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PKA turnover by the REG γ -proteasome modulates FoxO1 cellular activity and VEGF-induced angiogenesis

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Abstract

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Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yjmcc.2014.02.007>.

The REG γ -proteasome serves as a short-cut for the destruction of certain intact mammalian proteins in the absence of ubiquitin-and ATP. The biological roles of the proteasome activator REG γ are not completely understood. Here we demonstrate that REG γ controls degradation of protein kinase A catalytic subunit- α (PKA α) both in primary human umbilical vein endothelial cells (HUVECs) and mouse embryonic fibroblast cells (MEFs). Accumulation of PKA α in REG γ -deficient HUVECs or MEFs results in phosphorylation and nuclear exclusion of the transcription factor FoxO1, indicating that REG γ is involved in preserving FoxO1 transcriptional activity. Consequently, VEGF-induced expression of the FoxO1 responsive genes, *VCAM-1* and *E-Selectin*, was tightly controlled by REG γ in a PKA dependent manner. Functionally, REG γ is crucial for the migration of HUVECs. REG $\gamma^{-/-}$ mice display compromised VEGF-instigated neovascularization in cornea and aortic ring models. Implanted matrigel plugs containing VEGF in REG $\gamma^{-/-}$ mice induced fewer capillaries than in REG $\gamma^{+/+}$ littermates. Taken together, our study identifies REG γ as a novel angiogenic factor that plays an important role in VEGF-induced expression of *VCAM-1* and *E-Selectin* by antagonizing PKA signaling. Identification of the REG γ -PKA-FoxO1 pathway in endothelial cells (ECs) provides another potential target for therapeutic intervention in vascular diseases.

Keywords

Angiogenesis; *E-Selectin*; FoxO1; PKA α ; REG γ ; *VCAM-1*

1. Introduction

Angiogenesis is a physiological process and essential for reproduction, development, and wound repair. It also plays an important role in pathological conditions, such as tumor growth and metastasis, rheumatoid arthritis, and diabetic retinopathy [1, 2]. Angiogenesis is a highly ordered process which is tightly controlled by key angiogenic factors, including vascular endothelial growth factor (VEGF). There is consensus that VEGF signaling to ECs occurs by engagement with its receptors and represents the crucial rate-limiting step during normal and pathophysiological angiogenesis [3]. However, VEGF-targeted therapies have been limited by insufficient efficacy, development of resistance, and inherent toxicities [4].

Known angiogenic mediators include soluble forms of vascular cell adhesion molecule-1 (*VCAM-1*) and endothelial-Selectin (*E-Selectin*) [5]. Both *VCAM-1* and *E-Selectin* are subject to regulation by VEGF and play a pro-angiogenic role, contributing to the cross-regulation of angiogenesis and inflammation [6, 7]. Elevated levels of soluble *E-selectin* have been found in patients with vasculoproliferative disorders such as rheumatoid arthritis [8] and in malignant tumors [9]. Serum *VCAM-1* has been proposed as a surrogate marker for angiogenesis in breast cancer [10]. *VCAM-1* gene expression is effectively controlled by an upstream regulator — Forkhead box O1 (FoxO1), a key modulator of numerous genes that govern a wide array of cellular functions [11]. FoxO1 function is regulated by post-translational modifications [12], phosphorylation of FoxO1 at specific sites promotes FoxO1 nuclear exclusion and impairs its transcriptional activity [13]. PI3K-Akt-mediated phosphorylation of FoxO1 is the most characterized signaling pathway to regulate intercompartmental FoxO1 shuttling [14]. However, there are additional kinases that

phosphorylate FoxO1 to regulate its function in a similar manner, including SGK (serum and glucocorticoid-regulated kinase) and CK1 (casein kinase 1) [15]. Recently, the protein kinase A catalytic subunit- α (PKA α) was demonstrated to interact with FoxO1 and directly phosphorylate FoxO1 to regulate *VCAM-1* gene expression in human aortic endothelial cells, suggesting an involvement of the PKA–FoxO1 pathway in angiogenesis [16].

PKA has been linked to angiogenesis as both a positive [17–20] and negative [21–25] regulator. So far, there is no detailed analysis on the regulation of PKA degradation. Forskolin-induced PKA stimulation enhances angiogenesis through PKA-dependent VEGF expression [18]. PKA inhibitor H89 blocks VIP-induced VEGF production to inhibit proliferation of ECs [17]. On the contrary, PKA antagonizes angiogenesis when the exogenous VEGF stimulation is involved [21–23]. Pharmacologic or genetic activation of PKA inhibits cell migration in endothelial and other cells [22, 26, 27]. Indeed, PKA plays a key role in the regulation of vascular sprouting by stimulating ECs adhesion and inhibiting cell migration [23], yet the molecular mechanism is only partially understood.

Recently, emerging mechanisms point to regulation of angiogenesis by the proteasome system. The ubiquitin proteasome pathway has been suggested to have potential therapeutic value in angiogenesis-associated diseases [28], but no linkage is known between angiogenesis and the ubiquitin independent proteasome pathway. The REG γ proteasome, a representative of this proteolytic mode, is drawing considerable attention nowadays. REG γ (also known as PA28 γ ; PSME3) is a member of the 11S family of proteasome activators and has been shown to promote the degradation of several intact cellular proteins, including *SRC-3* and *p21* [29–32], in a ubiquitin independent manner. Apart from previous studies showing reduced body size in REG $\gamma^{-/-}$ mice and decreased cell proliferation in REG $\gamma^{-/-}$ MEFs [33, 34], REG γ has been related to cancer progression [35, 36]. In tumor development, angiogenesis is a necessary and required step in tumor growth and metastasis [37]. Understanding the precise regulation of angiogenesis is fundamental for the identification of new therapeutic strategies for cancers [38] and vascular abnormalities. Previous studies indicated anti-angiogenic and anti-tumor properties of proteasome inhibitors, such as PS-341 and MG132, indicating that limiting proteasome activity restricted capillary growth [39, 40]. Thus, it is of interest to explore whether regulation of the ubiquitin-dependent proteasome pathway can also benefit patients with vascular diseases.

Here we report, for the first time, that the proteasome activator REG γ is required for *VCAM-1* and *E-Selectin* gene expression through PKA–FoxO1 pathway and controls EC migration as well as VEGF-induced angiogenesis. Mechanistically, REG γ interacts with PKA catalytic subunit α (PKA α) and reduces PKA α intracellular stability. In HUVECs and MEFs depleted of REG γ , PKA α accumulation accelerated phosphorylation of the transcription factor FoxO1 and its subsequent nuclear exclusion. As a result, REG γ positively regulated VEGF-mediated expression of the FoxO1 downstream genes, *VCAM-1* and *E-Selectin*, promoting HUVECs migration. In the mouse models, REG $\gamma^{-/-}$ mice had poor responses to VEGF-induced angiogenesis in the cornea and the aortic ring, as well as during hypoxia-induced angiogenesis in the retina. VEGF-matrigels implanted in REG $\gamma^{-/-}$ mice induced fewer capillaries than in REG $\gamma^{+/+}$ control mice. Collectively, our results

identify REG γ as a new regulatory mediator for VEGF-induced expression of *VCAM-1* and *E-Selectin* genes via the PKA/FoxO1 pathway and substantiate an important role for REG γ in ECs migration and angiogenesis.

2. Materials and methods

2.1. Animals

REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ mice were kindly provided by Dr. John Monaco at the University of Cincinnati College of Medicine [33]. Animal procedures were carried out according to the rules of the American Association for the Accreditation of Laboratory Animal Care International and approved by the Animal Center of the school.

2.2. Cell lines and cell culture

Primary HUVECs were purchased from ScienCell Research Laboratories and cultured in ECM (ScienCell, Carlsbad, CA, USA). Primary REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ MEFs were previously generated [29]. Immortalized REG $\gamma^{+/+}$ (which behaves similar to REG $\gamma^{+/+}$) and REG $\gamma^{-/-}$ MEFs were kindly provided by James M. Roberts (Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle) [41]. 293T, A549, HMEC-1 and HeLa cells were purchased from ATCC and maintained at the Cell culture Core in our department. All cells were cultured under standard conditions described by the ATCC.

2.3. Plasmids and reagents

Flag-REG γ was previously generated [36]. GFP-PKA α was generated in pEGFP-n1-vector (Addgene). Antibodies were purchased from Invitrogen (PA28 γ), Cell Signaling (FoxO1, Phospho-FoxO1/Ser²⁵⁶, Thr²⁴, Ser³¹⁹), Sigma (β -actin, M2 anti-Flag), Santa Cruz (PKA α cat, HA, GFP), GeneTex (E-Selectin, VCAM-1). Other purchased reagents include VEGF-A165 (PeproTech Inc), cycloheximide (Amresco). All the experiments shown in the study were repeated at least three times.

2.4. In vivo and ex vivo experiments

REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ mice were prepared for aortic ring assay [42], oxygen-induced retinopathy assay [43], mouse corneal micropocket assay [44] and matrigel plug assay [45] following the procedure (see Supplementary methods online).

2.5. Wound healing, transwell assay, MTT assay and pulse-chase labeling experiment

See supplementary methods online [30, 44].

2.6. siRNA transfection

All siRNA duplexes were purchased from Dharmacon (Lafayette, CO). siRNA targeting REG γ (catalog #L-012133-00-0010), PKA α (catalog #L-004649-00), FoxO1 (catalog #M-003006-01-05) or control siRNA (catalog #D-001810-10-20) were transfected into cells by 48–72 h using Lipofectamine 2000 (Invitrogen) following the instructions provided by the company.

2.7. Immunoprecipitation, Western blot, immunostaining and real-time qPCR

Immunoprecipitation, Western blot, immunostaining and qPCR were performed as described [29, 30].

2.8. Data collection and statistical analysis

The intensity of the Western blot results was analyzed by densitometry using Bio-Rad Quantity One 4.4.0 software and normalized to the band with the least intensity which was arbitrarily set as 1. The results were expressed as the mean $\pm$ SD. Statistical analyses of all experimental data in this study were performed using the two-tailed Student's t-test or one-way ANOVA (Tukey's Multiple Comparison Test). A *p* value of less than 0.05 was considered statistically significant.

3. Results

3.1. REG γ mediates the degradation of PKA α

Majority of the proteins that are degraded by the REG γ -proteasome pathway remain unknown. In an effort to search for REG γ target proteins, our large-scale antibody microarray analysis on whole cell lysates of REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ MEFs indicated that PKA α was negatively correlated with REG γ [31]. We then validated REG γ -mediated regulation of PKA α in different cells/tissues *in vitro* and *in vivo*. Silencing REG γ in HUVECs resulted in an almost 3-fold increase in PKA α protein level compared with samples treated with a control siRNA (Fig. 1A). Similar results were observed in immortalized Human Microvascular Endothelial Cell-1 (HMEC-1 cells) (Fig. S1A). To evaluate *in vivo* effects of REG γ on PKA α expression, we isolated primary MEFs from REG $\gamma^{-/-}$ and REG $\gamma^{+/+}$ mice. Consistent with our expectation, PKA α protein level was elevated in REG $\gamma^{-/-}$ MEFs compared to REG $\gamma^{+/+}$ MEFs (Fig. 1A, right panel). Similar results were observed in immortalized REG $\gamma^{-/-}$ MEFs which showed the increase of PKA α (Fig. 2A). Furthermore, we measured PKA α expression in several mouse tissues. As shown in Fig. 1B and Fig. S1B, expression of PKA α proteins in REG $\gamma^{-/-}$ bone marrow and spleen (2-week-old) was more abundant than that from REG $\gamma^{+/+}$ littermates. We also noticed that the regulation of PKA α by REG γ appears to be tissue/cell specific, since REG γ had no effect on PKA α in A549 cells (Fig. S1C) or in mouse liver (Fig. S1D). To exclude a potential mechanism involving transcriptional regulation by REG γ , expression of PKA α mRNA was assessed by real-time RT-PCR analyses. As shown in Fig. 1C, REG γ -depletion in HUVECs (lane 1), MEFs (lane 2), bone marrow and spleen (lane 3 and 4) had no effect on PKA α mRNA levels, indicating that REG γ likely regulates PKA α by enhancing turnover of PKA α protein.

To further understand if REG γ patrols PKA α stability, we tested the degradation kinetics of PKA α . Following cycloheximide treatment for various periods of time, the degradation of PKA α in REG $\gamma^{-/-}$ MEFs was nearly blocked, indicating that the REG γ -proteasome could be the major mechanism controlling PKA α protein turnover in test cells (Fig. 1D). By pulse-chase experiments, we verified that REG γ indeed is responsible for accelerated degradation of PKA α protein (Fig. S1E).

All of the REG γ target proteins identified to date can interact with this proteasome activator. Therefore, we performed a co-immunoprecipitation assay to define the intracellular interactions between REG γ and PKA α . Endogenously expressed REG γ and PKA α in HUVECs were immunoprecipitated using either anti-PKA α or anti-REG γ antibody. Western blotting detected co-precipitation of REG γ (Fig. 1E) or PKA α (Fig. 1F), respectively. We also validated the interaction by over-expression and reciprocal immunoprecipitation of REG γ and PKA α in 293T cells (Fig. S1F&G). Taken together, our results suggest that REG γ directly mediates PKA α turnover.

3.2. REG γ controls PKA α -mediated FoxO1 phosphorylation and cellular localization

Since the role of PKA signaling in ECs involves phosphorylation of one or more of the factors responsible for regulating the expression of downstream genes, we next wished to explore the effectors downstream of PKA that are also regulated by REG γ . We screened the expression of a panel of genes in HUVECs before and after silencing REG γ (Fig. S2A). Among the differentially expressed factors were *VCAM-1* and *E-Selectin*, whose expressions were negatively regulated by cAMP/PKA signaling [46]. More recent studies linked PKA-FoxO1 to the regulation of *VCAM-1* and *E-Selectin* in vascular ECs [16]. Therefore, we hypothesized that the PKA-FoxO1 pathway might be required for REG γ -dependent regulation of *VCAM-1* and *E-Selectin* gene expression. To test our hypothesis, we examined PKA-dependent phosphorylation of FoxO1 in immortalized REG $\gamma^{-/-}$ MEFs and HUVECs. As shown in Fig. 2A, REG γ knockout caused a dramatic increase in the phosphorylation of FoxO1 at Ser²⁵⁶ along with marked elevation of PKA α (Fig. 2A, lane 4). Similar results were observed at Ser³¹⁹ and Thr²⁴ (Fig. S2B). To determine whether the increases are mediated by PKA signaling, we monitored phosphorylation of FoxO1 following treatment with forskolin or H89. Consistent with previous reports, forskolin-mediated PKA activation dramatically elevated phosphorylation of FoxO1 by 30 min in REG $\gamma^{+/+}$ cells (Fig. 2A, lane 2). In contrast, there was no further phosphorylation of FoxO1 observed in REG $\gamma^{-/-}$ MEFs upon forskolin treatment (Fig. 2A, lane 5, compare with lane 4). Similar results were observed in HUVECs (Fig. 2B, lanes 2 and 5), suggesting that PKA signaling has been activated and phosphorylation of FoxO1 is already saturated in cells lacking REG γ . We next examined the phosphorylation of FoxO1 following H89 treatment. Elevated phosphorylation of FoxO1 in REG $\gamma^{-/-}$ MEFs was almost eradicated by inhibiting PKA activity (Fig. 2A, lane 6), indicating that enhanced phosphorylation of FoxO1 in REG γ deficient cells was mostly mediated by a sustained and activated PKA signaling. To ensure the specificity of REG γ -PKA regulation of FoxO1 phosphorylation, both REG γ and PKA α were simultaneously silenced by RNA interference in HUVECs. Knocking down PKA α in HUVECs (Fig. 2B, lanes 3 and 6) dramatically attenuated phosphorylation of FoxO1 regardless of REG γ levels, validating that REG γ modulates FoxO1 phosphorylation in a PKA-dependent manner.

Post-translational modification of FoxO1 has been known to regulate its translocation between the nucleus and cytoplasm [15]. To determine whether REG γ -PKA pathway regulates subcellular localization of FoxO1, immunofluorescence studies were performed in immortalized REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ cells in the presence or absence of PKA modulators. As shown in Fig. 2C, FoxO1 was mainly located in the nucleus in REG $\gamma^{+/+}$ MEFs, whereas FoxO1 was mostly cytoplasmic in REG $\gamma^{-/-}$ cells (Fig. 2C, top panels), indicating that REG γ

is a key factor regulating FoxO1 subcellular localization. Activation of PKA by forskolin induced nuclear exclusion of FoxO1 in REG $\gamma^{+/-}$ MEFs but had minimal effects on FoxO1 nuclear export in REG $\gamma^{-/-}$ MEFs (Fig. 2C, middle panels), consistent with the changes in FoxO1 phosphorylation status in Fig. 2A. However, blockage of PKA signaling by H89 prevented FoxO1 export to cytoplasm in REG $\gamma^{-/-}$ MEFs (Fig. 2C, lower panels), substantiating that the cytoplasmic localization of FoxO1 in the absence of REG γ was mediated by the action of PKA. All of the experimental results for subcellular localization were quantitatively analyzed (Fig. 2D). We further confirmed our observation of FoxO1 distribution by cell fractionation and Western blot analysis (Fig. S2C). The influence of PKA signaling on the dynamic changes in FoxO1 subcellular localization in HUVECs also was validated in the presence of forskolin (Fig. S2D). Taken together, our results demonstrate that REG γ regulates FoxO1 phosphorylation and cellular localization in a PKA-dependent manner.

3.3. REG γ affects the expression of *VCAM-1* and *E-Selectin* genes through the PKA-FoxO1 pathway

As described above, *VCAM-1* and *E-Selectin* were among the genes differentially regulated in HUVECs with or without REG γ silencing (Fig. S2A). Similar results were observed in HUVECs (Fig. 3A, lanes 1&2) and HMEC-1 cells (Fig. 3B, lanes 1&4). We next sought to understand how REG γ regulates *VCAM-1* and *E-Selectin* mechanistically. Given that REG γ regulates FoxO1 in a PKA dependent manner, we tested whether REG γ mediated regulation of *VCAM-1* and *E-Selectin* could be affected by manipulation of PKA levels. Interestingly, ablation of PKA activity by H89 in siREG γ -treated HUVECs (Fig. 3A, lanes 4) or HMEC-1 cells (Fig. 3B, lanes 6) enhanced the expression of *VCAM-1* and *E-Selectin* genes, indicating an inhibitory role for PKA pathway in the regulation of these FoxO1 responsive genes [47]. Similarly, simultaneous silencing REG γ and PKA in HUVECs resulted in derepression of *VCAM-1* and *E-Selectin* genes (Fig. 3A, compare lanes 2 and 6). We also diminished forkhead activity by RNA interference against FoxO1 and demonstrated that *VCAM-1* and *E-Selectin* indeed requires FoxO1 for their transcription in HUVECs (Fig. 3C). To further ensure that FoxO1 dependent transcription of *VCAM-1* and *E-Selectin* can be modulated by REG γ -PKA signaling, we used a PKA activator to promote FoxO1 nuclear export (Fig. 2C) and to “switch off” FoxO1 activity. Upon forskolin treatment, expression of *VCAM-1* and *E-Selectin* genes was reduced in HMEC-1 cells (Fig. 3B, lane 2) and HUVECs (Fig. S3C), whereas no significant effects were noted in cells depleted of REG γ (Fig. 3B, compare lanes 4 and lane 5; Fig. S3A). In addition, there was corresponding regulation of *VCAM-1* and *E-Selectin* protein expression by REG γ (Fig. S3B). In summary, these results demonstrate that REG γ regulates the expression of *VCAM-1* and *E-Selectin* genes through the PKA-FoxO1 pathway.

3.4. REG γ facilitates VEGF-induced expression of pro-angiogenic genes by repressing PKA signaling

VEGF-mediated up-regulation of *VCAM-1* can be inhibited by a PI3K-Akt agonist that facilitates FoxO1 phosphorylation and nuclear exclusion [48]. Given the similar effect of PI3K-Akt and PKA in promoting FoxO1 phosphorylation, we analyzed whether PKA signaling may also interfere with VEGF-mediated expression of downstream responsive

genes. As expected, VEGF stimulation induced robust expression of *VCAM-1* and *E-Selectin* genes in HUVECs (Fig. 3D, lanes 3). Pretreatment with forskolin interrupted VEGF-mediated gene transcription (Fig. 3D, lanes 4), demonstrating an inhibitory role for activated PKA in VEGF-induced expression of *VCAM-1* and *E-Selectin* genes. Likewise, VEGF-mediated induction of *VCAM-1* and *E-Selectin* genes expression was also impaired in REG γ deficient HUVECs both on mRNA and protein levels (Fig. 3D, lanes 5 & Fig. S3C), substantiating that REG γ is required for efficient VEGF-stimulated expression of FoxO1 responsible genes. To test if there is a feedback regulation by VEGF signaling, we examined the effect of VEGF stimulation on REG γ expression. As shown in Fig. S3D, REG γ expression was not altered upon VEGF treatment, suggesting that a steady expression of REG γ may be required to balance the VEGF signaling and the suppressive PKA activity.

Numerous studies have shown that VEGF activates PI3K/Akt, which can lead to phosphorylation and nuclear export of FoxO proteins [48]. To investigate the seemingly contradictory actions between the VEGFAKT and the PKA signalings, we compared the effects of VEGF-stimulation vs. PKA-activation on FoxO1 phosphorylation in HUVECs (Fig. S3E). VEGF-treatment induced FoxO1 phosphorylation (about 1.3-fold increase) in 15 min, and then quickly decreased to basal level in 45 min; however, forskolin induced a late onset but higher phosphorylation in FoxO1 at 30 min, which was prolonged to 45 min. Based on these observations, we believe that the effect of VEGF on FoxO1 nuclear localization is minor and transient; however, activation of PKA promotes more intense and prolonged FoxO1 phosphorylation. Given that VEGF-dependent regulation of *VCAM-1* and *E-Selectin* is likely mediated by TNF α [49], only stronger and sustained phosphorylation in FoxO1 may be sufficient to antagonize VEGF action on *VCAM-1* and *E-Selectin* expression.

3.5. REG γ is involved in ECs migration

Both *VCAM-1* and *E-Selectin* genes are known to play a role in angiogenic process [50, 51]. Based on the observation that REG γ regulates the expression of *VCAM-1* and *E-Selectin* genes via PKA, whose activity is tightly associated with ECs movement [23], we carried out transwell and wound-healing assays to assess REG γ 's capability in regulating ECs migration. Silencing REG γ in HUVECs with transient expression of specific siRNAs resulted in a significant reduction in cell migration in both the transwell and wound-healing assays (Fig. 4A and B, upper panels). In contrast, overexpressing REG γ in HUVECs by lenti-viral (plvx-REG γ) infection remarkably enhanced cell migration (Fig. 4A and B, lower panels). To better understand the biological functions of the REG γ -PKA pathway in ECs migration, a HUVECs transwell assay was performed in the absence or presence of forskolin. Indeed, the number of migrated cells was decreased significantly by forskolin in a dose-dependent manner, substantiating that PKA signaling inhibits migration of ECs (Fig. 4C). Furthermore, inhibition of PKA by H89 restored HUVECs migration when REG γ was depleted (Fig. 4D), demonstrating that PKA is the major effector in REG γ -regulated HUVECs migration. To test whether the ECs proliferation was affected in the above experiments, we analyzed proliferation of HUVECs at the same condition in the cell migration assays. Neither REG γ knockdown nor manipulation of PKA activity affected ECs

proliferations by MTT assays (Fig. S4B). Taken together, the above experimental results demonstrate that REG γ is a novel modulatory factor in ECs migration.

3.6. REG γ ablation attenuates VEGF-induced angiogenesis in vivo/ex vivo

The discovery of REG γ 's ability in the regulation of VEGF-induced *VCAM-1* and *E-Selectin* gene expression in vascular ECs prompted us to test if REG γ may be involved in VEGF-induced angiogenesis in our mouse models. The mouse corneal micropocket assay has been widely accepted as a gold standard for *in vivo* study of angiogenic factors. Hence, we inserted a VEGF-containing slow-release pellet into a preformed mouse cornea micropocket, and watched for the outgrowth of new limbus vasculature. Seven days later, we visualized robust VEGF-induced corneal angiogenesis in the REG $\gamma^{+/+}$ eyes whereas this phenomenon was restrained in the REG $\gamma^{-/-}$ groups (Fig. 5A). Significant and consistent differences between REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ corneal angiogenesis appeared by all of the three criteria employed in this model, including vessel length, clock number and coverage area (Fig. 5A, lower panel), thereby reflecting an important role for REG γ in VEGF-induced angiogenesis.

Next, we injected VEGF-containing matrigels into REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ mice and analyzed the results 7 days later. As shown in Fig. 5B, there were more blood vessels in the gel-plugs peeled from REG $\gamma^{+/+}$ mice than found in REG $\gamma^{-/-}$ mice. Quantification of migrated ECs within the plugs embedded in paraffin [45] also disclosed inhibition of VEGF-induced angiogenesis in the absence of REG γ (Fig. 5B, right panel). To further evaluate the blockade of REG γ deficiency on physiological angiogenesis, we performed a mouse aortic ring assay, a classical and reliable *ex vivo* angiogenesis model [42]. Freshly isolated REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ aortas were explanted into VEGF-soaked or control collagen. The outgrowth of micro-vessels from REG $\gamma^{+/+}$ aortas were more prominent than that in REG $\gamma^{-/-}$ aortas in the presence of VEGF (Fig. 5C). To verify that the outgrowth emanating from aortic rings contains differential endothelial-cell numbers in WT and KO samples, we immunostained aorta with endothelial-cell-specific marker vWF (Fig. S5A). The result showed that the ECs stimulated by VEGF are more abundant in the aorta from REG $\gamma^{+/+}$ mice. Furthermore, to test the role for REG γ in pathological angiogenesis, we carried out experiments using the model of oxygen-induced retinopathy, which has been well-documented for the study of pathological angiogenesis in mouse Retinopathy of prematurity (ROP) [43]. As expected, pathological neovascularization (Fig. 5D, mid-upper panel) was induced in REG $\gamma^{+/+}$ retina when the mice were shifted from high-oxygen cages back to “relative hypoxia” (room air) cages. In contrast, REG γ ablation reduced the pathological vascular plexuses characteristic of excessive angiogenesis (Fig. 5D, mid-lower panel) occurring during the relative hypoxic stress. These results above identify a novel function for REG γ in VEGF-mediated angiogenesis.

Finally, we performed aortic ring assays to characterize if PKA signaling antagonizes VEGF-induced angiogenesis. In the presence of VEGF, we observed a vigorous growth of endothelial-cell-specific microvessels (Fig. 6A and Fig. S5B, upper panel). However, co-culture of the aortic ring with VEGF in the presence of forskolin attenuated VEGF-induced microvessel growth (Fig. 6A and Fig. S5B, lower panel). These results demonstrate that PKA signaling negatively regulates VEGF-induced angiogenesis.

4. Discussion

The proteasome activator REG γ has emerged as a master regulator in certain biological processes [29, 36]. Yet the vast majority of its biological functions remain unknown. In this study, we present evidence that REG γ is required for *VCAM-1* and *E-Selectin* genes expression via the PKA-FoxO1 pathway and crucial for ECs migration, as well as VEGF-induced angiogenesis. This study has elaborated a regulatory signaling cascade emanating from REG γ -proteasome mediated regulation of PKA, which turns on/off FoxO1 activity by phosphorylation-dependent subcellular localization, thereby promoting or attenuating the transcription of downstream pro-angiogenic genes. Our results reveal a previously unrecognized regulatory mechanism upstream of PKA and uncover new molecular links in a REG γ -PKA-FoxO1 angiogenic pathway (Fig. 6B).

VCAM-1 and *E-Selectin* genes are well known to be associated with pathological angiogenic responses involving inflammation [52]. Expression of *VCAM-1* is effectively controlled through PKA-FoxO1 pathway [16]. Due to limited prior studies, the effects of PKA-FoxO1 pathway on angiogenesis are largely unknown. FoxO1 plays roles in the regulation of vascular homeostasis in response to insulin and other growth factors [53]; FoxO1 knockout mice die around embryonic day 11 because of defects in their bronchial arches and remarkably impaired vascular development of embryos and yolk sacs due to an abnormal response of ECs to VEGF stimulation [54]. Consistent with the biochemical evidence for PKA-dependent phosphorylation of FoxO1 [16], we recapitulated the regulation of subcellular localization of FoxO1 in response to the REG γ -PKA activities. In addition to *VCAM-1*, a well-known target of FoxO1, we found that REG γ also is required for the expression of two other endothelial adhesion molecules, *E-Selectin* and *ICAM-1* (Fig. S2A). Our results are endorsed by the fact that endothelial adhesion molecules facilitate the entry of leukocytes into inflamed tissues and thereby promote angiogenesis [55]. To our surprise, regulation of *ICAM-1* by REG γ is distinct from *VCAM-1* and *E-Selectin* since this regulation does not require PKA signaling (Fig. S4A), indicating involvement of additional mechanisms for REG γ -mediated angiogenesis.

Numerous *in vivo* studies have shown that expression of VEGF is up-regulated during angiogenesis under physiological and pathological processes [56, 57]. Different mechanisms appear to participate in the regulation of VEGF mRNA expression, including PKA-mediated signals [58]. Thus, activated PKA may facilitate angiogenesis by promoting endogenous expression of VEGF, or through Epac-dependent ERK activation and PI3K/Akt/eNOS/NO signaling [17, 18]. Furthermore, activated PKA may promote angiogenesis during early developmental. Two studies have implicated PKA activity in postnatal angiogenesis and ischemic angiogenesis [19, 20]. However, there are also several mechanisms involved in PKA-mediated negative regulation of angiogenesis. Activation of PKA signaling can not only attenuate VEGF signaling in physiological conditions by blocking Raf activation [21] or through their actions on pp60Src [23], but also prohibit VEGF-induced increase in vascular permeability during cancer progression [22]. Paradoxically, distinct from previous demonstration that PKA promotes VEGF secretion, PKA may inhibit VEGF production by antagonizing Rap1 to regulate tumorstromal induction of angiogenesis in prostate cancer [24]. Besides the inhibitory role of PKA on VEGF signaling, direct activation of PKA

signaling can induce EC apoptosis, resulting in the inhibition of angiogenesis in vivo [25]. It has been reported more recently that PKA phosphorylates FoxO1 directly in vascular ECs [16], whose effect is quite similar to hepatocyte growth factor to promote FoxO1 nuclear exclusion [48], suggesting that PKA may also prohibit the effect of VEGF on FoxO1-dependent gene expression. In our present study, since the post-translational modification of FoxO1 and the subsequent activation/inactivation of cell adhesion molecule is the major mechanism affected by REG γ -mediated regulation of PKA, we propose that the major PKA function may be to inhibit angiogenesis under a physiological condition. Nevertheless, the role of PKA on angiogenesis can be altered, depending upon different cell context and various signal cascades in physiological or pathological environments.

Protein degradation has been documented previously to play an important role in angiogenesis [28]. However, prior studies represent an incomplete understanding of the impact that protein degradation pathways play in the regulation of angiogenesis. The pro-angiogenic role of REG γ -proteasome further substantiates the importance of the proteasome system for fine-tuning the functions of core angiogenic proteins. The discovery that REG γ negatively regulates the PKA pathway in a cell specific manner highlights a novel function of this proteasome pathway and demonstrates the complexity existing in the regulation of angiogenesis. Although we are aware of no publication that describes the ubiquitin proteasome system as a key mediator of degradation of PKA α , we can't exclude the possibility that REG γ -proteasome is not the only pathway required for decay of PKA α . Since we observed that elevated PKA α levels in REG γ deficient cells are coupled with a dramatic increase of PKA activity, perhaps the REG γ -proteasome has been designed by nature to guard against nuclear accumulation of activated PKA α molecules that are either "leaked" or prematurely released from their regulatory subunits upon inadvertent cAMP surges (Fig. 6B). Despite the identification of PKA α as a primary target for REG γ 's regulation of angiogenesis, our study does not exclude the possibility of additional REG γ substrates involved in this function. In fact, we recently found casein kinase 1 (CK1) to be a novel REG γ substrate for its effect on cellular aging [31]. CK1 also has been reported to phosphorylate FoxO1 within several intramolecular domains to promote nuclear exclusion of FoxO1 [15]. In addition, a recent study suggests that the ubiquitin E3 ligase SCF $^{\beta}$ -TRCP promotes ubiquitination and destruction of VEGF2 in a CK1-dependent manner and subsequently suppresses angiogenesis [59]. The totality of these data indicates that diverse mechanisms underlie the regulation of angiogenesis by REG γ .

In conclusion, the present study that provides evidence that REG γ is a novel positive regulator for *VCAM-1* and *E-Selectin* gene expression, plays an important role in ECs migration and VEGF-induced angiogenesis. REG γ antagonizes the PKA pathway and facilitates VEGF-induced expression of pro-angiogenic genes by modulating FoxO1 phosphorylation and sub-cellular localization (Fig. 6B). Our findings provide the missing upstream links for PKA regulation of its downstream angiogenic effectors. This novel angiogenic function of REG γ provides another potential molecular basis for future therapeutic applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

REGγ controls PKAα stability. (A–B) REGγ negatively regulated PKAα level in multiple cell types. Primary HUVECs were treated with 20 nM siREGγ or a negative control (siNeg) (A, left panel). Primary REGγ^{+/+} and REGγ^{-/-} MEFs from the third passage were used for Western blot analysis (A, right panel). Bone marrows (B, left panel) collected from day 14 REGγ^{+/+} and REGγ^{-/-} mice were prepared for Western blot analysis. Relative PKAα levels were quantitated and normalized to β-actin as indicated by the numbers. (C) REGγ deficiency had no effect on PKAα mRNA levels. Total RNA extracted from primary HUVECs treated with 20 nM siREGγ or siNeg, primary MEFs, bone marrows, or spleens from REGγ^{+/+} and REGγ^{-/-} mice, were prepared for real-time RT-PCR analysis. Data are shown as mean ± SD of three independent experiments. (D) REGγ dictated degradation kinetics of PKAα. Primary REGγ^{+/+} and REGγ^{-/-} MEFs were treated with 100 μg/ml cycloheximide for different periods of time as indicated. Total cell lysates were extracted for Western blot (upper panel). Quantitated results of the Western blot analysis were plotted against indicated time course (lower panel). (E–F) Intracellular interactions between REGγ and PKAα. Immunoprecipitation was performed with whole cell lysates of HUVECs in the presence of MG132 using an anti-PKAα (E) or an anti-REGγ (F) antibody. The immuno-

precipitated complexes were separated by SDS-PAGE and detected by an antibody against PKA α or REG γ .

Fig. 2. REGγ modulates phosphorylation and subcellular distribution of FoxO1. (A–B) REGγ depletion enhanced FoxO1 phosphorylation through activating PKA signaling. Immortalized REGγ^{+/-} and REGγ^{-/-} MEFs were serum-starved overnight and treated with DMSO, 20 μM forskolin (FSK) or 20 μM H89 for 30 min (A). Primary HUVECs were treated with/without siREGγ alone or in combination with siPKAα, then serum-starved overnight following incubating with 20 μM forskolin or DMSO for 30 min (B). Protein expression of endogenous p-FoxO1(Ser²⁵⁶), PKAα, FoxO1, REGγ, β-actin was detected by Western blot. (C) REGγ deficiency promoted FoxO1 nuclear export by activating PKA. Immunofluorescence staining was performed in REGγ^{+/-} and REGγ^{-/-} MEFs. Cells were

serum-starved overnight before treatment with DMSO, 20 μ M forskolin, or 20 μ M H89, for 30 min. Cells were immunostained with anti-FoxO1 antibody (red) and the nuclei were counterstained with DAPI (blue). (D) The bar graph shows the corresponding quantitative analyses of the subcellular localization of FoxO1 and was generated by manually counting nuclear and cytoplasmic localization of FoxO1 in 400 FoxO1-positive staining MEFs for each condition. The area of the nucleus was determined using DAPI staining (nuclear staining). Data are shown as mean $\pm$ SD of quantitated results from three independent experiments (* p < 0.05 relative to DMSO-treated REG $\gamma^{+/-}$ MEFs; *, p < 0.05 relative to DMSO-treated REG $\gamma^{-/-}$ MEFs).

Fig. 3.

REG γ tightly controls the expression of *VCAM-1* and *E-Selectin* genes. (A) Cells lacking REG γ prohibited optimal expression of *VCAM-1* and *E-Selectin* via activation of PKA in HUVECs. Total RNA extracted from primary HUVECs separately treated with control siNeg (lane 1), siREG γ (lane 2), siNeg + H89 (20 μ M, 6 h) (lane 3), siREG γ + H89 (20 μ M, 6 h) (lane 4), siNeg + siPKA (lane 5) or siREG γ + siPKA (lane 6), was prepared for real-time RT-PCR analysis. Cells were serum-starved overnight before H89 treatment. Statistical analyses were performed using the one-way ANOVA. Data are shown as mean $\pm$ SD of three independent experiments (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (B) Depleted of REG γ prohibited optimal expression of *VCAM-1* and *E-Selectin* via activation of PKA in HMEC-1 cells. HMEC-1 cells separately treated with 20 nM siNeg or siREG γ , following incubated with DMSO (lanes 1&4), 20 μ M forskolin (lanes 2&5) or 20 μ M H89 (lanes 3&6) for 6 h. Cells were serum-starved overnight before forskolin or H89 treatment. Total RNA extracted from HMEC-1 was prepared for real-time RT-PCR analysis. Statistical analyses were performed using the one-way ANOVA. Data are shown as mean $\pm$ SD of three independent experiments (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (C) FoxO1 is required for the expression of *VCAM-1* and *E-Selectin* genes. Total RNA from primary HUVECs treated with 20nM siNeg or siFoxO1 for 48 h was prepared for real-time RT-PCR analysis. Cells were serum-starved overnight before RNA extraction. Data are shown as mean $\pm$ SD of three independent experiments (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (D) REG γ is responsible for VEGF-induced expression of *VCAM-1* and *E-Selectin* genes. Primary HUVECs treated with 20 nM control siRNA (siNeg) were incubated with either vehicle (lane 1), 20 μ M forskolin (lane 2) for 6 h, 20 μ g/ml VEGF (lane 3) for 2 h, or a combination of 20 μ M forskolin (6 h) and 20 μ g/ml VEGF treatment (2

h) (lane 4). HUVECs transfected with 20nM siREG γ were treated with 20ug/ml VEGF for 2 h (lane 5). Cells were serum-starved overnight before forskolin and VEGF treatment. Total RNA extracted from HUVECs was prepared for real-time RT-PCR analysis. Statistical analyses were performed using the one-way ANOVA. Data are shown as mean $\pm$ SD of three independent experiments (*, $p < 0.05$; ***, $p < 0.001$).

Fig. 4.

REG γ regulates migration of HUVECs. (A–B) REG γ is crucial to HUVECs migration. Transwell assays (A) and wound healing experiments (B) were performed in HUVECs following REG γ overexpression by virus infection (plvx-REG γ lentivirus) or transient knockdown of REG γ by siRNA. The migrated HUVECs were pictured using an Olympus inverted microscope and counted. Data are shown as mean $\pm$ SD of three independent experiments (**, $p < 0.01$; ***, $p < 0.001$). The upper y-axis represents migrated HUVECs (% of siNeg), and the lower y-axis refers to migrated HUVECs (% of Mock) in the quantitation bar graphs. (C) Activation of PKA by forskolin inhibited the migration of HUVECs. HUVECs were seeded in the upper chamber containing different doses of forskolin in the presence of 20 ng/ml VEGF. Migration of HUVECs to the other surface in the lower chamber of the transwell was recorded in 24 h (left panel) and statistically analyzed (right panel). Statistical analyses were performed using the one-way ANOVA. Data are shown as mean $\pm$ SD of three independent experiments (*, $p < 0.05$; **, $p < 0.01$). (D) Inhibition of PKA by H89 restored the HUVECs migration during REG γ depletion. Wound healing initiated 48 h after REG γ siRNA transfection in HUVECs, following incubation with different doses of H89 by 5 h. The migrated HUVECs were pictured using an Olympus inverted microscope and quantified. Statistical analyses were performed using

the one-way ANOVA. Data are shown as mean $\pm$ SD of three independent experiments (**, $p < 0.01$).

Fig. 5.

REG γ plays a crucial role in VEGF-induced angiogenesis *in vivo*. (A) REG γ depletion compromised VEGF-induced neovascularization in corneal pocket model. VEGF-containing pallets were placed into cornea in REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ mice (indicated by yellow arrows) for 7 days and then the corneal angiogenesis were imaged (Upper panel). White arrow indicates the VEGF-induced microvessels. The bar graphs show the results of quantitative analysis including vessel length, clock number and vessel area (Lower panel). Data are shown as mean $\pm$ standard deviation (SD) of four pairs of individual animals (*, $p < 0.05$). (B) REG $\gamma^{-/-}$ mice revealed poor angiogenesis in VEGF-matrigel plug assays. A total of 500 μ l matrigel containing 50 ng VEGF was injected subcutaneously into the ventral abdomen of REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ mice. Pictures of matrigel plugs were taken 8 days after the plugs were removed (left panel). The embedded matrigels were cut into 5 μ m slides and were analyzed by H&E staining to visualize and quantify the number of invaded ECs (lower panel and right panel). Data are shown as mean $\pm$ SD of four independent experiments (**, $p < 0.01$). (C) REG $\gamma^{-/-}$ mice had impaired aortic angiogenesis. Three aortic rings for each treatment in total from 3 pairs of REG $\gamma^{+/+}$ and REG $\gamma^{-/-}$ mice were cut into rings. The rings was starved overnight and then fed with opti-MEM with/without 30 ng/ml VEGF. Pictures were taken at day 7 (left panel). The number of microvessels emanating from aortic rings

were counted (right panel). Statistical analyses were performed using the one-way ANOVA. Data are shown as mean $\pm$ SD of the results using three pairs of mice (*, $p < 0.05$). (D) $REG\gamma^{-/-}$ mice displayed attenuated oxygen-induced retinopathy. Postnatal day 7 $REG\gamma^{+/+}$ and $REG\gamma^{-/-}$ mice were exposed to hyperoxia for 5 days followed by another 5 days of room air exposure. Neovascular proliferative retinopathy was imaged. Red squares represent invisible areas in the original scale and are amplified in lower panels. Arrows points to twisted vascular plexuses. Quantitated results of three pairs of animals for nodes area/nodes numbers as percentage of $REG\gamma^{+/+}$ are shown as mean $\pm$ SD.

Fig. 6.

PKA signaling antagonizes VEGF-induced angiogenesis. (A) Activation of PKA by forskolin inhibited VEGF-induced aortic angiogenesis. Three aortic rings for each treatment in total from 3 pairs of $REG\gamma^{+/+}$ mice were cut into rings and fed by opti-MEM containing 30 ng/ml VEGF followed by treated with DMSO or 20 μ M forskolin. Three pairs of aortic rings (forskolin treated vs. controls) were quantified by Image-Pro Plus 6.0. Data are shown as mean $\pm$ SD of three independent experiments (*, $p < 0.05$). (B) A working model for the role of $REG\gamma$ in regulating *VCAM-1* and *E-Selectin* genes expression. PKA catalytic subunit

α (PKA α) released from inactive PKA complex is translocated to the nucleus in the presence of cAMP. Activated PKA directly phosphorylates FoxO1 and induces its nuclear exclusion, which negatively regulates the expression of *VCAM-1* and *E-Selectin*. REG γ down-regulates PKA α levels by decreasing its protein stability, which in turn preserves FoxO1 transcriptional activity and facilitates down-stream responsive genes expression.