDGF-BB (40 ng/ml) for 0–24 h at 37 °C, washed with PBS (in mM: NaCl 137; KCl 6; $MgCl_2$ 1.2; $CaCl_2$ 2; glucose 10; HEPES 10 at pH 7.4 with Tris), fixed with 4% PFA for 10 min and permeabilized with TritonX100 0.5% for 5 min. Cells were incubated for 1 h with StartingBlock blocking buffer (Thermoscientific) and stained with primary antibody anti-vinculin (1/200, Sigma), anti-phospho-ERM (1/100, Cell Signaling) or rhodamine-phalloïdin (20 nM, F-actin visualization kit, Cytoskeleton) and thereafter probed with the corresponding secondary antibody (Alexa 488-labeled donkey anti-rabbit). Cells were mounted on the coverslips with Prolong Gold antifade reagent (Invitrogen) and nuclei were stained with DAPI. Cells were observed with a Zeiss Axiovert S-100 microscope and individual images were acquired with a Zeiss Axiocam camera and a 20× objective (Zeiss Fluar, 0.75 NA) at room temperature. Images were analyzed with ImageJ software, background signal was reduced using the "subtract background" tool (ball radius: 200 pixels). Cell images of vinculin staining were analyzed with the particle analysis tool from ImageJ software.

2.4. Cell adhesion

96 wells plates were coated with fibronectin (1–20 μg/ml PBS – 5–30 min), washed with PBS and blocked with heat-denaturated BSA (10 mg/ml). Cells were trypsinized, resuspended in serum-free medium with 1 mg/ml BSA and plated at 30×10^3 cells per well. After different incubation times at 37 °C, adherent cells were washed three times with PBS and quantified by the acid phosphatase assay [17].

2.5. Cell de-adhesion

De-adhesion was measured according to the centrifugation assay described by McClay et al. [18]. 96 wells plates were coated with fibronectin (20 μg/ml, for 2 h at room temperature), washed with PBS, and blocked with BSA (10 mg/ml). Cells suspended in serum-free medium were plated at 30×10^3 cells per wells (or 50×10^3 when test was performed at 4 °C). The wells were filled with serum-free medium containing 1 mg/ml BSA and sealed with PCR sealing film. Cells were seeded by centrifugation of the plates at 30 g for 3 min at 37 °C or at 4 °C, and further incubated at 37 °C for 10 min or at 4 °C for 30 min. The plates were then inverted and centrifuged at 50*g* for 3 min. Cells remaining attached were quantified by the acid phosphatase assay. Background cell binding was measured in wells blocked with 10 mg/ml BSA.

2.6. Cell migration

Cells were transfected with corresponding siRNA and grown to confluence for 96 h. FBS was removed from the culture medium 24 h before experiment. Cell layer was scratched with a sterile pipette tip. Cells were fixed with 4% formaldehyde and colored with crystal violet after 0, 18 or 24 h of culture in FBS-free DMEM culture medium containing 40 ng/ml of PDGF-BB (Cell Signaling). Scratch line was imaged with a 4× objective (Zeiss Achroplan, 0.10 NA) at room temperature. Image analysis and estimation of the refilling of the wound were done using the software Tscratch (CSE laboratory, ETH Zurich, Switzerland) [19].

2.7. Cell proliferation

The Click-iT™ Edu flow cytometry assay was used to measure cell proliferation (Molecular Probes), according to manufacturer's recommendations.

2.8. Cytosolic calcium determination

VSMC cultured on glass coverslip were washed with PBS and loaded with 2.5 μM fura-2 AM (Calbiochem) for 1 h 30 at room temperature. Coverslip was mounted in a fluorimeter (CAF100, JASCO, Japan) and calcium signal was measured as described [20]. After 10 min washing, cells were stimulated by adding PDGF-BB (40 ng/ml) into the bathing solution. After washing PDGF-BB and return of the Ca^{2+} signal to the baseline, cells were stimulated with vasopressin (10 nM).

2.9. Rac1 activity

For measurement of Rac1 activation, the Rac G-Lisa Activation Assay Biochem kit (Cytoskeleton) was used according to the manufacturer's recommendations. Total Rac1 protein level was measured by Western blot analysis with Rac1 antibody (Cytoskeleton) and normalized to the expression of actin.

3. Results and discussion

3.1. ERM proteins localize in polarized structures in response to PDGF stimulation

Serum starved VSMC have a polygonal shape and connect each other to form pluricellular islands. They also possess thick parallel actin bundles, which cross the entire cell (Fig. 1). In this unstimulated condition, some level of phosphorylated ERM proteins was detected, indicating the existence of a basal phosphorylation mechanism (Fig. 1A), but phospho-ERM proteins did not seem to

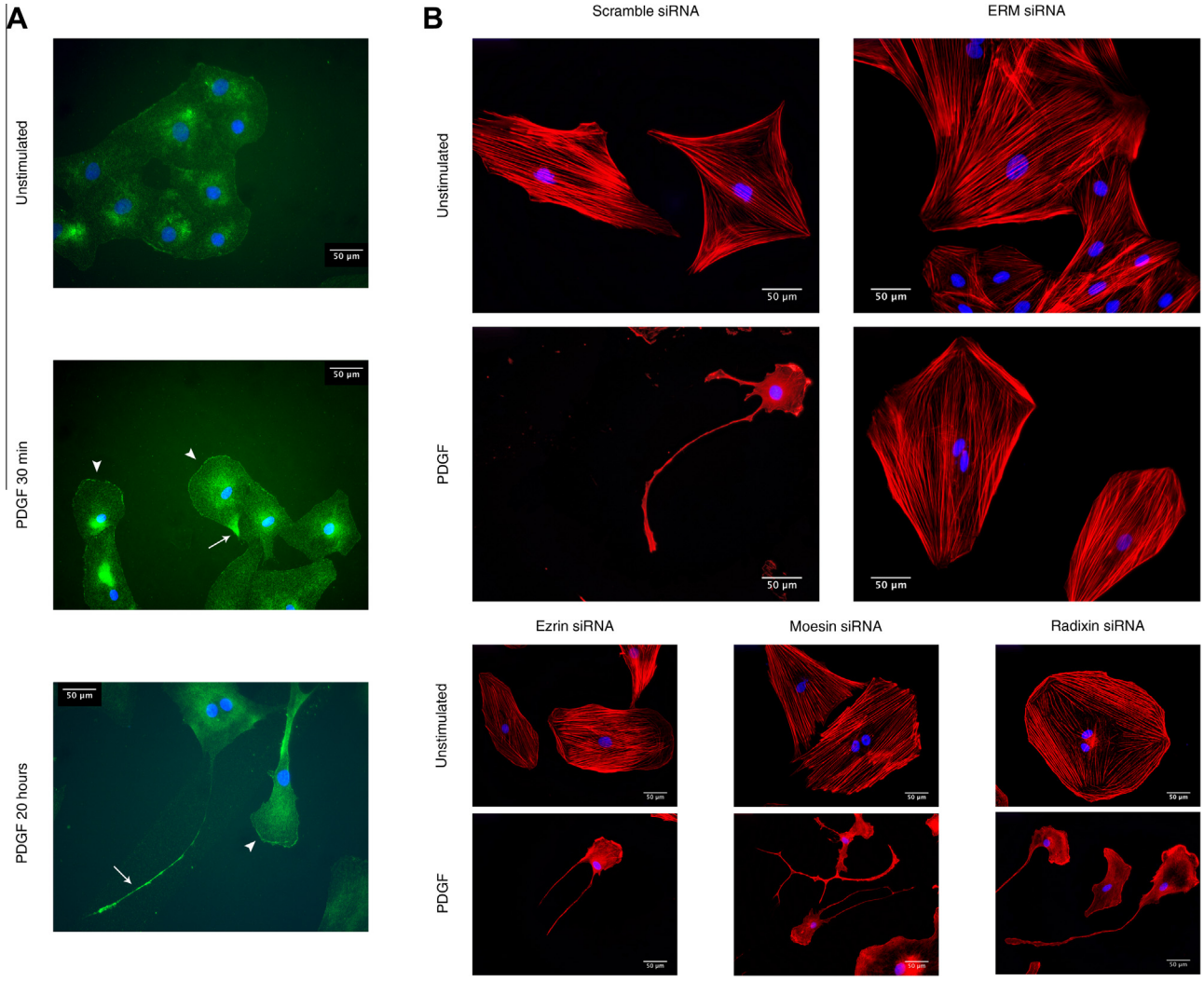

Fig. 1. ERM proteins contribute to the formation of lamellipodia and training tail in response to PDGF. (A) VSMC stained for phospho-ERM before and after stimulation with PDGF (40 ng/ml). Arrowheads indicate lamellipodia and arrows indicate trailing tails. Nuclei were stained with DAPI. (B) Representative pictures of VSMC stained with phalloidin (F-actin) after transfection of a scramble siRNA, anti-ERM-siRNAs or siRNA directed against ezrin, radixin or moesin. These cells were either unstimulated in serum-free media or stimulated for 24 h with PDGF (40 ng/ml).

have a specific localization. Stimulation with 40 ng/ml of PDGF induced structural changes with the appearance of specific polarized structures associated with cell migration: lamellipodia at the front of the cell and long trailing tails at the back. In parallel, phosphorylated ERM proteins tended to localize to the leading edge of the lamellipodia and to trailing tails (Fig. 1A). A similar localization of phosphorylated ERM proteins has been observed in EGF stimulated epithelial cells [21]. This polarized localization suggested that ERM proteins may be involved in the formation of these structures involved in migration.

3.2. ERM proteins are required for the formation of lamellipodia and trailing tails

In cultured VSMC, both ezrin, radixin, and moesin are expressed, so are they in intact arteries [14]. Transfection of two different siRNA targeting ezrin mRNA led to the inhibition of 96 ± 1% and 91 ± 2% of ezrin expression ($n = 6$). The inhibition of radixin expression by transfection of two siRNA directed against radixin mRNA was of 98 ± 1% and 99 ± 1% ($n = 5$ and 6) and the use of two siRNA against moesin led to the inhibition of 92 ± 1% and 94 ± 1% of moesin expression ($n = 4$ and 6) (Supplemental Fig. 1). The selectivity of the inhibition among ERM proteins was indicated by the absence of change in ezrin and radixin expression following moesin depletion with a moesin siRNA while moesin expression was nearly totally abolished (Supplemental Fig. 2). Since these proteins share a very similar structure, suggesting that they could be functionally redundant, we also targeted the three proteins together by transfecting VSMC with a cocktail of 3 siRNAs, each targeting one of the three ERM proteins (anti-ERM siRNA), leading to a global inhibition of 98 ± 1% of ERM proteins expression (Supplemental Fig. 1). As no difference was observed in the efficacy of the different siRNA tested, they were used without distinction in further experiments.

We first tested the consequence of ERM proteins knock-down on the structural changes associated with PDGF stimulation. As depicted in Fig. 1B, neither individual knock-down of ezrin, radixin or moesin, nor inhibition of these three proteins together did affect the actin bundles structure and distribution in unstimulated cells. Incubating VSMC transfected with a scramble siRNA with PDGF (40 ng/ml) triggered the formation of lamellipodia and trailing tails and so did the same stimulation on VSMC transfected with a siRNA against either ezrin, radixin or moesin. However, inhibiting the expression of the three ERM proteins together, by using a siRNAs

cocktail, altered the formation of these polarized structures induced by PDGF. Indeed, cell architecture of PDGF-stimulated cells depleted in all three ERM proteins remained similar to the one observed in unstimulated cells. The lack of effect of single knock-down suggested a redundant role of these proteins.

3.3. ERM proteins control PDGF-induced cell migration in a redundant manner

To confirm the previous observations, we performed wound healing assay in order to measure cell ability to migrate in response to PDGF. Cells transfected with a scramble siRNA quickly covered the wounded area in response to PDGF (Fig. 2). Wound recovery after PDGF stimulation was significantly lower ($p = 0.0012$, $n = 6$) for cells depleted for the three ERM proteins (Fig. 2A). Such a difference was not observed after depleting VSMC for ezrin ($p = 0.78$, $n = 3$), radixin ($p = 0.09$, $n = 3$) or moesin ($p = 0.63$, $n = 3$) alone (Fig. 2B), confirming the previous observations on the lamellipodia formation and suggesting a redundant role of these proteins in the control of PDGF-induced cell migration. The redundancy of ERM proteins function is in agreement with the observations that knock-out mice for each of these proteins only exhibit defects in tissues or cells that selectively express one of the three ERM proteins [10,22,23]. However, redundancy of ERM function has been questioned in other studies where selective role for moesin, radixin or ezrin has been reported [9].

3.4. Cells depleted for ERM proteins still respond to PDGF

The lack of cytoskeleton rearrangement and thus, the decrease in cell migration induced by PDGF after ERM depletion could result from a deficiency in PDGF signaling. The formation of lamellipodia and the induction of migration require the activation of Rac1, a small Rho GTPase [24]. Then, basal and PDGF-stimulated Rac1 activity was estimated by measuring the amount of GTP-Rac1, in scramble siRNA transfected and ERM knock-down cells. PDGF stimulation for 30 min produced a significant increase in GTP-Rac1-level in both scramble ($p < 0.001$, $n = 3$ independent experiments) and ERM siRNA ($p < 0.0001$, $n = 3$ independent experiments) transfected cells (Fig. 3A), indicating that this mechanism was preserved after ERM proteins knock-down. No significant difference was observed between scramble or ERM siRNA transfected cells, either in unstimulated condition or after stimulation with PDGF. Normalization of Rac1 activity for total Rac1 expression did not change the result (Supplemental Fig. 3).

In addition, we measured cell proliferation in serum-starved cells, non-stimulated or stimulated with 40 ng/ml of PDGF. Even in the absence of PDGF and following 24 h of serum starvation, VSMC proliferate and this basal proliferation was significantly increased in cells depleted for ERM proteins (Fig. 3B, $p < 0.01$, $n = 6$). In cells transfected with a scramble siRNA, PDGF stimulation for 20 h induced a small increase of cell proliferation, while a significantly larger increase in proliferation was observed in cells depleted in ERM proteins (Fig. 3B, $p < 0.001$, $n = 6$). PDGF-induced VSMC proliferation was then preserved and even potentiated, in cells lacking ERM proteins.

As many other chemical agonists, PDGF triggers a quick increase in the cytosolic calcium concentration. We measured the change in cytosolic calcium after PDGF stimulation in control cells or in ERM-depleted cells (Fig. 3C). PDGF induced an increase in cytosolic calcium concentration in VSMC, although of a lower amplitude than vasopressin. No difference was seen between cells transfected with a scramble siRNA or a siRNA cocktail against the three ERM proteins for the basal concentration of cytosolic calcium (Fig. 3C, $n = 6$). We neither observed any statistical difference in calcium increase following either PDGF or vasopressin stimulation (Fig. 3C, $n = 6$).

All these observations clearly indicated that the lack of cytoskeleton reorganization in response to PDGF stimulation after ERM proteins depletion did not rely on a defective PDGF-dependent signalization as PDGF was still able to trigger proliferation, calcium signal, and Rac1 activation.

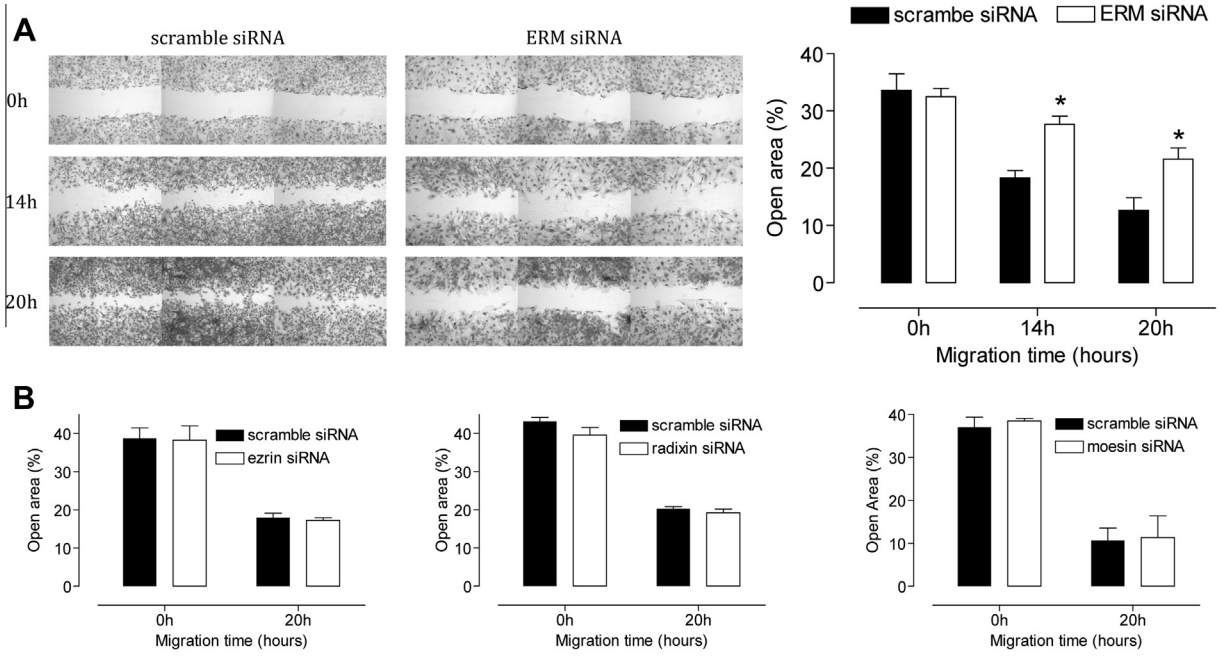

Fig. 2. ERM proteins control PDGF-induced cell migration in a redundant manner. (A) Representative images of cell migration after wound healing and stimulation with 40 ng/ml of PDGF for 0, 14 and 20 h. Cells were transfected with either scramble or anti-ERM siRNAs. Right panel: mean values of the wound healing, expressed as the percentage of the open area after 0, 14 or 20 h of stimulation with PDGF ($n = 6$, $^*p < 0.05$). (B) mean values of the wound healing, expressed as the percentage of the open area after 0, 14 or 20 h of stimulation with PDGF, in cells transfected with a scramble siRNA or a siRNA directed against ezrin, radixin or moesin. ($n = 3$).

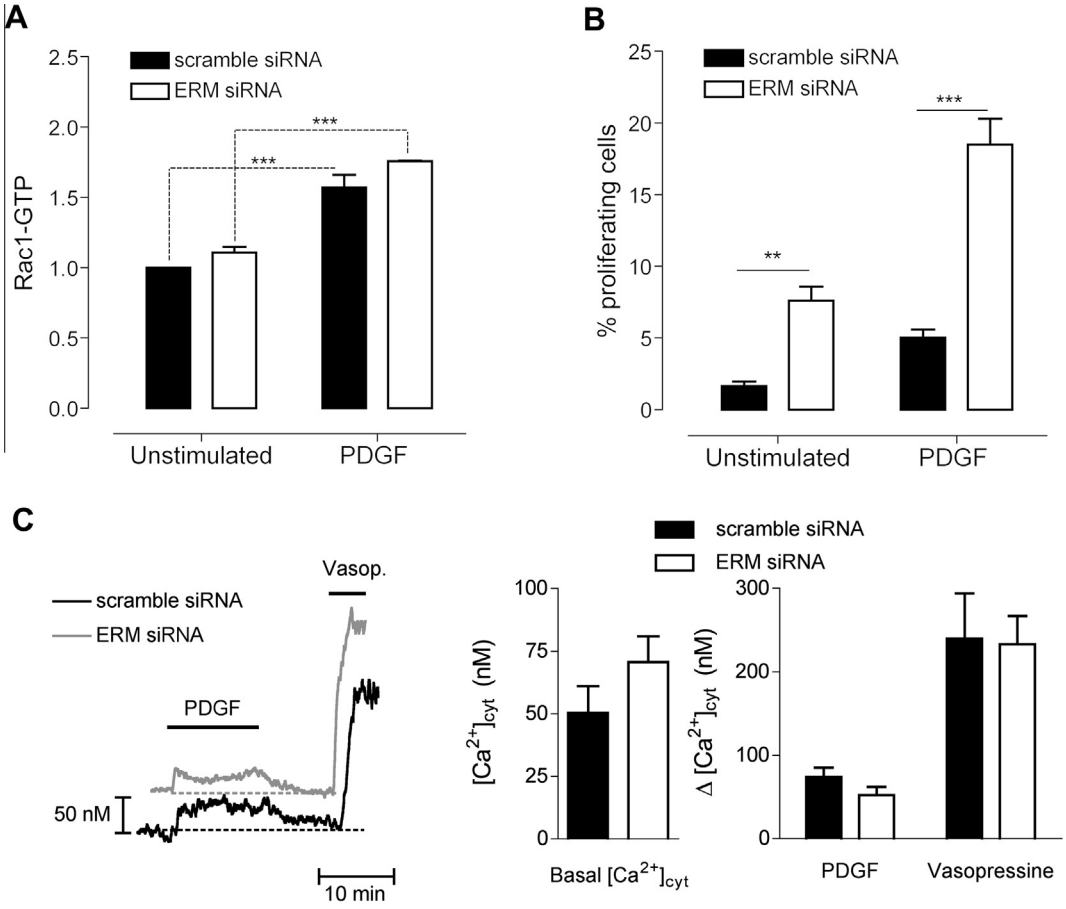

Fig. 3. PDGF-dependent activation of Rac1, proliferation, and Ca^{2+} signaling are preserved in ERM knock-down cells. (A) Rac1-GTP levels in cells transfected with scramble or anti-ERM siRNAs, with or without stimulation with PDGF (40 ng/ml) for 30 min. Data were normalized to unstimulated scramble siRNA transfected cells and expressed as mean ± SEM (***$p < 0.001$, $n = 3$). (B) Quantification of proliferating cells by measuring the incorporation of AlexaFluor 488 EdU in VSMC transfected with a scramble siRNA or anti-ERM siRNAs ($n = 3$, **$p < 0{,}01$, ***$p < 0{,}001$). (C) Typical records of the cytosolic Ca^{2+} concentration in fura-2 loaded VSMC transfected with a scramble siRNA or with anti-ERM-siRNAs. PDGF (40 ng/ml) and vasopressin (10 nM) were applied as indicated. Bar charts: Basal cytoslic Ca^{2+} concentation (left panel) or changes in cytosolic Ca^{2+} concentration (right panel) induced by PDGF and vasopressin in VSMC transfected with a scramble siRNA or with anti-ERM-siRNAs. Data are mean values ± SEM from 6 determinations.

3.5. ERM proteins regulate focal adhesion turn-over and adhesion to substrate

To explain the lack of lamellipodia formation and migration properties in ERM-depleted VSMC, we then hypothesized that ERM knock-down cells may exhibit a defective focal adhesion turnover and increased adhesion to the ECM, slowing down considerably the migration and inhibiting the cytoskeleton reorganization. To analyze VSMC focal adhesions, we stained cells for vinculin (Fig. 4A). Scramble siRNA transfected cells exhibited small, circular, individual, focal adhesions while cells depleted for ERM proteins exhibited larger fibrillar focal adhesions often grouped in larger complexes, suggesting a decreased focal adhesion turnover [25]. As depicted in the graph 4B, only cells lacking the three ERM proteins, and not cells transfected with one siRNA only, exhibited statistically significant larger adhesion complexes compared to control cells ($p < 0.0001$, $n \geqslant 23$ pictures analyzed in each condition), confirming the redundant role of ERM proteins in the control of the focal adhesion turn-over.

We then tested VSMC adhesion to substrate to confirm this observation (Fig. 4C). While the seeding density was similar, we observed that ERM-depleted cells exhibited a better adhesion to fibronectin (10 and 20 μg/ml gave similar results) compared to scramble siRNA-transfected cells ($p < 0.05$, $n = 10$ in 3 independent experiments). As this protocol required washing to remove non-adherent cells, inducing shear forces able to detach cells, a greater resistance to detachment may explain the difference in the number of adherent cells 30 min after seeding between control and ERM knock-down cells. To measure cell adhesion strength, we then performed a different protocol based on a modified version of the centrifugal adhesion assay developed by McClay et al. [18]. When cells were first allowed to attach to fibronectin (20 μg/ml) at 37 °C, more ERM-depleted cells resisted detachment compared to control cells (Fig. 4D, $p < 0.0001$, $n = 50$ in 5 independent experiments). Such a difference was not observed when cell adhesion was performed at 4 °C (Fig. 4D, $p = 0.65$, $n = 26$ in 2 independent experiments), suggesting that the number of adhesion receptors was similar in both cells. Individual knock-down of ezrin, radixin or moesin also increased cell adhesion strength at 37 °C (Fig. 4D, ezrin- and moesin-siRNA $p < 0.01$, radixin-siRNA $p > 0.05$ vs scramble siRNA, $n = 19$ in 2 independent experiments) but this increase was significantly lower compared to the increased adhesion strength obtained by knocking-down the three proteins together (Fig. 4D, $p < 0.001$ vs ERM-siRNA). This observation indicated that ERM proteins may play an important role in the control of focal adhesion disassembly, rather than the expression of adhesion receptors. In the absence of ERM proteins VSMC possess such strong and thick focal adhesions that prevent formation of structures required for migration.

PDGF stimulation triggers a complex signaling cascade involving calcium entry and Rac1 activation, inducing the formation of

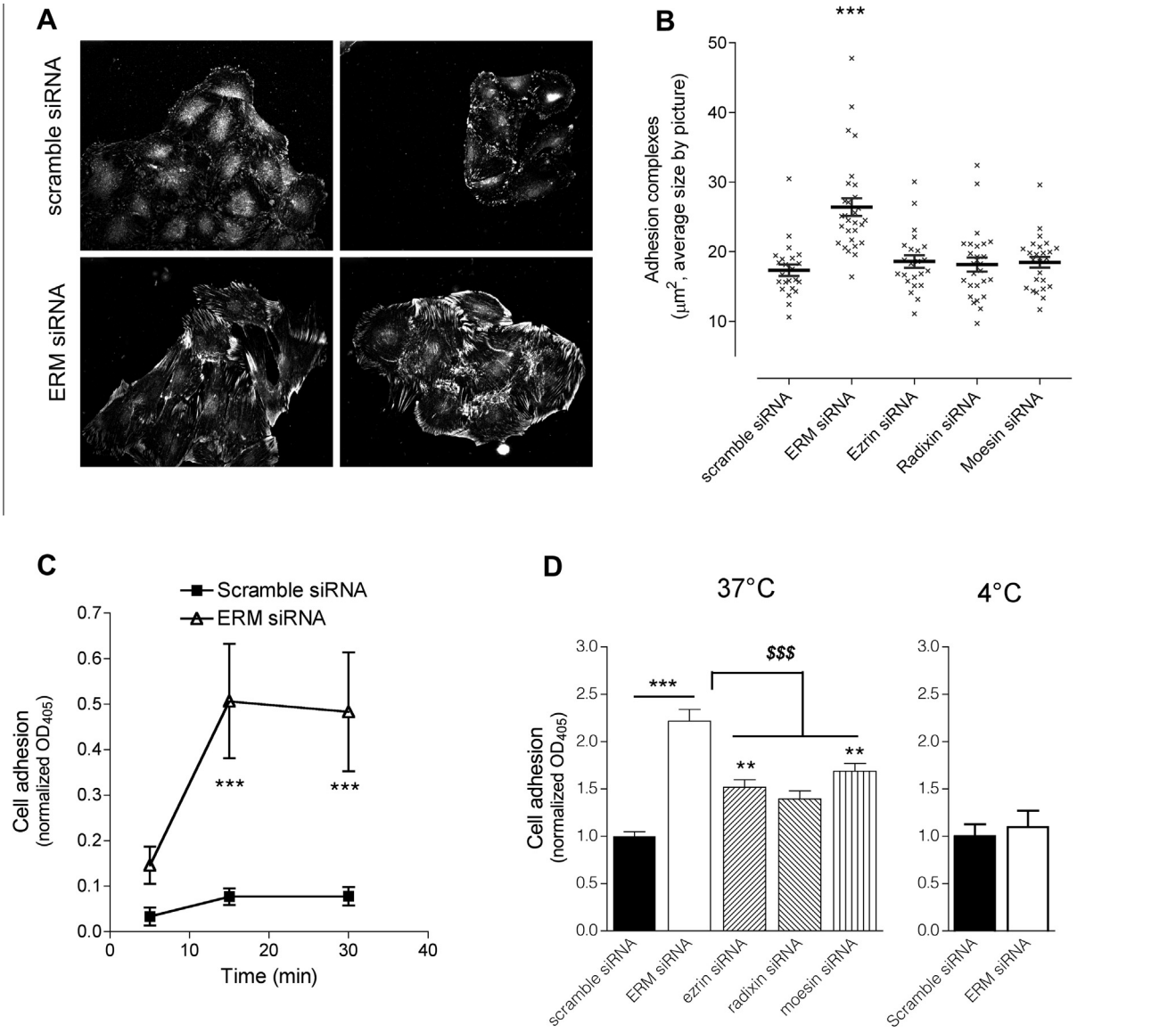

Fig. 4. ERM proteins regulate cell adhesion. (A) VSMC transfected with a scramble siRNA or anti-ERM-siRNAs stained for vinculin. (B) Quantification of adhesions size in VSMC transfected with scramble, anti-ERM or anti-ezrin, radixine or moesin siRNA. (C) Time-course of attachment to fibronectin (10 μg/ml) of scramble siRNA or ERM-siRNA transfected VSMC (n = 10 in 3 independent experiments). (D) Adhesion of cells transfected with a scramble siRNA, anti-ERM siRNA or a siRNA directed against ezrin, radixin or moesin determined by their resistance to removal force. (n = 19–50, in 2 to 5 independent experiments). ($^{***}p < 0.001$ $^{**}p < 0.01$ vs scramble siRNA transfected cells; $^{\$\$\$}p < 0.001$ vs ERM siRNA transfected cells).

polarized structures inside the cell. Several recent reports pointed out that the control of cell polarity and migration on a rigid substrate may be dependent on cell adhesion strength to this substrate and that the size of focal adhesions controls cells migration speed [25,26]. Also, cell migration involves several coordinated steps requiring the adhesion, disassembly and readhesion of focal adhesions to allow cell movement [15]. A defect in focal adhesions disassembly may then totally impair cell polarity and migration even if the other components of the pro-migratory signaling cascade are still effective. FAK is an important component of focal adhesions and could be a good target for the control of adhesion by ERM proteins. FAK activation was indeed decreased in cells depleted for ERM proteins. However, this effect cannot be the only way for ERM proteins to control migration, as depletion in radixin alone produced a similar effect as depletion of the three ERM proteins together (Supplemental Fig. 4).

In conclusion, this study shows that ERM proteins may control VSMC migration in a redundant manner. By regulating focal adhesion turn-over and cell adhesion to substrate ERM proteins can play an important role in artery wall remodeling involved in the development of vascular pathologies.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.118.

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