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LPA/PKD-1-FoxO1 signaling axis mediates endothelial cell CD36 transcriptional repression and proangiogenic and proarteriogenic reprogramming

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Abstract

Objective—CD36 is a scavenger and antiangiogenic receptor that is important in atherothrombotic diseases, diabetes, cancer and obesity. Lysophosphatidic acid (LPA), a phospholipid signaling mediator, abolishes endothelial cell responses to antiangiogenic proteins containing thrombospondin type 1 homology domains by down-regulating endothelial *CD36* transcription *via* protein kinase D1 (PKD-1) signaling. We aimed to understand mechanisms by which LPA-mediated angiogenic signaling is integrated to regulate *CD36* transcription and endothelial cell function *via* a nuclear transcriptional complex.

Approach and Results—Microvascular endothelial cells (MVECs) expressing *CD36* were used for studying angiogenic signaling and *CD36* transcription. Gene transfection and transduction, RT-qPCR, avidin–biotin-conjugated DNA-binding assay, chromatin immunoprecipitation assay, co-immunoprecipitation, proximal ligation assay and immunofluorescence microscopy showed that LPA-mediated *CD36* transcriptional repression involved PKD-1 signaling mediated formation of FoxO1-HDAC7 complex in the nucleus. Unexpectedly, turning off *CD36* transcription initiated reprogramming MVECs to express ephrin B2 (EFNB2), a critical "molecular signature" involved in angiogenesis and arteriogenesis. Spheroid-based angiogenesis and *in vivo* Matrigel angiogenesis assays indicated that angiogenic branching morphogenesis and *in vivo* angiogenesis were dependent on PKD-1 signaling. A mouse tumor angiogenesis model revealed enhanced PKD-1 signaling and expression of EFNB2 and smooth muscle actin (SMA) in neovessels of Lewis Lung Carcinomas, along with low CD36 expression or *CD36* deficiency.

Conclusions—LPA/PKD-1 signaling leads to nuclear accumulation of HDAC7, where it interacts with FoxO1 to suppress endothelial *CD36* transcription and mediates silencing of

Disclosures. None.

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antiangiogenic switch, resulting in proangiogenic and proarteriogenic reprogramming. Targeting this signaling cascade could be a novel approach for ischemic cardiovascular disease and cancer.

Introduction

A dynamic balance between pro- and anti-angiogenic signals determines neo-vessel formation during physiological and pathological conditions. Broadly and constitutively expressed in microvascular beds, CD36 functions as a receptor that can initiate an antiangiogenic response by interaction with thrombospondin-1 and related proteins containing thrombospondin type 1 repeat (TSR) domains1–4. TSR binding to CD36 provides a "switch" that blunts vascular endothelial growth factor receptor signals[5, 6] and converts growth factor–mediated proangiogenic responses into antiangiogenic, proapoptotic responses[1–4, 6–9]. Diminishing CD36 expression, for example in tumor vasculature, may tip the balance in favor of proangiogenic responses under certain pathological circumstances.

CD36 transcription in monocyte/macrophages, adipocytes and myocytes is tightly regulated by various cellular stimuli including cytokines, lipids and lipoproteins, reactive oxygen species, and differentiation and adhesion events[10]. The expression is also regulated post-transcriptionally. Accelerated atherosclerosis in diabetics has been linked to increased translation of macrophage CD36 transcripts under high glucose conditions[11]. Despite the biological importance of CD36 in angiogenesis, little is known regarding how endothelial cell-specific CD36 transcription is regulated or how that might relate to endothelial cell heterogeneity and function. In retinal MVECs, CD36 transcription is up-regulated in response to hypoxia *via* HIF-1 transcription factor[12]. The antiangiogenic pathway triggered by HIF-1 signaling counteracts hypoxia-driven proangiogenic pathways, providing a "homeostatic brake" to prevent pathological neovascularization. Recently we showed that LPA, a phospholipid signaling mediator and angiogenic factor[13–19], led to long-term CD36 transcriptional repression via PKD-1 signaling in MVECs to promote angiogenesis[7]. The precise mechanisms, however, by which LPA mediated PKD-1 signaling regulates CD36 transcription and functions remain largely unknown.

PKD-1 is a serine/threonine protein kinase that is classified as a member of the calcium/calmodulin-dependent protein kinase family. PKD-1-initiated signaling is essential for angiogenesis, inflammatory responses, cardiac hypertrophy and tumor progression, and related to its functions in regulating cell proliferation and death, migration, and differentiation[7, 20–27]. We have hypothesized that PKD-1 signaling triggered by LPA receptors initiates assembly of a nuclear transcriptional complex, which suppresses CD36 transcription and thereby turns off the anti-angiogenic switch. We found that Forkhead box protein O1(FoxO1) controls basal MVEC CD36 transcription and that PKD-1 signaling induces recruitment of a co-repressor, nuclear receptor co-repressor 1 (NCoR1), and a specific histone deacetylase (HDAC), HDAC7, to the FoxO1 nuclear complex, resulting in CD36 transcriptional repression. Intriguingly and unexpectedly, down-regulation of CD36 transcription was associated with reprogramming of MVEC transcription towards pro-angiogenic and arteriogenic gene expression, which is partially dependent on CD36 expression. Furthermore, the activation of this signaling pathway in the tumor vasculature of mice could be associated with CD36 deficiency and pro-angiogenic and pro-arteriogenic

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responses in the tumor microenvironment. Our study suggests that there is a plasticity in EC differentiation and postnatal angiogenesis, which is regulated by the LPA/PKD-1-FoxO1-CD36 signaling pathway. Targeting this pathway could provide a novel therapeutic strategy in ischemic heart and vascular diseases, cancer, and other disorders such as diabetes and obesity.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

CD36 transcription in endothelial cells is regulated by histone deacetylase 7

HMVECs transfected with a luciferase reporter plasmid (pGL3-CD36-luc) encoding a 3519 bp sequence of the *CD36* gene immediately proximal to the transcriptional start site[12] showed significant basal luciferase activity (Figure 1A) compared to cells transfected with the control pGL3 plasmid. Activity was decreased by over 80% after LPA exposure for 24 hrs ($p < 0.01$). Moreover, *CD36* transcriptional repression was long lasting; removal of LPA from MVEC cultures after 24 hrs followed by reconstitution in complete media for 48 hrs did not restore the CD36 transcription (Figure 1B). There was also a significant down-regulation of CD36 in primary human cardiac MVEC (HMVEC-C), which was not seen in cells treated with a pharmacologic inhibitor of the G protein coupled LPA receptors LPA_1 and LPA_3 (Figure 1C). To determine whether this long lasting effect was associated with chromatin remodeling, we assessed CD36 promoter specific histone acetylation status by chromatin immunoprecipitation analyses and found that nucleosome histone H3K9 enrichment in the CD36 promoter was reduced in response to LPA for 24 hrs (Figure 1D), suggesting that CD36 transcriptional repression by LPA may be regulated by histone deacetylases.

Class IIa HDACs including HDAC5 and HDAC7 are signal-dependent modulators of transcription[28] and previous studies indicated that HDAC5[29] and HDAC7[30] play roles in PKD signaling and gene transcription that regulates EC functions[30, 31]. We hypothesized that LPA suppression of CD36 transcription was *via* either one or both of these HDACs. To test this hypothesis we pretreated HMVECs with class IIa HDAC inhibitors, suberoylanilide hydroxamic acid or trichostatin A, and then exposed them to LPA. Both inhibitors abolished LPA-mediated down-regulation of CD36 protein expression (Figure 1E). By using immunofluorescence microscopy and biochemical cell fractionation we found that LPA exposure increased the nuclear accumulation of HDAC7 but not HDAC5 (Figure 2A).

To define the role of specific HDACs in LPA-mediated CD36 transcriptional repression, we knocked down HDAC7 expression in human MVECs transfected with pGL3-CD36-luc by using a specific shRNA (Figure 2B upper panel) and showed that LPA-inhibited *CD36* promoter activity was partially attenuated (Figure 2B lower panel; $p < 0.01$). Moreover, LPA-mediated CD36 mRNA down-regulation was blocked by transfection with shRNA plasmids targeting HDAC7 (Figure 2C). Knockdown of HDAC5 had no effect in these assays (data not shown).

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Transcription factor FoxO1 is required for constitutive CD36 transcription in MVECs

We next sought to identify transcription factors responsible for endothelial cell (EC) CD36 transcription. The analysis of the *CD36* gene promoter region revealed three putative FoxO-responsive elements (FHRE) located between −3500 to −250 bp relative to transcription start site (Figure 3A upper panel). FoxO1, a member of Forkhead transcription factor family, is known to be important in vascular development and postnatal angiogenesis[32, 33]. We deleted proximal promoter sequences containing FHRE at 258 bp relative to transcriptional start site in the pGL3-CD36-luc plasmid. The deletion of this regulatory element significantly reduced basal promoter activity in HMVECs (Figure 3A lower panel). Similar results were seen when this FHRE was subjected to site-directed mutagenesis (Figure 3A lower panel). Chromatin immunoprecipitation analyses revealed that endogenous FoxO1 in HMVECs bound specifically to a sequence within regulatory regions of the *CD36* gene (Figure 3B upper panel). Whereas antibody to the transcription factor the nuclear receptor subfamily 4 group A member 1 used as a control did not precipitate CD36 promoter sequences in these experiments, demonstrating specificity.

To investigate mechanisms by which FoxO1 might play a role in LPA-mediated *CD36* repression, ChIP analyses were performed to determine whether the interaction between FoxO1 and the CD36 gene was altered in response to LPA. Unexpectedly, no significant change was seen (Figure 3B lower panel). However, using an alternative avidin-biotin conjugated DNA and protein binding assay we found that LPA induced increased FoxO1 interaction with the *CD36* proximal promoter but not distal promoters *in vitro* (Figure 3C). This is consistent with the data (Figure 3A) showing that deletion or mutation in the proximal FHRE abrogated basal promoter activity. Further analyses revealed that deletions in the distal promoter removing sequences from −2799 to −396 did not have a significant impact on promoter activity in MVECs (Figure 3D). These results suggest other mechanisms are involved in the regulation of FoxO1 transcriptional activity by LPA.

Having shown that the proximal FHRE is essential for basal *CD36* promoter activity in HMVECs and that LPA actually increased FoxO1 interaction with the promoter we reasoned that the inhibitory effect of LPA was due to recruitment of a transcriptional repressor. Indeed LPA treatment of HMVECs induced interaction of the co-repressor NCoR1 with FoxO1. Whereas, this interaction was attenuated by pharmacologic inhibition of PKD-1 (Figure 3E), the kinase that we showed was downstream of LPA signaling in the *CD36* transcriptional repression pathway[7].

LPA/PKD-1 signaling mediates FoxO1-HDAC7 interaction

To explore potential mechanisms by which LPA/PKD-1 signaling contributes to endothelial *CD36* transcription, we generated lentiviral vectors encoding green fluorescence protein (GFP), wild type PKD-1 (PKD-WT) or constitutively active PKD (PKD-CA) fused to GFP (Figure 4A)[34] using both tetracycline inducible and constitutive systems. Tumor associated endothelial cells (TAECs) that express abundant CD36 were transduced by the inducible PKD-CA lentivirus and then exposed to doxycycline. The doxycycline treated cells showed down-regulated CD36 protein expression (Figure 4A) to levels similar to that seen in HMVECs exposed to LPA.

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Since specialized roles of PKD-1 may depend on both its localization and substrate proximity[27] we examined PKD-1 intracellular localization by fluorescence microscopy (Figure 4B left panels). LPA exposure increased nuclear PKD-1 expression and the phosphorylated PKD-1 was also increased in the nucleus in response to LPA treatment by fluorescence confocal laser scanning microscopy (Figure 4B right panels). To confirm the microscopy data we performed cell fractionation to isolate nuclear components. Western blots of cell fractions showed that LPA increased nuclear accumulation of PKD-1 (Figure 4C). Similarly, LPA exposure increased nuclear FoxO1 levels in HMVECs as determined by Western blots and immunofluorescence microscopy (Figure 4D).

Having shown that PKD-1 and its substrate HDAC7[20, 35] as well as FoxO1 accumulate in the MVEC nuclei in response to LPA, we hypothesized that PKD-1 mediates formation of a nuclear signaling complex to regulate *CD36* transcription. HMVECs were thus transfected with Flag-tagged FoxO1 to assess FoxO1-HDAC7 interactions in response to LPA. Co-immunoprecipitation experiments showed that FoxO1 interacted with endogenous HDAC7 at a basal level and that LPA exposure enhanced the interaction (Figure 4E). Furthermore, inducing expression of either PKD-WT or PKD-CA by doxycycline promoted FoxO1-HDAC7 interactions, but PKD-CA promoted interaction to a greater extent (Figure 4F). To determine whether PKD-1 is essential for this interaction, we knocked down PKD-1 expression in the cells with RNAi plasmids. FoxO1-HDAC7 interactions in response to LPA were significantly attenuated with PKD-1 knockdown (Figure 4G). To assess interactions of endogenous FoxO1 and HDAC7 in HMVEC-C we utilized a fluorescence-based immunologic cross linking assay (Proximity Ligation Assay/PLA) that uses antibodies to the 2 proteins derived from different animal species and detects proteins in close proximity (<40nm) microscopically as bright red fluorescent dots. As seen in Figure 4H, multiple bright dots were seen in cell nuclei after LPA treatment, but not in the control untreated cells (Figure 4I, $p < 0.01$). As an additional control, no signal was detected using antibodies to FoxO1 and HDAC5 (Supplemental Figure I). Co-immunoprecipitation confirmed the increased interaction between endogenous HDAC7 and FoxO1 (Figure J) and this interaction was attenuated partially by treating the cells with a pharmacological PKD inhibitor (Figure 4J).

Regulation of pro-arteriogenic and angiogenic responses by LPA/PKD-1 signaling

To determine whether the repression of CD36 transcription induced by LPA/PKD-1 signaling is part of a broader program of angiogenic gene regulation, we performed a focused angiogenic gene expression array using real-time qPCR. In response to LPA exposure the expression levels of 20 angiogenic genes were up-regulated over 3–fold, including *EFNB2, neuropilin 1 and flt1* (Supplemental Figure II, A and B). These data suggest that down-regulation of the anti-angiogenic receptor CD36 was linked to the up-regulation of specific pro-angiogenic genes. EFNB2 is an arterial endothelial cell marker that is important in angiogenesis and arteriogenesis[36–38]. To validate the qPCR profiling data we showed significant increased expression of EFNB2 mRNA by qPCR and of protein by western blots in the PKD-CA transduced HMVEC-C and mouse MVEC lines (Figure 5A & 5B). Moreover, over-expressing PKD-CA in HMVEC-C significantly increased expression of additional pro-arteriogenic genes including delta-like ligand 4 and HEY2, but not

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neuropilin1 (Figure 5C). Over-expressing *CD36* down-regulated EFNB2 expression in HMVEC-C and LPA treatment significantly reversed CD36-mediated down-regulation (Figure 5D). Intriguingly, *CD36* overexpression did not significantly change delta-like ligand 4, HEY2 or neuropilin1 expression (Figure 5E).

To show functional significance of this transcriptional program switch we studied the impact of LPA on angiogenic differentiation in mouse heart MVECs expressing CD36. Exposure to LPA promoted endothelial sprout formation *in vitro* as measured in a three-dimensional spheroid assay; the spheroids showed robust branching morphogenesis compared to the control ($p<0.01$, Figure 6A) and this was partially attenuated by addition of a selective PKD inhibitor ($p>0.05$, Figure 6A). Furthermore, an *in vivo* Matrigel assay showed that pharmacologic inhibition of PKD signaling reduced the inhibitory effect of LPA on TSR/CD36-mediated anti-angiogenic effects in response to VEGF *in vivo* (Figure 6B left panel). LPA, when incorporated into VEGF containing Matrigel plugs induced formation of functional vascular networks *in vivo* as demonstrated by the presence of hematopoietic cells within the neo-vessels (Figure 6B upper right panel). Pharmacologic inhibition of LPA signaling did not significantly change microvascular density (Figure 6B lower right panel). However, LPA significantly promoted microvascular remodeling as shown by increased number of cells expressing alpha SMA (Figure 6C, upper and lower left panel, $p < 0.05$). Furthermore, conditioned media from LPA-treated HMVECs significantly increased proliferation of VSMCs in culture compared to cells grown in standard media (Figure 6C, lower right panel, $p < 0.05$). Vascular smooth muscle cell (VSMC) proliferation in the presence of conditioned media from HMVECs treated with PKD inhibitor was not different from cells treated with standard media alone (Figure 6C, lower right panel, $p>0.05$). These data suggest the LPA signaling through PKD pathway in MVECs can impact VSMC function via secreted factors. We also examined tumor angiogenesis and relevant signaling pathways in a syngeneic tumor transplant model using Lewis Lung Carcinoma cells in wild type and *cd36* null mice. Immunofluorescence microscopy revealed extensive angiogenesis within the tumors detected with anti-VEGFR2 antibody (Figure 6D and 6E lower panels). The angiogenic response was associated with lower levels of expression of CD36 in the neo-vessels compared to nearby cutaneous vessels (Figure 6D and 6E) and with expression of phospho-PKD-1, EFNB2 and alpha SMA in the tumor vasculature (Figure 6E, 6F and 6G). There was no significant difference in the number of alpha SMA expressing cells in tumor vessels in wild type and *cd*36 null mice (Figure 6G right panel, $p > 0.05$).

Discussion

CD36 is an important angiostatic receptor on MVECs that interacts with TSP-1 and other anti-angiogenic proteins containing TSR domains[1–4] to modulate VEGF signaling and promote endothelial cell apoptosis. Our previous work demonstrated that LPA promotes angiogenesis in the presence of antiangiogenic TSR-containing proteins by down-regulating *CD36* gene transcription *via* a protein kinase PKD-1 pathway[7]. In the current study we made the novel observations that the transcription factor FoxO1 is required for constitutive *CD36* expression in MVECs and, as shown in the model in Figure 7, that LPA-mediated PKD-1 activation and nuclear translocation results in recruitment of the class II histone deacetylase HDAC7 to the *CD36* promoter driving formation of an HDAC7/NCoR1/FoxO1 nuclear

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complex and subsequently triggering *CD36* transcriptional repression. Furthermore, turning off CD36 transcription was associated with transcriptional reprogramming that up-regulated expression of arterial genes, including *EPHB2*. On a functional level, our data suggest that activating this pathway *in vivo* may promote microvascular remodeling and functional stability (Figure 6), and in murine tumor model systems absence of CD36 was associated with increased expression of the arterial marker in the tumor microvasculature.

These results also support the concept that terminally differentiated MVECs are developmentally plastic, and in the setting of specific environmental cues can be re-programmed[39–42] towards an arteriogenic phenotype that includes change in expression of surface proteins and secretion of factor(s) that support VSMC proliferation and migration. LPA is likely to be an important such cue as it is often generated in angiogenic microenvironments associated with inflammation, ischemia, platelet activation, cancer and obesity. Consistent with previous reports[43, 44], our results show unsaturated LPA promoted SMC entry into Matrigel plugs *in vivo*. While our data suggest that LPA in the microenvironment of lung cancers may reduce MVEC CD36 expression and contribute to microvascular remodeling comparable to that seen in the *cd36* null mice at the late stages of tumor development examined in these studies, we did not detect differences in VSMC investment in neovessels, as detected by anti-alpha SMA staining. Our data also suggest that *CD36* transcriptional status might be more than just a marker for the loss of capillary endothelial phenotype. Forced expression of CD36 in MVECs abrogated the arteriogenic "switch" that may be turned on by LPA, perhaps implicating CD36 in the regulatory pathway.

PKD-1-mediated signaling has been shown to regulate cardiovascular functions including myocardial responses to ischemia, angiogenesis, and arterial remodeling,[7, 20–24, 42, 45] and the transcription factor FoxO1 is well known to be involved in vascular development, postnatal angiogenesis,[32, 33] and regulation of *EPHB2* expression[46]. Our study now links the PKD-1 signaling pathway initiated by specific LPA receptors to FoxO1 in MVECs and demonstrates a critical mechanistic role for nuclear accumulation of activated PKD-1 in recruiting a specific class II histone deacetylase, HDAC7, to the FoxO1 transcriptional regulatory complex. HDAC7, which is known to be highly expressed in ECs[45, 47], was previously shown by Chang and colleagues[31] to be important in maintenance of vascular integrity during embryonic development *via* a mechanism that involves inhibition of a transcriptional activator, myocyte enhancer factor-2 (MEF-2). This interaction results in transcriptional repression of the matrix metalloproteinase-10 gene and promotes stability of the extracellular matrix. Thus these studies and our new data show that HDAC7 interacts with specific EC transcription factors (FoxO1 and MEF-2) that function in at least two pathways related to vascular development and stability. Our studies using pharmacologic HDAC inhibitors or specific shRNA (Figures 1E, 2B and 2C) also suggest that HDAC7 could be a therapeutic target to de-stabilize angiogenesis and modulate EC differentiation[48].

CD36 is a multifunctional transmembrane protein that on phagocytic cells serves as a scavenger receptor for endogenous danger-associated molecular patterns (e.g. oxidized low density lipoproteins) and exogenous pathogen-associated molecular patterns (e.g. staphylococcus lipoteichoic acid)[3]. On fat, muscle, and gut epithelial cells CD36 functions

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as a transporter of free fatty acids. Interestingly, CD36 transcriptional regulation in MVECs is very different than in these other cell types, in which expression is regulated both positively and negatively by metabolic factors, cytokines, bacterial components, reactive oxygen species, and adhesion[3, 10]. Our studies have not revealed any response of CD36 expression in MVECs to those factors that up-regulated CD36 in phagocytic cells, fat and muscle (e.g. PPARγ agonists and cytokines) or that down-regulated expression in these cells (e.g. bacterial endotoxin), suggesting that the expression of CD36 in MVECs may have evolved for a different function; *i.e.* as a receptor for TSR proteins and a regulator of both angiogenesis and arteriogenesis[38], rather than as a scavenger receptor or fatty acid transporter. Further study of the precise mechanisms linking CD36 expression to EC differentiation and of the *in vivo* relevance of this regulatory pathway may aid in discovering novel therapeutic targets against ischemic diseases, cancer, obesity and diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FHRE	FoxO-responsive elements
PKD-CA	constitutively active protein kinase D1
TAEC	tumor associated endothelial cell
HMVEC-C	cardiac human microvascular endothelial cells
PKD-WT	wild type PKD-1
TSR	thrombospondin type 1 repeat

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Significance

FoxO1, a transcription factor targeting genes in angiogenesis, vascular remodeling and homeostasis, may be required for endothelial cell CD36 transcription whereas a novel LPA/PKD-1-HDAC7-FoxO1 signaling axis is involved in CD36 transcriptional repression in microvascular endothelial cells. Intriguingly, the endothelial cells are reprogrammed to express arteriogenic genes once CD36 transcription is turned off. Our study defines a novel mechanistic pathway of CD36 transcriptional repression and implicates the potential importance of this repression in proarteriogenic responses and microvascular remodeling under ischemic conditions. Our study also suggests the plasticity of angiogenic gene transcription during postnatal angiogenesis, and this plasticity could be controlled *via* cellular context-dependent transcriptional switch of FoxO1 regulated by LPA/PKD1-HDAC7 signaling. PKD-1-FoxO1 signaling could serve as a reprogramming factor for modulating arterial development and arteriogenesis.

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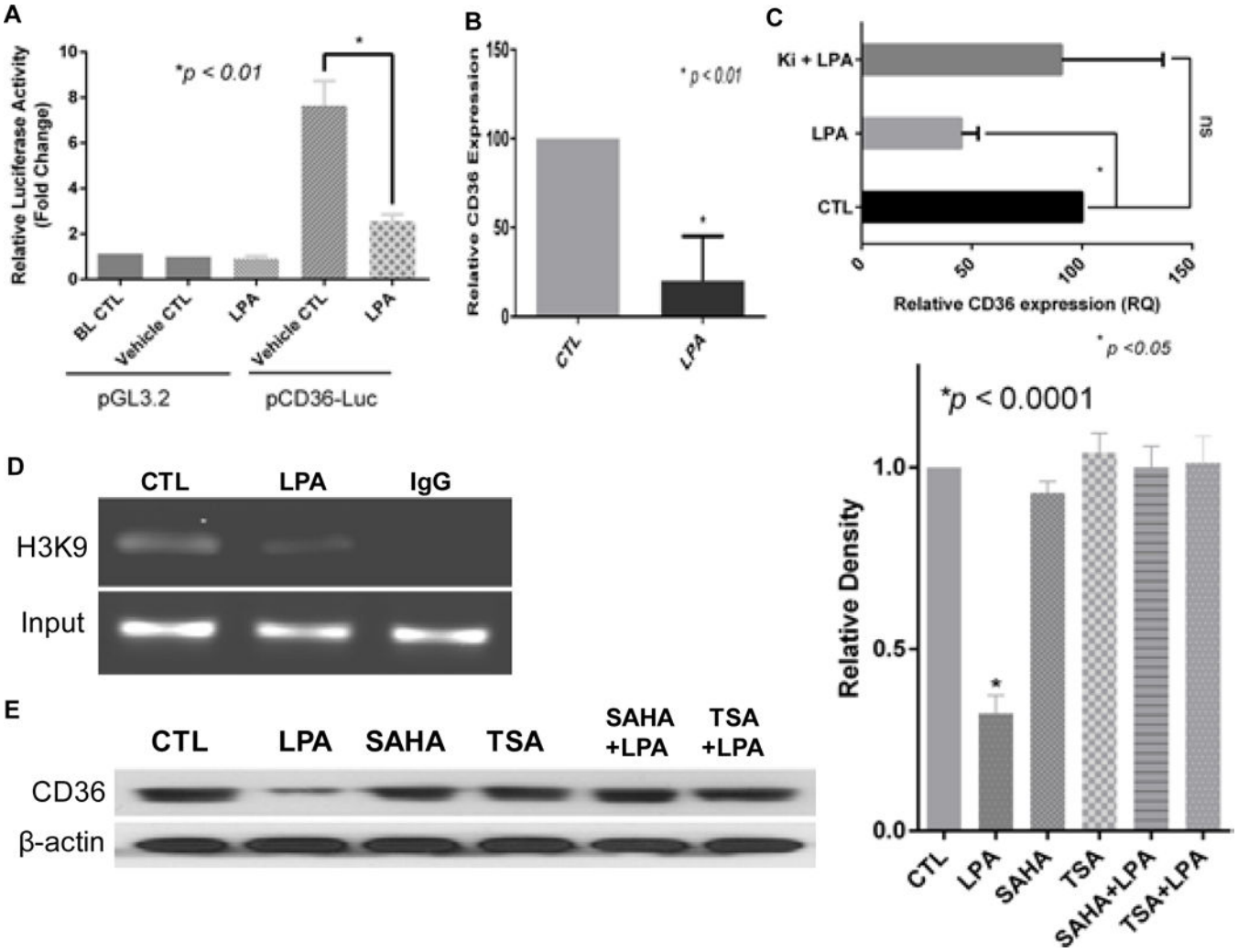

Figure 1.

LPA-mediated sustained CD36 transcriptional repression may be associated with histone deacetylase activity in endothelial cells. **A**, LPA exposure suppresses CD36 promoter activity. Primary HMVECs were transfected with reporter constructs containing the CD36 proximal promoter fused to the pGL3-basic (pCD36-luc) for 24 hrs with co-transfection of pRL Renilla luciferase for normalization. The cells were then exposed to LPA for 24 hrs before measurement of luciferase activity with Dual-Luciferase® Reporter Assay System. Promoter activity was expressed as the fold-change in relative luciferase activity compared with pGL3.2 basic control (mean ± S.E. of three independent experiments). **B**, LPA mediates sustained CD36 mRNA downregulation. Primary HMVECs were exposed to LPA (10 μM) for 24 hrs, and then LPA was removed and replaced with complete media for 48 hrs. The mRNA level was assayed with quantitative real time RT-PCR. Triplicate experiments were performed ($p < 0.01$). **C**, LPA signaling significantly reduces CD36 mRNA expression in cardiac microvascular endothelial cells (HMVEC-C). Primary HMVEC-Cs (passage 3) were exposed to LPA (10 μM) for 24 hrs or pretreated with LPA receptor1,3 antagonist Ki16425 (2 μM) for 30 min followed by LPA (10 μM) for 24 hrs. Total RNA was obtained for quantitative real time RT-PCR. **D**, ChIP analyses targeting the CD36 proximal promoter in

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LPA-treated (10 μM, 24 hrs) HMVECs using antibodies against AcH3 (K9). Input shows DNA isolated from the lysate before immunoprecipitation (10% of the total chromatin was used for the PCR reactions). Lane labeled "IgG" is ChIP performed with a non-immune IgG. **E**, CD36 expression in primary HMVECs treated with LPA and histone deacetylase inhibitors suberanilohydroxamic acid (SAHA) or trichostatin (TSA). The HMVECs were pre-exposed to 500 nM of SAHA or 500 ng/ml of TSA for 30 min followed with 10 μM of LPA for 24 hrs. Cell lysates were subjected to Western blots for CD36 protein expression (right panel). Densitometry was performed and relative expression levels calculated for triplicate experiments (left panel).

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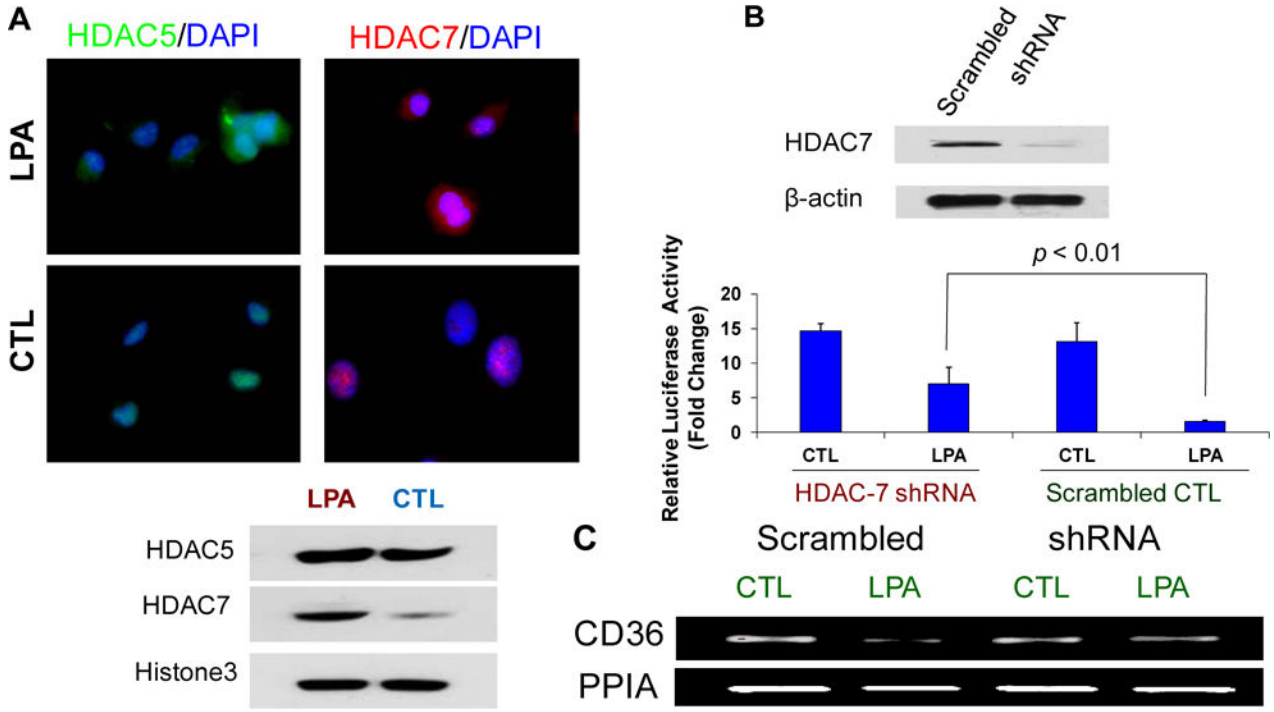

Figure 2.

HDAC7 regulates CD36 transcription in endothelial cells. **A**, Nuclear HDAC7 translocation is increased in response to LPA exposure. MVECs were exposed to LPA (10 μM) for 24 hrs and then incubated with antibodies to HDAC5 or HADC7 followed by Alexa fluor® conjugated secondary antibodies. Cells were co-stained with DAPI to mark nuclei. In some studies cells were fractionated and the nuclear fractions isolated and subjected to Western blots for HDAC5 or HDAC7 expression. Representative results are shown from triplicate experiments. **B**., Knocking down endogenous HDAC7 attenuates LPA-mediated CD36 transcriptional repression in HMVECs. Western blots showed that HDAC 7 shRNA plasmids transfection effectively reduced the endogenous HDAC7 expression (upper panel). Cells transfected with HDAC7 shRNA plasmids or scrambled control were co-transfected with pGL3-CD36-luc and Renilla plasmids for 48 hrs. The cells were exposed to LPA (10 μM) or vehicle control for 24 hrs and luciferase activity measured as in Figure 1A ($p < 0.01$). **C**, HMVECs were transfected with a pool of HDAC7 shRNA plasmids for 28 hrs, and the cells were exposed to LPA (10 μM) for 18 hrs. The cells were harvested and total RNA was isolated for RT-PCR for CD36 mRNA expression. The image shows a representative gel from triplicate experiments.

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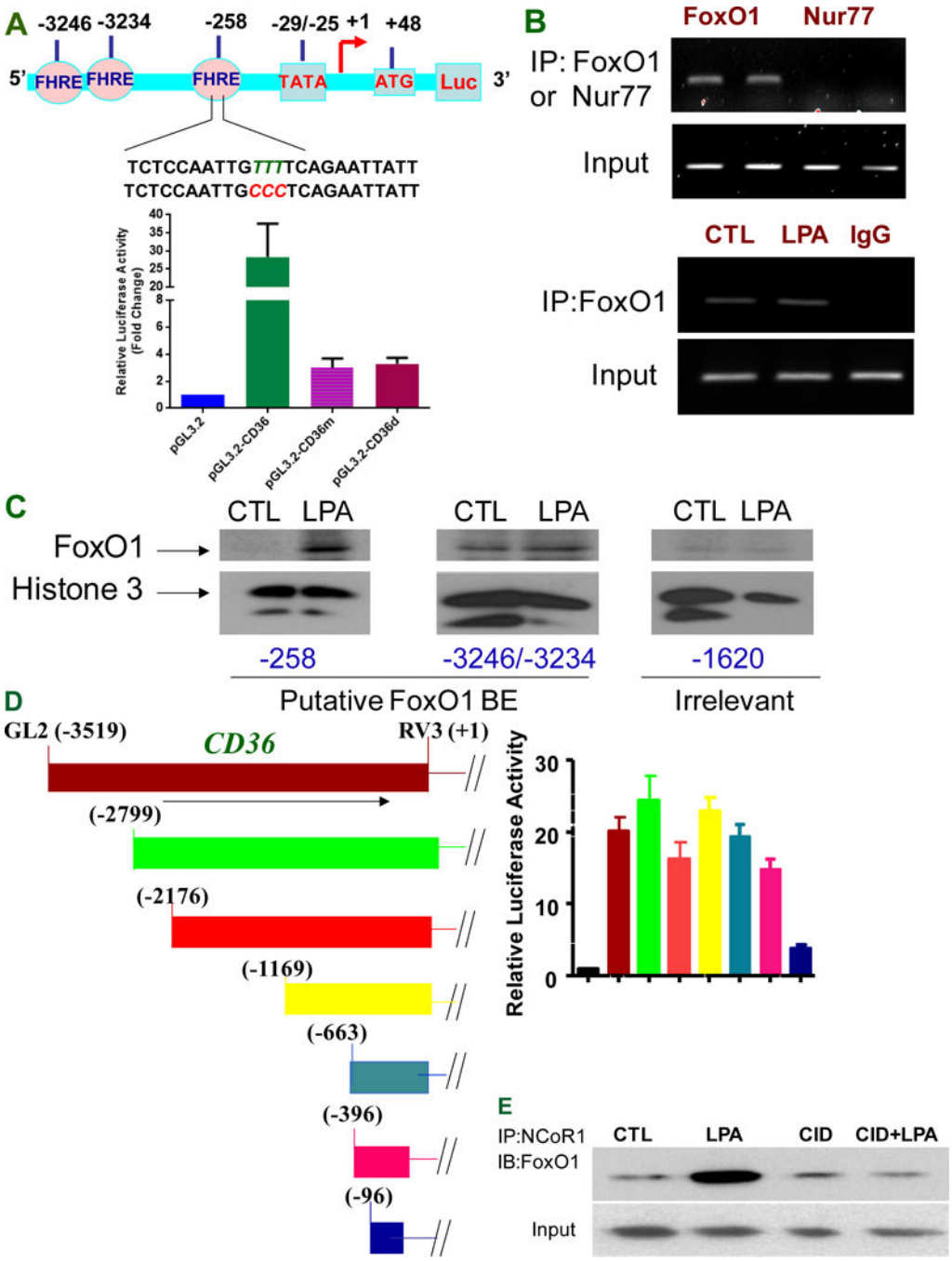

Figure 3.

FoxO1 transcriptional activities are associated with CD36 transcription. **A**, FoxO1 regulates activity of the CD36 promoter through recognition of FoxO1 responsive element (FHRE) at the proximal promoter. The promoter analysis shows three putative forkhead response elements (FHREs), respectively located at −258, −3234, and −3246 relative to the transcription start site. The TTT were mutated into CCC in FHRE sequence contained at proximal promoter (upper panel). Proximal promoter mutagenesis at −258 (pGL.3.2-CD36m) or deletion of proximal promoter (pGL3.2-CD36d) attenuated CD36 promoter

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activity (lower panel). Data represent mean ± SD of three independent experiments. **B**, FoxO1 binds CD36 proximal promoter (upper panel) but LPA does not enhance this binding *in vivo* (lower panel). HMVECs were exposed to LPA (10 μM) for 24 hrs and subjected to Chromatin immunoprecipitation analyses. Nur77 (nuclear receptor subfamily 4 group A member 1) is used as a control. Representative results are shown in triplicate experiments. **C**, LPA increases the interactions of FoxO1 FHRE with the CD36 proximal promoter. Based on putative binding sites for FoxO1 at CD36 promoter, biotin-labeled probes targeting FHRE respectively at proximal, distal promoter or irrelevant site were designed for detecting FoxO1 interaction with FHREs. HMVECs were exposed to LPA (5 μM) for 24 hrs, and the nuclear components were isolated and subjected to an avidin-biotin conjugated DNA binding assay. **D**, Distal promoter deletion does not alter CD36 promoter activity. The CD36 promoter variants in the luciferase reporter system were prepared as shown in left panel and the basal CD36 promoter activity with various deletion mutations were assayed in the HMVECs as in Figure 1A (right panel). **E**, PKD-1 signaling regulates recruitment of co-repressor NCoR1 to FoxO1 complex in response to LPA. HMVECs were exposed to LPA (10 μM) for 24 hrs or pre-exposed to a selective PKD inhibitor CID 755673 (25 μM) for 30 min followed with LPA (10 μM) for 24 hrs before the cell lysates were collected and subjected to co-immunoprecipitation (IP). Representative result is shown.

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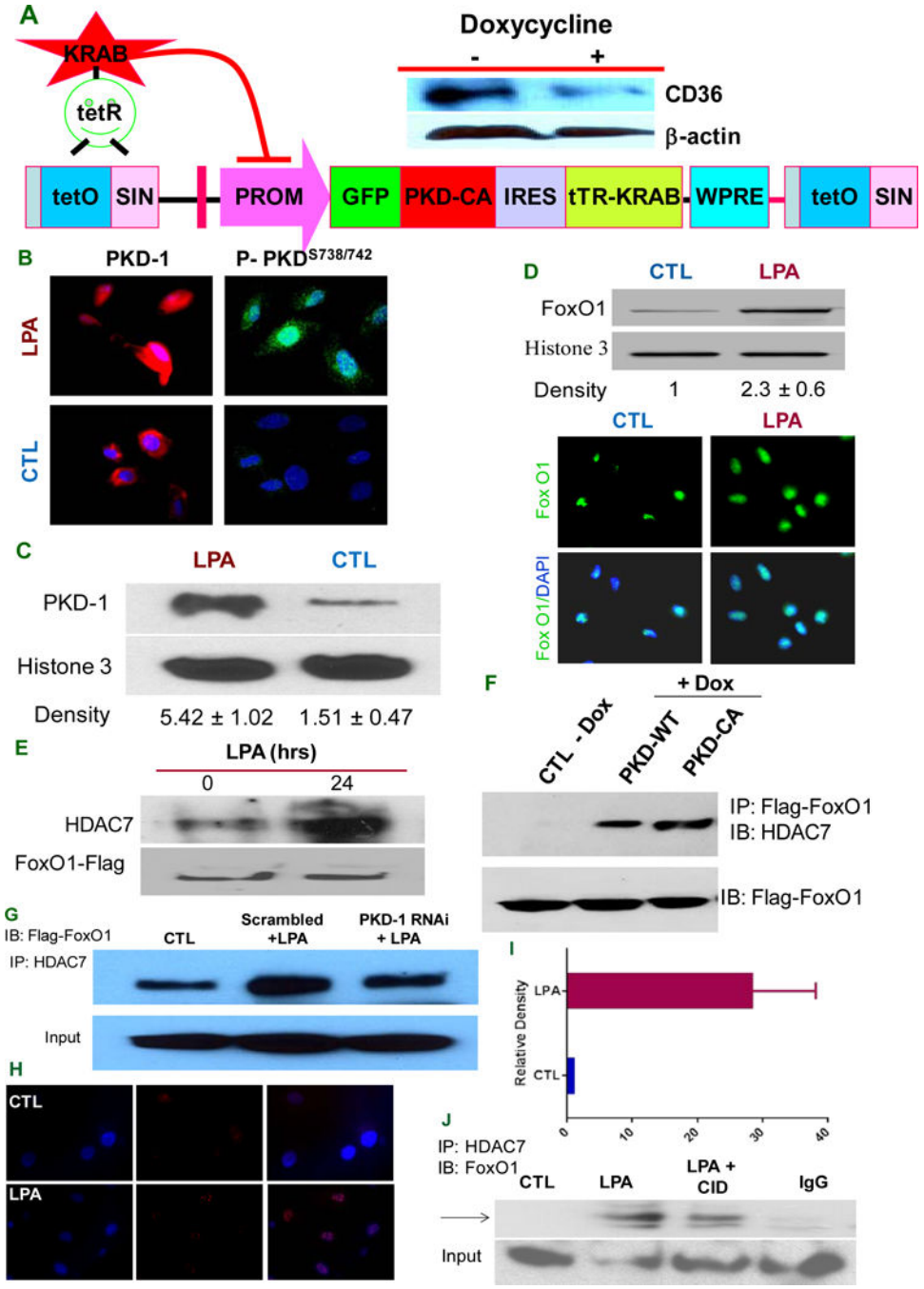

Figure 4.

PKD-1 signals are required for HDAC7/FoxO1/interactions to regulate CD36 expression. **A**, PKD-CA expression inhibits CD36 expression in TAECs. A doxycycline-controllable single Lentiviral vector system containing pLVET-GFP: PKD-CA was established. TAECs were transduced with the vector and GFP positive clones were selected. Clone 3 cells were grown in the presence of 1 μg/ml doxycycline for 36 hrs and cell lysates were processed and subjected to Western blots. **B**, PKD-1 is translocated to the nucleus in response to LPA. HMVECs were exposed to LPA (5 μM) or vehicle for 24 hrs (left panels) or for 30 min

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(right panels) and incubated with anti-PKD-1 or anti-phospho-PKD-1 antibodies followed by Alexa Fluor® 2nd antibody. Images were obtained with a immunofluorescence microscopy or confocal microscopy. **C**, HMVECs were exposed to LPA (5 μM) for 24 hrs, the nuclear components were isolated and subjected to Western blots for total PKD-1 and histone 3 antibodies were used to determine nuclear protein loading (left panel). The relative PKD-1 level was determined by densitometry for triplicate experiments (right panel). **D**, FoxO1 level increases in the nucleus with LPA exposure. After LPA (10 μM) treatment for 24 hrs, ECs were subjected to immunofluorescence microscopy using anti-FoxO1 antibody (lower panel). In some studies nuclear components were isolated from the cells and subjected to Western blots for FoxO1 protein expression and relative density was calculated from triplicate experiments (upper panel). **E**, LPA promotes HDAC7-FoxO1 interaction. HMVECs were transfected with FoxO1-flag for 36 hrs and then exposed to LPA (5 μM) for 18 hrs. Lysates were immunoprecipitated with HDAC7 antibody and re-probed with anti-Flag antibody. Total cell lysate was subjected to Western blots for Flag-tag. **F**, Overexpressing PKD-1 increases HDAC7 interaction with FoxO1. TAECs transduced with pLVET-GFP: PKD1-WT or pLVET-GFP: PKD1-CA plasmids were co-transfected with FoxO1-Flag in the absence or presence of doxycycline (1 μg/ml) for 36 hrs. Cell lysates were collected and immunoprecipitated with anti-Flag antibody, followed by Western blot analyses using anti-HDAC7 or anti-Flag antibody. **G**, PKD-1 is required for HDAC7-FoxO1 interactions. HMVECs transfected with scrambled RNAi plasmids (Scramble), or pSUPER PKD-1 RNAi and co-transfected with FoxO1 for 36 hrs were treated with LPA (5 μM) for 18 hrs and analyzed by Co-IP for FoxO1-HDAC7 interactions as in panel E. Representative results are shown in the figure. **H**, LPA exposure promotes interaction of endogenous HDAC7 and FoxO1 in the nucleus of HMVEC-C. In response to LPA (10 μM), there was a significant increase in the HDAC7-FoxO1 interaction in early passages of HMVEC-C (P3) detected as bright red dots in the cell nuclei by fluorescence microscopy using the Proximity Ligation Assay (PLA). **I**, Increased relative density of red fluorescent dots in the PLA indicating HDAC7-FoxO1 interactions in HMVEC-C exposed to LPA (10 μM). **J**, LPA-induced HDAC7-FoxO1 interaction was partially attenuated by PKD inhibitor. HMVEC-C (P3) wasexposed to LPA (10 μM), LPA (10 μM) plus a selective PKD inhibitor CID755673 (25 μM) or buffer control and the nuclei were isolated and subjected to immunoprecipitation with anti-HDAC7 followed by immunoblot with anti-FoxO1 to detect endogenous interactions.

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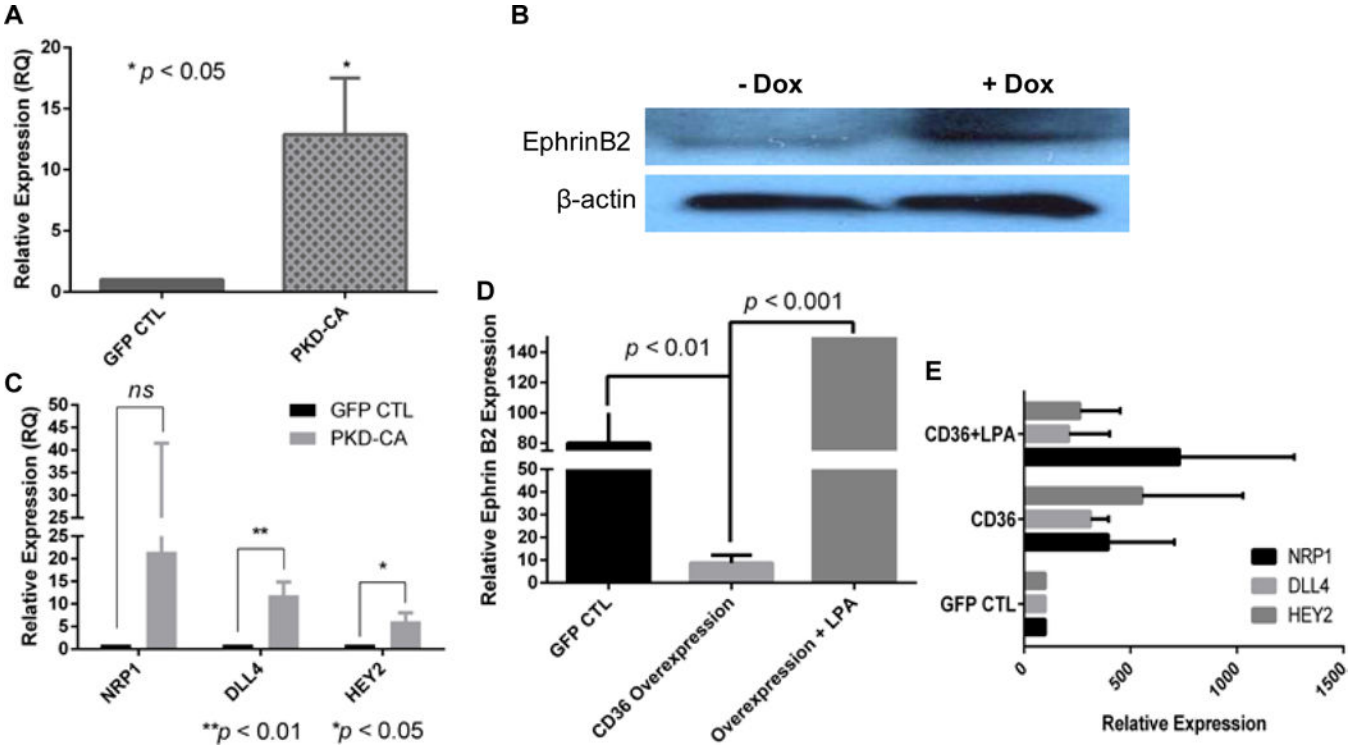

Figure 5. LPA/PKD-1-CD36 signaling axis regulates the expression of angiogenic and arteriogenic genes

A, Overexpression of PKD-CA increases the expression of EFNB2 mRNA level. HMVEC-C (passage 2) transduced with GFP or PKD-1:GFP were processed for total RNA and mRNA expression by quantitative real time RT-PCR. **B**, Clone 3 TAEC cells were processed as in Figure 4A and cell lysates collected and subjected to Western blots with anti-ephrinB2 antibody. Shown is a representative result from duplicate experiments. **C**, The impact of PKD-CA overexpression on angiogenic gene expression. HMVEC-C transduced with GFP or PKD-1:GFP were processed for total RNA and mRNA expression of NRP1, DLL4 and HEY2 by quantitative real time RT-PCR. **D**, Overexpression of human CD36 downregulates EFNB2 expression in HMVEC-C. HMVEC-C (passage 2) transduced with GFP or hCD36:GFP were processed for total RNA and mRNA expression by quantitative real time RT-PCR. **E**, Overexpression of human CD36 does not impact the expression of NRP1 (neuropilin1), DLL4 (delta like ligand 4) and HEY2 in HMVEC-C. HMVEC-C (passage 2) was processed as described in D for quantitative real time RT-PCR.

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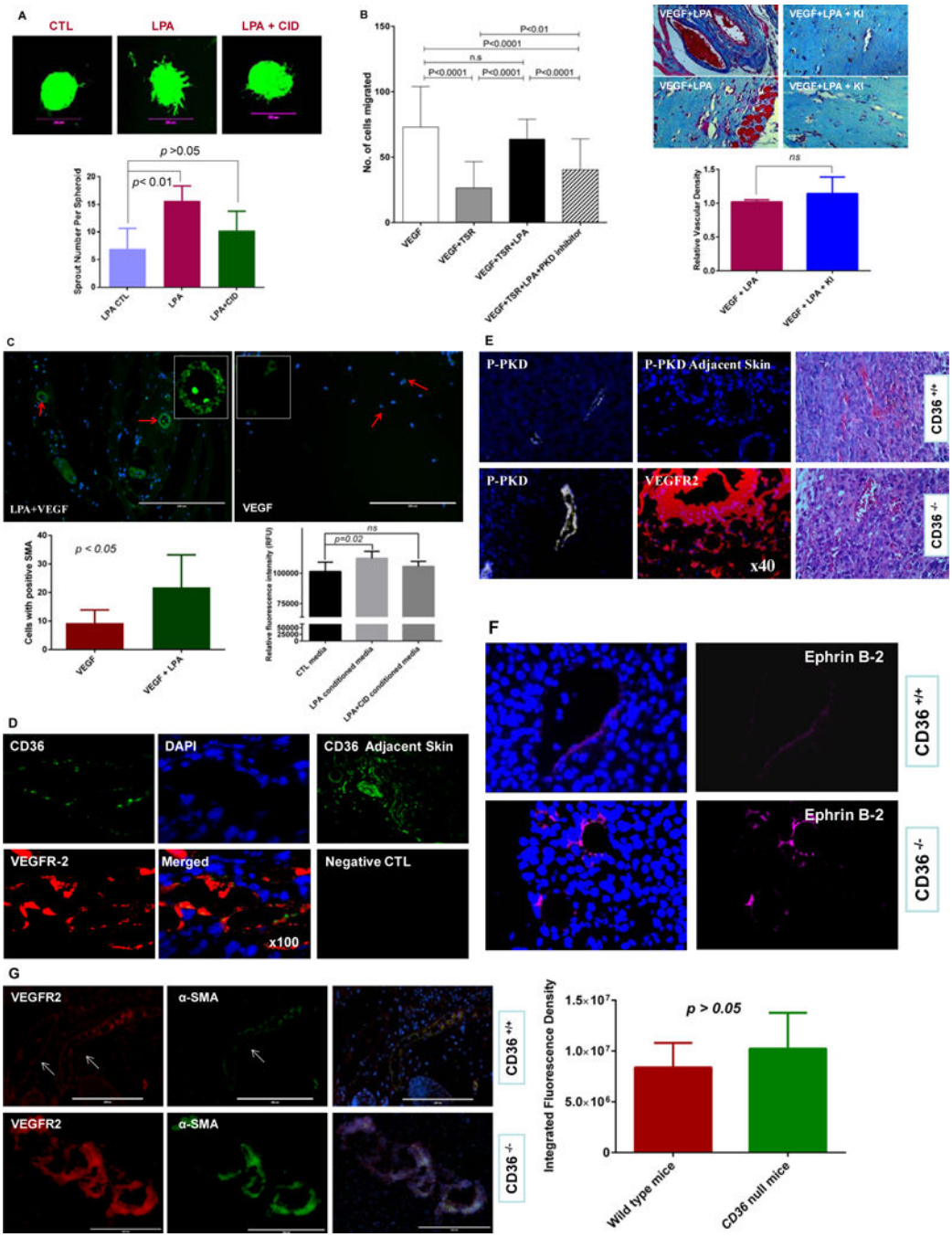

Figure 6. PKD-1 signaling promotes proangiogenic and proarteriogenic responses

A, PKD-1 signaling is associated with LPA-stimulated sprouting morphogenesis in primary MHECs. MHECs were exposed to LPA and used in a three-dimensional spheroid assay. LPA (10 μM) was added to the media for 48 hrs to induce branching morphogenesis, and a selective PKD inhibitor CID755673 (25 μmol/L) was added in a group with LPA treatment with vehicle as control. Branching morphogenesis was observed under fluorescence microscope and pictures were taken with a digital camera linked to MetaMorph® 7.0 software and representative pictures are shown, and scale bar is 200 μm (upper panel).

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Sprouting described in B was quantified microscopically by counting sprouts per spheroid at 48 hrs in response to LPA (lower panel). **B**, LPA inhibition of TSR-induced inhibition of vascular invasion is partially dependent on PKD-1 signaling. Matrigel plugs mixed with a combination of VEGF (50 ng/ml), recombinant TSR (10 nM), LPA (10 μM) and CID 755673 (25 μmol/L) were injected into mice and analyzed histologically after 10d. Bar graph shows the relative angiogenic responses and vascular invasion in the Matrigel plugs (left panel). Additionally, LPA signaling promotes vascular remodeling. Matrigel plugs mixed with a combination of VEGF (50 ng/ml) and LPA (10 μM) or VEGF and LPA plus LPA receptor 1,3 antagonist Ki16426 (2 μM) were respectively injected into mice and analyzed histologically after 10d. Representative Masson's Trichrome stained images are shown and bar graph shows vascular invasion relative to VEGF stimulation (right panel; p >0.05; bar 50 μm). Images were acquired with a Nikon Eclipse E600 microscope and NIH Image J was used for the analysis of vascular invasion. ns: no significant statistical difference. **C**, LPA stimulates the formation of SMA positive vessels. Matrigel plugs were sectioned and examined with an EVOS®FL imaging system using antibodies to alpha SMA (green) and nuclei was stained with DAPI (blue), and representative images are shown, bar = 200 μM. Images with positive SMA staining were randomly collected and the positive cells counted with NIH image J. The average cell number was calculated in each visual field and compared between VEGF and LPA plus VEGF groups (p <0.05, upper and lower left panel). Additionally, VSMCs were exposed to the conditioned media from HMVECs treated with LPA or LPA and PKD inhibitor, and cell proliferation was assessed by an alamarBlue® Cell viability assay (lower right panel). Two independent experiments were performed, and the cell proliferation is represented as relative fluorescence intensity. LPA conditioned media significantly increases VSMC proliferation (p<0.05; ns: no significant statistical difference). **D**, Tumors harvested from C57Bl and *cd*36$^{+/+}$ mice after transplantation of Lewis Lung carcinoma cells were sectioned and examined by immunofluorescence microscopy using antibodies to VEGFR2 (red) and CD36 (green). Nuclei were stained with DAPI (blue) (100×). CD36 expression in skin adjacent to the tumors and sections incubated with CD36 antibodies or non-immune IgG are shown as positive and negative controls. Representative results are shown. **E**, Tumor vessels as in Panel D were examined by immunofluorescence using antibodies to phospho-PKD-1 (white) in wild type or the *cd36* null mice, and no PKD-1 phosphorylation was shown in the skin adjacent to the tumor tissues. VEGFR-2 (red) staining was shown in the tumor tissues to visualize the angiogenic response in the *cd36* null mice. H&E staining is used to show tumor tissues in both wild type and *cd36* deficiency mice (40×). Representative images are shown. **F**, Tumor vessels were examined by immunofluorescence using antibodies to EFNB2 (magenta) in wild type or the *cd36* null mice. Representative images are shown. **G**, Tumor vessels were examined by immunofluorescence using antibodies to alpha SMA and VEGFR2 and DAPI was used for staining the nuclei (VEGFR2 red, SMA green, DAPI blue) in wild type or the *cd36* null mice. Images were randomly acquired with a Zeiss Axioskop Microscope with Photometrics Cool SNAP ES Camara system using Metamorph v7 software or EVOS®FL cell imaging system, and representative images are shown (left panel). NIH Image J was used to analyze the integrated fluorescence density (IFD) of SMA positive cells (green) in the hypervascular areas of the tumor microenvironment. The average IFD was used to assess the relative number of SMA positive cells in the neovessels and compared between wild type and *cd*36

null mice. No significant statistical difference was found between these two groups ($p > 0.05$, right panel).

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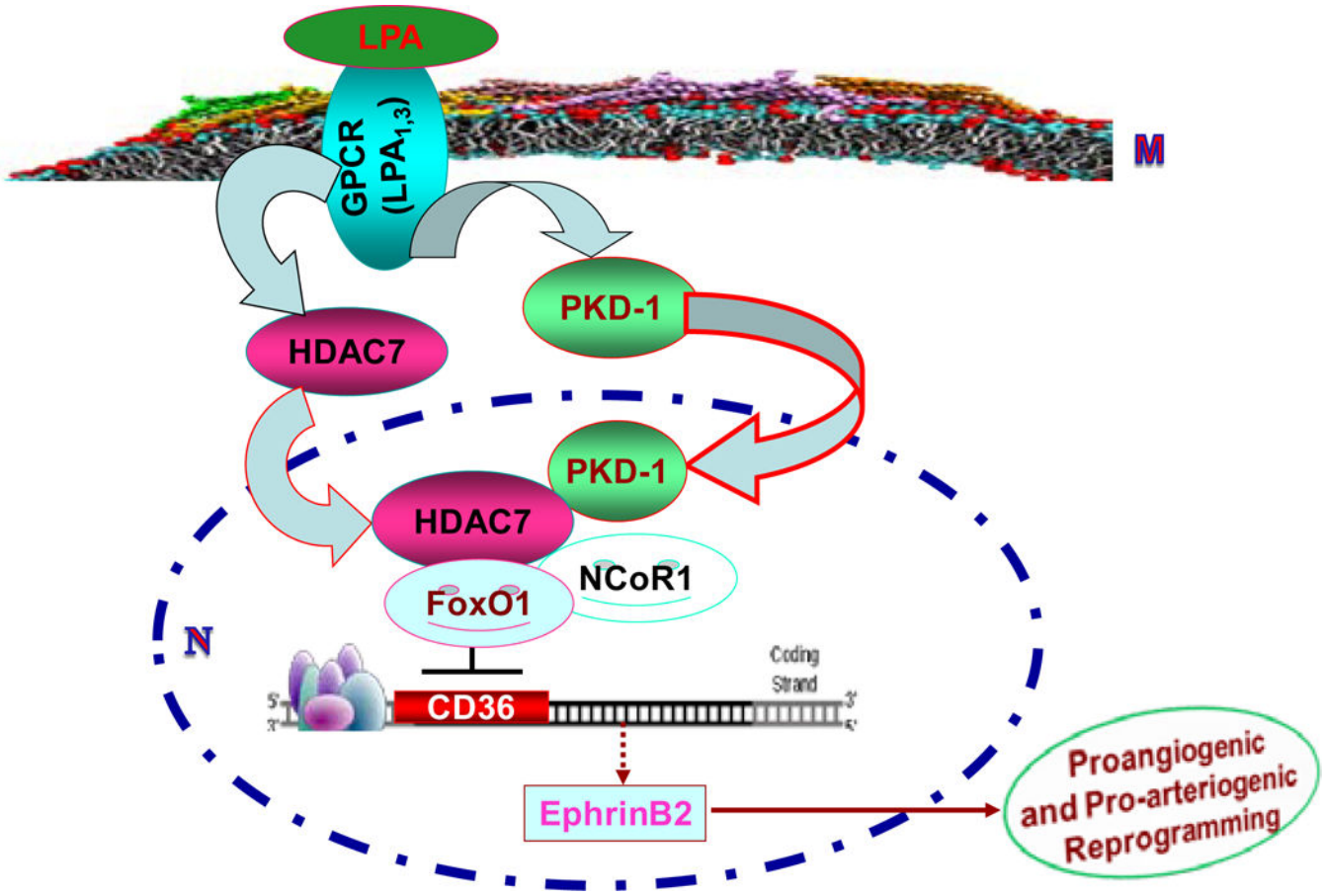

Figure 7.

Model showing potential mechanism by which an LPA/PKD-1-HDAC7-FoxO1 signaling axis regulates EC CD36 transcription and angiogenesis. LPA signaling in MVEC is mediated by G-protein coupled LPA receptors 1 and 3 at the EC membrane (M) which activate protein kinase PKD-1, leading to PKD-1 and HDAC7 translocation into the nucleus (N) where they interact with the transcription factor FoxO1 and the co-repressor NCoR1. This results in *CD36* transcriptional repression and reprogramming EC to express ephrinB2 which promotes angiogenesis, arteriogenic differentiation and microvascular remodeling.