



The antagonistic roles of PDGF and integrin $\alpha v \beta 3$ in regulating ROS production at focal adhesions

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ABSTRACT

Reactive oxygen species (ROS) have been shown to play crucial roles in regulating various cellular functions, e.g. focal adhesion (FA) dynamics and cell migration upon growth factor stimulation. However, it is not clear how ROS are regulated at subcellular FA sites to impact cell migration. We have developed a biosensor capable of monitoring ROS production at FA sites in live cells with high sensitivity and specificity, utilizing fluorescence resonance energy transfer (FRET). The results revealed that platelet derived growth factor (PDGF) can induce ROS production at FA sites, which is mediated by Rac1 activation. In contrast, integrins, specifically integrin $\alpha v \beta 3$, inhibits this local ROS production. The RhoA activity can mediate this inhibitory role of integrins in regulating ROS production. Therefore, PDGF and integrin $\alpha v \beta 3$ coordinate to have an antagonistic effect in the ROS production at FA sites to regulate cell adhesion and migration.

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1. Introduction

Living cells are continuously exposed to environmental cues. It becomes clear that the changes in microenvironments play crucial roles in regulating normal cellular functions as well as the progression of diseases [1–3]. Recent papers revealed that matrix mechanics controls the cell fate by modulating the density and types of the bonds between membrane receptor integrins and extracellular matrix (ECM) proteins coupled to the materials [4,5]. However, it remains elusive on how specific ECM proteins interact with integrins to mediate the transmission of the environmental cues. Reactive oxygen species (ROS) such as hydrogen peroxide and

superoxide anion are crucial secondary messengers in cellular adhesion [6], spreading [7], and migration [8]. It has been shown that ROS can regulate these processes via focal adhesions (FA) and focal complexes mediated by the ligation of integrins [8], heterodimeric transmembrane glycoprotein receptors composed of non-covalently linked α and β subunits. ROS induction has also been shown to significantly reduce the surface expression of the αv and $\beta 3$ integrins [9,10]. Conversely, the overexpression of integrin $\alpha v \beta 3$ can increase glutathione (GSH), indicating an inhibitory role of integrin $\alpha v \beta 3$ in ROS production [9].

Platelet derived growth factor (PDGF) was shown to cause an ROS increase in smooth muscle cells [11] and induce tyrosine phosphorylation events by inhibiting protein tyrosine phosphatase (PTP) [12]. Consistently, the inhibition of intracellular ROS by scavengers can block the PDGF-stimulated signal transduction [11]. These PDGF effects on ROS can be regulated by integrins and their mediated cell adhesion on extracellular matrix (ECM) proteins [13]. In fact, integrins have been shown to directly regulate growth factor receptors by changing their expressions or localizations/functions at focal contacts, subcellular regions where integrins co-localize

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Abbreviations

5-LOX	5-lipoxygenase
Cys	cysteine
DTT	dithiothreitol
ECFP	enhanced cyan fluorescent protein
ECM	extracellular matrix
FBS	fetal bovine serum
FN	fibronectin
FA	focal adhesion
FRET	fluorescence resonance energy transfer
GSH	glutathione

HEK	human embryonic kidney
IPTG	isopropyl- β -D-thiogalactopyranoside
LMW-PTP	low molecular weight- protein tyrosine phosphatase
MEF	mouse embryonic fibroblast
NAC	N-acetyl cysteine
PDGF	platelet derived growth factor
PDGFR	PDGF-receptor
ROS	reactive oxygen species
Redox	reduction–oxidation
His6	six histidines
Ypet	yellow fluorescent protein

with signaling and cytoskeletal molecules [13–16]. For example, cell adhesion on ECM protein fibronectin can enhance the PDGFR- β auto-phosphorylation and protein levels [17]. Integrin $\alpha v \beta 3$ and PDGFR- β were also shown to interact physically for the regulation of cell migration [18]. Consistently, PDGFR- β activation increases endothelial cell migration on vitronectin, a ligand for integrin $\alpha v \beta 3$ [18]. Migration of mesenchymal stem cells is also promoted by the crosstalk between PDGFR- β and integrin $\alpha 5 \beta 1$ in the presence of fibronectin [19].

Rac1 and RhoA can act downstream to integrin and PDGFR signaling [20], and trigger a variety of opposite responses [21–23]. For example, high concentration of fibronectin can downregulate Rac1 but upregulate RhoA, and subsequently inhibit migration [20]. ROS has also emerged as a crucial mediator of Rac1 and RhoA signaling by covalently modifying specific cysteine residues. Although both Rac1 and RhoA contain the GXXXXGK(S/T)C motif that can respond to ROS, RhoA has an extra cysteine (Cys20) in the redox motif, which can be oxidized by ROS to promote the formation of an intramolecular disulfide bond and cause RhoA inactivation [24]. The activation of Rac1 can also induce the downregulation of RhoA via the ROS-dependent inactivation of LMW-PTP (Low molecular weight-protein tyrosine phosphatase) [25]. As such, ROS can mediate the antagonistic crosstalk between Rac1 and RhoA [26–29].

We have developed a ROS sensor based on fluorescence resonance energy transfer (FRET) that allows the real-time assessment of intracellular reduction–oxidation (redox) conditions at subcellular focal adhesion (FA) sites. Herein, we report the design, generation, and investigation of this FRET-based redox sensor. The FRET sensor displayed increased redox sensitivity upon integration of paxillin, which acts to localize the sensor specifically to FA sites. In addition to the detection of ROS at FA sites in response to exposure to oxidants such as diamide or hydrogen peroxide, we can monitor intracellular activities of signaling pathways involving Rac1 and RhoA. The ROS production at FA sites of mouse embryonic fibroblasts was investigated upon stimulation by PDGF of cells cultured on different concentrations of extracellular matrix protein (fibronectin). Finally, we examined the relationship between PDGF and integrins (specifically $\alpha v \beta 3$ or $\alpha 5 \beta 1$) in regulating ROS production as mediated by Rac1 and RhoA signaling at FA sites.

2. Materials and methods**2.1. Gene construction and plasmids**

The gene encoding the cytosolic ROS sensor includes a central fragment consisting of the peptide sequence CEGGSTSGSGKPGSGEGSTKGCEG flanked by a SphI site at the N terminus and a SacI site at the C terminus, which was further fused in between an N-terminal ECFP and a C-terminal YPet (Table 1). This cytosolic ROS sensor was cloned into pRsetB (Invitrogen), and the insert was sequenced (W. M. Keck Center for Functional and Comparative Genomics, University of Illinois at Urbana-Champaign) to verify the integrity of the coding sequence. The plasmid has the coding sequence in-frame with six histidines (His₆-tag), and hence when expressed, the product carried an N-terminal His₆-tag to allow Ni column purification. The DNA insert was then digested by BamHI/EcoRI and ligated into a pcDNA3 vector (Invitrogen) to create the resultant ROS sensor for mammalian cell expression (Fig. 1). The ROS sensor mutant was generated by site-specific mutations of cysteine to serine on both CEG regions, thus resulting in its resistance to oxidative modification. The ROS-paxillin sensor was constructed by adding the paxillin gene to the C-terminus of the cytosolic ROS sensor. Recombinant plasmids pRsetB and pcDNA3.1 were from Invitrogen. Dominant negative forms of Rac1 (RacN17) and RhoA (RhoN19), and the constitutively active forms of Rac1 (RacV12) and RhoA (RhoV14) were gifts from Dr. Alan Hall (Memorial Sloan–Kettering Cancer Center, NY, USA).

2.2. Materials and reagents

Phusion DNA polymerase and enzymes for DNA digestion were from NEB (MA, USA). Ni-NTA agarose, QIAprep spin miniprep and QIAquick gel purification kits were from Qiagen (CA, USA). The Bradford protein assay kit was from BioRad (CA, USA). Oligonucleotides were obtained from Invitrogen (CA, USA). Dithiothreitol (DTT), an anti-oxidant used to stabilize proteins containing sulfhydryl groups, was purchased from Promega (WI, USA). Diamide as a thiol oxidizing agent and N-acetyl cysteine (NAC) as a ROS scavenger were purchased from Sigma (MO, USA). Antibodies for blocking integrins $\alpha v \beta 3$ (clone LM609) and $\alpha 5 \beta 1$ (Clone BMB5) were from Millipore (MA, USA).

2.3. Protein purification

The ROS sensors were expressed in *Escherichia coli* (BL21 strain) as fusion proteins with an N-terminal His₆-tag and purified by nickel chelation chromatography. In brief, BL21 cells expressing the ROS sensor in the pRsetB vector were grown in LB medium containing ampicillin (100 mg/L) at 37 °C until OD₆₀₀ measured around 0.2. Isopropyl- β -D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.2 mM, and the culture was incubated for another 16 h at 25 °C. Cells were collected by centrifugation, and re-suspended in 10 ml binding buffer (50 mM Tris-HCl, 200 mM NaCl, 10 mM imidazole, pH 7.4) and lysed by B-PER protein extraction reagents (Thermo Scientific). The cell lysate was clarified by centrifugation and subjected to the incubation with nickel-NTA beads. The protein-coated beads were washed with the binding buffer and the proteins were then eluted with 5 ml elution buffer (50 mM Tris, 200 mM NaCl, 200 mM imidazole, pH 7.4).

Table 1
Description of ROS sensor constructs.

Construct name	Construct features
Cytosolic ROS sensor	His ₆ tag-ECFP-Cys-EGGSTSGSGKPGSGEGSTKG-Cys-EG-YPet
Cytosolic ROS mutant	His ₆ tag-ECFP-Ser-EGGSTSGSGKPGSGEGSTKG-Ser-EG-YPet
ROS-paxillin sensor	His ₆ tag-ECFP-Cys-EGGSTSGSGKPGSGEGSTKG-Cys-EG-YPet-Paxillin
ROS-paxillin mutant	His ₆ tag-ECFP-Ser-EGGSTSGSGKPGSGEGSTKG-Ser-EG-YPet-Paxillin

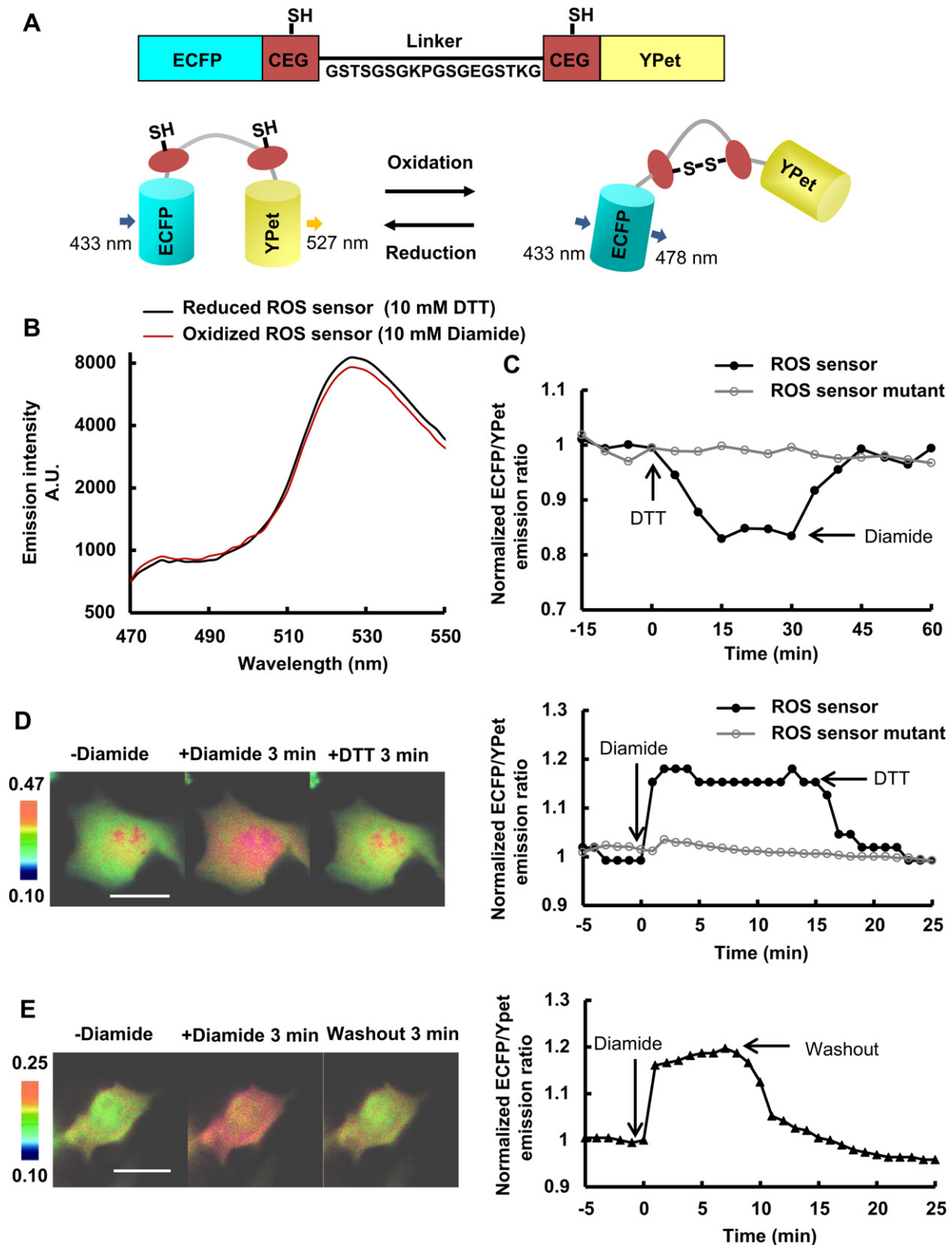


Fig. 1. The design and characterization of FRET-based cytosolic ROS sensor. (A) The design of cytosolic ROS sensor. The ROS sensor is composed of ECFP serving as the donor, two CEG peptides flanking a flexible peptide linker, and YPet serving as an acceptor. ROS is expected to cause the formation of a disulfide bond between the two cysteine residues of the CEG peptides that leads to a change in the conformation of cytosolic ROS sensor. (B) Emission spectra of the 10 mM DTT pretreated cytosolic ROS sensor before (black line) and after (red line) 10 mM diamide treatment. (C) The time courses of ECFP/YPet emission ratio of 0.5 μ M purified cytosolic ROS sensor (black circles) and its negative C to S mutant (gray circles) incubated with 10 mM DTT and 10 mM diamide as indicated. The ECFP/YPet ratio values were normalized against the averaged values before the treatment. (D) Left: the ECFP/YPet ratio images and values of cytosolic ROS sensor expressed in HEK cells. Right: the time course of the normalized ECFP/YPet emission ratio of cytosolic ROS sensor (black circles) and its mutant (gray circles) incubated with 0.5 mM diamide prior to 4 mM DTT treatment. (E) The ratio images and time courses of cytosolic ROS sensor when the HEK cells were incubated with 0.5 mM diamide followed by a washout (Scale bar, 10 μ m). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.4. Cell culture and reagents

Human embryonic kidney (HEK) and mouse embryonic fibroblast (MEF) cell lines were maintained in DMEM (Gibco BRL) medium with 10% fetal bovine serum (FBS) (Gibco BRL), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1 mM sodium pyruvate (Gibco BRL). Cells were grown in culture dishes in a 5% CO₂ incubator at 37 °C. Lipofectamine 2000 (Invitrogen) was used for the transfection of DNA plasmids. The transfected HEK and MEF cells expressing a ROS sensor were cultured in 10% FBS for 36–48 h before subjected to diamide (0.5 mM) or H₂O₂ (1 mM) stimulation. For PDGF experiments, cells were plated and incubated for 24 h in growth medium with 0.5% FBS before PDGF stimulation.

2.5. In vitro spectroscopy

Fluorescence emission spectra of the purified ROS sensors were measured with an excitation wavelength of 433 nm by a fluorescence plate reader (TECAN, Sapphire II). The emission ratios of donor/acceptor (478 nm/527 nm) of the recombinant ROS sensor (1 µg/ml) were measured before and after the addition of DTT (10 mM). 10 mM diamide was added 30 min later and the emission ratios were continuously measured for another 30 min.

2.6. Microscopy and image acquisition

During imaging, cells were cultured in cover-glass-bottom dishes (Cell E&G, Houston, TX) and maintained in DMEM medium containing 0.5% FBS. The microscope is equipped with an environmental chamber that is temperature controlled at 37 °C and contains humidified 5% CO₂ air. Images were collected by a Nikon eclipse microscope using MetaFluor 6.2 and MetaMorph software (Universal Imaging) with a 420DF20 excitation filter, a 450DRLP dichroic mirror, and two emission filters controlled by a filter changer (475DF40 for ECFP and 535DF25 for YPet). The excitation filter for ECFP at 420 ± 20 nm shifts the excitation toward the blue to reduce the cross-excitation of YPet and the effect of bleed-through on the FRET channel. The majority of the cell body was selected as the region of interest to collect signals and conduct quantification. All the images were background-subtracted and smoothed using a median-filter with a window size of 3 × 3 pixels. The pixel-by-pixel ratio images of ECFP/YPet were calculated based on the background-subtracted fluorescence intensity images of ECFP and YPet by using the MetaFluor software. These ratio images were displayed in the intensity modified display mode in which the color and brightness of each pixel is determined by the ECFP/YPet ratio and ECFP intensity, respectively.

3. Results

The cytosolic ROS sensor was engineered to contain a ROS sensitive peptide CEGGSTSGSGKPGSGEGSTKGCCEG concatenated between ECFP and YPet, two fluorescent proteins serving as a FRET-sensitive pair (Fig. 1A) [30]. This cytosolic ROS sensor purified by affinity chromatography was first incubated with 10 mM reducing reagent DTT for 30 min to convert into the reduced form. The emission spectrum of 0.5 µM purified ROS sensor revealed a relatively weak peak for ECFP (478 nm) and a strong peak for YPet (527 nm) emission, indicating a high basal FRET level of the sensor in the reduced form (Fig. 1B). The conversion of the biosensor to the reduced form by DTT also clearly caused a FRET increase and a decrease in ECFP/YPet ratio (Fig. 1C). In contrast, the oxidizing reagent diamide caused an oxidation of the sensor and induced a disulfide bond formation between the reactive thiol groups of the two cysteines, which resulted in a FRET loss and a 20% increase in ECFP/YPet emission ratio (Fig. 1B–C). The FRET signal of the freshly purified cytosolic ROS sensor is not sensitive to 10 mM diamide but can be changed by DTT incubation (Fig. 1C), suggesting an oxidized state of the purified sensor before any treatment. A negative mutant of the sensor with serine (S) replacing the two cysteine (C) residues in the CEG regions had no response to DTT or diamide treatment (Fig. 1C), confirming that the responses of the ROS sensor are specifically caused by the oxidation/reduction event of the disulfide bond. Therefore, these results suggest that our ROS sensor can report the reversible oxidation and reduction events *in vitro*.

The cytosolic ROS sensor was then transfected into human embryonic kidney (HEK) cells to monitor the oxidative stress in live cells. The concentration of glutathione is relatively high inside cells to maintain a reduced cellular environment and set the sensor in an

initial high-FRET state with low ECFP/YPet emission ratio. Diamide can rapidly penetrate across the plasma membrane and deplete the intracellular glutathione within minutes, resulting in the oxidation of the ROS target proteins [31]. Indeed, 0.5 mM diamide caused a rapid increase of the ECFP/YPet emission ratio, which was reversed by the addition of 4 mM DTT (Fig. 1D). Notably, the negative mutant of the ROS sensor did not respond to diamide or DTT (Fig. 1D). Moreover, mammalian cells were able to restore a reduced intracellular redox environment on a time scale of a few minutes after the removal of the oxidizing reagent diamide (Fig. 1E). Therefore, our cytosolic ROS sensor can report the reversible and dynamic changes of ROS in mammalian cells.

We then designed a subcellular targeted ROS sensor by fusing paxillin at the C-terminus of the cytosolic sensor to visualize ROS signaling at FA sites in mouse embryonic fibroblasts (MEFs) where ROS play crucial roles in regulating FA proteins and cellular functions (Fig. 2A and Table 1). This ROS-paxillin sensor can be clearly observed to co-localize with paxillin fused to mCherry when co-transfected in MEFs (Supplementary Fig. 1A). We also examined the cytosolic ROS sensor in MEFs. The results revealed that MEFs have similar response to ROS as is the case in HEK cells visualized by the cytosolic ROS sensor (Supplementary Fig. 1B), suggesting that our ROS sensors are non-cell-type specific and have wide applicability. Consistently, either diamide (0.5 mM) or H₂O₂ (0.5 mM) treatment caused oxidation and a significant 20–40% change in the ECFP/YPet ratio of the ROS-paxillin sensor in MEFs (Fig. 2B–C). When MEFs were cultured on a fibronectin (FN) concentration of 2.5 µg/ml and exposed to 25 ng/ml PDGF, a known stimulator of cell motility and FA modulation, a more than 50% change in ECFP/YPet ratio of the ROS-paxillin sensor can be observed whereas only around 10% change can be observed with the cytosolic ROS sensor (Fig. 3A–B). Pretreating ROS-paxillin sensor transfected MEFs with an anti-oxidant N-acetyl cysteine (NAC), a free radical and ROS scavenger, eliminated any change in FRET response (Fig. 3A–B). Again, the negative mutants of ROS sensors (Table 1) had no detectable change of FRET signals (Fig. 3B), confirming that the sensing mechanism of ROS sensors is indeed due to the ability to form the intramolecular disulfide bridge. These results suggest that the ROS-paxillin sensor can specifically monitor the ROS regulation at FA sites in response to PDGF with a higher sensitivity compared to the cytosolic sensor, possibly reflecting a highly concentrated ROS dynamic at the subcellular FA sites.

Since the ligation of ECM and integrins is crucial for FA and ROS regulation, we reasoned that the PDGF-induced ROS modulation at FA sites may be regulated by the cell-ECM adhesion. We therefore examined the different concentrations of PDGF and ECM protein FN in regulating ROS production. MEFs transfected with the ROS-paxillin sensor were responsive only when cultured on a low FN concentration (2.5 µg/ml) and exposed to a high PDGF concentration (25 ng/ml) (Fig. 3C, red line). Either lowering the PDGF dosage to 10 ng/ml or raising the FN concentration to 10 µg/ml can inhibit this PDGF-induced ROS production (Fig. 3C). These results suggest that PDGF can promote intracellular ROS production at FA sites whereas ligation of integrins to FN may have an inhibitory effect. Since both integrins αvβ3 and α5β1 interact with FN, we further examined which integrin subtype is involved in inhibiting ROS production under PDGF stimulation. MEFs cultured on a high concentration of FN (10 µg/ml) were pretreated for 2 h with a function-blocking antibody against either integrin αvβ3 or α5β1, two primary integrin subtypes serving as the receptors of FN. The antibody against integrin αvβ3 eliminated the ROS inhibitory effect of 10 µg/ml FN, with a marked FRET response of the ROS-paxillin sensor upon 25 ng/ml PDGF stimulation (Fig. 3D and Supplementary movie 1). In contrast, the antibody against integrin α5β1 did not have any detectable effect (Fig. 3D and Supplementary movie

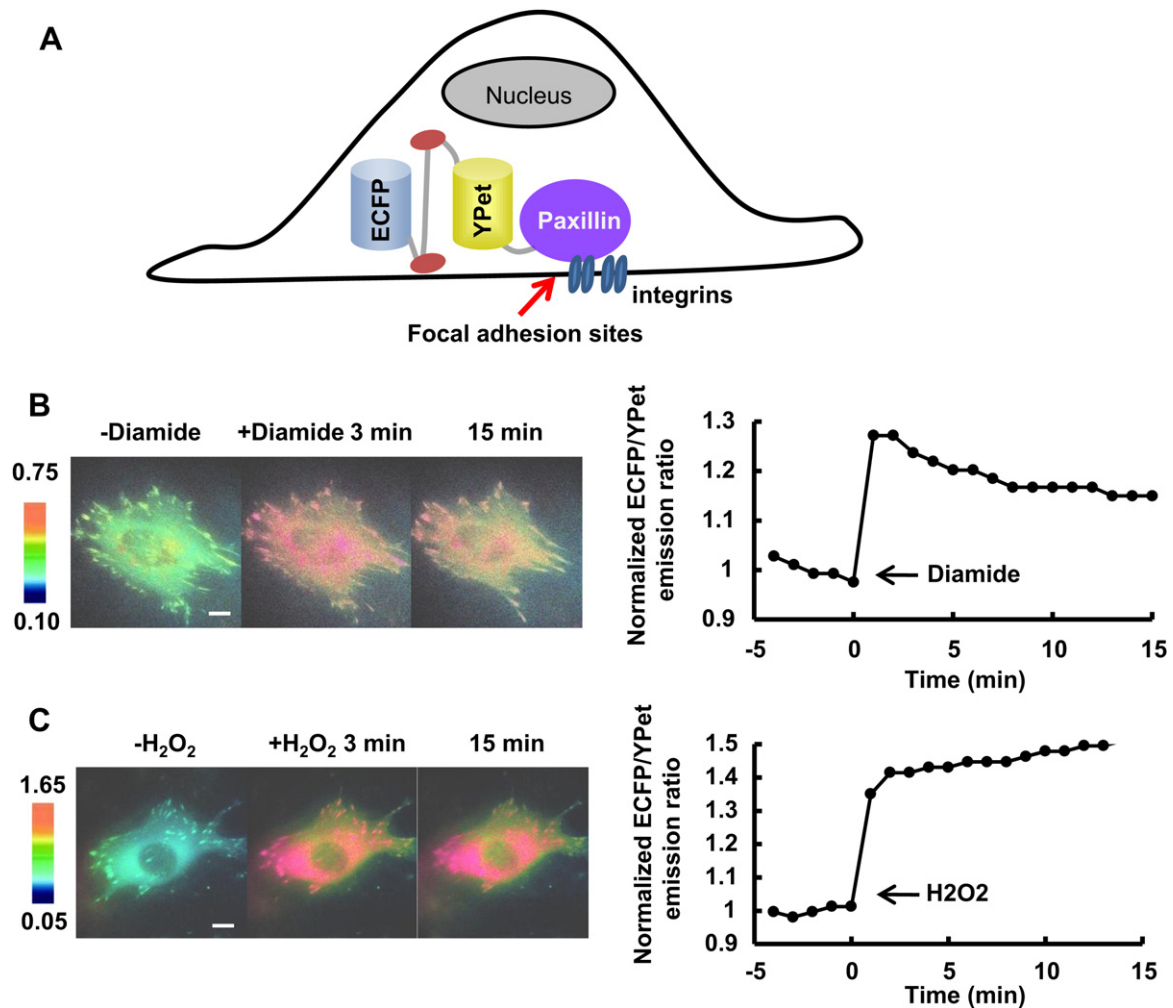


Fig. 2. The ROS sensor targeted to FA sites. (A) The design of FA-targeting ROS sensor. The C-terminal paxillin can direct the ROS sensor to localize at FA sites. (B–C) The ratio images (left) and time courses of the normalized ECFP/YPet ratio (right) of ROS-paxillin sensor expressed in MEFs treated with (B) diamide (0.5 mM) or (C) H₂O₂ (0.5 mM) as indicated (Scale bar, 10 μ m).

2). These results suggest that integrin α v β 3, but not α 5 β 1, serves as the negative regulator for ROS generation at FA sites upon PDGF stimulation.

Supplementary video related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2013.01.092>.

Rac1 and RhoA are downstream effectors for PDGF and integrins. Previous reports suggest that PDGF may induce ROS by activating Rac1 [32]. Consistently, when constitutively active (RacV12) or dominant negative (RacN17) mutants of Rac1 were co-transfected with the ROS-paxillin sensor in MEFs, RacV12 caused a stronger sensor response to 25 ng/ml PDGF stimulation in cells seeded on 10 μ g/ml FN (Fig. 4A–B). Conversely, RacN17 suppressed the sensor response to 25 ng/ml PDGF stimulation in cells on 2.5 μ g/ml FN. These results suggest that Rac1 mediates and promotes the PDGF-induced ROS production at FA sites. On the other hand, integrins and their interaction with FN have been shown to promote RhoA activities [20]. Consistently, a higher FN concentration caused a higher RhoA activity measured by a RhoA FRET biosensor in our study (Supplementary Fig. 2). The PDGF-induced ROS production at FAs was inhibited by a constitutively active RhoA mutant (RhoV14) on 2.5 μ g/ml FN but enhanced by a dominant negative Rho mutant (RhoN19) on 10 μ g/ml FN (Fig. 4C–D). Therefore, the ROS production at FAs can be induced by PDGF via Rac1 activation but inhibited by integrin α v β 3 and its activation of RhoA (Fig. 4E).

4. Discussion

ROS serve as crucial secondary messengers for various signaling cascades controlling the cell–environment interactions [6,33–35]. It becomes clear that ROS production is largely dependent on the subcellular location, possibly due to different sets and concentrations of molecular intermediates at these regions [8,36,37]. In this study, we designed a new FRET-based ROS-paxillin sensor capable of monitoring ROS production at FA sites. While excellent FRET-based sensors have been developed to monitor the cytosolic redox conditions [38,39], our ROS-paxillin sensor allowed the immediate and continuous detection of ROS at specific FA sites with high sensitivity. With this ROS-paxillin sensor, we found a coordinated molecular crosstalk between the signaling pathways of growth factors and integrins at FA sites. Our results suggest that PDGF can induce the ROS production via Rac1 activation at FA sites. In contrast, ECM proteins and their ligation to integrins, specifically integrin α v β 3, can suppress the local ROS production by the activation of RhoA. Therefore, these results should shed new lights on the roles of matrix proteins at the interface between environmental materials and cells in regulating cellular responses.

PDGF causes FA disassembly to promote cell migration [40,41]. Indeed, PDGF and the subsequent activation of PDGFR can induce the translocation of Rac1 toward the plasma membrane and its

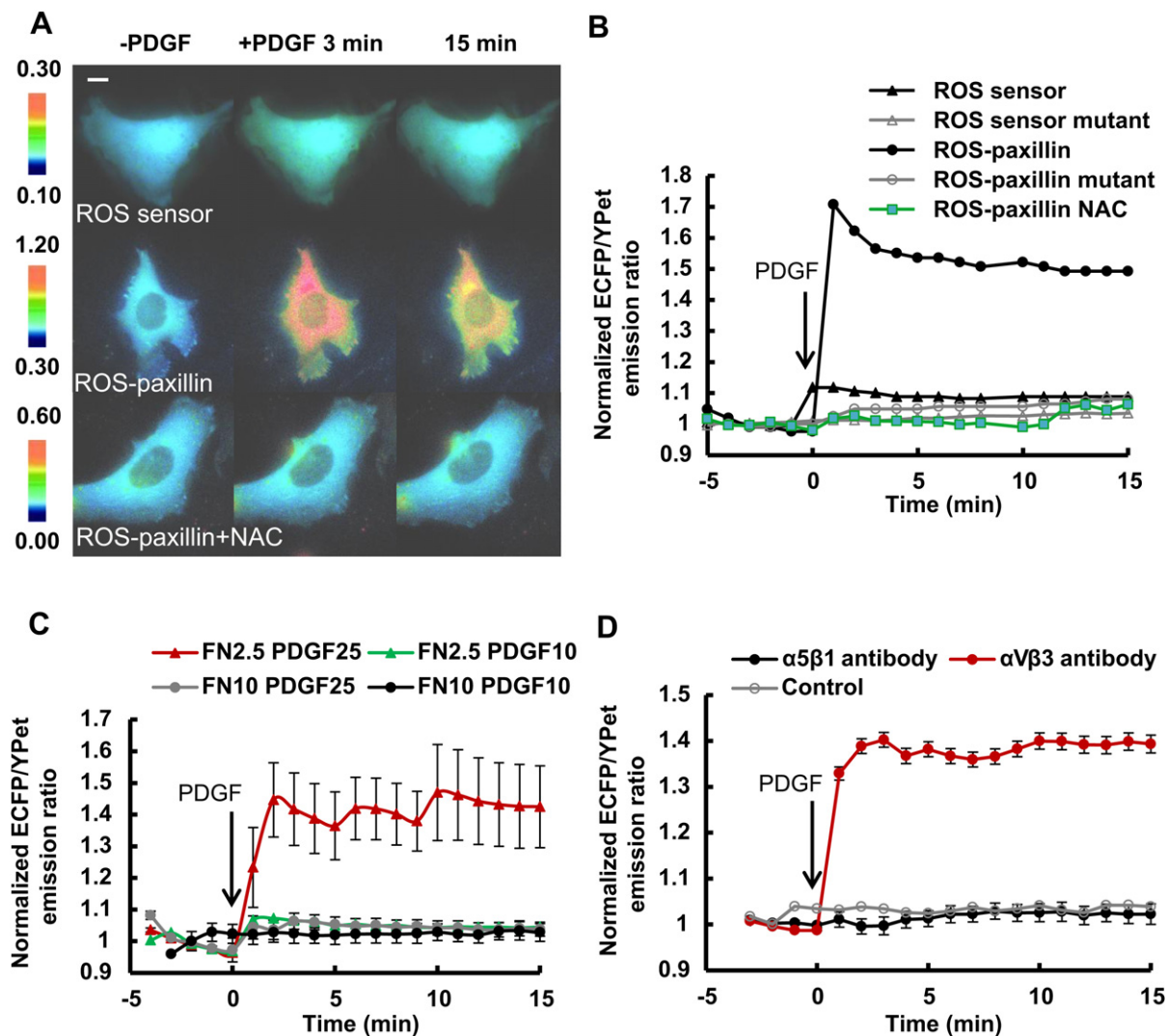


Fig. 3. The PDGF-induced ROS production at FA sites. (A) The representative ECFP/YPet ratio images of MEFs expressing either cytosolic ROS sensor or ROS-paxillin sensor with or without 25 mM NAC pretreatment for 30 min before 25 ng/ml PDGF stimulation for various periods of time (Scale bar, 10 μ m). The color bars reflect ECFP/YPet ratio values. (B) The ECFP/YPet ratio time courses of cytosolic ROS sensor and its mutant, ROS-paxillin sensor and its mutant with or without NAC pretreatment as indicated upon 25 ng/ml PDGF stimulation. (C) The time courses of normalized ECFP/YPet emission ratios (mean $\pm$ s.e.m.) of MEFs expressing ROS-paxillin sensor exposed to 10 or 25 ng/ml PDGF when the cells were cultured on 2.5 or 10 μ g/ml FN as indicated (red triangles, $n = 15$ for 2.5 μ g/ml FN and 25 ng/ml PDGF; green triangles, $n = 8$ for 2.5 μ g/ml FN and 10 ng/ml PDGF; gray circles, $n = 9$ for 10 μ g/ml FN and 25 ng/ml PDGF; and black circles, $n = 14$ for 10 μ g/ml FN and 10 ng/ml PDGF). (D) The time courses of normalized ECFP/YPet emission ratios (mean $\pm$ s.e.m.) of MEFs expressing ROS-paxillin sensor exposed to 10 ng/ml PDGF stimulation when they were cultured on 10 μ g/ml FN without (control gray circles) or with the pretreatment of inhibitory antibodies against integrin α v β 3 (red circles, $n = 13$) or α 5 β 1 (black circles, $n = 4$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

activation, thus inducing actin polymerization at the leading edge to result in membrane ruffling and protrusion [42,43]. This Rac1 activation can promote the ROS production to inhibit RhoA activity [25,29]. Since Rac1 promotes the formation of nascent focal adhesion and protrusion at the leading edge whereas RhoA induces contractility and formation of stable focal adhesions [44,45], this Rac1 activation and the subsequent ROS-mediated inhibition of RhoA upon PDGF stimulation may regulate the spatially coordinated FA disassembly and hence promote cell migration. On the other hand, integrin α v β 3 and the ligation to ECM proteins can promote RhoA activation to suppress ROS production and stabilize FAs. As such, PDGF and integrin α v β 3 signaling events can antagonistically control the production of ROS to orchestrate the FA dynamics and hence cell migration. This tight regulation of ROS production balanced by PDGF and integrin α v β 3 can also limit the abnormal production of ROS and subsequent cell damage.

Previous studies have shown that ROS is involved in regulating integrin-mediated adhesion processes. Indeed, integrin activation was shown to trigger a transient and localized burst of ROS during cell adhesion processes on ECM proteins by activating 5-lipoxygenase (5-LOX) [8,46]. Our results demonstrated that integrin α v β 3 and its ligation to ECM proteins can inhibit ROS production at FA sites upon PDGF stimulation to induce cell migration (Fig. 3). These results suggest that integrins may play distinct roles in regulating the ROS production during different cellular events.

ROS can promote the phosphorylation of focal adhesion kinase (FAK) by inhibiting the phosphatases such as LMW-PTP and SHP-2 [47]. Since FAK is a crucial molecule controlling FA turnover and cell migration, the application of the ROS-paxillin sensor to monitor ROS dynamics at FA sites should offer new molecular insights on how the spatiotemporal patterns of ROS signaling govern cell migration under different pathophysiological conditions. ROS are

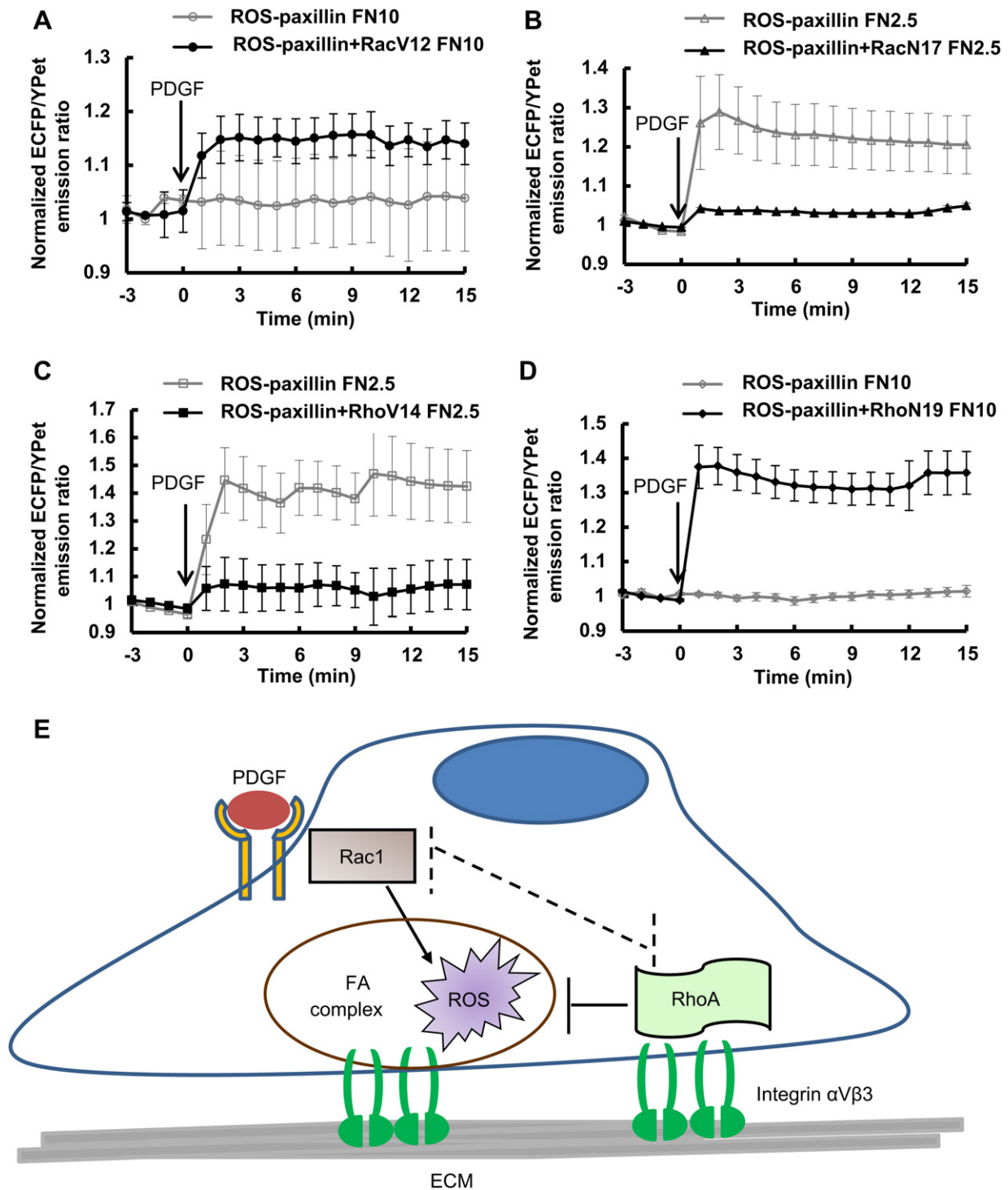


Fig. 4. The effects of Rac1 and RhoA on the PDGF-induced ROS production at FA sites. (A) The time courses of normalized ECFP/YPet emission ratios (mean $\pm$ s.e.m.) of MEFs expressing ROS-paxillin sensor with (black circles) or without the co-transfection of an active Rac1 mutant RacV12 (gray circles). Cells were cultured on 10 $\mu\text{g}/\text{ml}$ FN and exposed to 25 ng/ml PDGF stimulation. (B) The time courses of normalized ECFP/YPet emission ratios (mean $\pm$ s.e.m.) of MEFs expressing ROS-paxillin sensor with (black triangles) or without the co-transfection of a negative Rac1 mutant RacN17 (gray triangles). Cells were cultured on 2.5 $\mu\text{g}/\text{ml}$ FN and exposed to 25 ng/ml PDGF stimulation. (C) The time courses of normalized ECFP/YPet emission ratios (mean $\pm$ s.e.m.) of MEFs expressing ROS-paxillin sensor with (black squares) or without co-transfection of an active RhoA mutant RhoV14 (gray squares). Cells were cultured on 2.5 $\mu\text{g}/\text{ml}$ FN and exposed to 25 ng/ml PDGF stimulation. (D) The time courses of normalized ECFP/YPet emission ratios (mean $\pm$ s.e.m.) of MEFs expressing ROS-paxillin sensor with (black diamonds) or without co-transfection of a negative RhoA mutant RhoN19 (gray diamonds). Cells were cultured on 10 $\mu\text{g}/\text{ml}$ FN and exposed to 25 ng/ml PDGF stimulation. (E) The mechanistic diagram depicting the ROS regulation at FA sites. ROS production at FA sites is induced by PDGF via Rac1 activation but inhibited by integrins, specifically integrin $\alpha\text{V}\beta\text{3}$, via RhoA activation.

also thought to be crucial factors involved in aging, cancer development, and other disease processes. Indeed, various tumor cells in culture were shown to have an increased intrinsic ROS level [48]. Constant production of large amounts of ROS can promote genetic instability and the development of drug resistance. Therefore, our sensitive ROS-paxillin sensor can provide a powerful tool to allow the monitoring of the localized redox conditions during the cellular interactions with environment, and the evaluation of therapeutic reagents or strategies targeting diseases involving ROS and cell–material interactions.

5. Conclusion

We report here a FRET-based sensor that is able to detect local accumulation of reactive oxygen species at FAs in live cells. Using this sensor we found that the growth factor PDGF induces ROS production at focal adhesions via Rac1. In contrast, ECM proteins such as FN that interface the material environment with cells can interact with the membrane receptor integrins, specifically integrin $\alpha v \beta 3$, to inhibit local ROS production via RhoA. As such, the cross-talk and antagonistic coordination between molecular signals from different environmental cues, such as growth factor PDGF and ECM protein FN, determine ROS formation at focal adhesions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2013.01.092>.

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