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Protein kinase D2 and heat shock protein 90 beta are required for *BCL6-associated zinc finger protein* mRNA stabilization induced by vascular endothelial growth factor-A

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Abstract Vascular endothelial growth factor (VEGF) is a major angiogenic factor that activates pro-angiogenic molecules to generate new vessels. Recently, we identified a VEGF-A-induced pro-angiogenic gene, *BCL-6 associated zinc finger protein* (*BAZF*), in endothelial cells. BAZF interacts with CBF1, a transcriptional regulator of Notch signaling, and downregulates Notch signaling by inducing the degradation of CBF1. A signal inhibition assay with a combination of chemical inhibitors and siRNA revealed that the protein kinase D (PRKD) family, mainly PRKD2, mediated *BAZF* gene expression by VEGF-A stimulation. A luciferase reporter assay showed that the promoter activity of the *BAZF* gene was unchanged by VEGF-A stimulation. However, we found that the stability of *BAZF* mRNA increased in a VEGF-A/PRKD2-dependent manner. In further studies to investigate the underlying mechanism, we successfully identified heat shock protein 90 beta (HSP90β) as a molecule that interacts with and stabilizes *BAZF* mRNA following VEGF-A/PRKD2 activation. These data suggest that HSP90β may positively regulate angiogenesis, not only as a protein chaperone, but also as an mRNA stabilizer for pro-angiogenic genes, such as *BAZF*, in a PRKD2 activity-dependent manner.

Keywords Angiogenesis · VEGF-A · BAZF · Protein kinase D · HSP90β · RNA stabilization

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Introduction

Angiogenesis is defined as new vascular formation from pre-existing blood vessels. This phenomenon arises in several physiological conditions, such as embryonic development, wound healing, and the female reproductive cycle in adults. Defects in angiogenesis also affect the progression of a wide range of diseases, including rheumatoid arthritis, psoriasis, diabetic retinopathy, and malignant tumors, collectively referred to as "angiogenesis-dependent diseases" [1]. Further understanding of angiogenic mechanisms may lead to novel therapeutics for such diseases. The regulation of angiogenesis depends on a balance between activation and inhibition [2]. When angiogenesis is required, activation can be achieved either by increasing pro-angiogenic activators and/or decreasing angiogenic suppressors. Vascular endothelial growth factor

(VEGF), in particular, is a potent pro-angiogenic growth factor that controls most embryonic vessels.

The mammalian VEGF family comprises 5 glycoproteins, VEGF-A, VEGF-B, VEGF-C, VEGF-D (also known as FIGF), and placenta growth factor (PlGF) [3, 4]. VEGF-A is the best characterized member of the VEGF family and is predominantly expressed in many solid human tumors. Members of the VEGF family associate with 3 types of receptor tyrosine kinases, termed VEGFR1 (also known as FLT1), VEGFR2 (also known as Flk1 in mouse or KDR), and VEGFR3 (also known as FLT4). VEGF-A has a high binding affinity with VEGFR1 and VEGFR2. Importantly, VEGFR2 is expressed primarily in the vasculature and is a critical mediator of VEGF-induced angiogenesis.

On the other hand, vessel-stabilizing pathways, such as the Notch signaling pathway, are also essential in angiogenic regulation. Recent studies have demonstrated that angiogenic sprouting is tightly regulated by the Notch signaling circuit of the VEGFR-Notch ligand, Dll4 [5–18]. Interestingly, endothelial cells (ECs) in pre-existing vessels express both Dll4 and Notch, and signaling is thought to be balanced between ECs. Therefore, VEGF-A stimulation could affect the Dll4-Notch balance in pre-existing vessels to rearrange the signaling direction in sprouting areas, upregulating Dll4 in tip cells and inducing Notch signaling in adjacent cells to become stalk cells [19]. However, the precise molecular mechanisms mediating this fine-tuning of the angiogenic "tug-of-war" signaling balance between VEGFR and Notch have remained unclear.

In a recent study, we identified a VEGF-A-induced critical angiogenic factor, BCL6-associated zinc finger protein (BAZF) [20]. VEGF-A-induced BAZF forms a complex with cullin3, an E3 ubiquitin ligase, to degrade CBF-1, a transcriptional factor involved in Notch signaling. The loss of CBF-1 in endothelial cells induces spontaneous angiogenesis in adult mice, revealing that Notch signaling play a key role in the maintenance of angiogenic quiescence [21]. Thus, we propose that BAZF is an important factor that can interrupt the "tug-of-war" balance between VEGFR and Notch signaling to promote angiogenesis.

We have already shown that levels of BAZF mRNA and protein peak in 2–4 h and 4–6 h after a VEGF-A stimulation, respectively [20]. In this study, we investigated the molecular mechanisms mediating VEGF-A-induced BAZF gene expression.

Materials and methods

Reagents and antibodies

Recombinant human VEGF and fibroblast growth factor-2 (FGF-2) were purchased from R&D Systems. RO31-8220, LY294002, PD98059, Gö6976, Gö6983, and protein kinase C beta (PKCβ) inhibitors were purchased from Calbiochem. A23187 and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Actinomycin D was purchased from MP Biomedicals. PU-H71 hydrate was purchased from Santa Cruz Biotechnology. Anti-protein kinase D1 and 2 (PRKD1/2) antibodies were purchased from Santa Cruz Biotechnology. Anti-PRKD3 antibodies were purchased from Bethyl Laboratories Inc. Anti-heat shock protein 90 beta (HSP90β) antibodies were purchased from Millipore, abcam, and EPITOMICS. Anti-HSP27 was purchased from Cell Signaling Technology, and growth factor reduced Matrigel was purchased from BD Biosciences. Anti-green fluorescent protein (GFP) was purchased from abcam.

Cell culture

Human umbilical vein endothelial cells (HUVECs) and human dermal microvascular ECs from Cell System and human coronary artery ECs and human retinal microvascular ECs from Applied Cell Biology Research were cultured in endothelial growth medium, EGM-2 (Lonza). These cells were used for experiments from passages 3–6.

Reverse transcription real-time quantitative PCR (RT-qPCR)

Total RNAs were prepared using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. One microgram of RNA was used for first-strand synthesis using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). Real-time PCR was performed (FastStart Universal SYBR Green Master ROX; Roche) with the ABI 7300/7500 Real-Time PCR system (Applied Biosystems).

Western blotting

Samples were separated by SDS-PAGE, followed by transfer to a nitrocellulose membrane. The membrane was then blocked with 5 % skim milk in 0.05 % Tween 20/PBS (-) (PBS-T) for 30 min, followed by incubation with a primary antibody. After washing with PBS-T, the membrane was incubated with appropriate horseradish peroxidase conjugated IgG antibodies (Promega). After antibody incubation, proteins were detected by enhanced chemiluminescence (GE Healthcare).

RNA interference

siRNAs targeting human PRKD1, PRKD2, and PRKD3 and a scrambled siRNA control were purchased from

QIAGEN. The transfection of siRNA was performed with Lipofectamine RNAiMAX (Invitrogen) at 20 nM according to the manufacturer's protocol.

Construction of promoter-luciferase reporter plasmids

A 5.5-kb fragment containing 5530 bp of the 5′ upstream region and the first basepair of exon I of the human *BAZF* gene was amplified from the genomic DNA of HUVECs and ligated into the *Kpn*I and *Nhe*I sites of the pGL4.1 vector (Promega) to obtain the −5530/+1 reporter construct.

Actinomycin D chase assay

HUVECs were treated with 2 mg/mL actinomycin D for 30 min (added at time 0), followed by treatment with 50 ng/mL VEGF-A and/or chemical inhibitors. HUVECs were collected at the indicated times, and total RNA was isolated as described above. RT-qPCR was performed as described, and mRNA levels were normalized using *18S* ribosomal RNA, with the mRNA level at time 0 set at 100 %.

Construction and production of lentivirus vectors

Lentivirus vectors were constructed by inserting mutant cDNAs encoding GFP-fused PRKD2 into the lentiviral expression vector CSII-CMV-MCS-IRES2-Bsd. Lentiviral vectors were produced in 293T cells. Mutant cDNAs for GFP-fused PRKD2 were kindly provided by Dr. Nishimura (Kumamoto University) [22]. Lentiviral expression and packaging vectors were kindly provided by Dr. Miyoshi (RIKEN BioResource Center).

Luciferase assay

The human NF-κB-responsive promoter-luciferase reporter gene was kindly provided by Dr. Yoshimura (Keio University). A reporter plasmid containing the firefly luciferase gene was transfected into HUVECs with pRL-TK (Promega) using Sugarfect (Medgel) according to the manufacturer's protocol. Luciferase activities were measured using a dual-luciferase reporter assay system (Promega) with a GloMax96 microplate luminometer (Promega) according to the manufacturer's instructions.

Biotinylated RNA pull-down assay

For biotinylated RNA pull-down assays, PCR fragments containing the T7 RNA polymerase promoter sequence [(T7): CTAATACGACTCACTATAGGGAGA] were used as a template for in vitro transcription of the coding region

(CR) and full-length (FL) *BAZF* using biotinylated CTP. Ten micrograms of biotinylated FL transcript was incubated with 100 μg protein from the cytoplasmic fraction, which was precleared with biotinylated CR transcript, for 1 h at room temperature, followed by isolation of the complexes with streptavidin-coated magnetic Dynabeads (Invitrogen). The pull-down material was separated by SDS-PAGE, and CBB staining was carried out with Bio-Safe Coomassie (BIO-RAD). Protein bands were excised from gels and subjected to in-gel digestion with sequence-grade modified trypsin (Promega), as previously described [20, 23]. Tryptic digests were reconstituted with 0.2 % (v/v) trifluoroacetic acid for mass spectrometric analysis. PMF and MS/MS analysis was performed using a MALDI-TOF/TOF mass spectrometer (Shimadzu AXIMA-TOF2). All MS or MS/MS spectrum data were submitted to the MASCOT program (Matrix Science) in order to obtain a protein candidate for each spot. Database searches were performed against the NCBInr database version 20070629 using the following parameters: (1) unlimited protein molecular weight and pI ranges; and (2) presence of protein modifications, including acrylamide modification of cysteine, methionine oxidation, protein N-terminal acetylation, and pyro-glutamic acid.

Protein-binding RNA detection assay

To detect HSP90β-associated *BAZF* mRNA, we used a previously reported method, with some modifications [24]. Briefly, VEGF-A-treated HUVECs were lysed using a cell lysis buffer [50 mM Tris (pH7.4), 150 mM NaCl, 1 mM MgCl$_2$, and 0.05 % Nonidet P-40], and cell lysates were then incubated with Dynabeads Protein G (Invitrogen) and anti-HSP90β antibodies (abcam) for 1 h at 4 °C. The beads were then treated with 20 U DNase and 0.5 mg/mL proteinase K for 30 min at 15 °C. Total RNA was isolated from the supernatant using an ethanol precipitation method, and *BAZF* mRNA was detected by RT-qPCR as described above. The primer sets are listed in Supplemental Table 1.

Two-dimensional gel electrophoresis

After infection of HUVECs with kinase-dead (KD) or constitutively active (CA) PRKD2-expressing lentivirus, cells were collected and lysed with IEF lysis buffer [8.5 M Urea, 4 % CHAPS, and 0.2 % Biolyte 3/10 (Bio-Rad)]. Twenty micrograms of protein from each sample was loaded onto Immobiline Dry Strips (pH 3–10, 13 cm in length; GE Healthcare). Two-dimensional gel electrophoresis was performed as previously described [23]. Western blotting with anti-HSP90β or anti-HSP27 was then carried out as described above.

Endothelial cell network formation assay

Matrigel (BD Biosciences) was prepared in 24-well culture plates (IWAKI). HUVECs were serum starved with EBM-2 containing 0.15 % FBS for 12 h. These cells were seeded onto solidified gels at a concentration of 1×10^4 cells/well in 500 μL of EBM-2 containing 0.15 % FBS and VEGF-A (50 ng/mL) with or without Pu-H71 (1 μM). After 8 h, photographs of the cells were taken using a microscope (Olympus). Total network length was measured and calculated using Image-Pro Plus software (Roper).

Statistical analysis

Data were analyzed by unpaired t tests. Differences were considered significant if p values were less than 0.05.

Results

VEGF-A induces *BAZF* expression through PRKD2 activation in HUVECs

To clarify which signaling pathway is responsible for the induction of *BAZF* mRNA in response to VEGF–stimulation in HUVECs, we examined the effects of various kinase inhibitors on VEGF-A-dependent *BAZF* gene expression. HUVECs were treated with the MEK inhibitor PD98059 (30 μM), pan-PKC inhibitor RO31-8220 (1 μM), phosphatidylinositol 3-kinase inhibitor LY294002 (10 μM), or p38 MAPK inhibitor SB202190 (20 μM) for 30 min before VEGF-A stimulation. Although PD98059 and RO31-8220 significantly inhibited VEGF-A-dependent *BAZF* mRNA induction, the PKC inhibitor was more effective at suppressing *BAZF* transcript levels (Fig. 1a). To determine whether PKC activation was involved in VEGF-A-induced *BAZF* mRNA expression in HUVECs, we examined the effects of PKC activators on *BAZF* expression. HUVECs were treated with the pan-PKC activator PMA (10 nM) or the cyclic PKC activator A23187 (5 μM) for 2 h. PMA, but not A23187, significantly upregulated *BAZF* mRNA (Fig. 1b). In addition, we examined which PKC isoform was important for VEGF-A-induced *BAZF* expression using various PKC inhibitors. HUVECs were treated with RO31-8220, Gö6976 (1 μM), Gö6983 (1 μM), or PKCβ inhibitor (1 μM) for 30 min prior to VEGF-A stimulation. Both RO31-8220 and Gö6976, but neither Gö6983 nor PKCβ inhibitor, significantly inhibited VEGF-A-dependent *BAZF* mRNA expression (Fig. 1c). Since Gö6976, but not Gö6983, selectively inhibits PKCβ and PRKD enzymes, these data suggested that PRKD activation regulated *BAZF* gene expression in HUVECs.

The PRKD family includes 3 isoforms, PRKD1, PRKD2, and PRKD3. To assess which PRKD isoform was required for *BAZF* mRNA induction by VEGF-A in HUVECs, we examined the effects of siRNAs targeting each *PRKD* mRNA on *BAZF* mRNA expression. When HUVECs were transfected with human *PRKD1*, *PRKD2*, or *PRKD3* siRNA at 20 nM, the expression of each PRKD protein significantly decreased (Fig. 1d). The *PRKD2* siRNA was also effective at inhibiting *PRKD3* mRNA and protein expression. *PRKD* siRNA-transfected HUVECs were treated with VEGF-A for 2 h. *PRKD2* and *PRKD3* siRNAs significantly inhibited *BAZF* mRNA expression both with and without VEGF-A treatment; *PRKD2* siRNA suppressed VEGF-A-induced gene expression to a greater extent (Fig. 1e), possibly due to the more broad specificity of this siRNA construct. These data indicated that both PRKD2 and PRKD3 mediated VEGF-A-induced *BAZF* mRNA expression; in contrast, because PRKD3 knockdown was less effective in a VEGF-A-stimulated state, PRKD3 may specifically regulate the steady state expression of *BAZF* mRNA. In addition, we examined effects of wild-type (WT), CA, or KD PRKD2 mutants on *BAZF* mRNA expression. HUVECs were infected with each PRKD2 mutant-expressing lentivirus at 1 multiplicity of infection (m.o.i.). As shown in Fig. 1f and g, the CA PRKD2 mutant induced *BAZF* mRNA expression in HUVECs. On the other hand, expression of the KD-PRKD2 mutant significantly suppressed the induction of *BAZF* mRNA. These data indicated that PRKD2 activation mainly regulated VEGF-A-induced *BAZF* gene expression.

A 5.5-kb *BAZF* promoter does not respond to VEGF-A stimulation

To analyze transcriptional regulation of the *BAZF* gene, a 5.5-kb fragment of the 5′ upstream region of human *BAZF* from the first base of exon 1 was cloned in a luciferase reporter plasmid pGL4.1 [pGL4-BAZF (−5530/+1)]. This reporter plasmid was then transfected into various cell types. Transcriptional activity tended to be strong in endothelial cells (ECs), and promoter activity also appeared to correlate well to *BAZF* mRNA expression in ECs (Fig. 2a). To determine the critical region for promoter activity in the 5.5-kb fragment, we generated a series of deleted *BAZF* promoter-containing plasmids. These plasmids were transfected into HUVECs, and the cells were assayed for luciferase activity 24 h post-transfection. pGL4-BAZF (−2548/+1), pGL4-BAZF (−2033/+1), pGL4-BAZF (−1603/+1), and pGL4-BAZF (−1218/+1) exhibited transcriptional activity, while pGL4-BAZF (−510/+1) did not (Fig. 2b). These data suggest that the critical region for promoter activity exists in the −1218/−510 region. Therefore, the region spanning −1218/+1
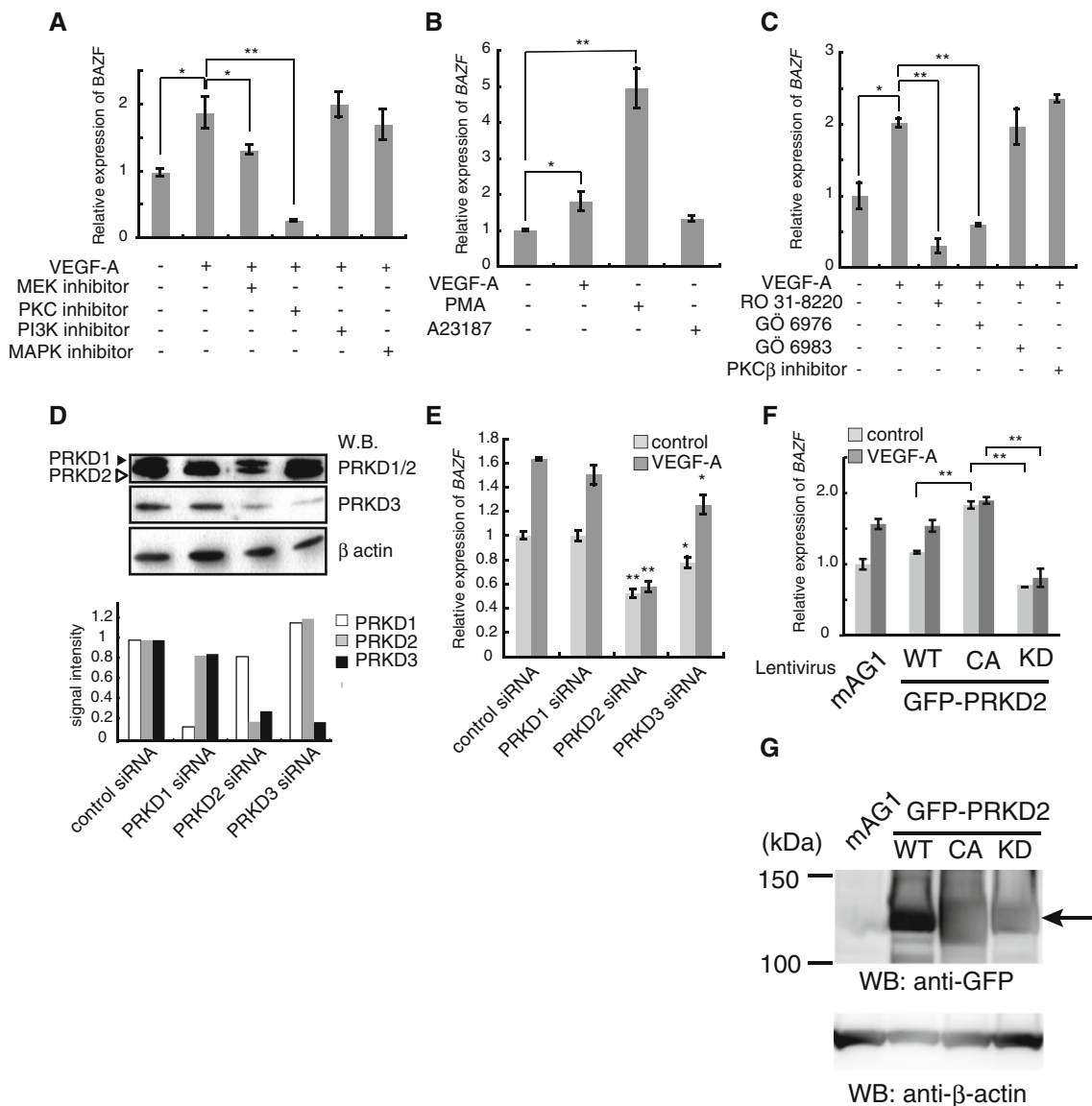
Fig. 1 VEGF-A induces *BAZF* expression through PRKD activation in HUVECs. **a** Identification of the VEGFR-mediated downstream pathway regulating *BAZF* gene activation. HUVECs were pretreated with the MEK inhibitor PD98059 (30 μM), PKC inhibitor RO31-8220 (1 μM), PI3 K inhibitor LY294002 (10 μM), or MAPK inhibitor SB202190 (20 μM). Inhibitor-treated HUVECs were stimulated with 50 ng/mL VEGF-A for 2 h, and *BAZF* mRNA was detected by reverse transcription real-time quantitative PCR (RT-qPCR). **b** The PKC family pathway stimulates *BAZF* gene expression. HUVECs were stimulated with 50 ng/mL VEGF-A, 10 nM phorbol 12-myristate 13-acatate (PMA), or 5 μM A23187 Calcium Ionophore for 2 h. **c** Identification of the PKC member that regulates the *BAZF* gene. HUVECs were pretreated with different PKC family inhibitors, including RO31-8220 (1 μM), Gö6976 (1 μM), Gö6983 (1 μM), or PKCβ inhibitor (1 μM) for 30 min before VEGF-A stimulation. The pretreated cells were then incubated with 50 ng/mL VEGF-A for 2 h. **d** Knockdown efficiency of siRNA against the PRKD family.

HUVECs were transfected with siRNAs (20 nM) targeting PRKD family members. The knockdown efficiency of each siRNA was checked at the protein level using western blotting analysis. Bands were normalized using a β-actin loading control. **e** PRKD2 is responsible for *BAZF* gene activation by VEGF-A. *BAZF* mRNA was measured in HUVECs after siRNA-mediated knockdown of PRKDs and subsequent stimulation with 50 ng/mL VEGF-A for 2 h. **f** Induction of *BAZF* mRNA by PRKD2 activation. HUVECs were infected with various mutant PRKD2-expressing lentiviruses at 1 multiplicity of infection (m.o.i.). Forty-eight hours after infection, the HUVECs were stimulated with 50 ng/mL VEGF-A for 2 h. mAG1; monomeric Azami Green 1, *WT* wild-type, *CA* constitutively active mutant, *KD*; kinase-dead mutant. The experiments were independently performed 3 times. *$p < 0.05$; ** $p < 0.01$. **g** The protein expression levels of exogenous PRKD2 between WT 1PRKD2 and the mutants in the infected HUVECs. The protein level of β-actin was shown as a loading control

could be a putative *BAZF* promoter. Hence, pGL4-BAZF (−1218/+1) was used for further studies. Next, we examined whether the *BAZF* promoter responded to

VEGF-A stimulation. pGL4-BAZF (−1218/+1)-transfected HUVECs were serum-starved for 12 h before VEGF-A stimulation, and luciferase activity was measured at 6 h

after VEGF-A stimulation. The *BAZF* promoter exhibited no response to VEGF-A, while NF-κB-mediating activation of the luciferase reporter responded to VEGF-A stimulation (Fig. 2c). These data suggested that endothelium-specific *BAZF* expression depended on its promoter activity, which is not enhanced by VEGF-A.

VEGF-A stabilizes *BAZF* mRNA through a PRKD2 signaling pathway

To determine whether *BAZF* mRNA was regulated by VEGF-A stimulation at post-transcriptional levels, we performed actinomycin D chase experiments. The half-life ($t_{1/2}$) of *BAZF* mRNA was analyzed by RT-qPCR following treatment with actinomycin D to inhibit *de novo* transcription. The $t_{1/2}$ of *BAZF* mRNA was found to be significantly longer after VEGF stimulation ($t_{1/2} = 3.47$ h) than without treatment ($t_{1/2} = 0.98$ h), and RO31-8220 inhibited *BAZF* mRNA stabilization by VEGF-A ($t_{1/2} = 0.98$ h), as shown in Fig. 3a. On the other hand, neither the $t_{1/2}$ nor the level of *GAPDH* mRNA was affected by VEGF-A, with or without RO31-8220 treatment (Fig. 3b). These data indicated that *BAZF* mRNA levels were stabilized by a VEGF-A stimulation through a PRKD-mediated signaling pathway.

## The *BAZF* 3′ UTR mediates regulation of mRNA stability by PRKD2

The 3′ untranslated region (3′UTR) of mRNA is known to be important for mRNA stability and translation. In particular, the AU-rich sequence element (ARE), AUUUA, in the 3′UTR was identified as a correlative region with mRNA decay [25]. *BAZF* mRNA also includes several ARE sites in its 3′UTR region. To investigate whether the 3′UTR of *BAZF* mRNA played a role in mRNA stability, we constructed a luciferase reporter plasmid containing the 3′UTR of *BAZF* mRNA (pCMV-luc-BAZF 3′UTR). Luciferase activity of pCMV-luc-BAZF 3′UTR significantly decreased as compared with the luciferase activity of the plasmid without the 3′UTR (pCMV-luc; Fig. 4a). PMA, a potent activator of PRKD2, significantly increased *BAZF* mRNA levels (Fig. 1b). Moreover, at 6 h post-PMA stimulation, luciferase activity of pCMV-luc-BAZF 3′UTR, but not that of pCMV-luc, increased, and this activity was attenuated by treatment with the PRKD inhibitor Gö6976 (Fig. 4b). Next, we examined whether the level of *luciferase* mRNA produced from pCMV-luc-BAZF 3′UTR was stabilized by PMA stimulation. These reporter plasmids were transfected into HUVECs, and the cells were treated with actinomycin D for 30 min before exposure to PMA. At 6 h post-PMA stimulation, the level of *luciferase* mRNA produced from pCMV-luc-BAZF

3′UTR, but not from pCMV-luc, increased (Fig. 4c). Moreover, downregulation of *PRKD2* by siRNA abrogated PMA-dependent luciferase activity of the *BAZF* 3′UTR-fused luciferase gene (Fig. 4d). On the other hand, overexpression of PRKD2 mutants revealed that expression of the CA PRKD2 construct enhanced the luciferase activity of pCMV-luc-BAZF 3′UTR both without and with actinomycin D treatment (Fig. 4e, f). These data suggest that the 3′UTR of *BAZF* is responsible for mRNA stability and that PRKD2 activity is involved in *BAZF* 3′UTR-mediated regulation of mRNA stability.

HSP90β binds *BAZF* mRNA and regulates mRNA stability

Previous reports have shown that 3′UTR binding proteins play a critical role in the regulation of mRNA stability [26, 27]. To identify molecules that bind to the 3′UTR of *BAZF* mRNA and regulate its stability, we precipitated *BAZF* mRNA binding proteins from HUVEC whole cell lysates with biotinylated *BAZF* mRNA and streptavidin-coated magnetic beads. The precipitated proteins were separated by SDS-PAGE and analyzed by mass spectrometry. We found that the interaction of HSP90β with *BAZF* mRNA increased after PMA stimulation (Fig. 5a). Other identified PMA-stimulation-dependent *BAZF* mRNA binding molecules were cytoskeleton-related proteins (data not shown). A recent report demonstrated that an HSP90 inhibitor destabilizes *B cell chronic lymphocytic leukemia/lymphoma 6* (*BCL6*) mRNA [28], indicating that HSP90β may stabilize *BCL6* mRNA; however, the details are still unclear. Therefore, we focused on analysis of HSP90β function in *BAZF* mRNA stabilization. To confirm binding of HSP90β to *BAZF* mRNA, we performed an immunoprecipitation (IP) assay using an anti-HSP90β antibody. The association of *BAZF* mRNA with HSP90β was monitored by RT-qPCR. The *BAZF* PCR product was enriched in the HSP90β IP sample in a VEGF-A-dependent manner, and an HSP90-specific inhibitor Pu-H71 (1 μM) [29] inhibited the enrichment of *BAZF* mRNA (Fig. 5b). To determine whether HSP90β was involved in stabilization of *BAZF* mRNA, we examined the effects of HSP90 inhibitors on *BAZF* mRNA stability after VEGF-A stimulation. HUVECs were treated with an HSP90-specific inhibitor Pu-H71 (1 μM) or 17-allylaminogeldanamycin (17-AAG; 1 μM) [30] for 30 min before exposure to VEGF-A. Both Pu-H71 and 17-AAG significantly inhibited *BAZF* mRNA stabilization by VEGF-A (Fig. 5c, d). Next, we performed an actinomycin D chase assay to investigate the effects of an HSP90β inhibitor on the $t_{1/2}$ of BAZF mRNA. The $t_{1/2}$ of *BAZF* mRNA significantly decreased from 1.72 to 0.74 h with Pu-H71 treatment (Fig. 5e). Next, we analyzed the influence of Pu-H71 on pCMV-luc-BAZF 3′UTR

Fig. 2 VEGF-A does not affect the transcriptional activity of *BAZF*. **a** Cell type-specific activity of the *BAZF* gene promoter. Several types of human primary endothelial cells (*CAEC* cardiac coronary artery endothelial cells, *DMVEC* dermal microvascular endothelial cells, *RMVEC* retinal microvascular endothelial cells), and non-endothelial cell lines were transfected with a BAZF promoter reporter, and luciferase activity was measured in each *cell line*. *BAZF* mRNA levels were measured by RT-qPCR. The level of *BAZF* mRNA expression was normalized to the *18S* ribosomal RNA (rRNA). **b** HUVECs were transfected with a reporter vector including various deleted *BAZF* gene promoters. Luciferase activity was assayed at 18 h post-transfection and was normalized to the activity of thymidine kinase promoter-driven renilla luciferase. **c** The *BAZF* promoter-containing reporter vector was transfected into HUVECs, and luciferase activity was assayed at 6 h post-VEGF-A stimulation (50 ng/mL). Luciferase activity of the NF-κB reporter was also measured as a positive control of VEGF-A stimulation. The experiments were independently performed at least 3 times. *p < 0.05
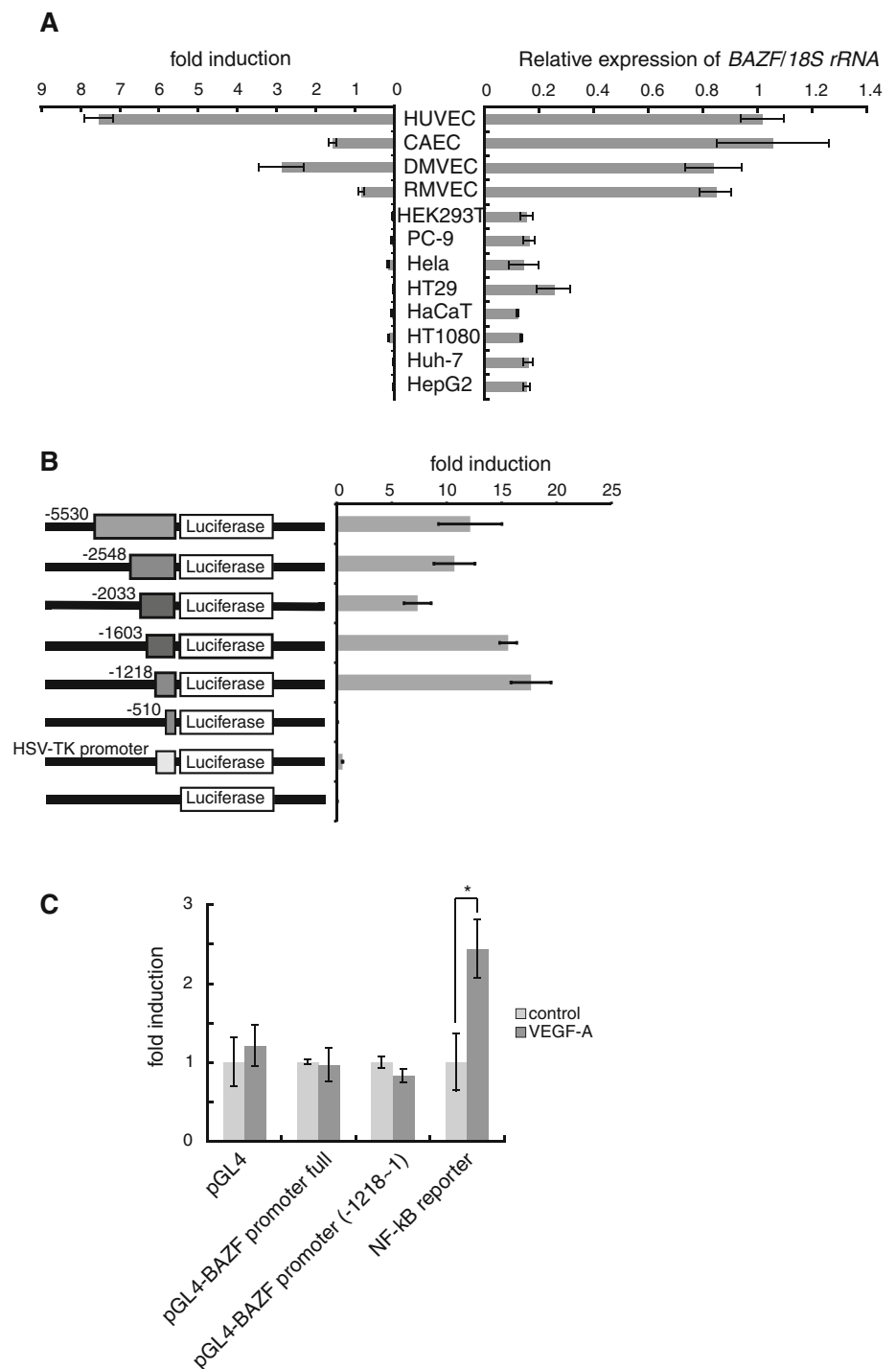
luciferase activity. Pu-H71 significantly inhibited PMA- and CA PRKD2-dependent luciferase activity of pCMV-luc-BAZF 3′UTR (Fig. 5f, g). These data suggested that HSP90β regulated the stability of *BAZF* mRNA through binding to the 3′UTR of *BAZF* mRNA in a VEGF-A/PRKD2 signaling pathway-dependent manner.

To clarify whether HSP90β is a substrate of PRKD2, we performed 2-dimensional electrophoresis and western blotting of KD or CA PRKD2-expressing HUVEC lysates. The data showed that there was no major protein shift for HSP90β between KD and CA PRKD2-expressing HUVECs, while acidic spots representing HSP27 increased in CA PRKD2-expressing HUVEC (Fig. 5h). In an in vitro kinase assay, we also found that the phosphorylation level of HSP90β did not increase in a CA PRKD2-dependent manner (data not shown). To further elucidate the relationship
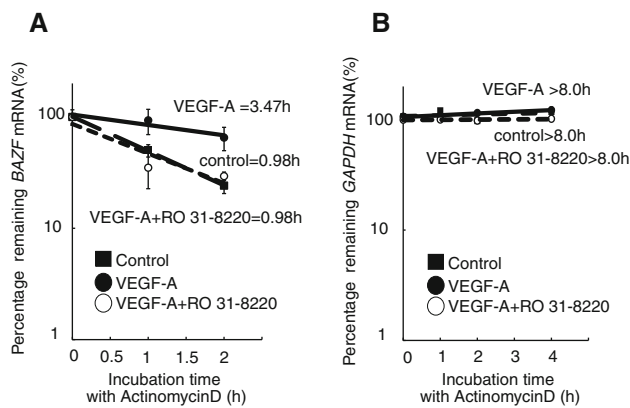
Fig. 3 VEGF-A stabilizes *BAZF* mRNA through a PRKD2-mediated pathway. The half-life of *BAZF* mRNA was measured after actinomycin D treatment to inhibit de novo mRNA synthesis. HUVECs were stimulated by VEGF-A at 30 min after actinomycin D treatment. Total RNA was collected at each indicated time, and *BAZF* (**a**) and *GAPDH* (**b**) mRNA levels were measured by RT-qPCR. Data were normalized to *18S* rRNA levels and represented as a percentage of the mRNA levels before actinomycin D treatment. The experiments were independently performed at least 3 times

between HSP90β and BAZF in angiogenesis, we performed a BAZF-expressing endothelial cell network formation assay using the HSP90 inhibitor Pu-H71. HUVECs were infected with adenovirus containing the cDNA of BAZF without the 3′UTR (AdBAZF) or of GFP (AdGFP). Adenovirus-infected HUVECs (250 m.o.i.) were seeded on Matrigel with or without Pu-H71. Network formation was completely inhibited by Pu-H71 in AdGFP-infected HU-VECs (Fig. 5i). Moreover, the inhibitory effect of Pu-H71 on network formation was partially rescued by BAZF-overexpression. These data suggest that HSP90β may mediate BAZF expression via a downstream angiogenic pathway.

Discussion

VEGF-A induces PRKD2 activation to regulate *BAZF* mRNA stability

The PRKD family is composed of 3 members: PRKD1 (also known as PKCμ [31, 32]), PRKD2 [33], and PRKD3 (also known as PKCν [34]). PRKD1, 2, and 3 share a similar molecular structure, including a tandem repeat of zinc finger-like cysteine-rich motifs at the N-terminus that display high affinity for diacylglycerol (DAG) or phorbol esters, a pleckstrin homology domain, and a catalytic domain in the C-terminal region that shares homology with the calmodulin-dependent kinases [35–37]. PRKD1 is the best characterized isoform of the family and is activated by a variety of growth factors and other stimuli [31, 32].

However, the biological functions of PRKD2 and PRKD3 remain unclear. In a recent study, researchers demonstrated that activation of PRKD1 and PRKD2 was required for VEGF-induced phosphorylation of HSP27, which mediated the migration of ECs [38, 39]. Hao et al. [40] proposed that PRKD2, but not PRKD1, plays a pivotal role in EC proliferation and migration and is necessary for angiogenesis, demonstrating that VEGF-activated PRKD2 modulates VEGFR2 and FGFR1 expression. In our current study, we found that PRKD2 signaling, but not PRKD1 signaling, modulated the expression of pro-angiogenic factor *BAZF*. This finding describes a novel function for PRKD2 and also suggests that PRKD2 activation represents a critical angiogenic signaling molecule, in accordance with the hypothesis of Hao et al.

Numerous mRNAs containing AREs are involved in angiogenesis

AREs are critical *cis*-acting short sequences in the 3′UTR that assemble a set of RNA-binding proteins and regulate mRNA stability [41]. Close to 15 % of the human transcriptome is composed of ARE-containing mRNAs [42], which include functionally diverse groups, such as those involved in the inflammatory and immune response, transcription, cell proliferation, RNA metabolism, development, and signal transduction [43]. AREs in the 3′UTR of mRNAs coding angiogenic genes, such as *VEGF* [44–48], basic FGF (*bFGF*) [49], interleukin-8 (*IL-8*) [47], hypoxia induced factor-α (*HIF1-α*) [50], endothelial nitric oxide synthetase (*eNOS*) [51], and cyclooxygenase (*COX-2*) [47, 52–54], regulate mRNA stability. *BAZF* mRNA also has 3 AREs in the 3′UTR, and we showed that VEGF-induced *BAZF* gene expression is regulated by stabilization of mRNA (Fig. 3a). Indeed, the regulation of pro-angiogenic gene expression through mRNA stabilization seems to be essential for angiogenesis.

The stability of mRNA is determined by not only *cis*-acting elements, such as AREs, but also *trans*-acting RNA-binding proteins that regulate the decay of targeted mRNA. Human antigen R (HuR) has been the most extensively studied mRNA stability factor. HuR is a member of the mammalian homologs of embryonic lethal abnormal vision (ELAV) family proteins. HuR binds to the AREs of several mRNAs coding for angiogenic genes, such as *VEGF*, *TNF-alpha*, *Cox-2*, and *IL-8*, to inhibit mRNA decay [45, 47, 48, 55]. In this report, we also identified HuR as a *BAZF* mRNA-binding protein (data not shown). However, PMA stimulation did not affect the interaction of HuR with the 3′UTR of *BAZF* mRNA. Taken together, these data suggest that HuR may not be a central player in the regulation of angiogenic gene expression by PRKD2 activation.
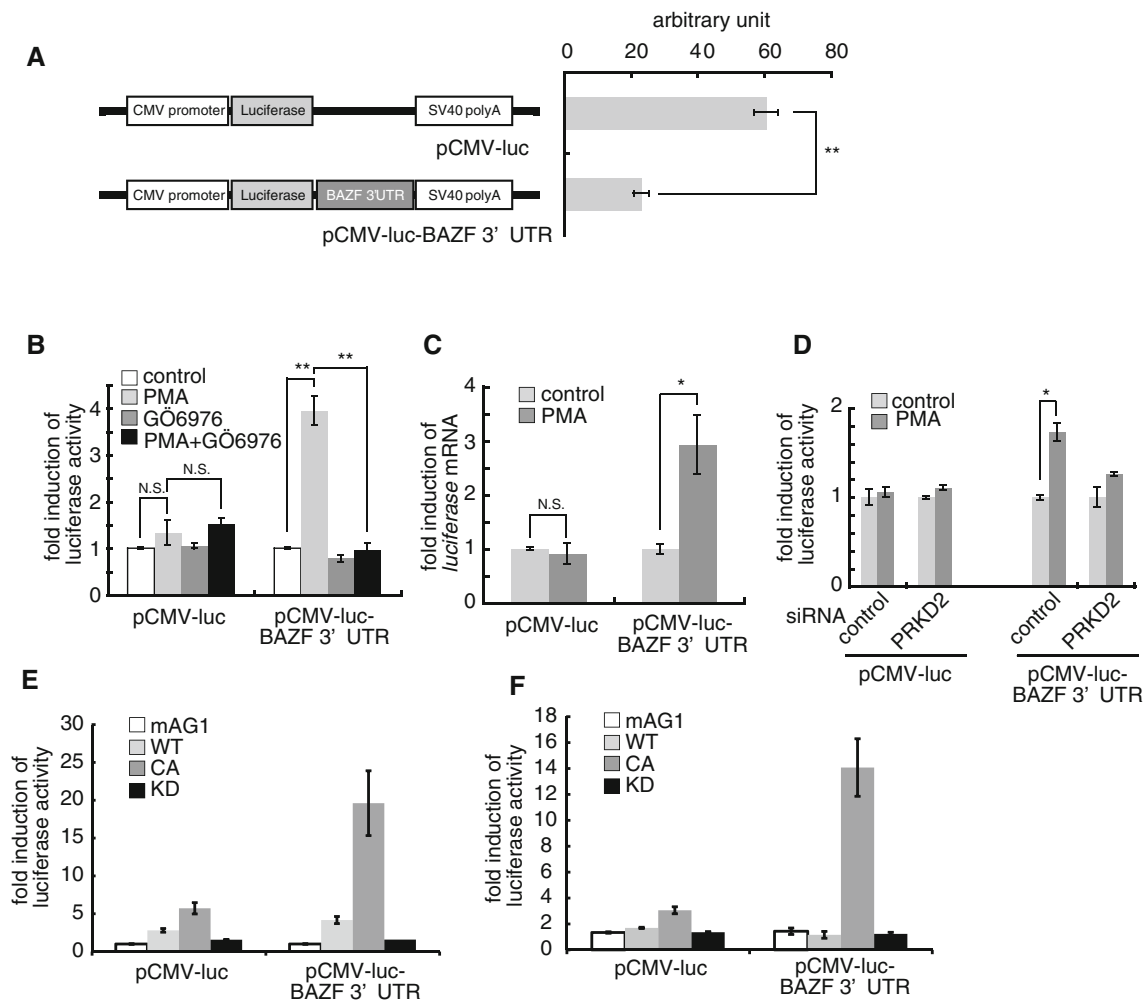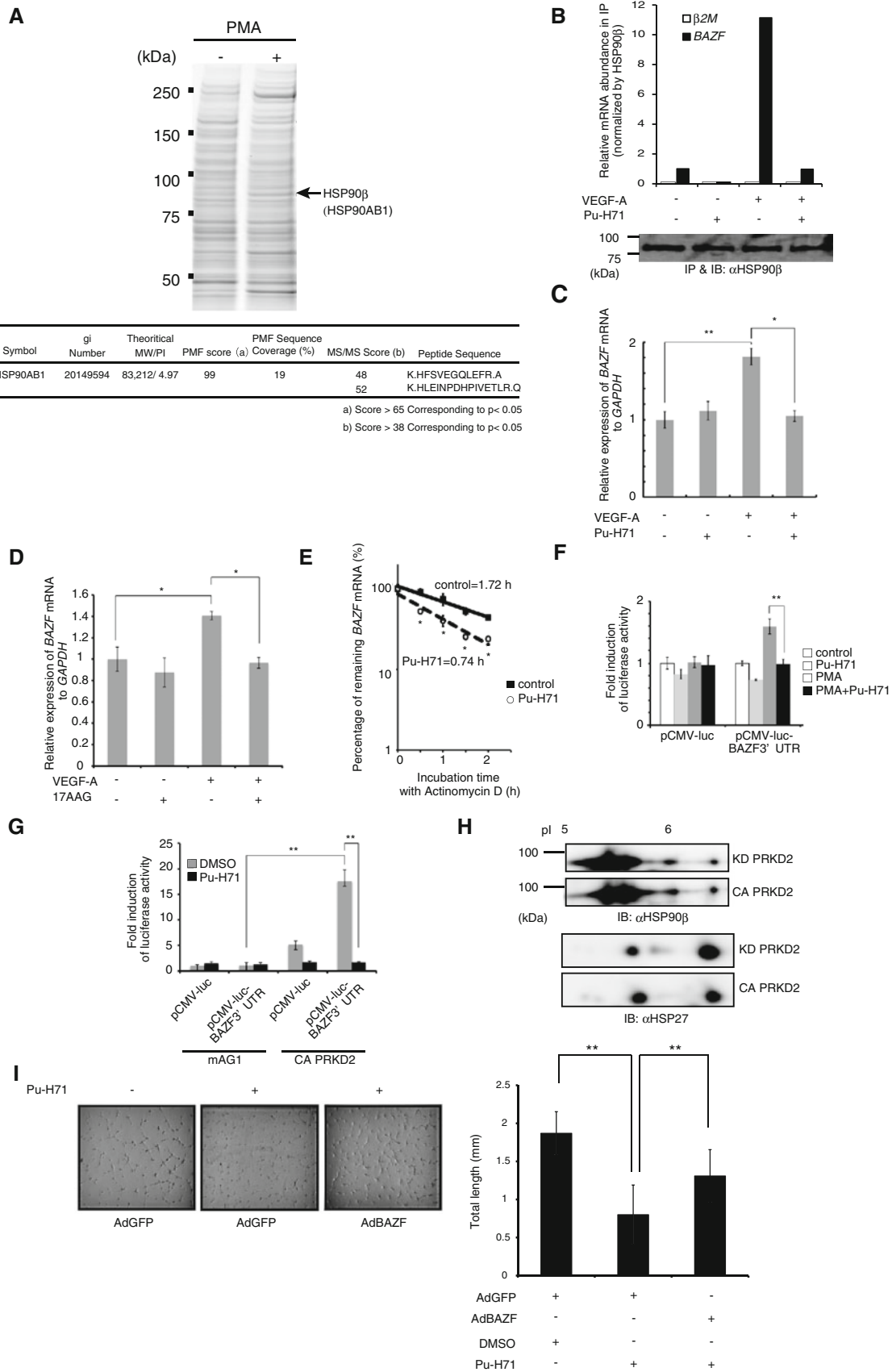
Fig. 4 VEGF-A-induced PRKD2 activation stabilizes *BAZF* mRNA via the 3′UTR. **a** HUVECs were transfected with a reporter vector with or without the 3′UTR of *BAZF* mRNA. Luciferase activity was assayed 18 h post-transfection and was normalized to the activity of thymidine kinase promoter-driven renilla luciferase. **b** The indicated reporter vector-transfected HUVECs were pretreated with Gö6976 for 30 min before PMA treatment and then stimulated with 10 nM PMA for 6 h. Luciferase activity was normalized to *18S* rRNA levels and represented as the fold induction relative to unstimulated cells. **c** Reporter vector-transfected HUVECs were pretreated with actinomycin D before PMA stimulation. **d** siRNA targeting PRKD2 and the indicated reporter genes were sequentially transfected into HUVECs. Next, the HUVECs were stimulated with PMA for 6 h. **e, f** HUVECs were infected with mAG1 or, WT, CA, or KD PRKD2 cDNA-coding lentiviruses. Cells were then transfected with the indicated reporter 30 h later. The HUVECs were incubated without (**e**) or with (**f**) actinomycin D. The experiments were independently performed at least 3 times. * $p < 0.05$; ** $p < 0.01$; *N.S.* not significant

HSP90β regulates mRNA stability of the pro-angiogenic factor *BAZF* via a VEGF/PRKD2 pathway in ECs

In the current study, we found that the *BAZF* 3′UTR interacted with HSP90β, which is regulated by VEGF-A. HSPs are molecular chaperones responsible for the maintenance of cell homeostasis and regulation of cell survival [56]. HSP90, a 90-kDa molecular chaperone, is one of many HSPs in eukaryotes and comprises 1–2 % of the total cellular protein under normal conditions [57–59]. HSP90 is the main functional component of a critical chaperone complex, and many tumors exhibit high expression of HSP90, allowing the activation of tumor-specific signaling pathways and buffering stress conditions in the tumor microenvironment [60]. Therefore, several groups have been working to develop HSP90 inhibitors to target a wide range of malignant tumors [61–63]. Additionally, HSP90 binds ATP in the N-terminal region. However, this ATP-binding function was not required in several chaperone assays, and ATP-nonbinding mutants, although compromised, retain some residual luciferase refolding activity [64]. Moreover, even though HSP90 contains an ATPase domain, the ATP-binding domain shows low intrinsic ATPase activity [65, 66]. These data from previous studies indicate that ATP interactions with HSP90 may play roles other than ATPase and protein refolding. In the current

A

PMA

(kDa)   −   +

250

150

100 → HSP90β (HSP90AB1)

75

50

Symbol	gi Number	Theoritical MW/PI	PMF score (a)	PMF Sequence Coverage (%)	MS/MS Score (b)	Peptide Sequence
HSP90AB1	20149594	83,212/ 4.97	99	19	48	K.HFSVEGQLEFR.A
					52	K.HLEINPDHPIVETLR.Q

a) Score > 65 Corresponding to $p < 0.05$
b) Score > 38 Corresponding to $p < 0.05$

B

Relative mRNA abundance in IP (normalized by HSP90β)

□ β2M
■ BAZF

VEGF-A   −   −   +   +
Pu-H71   −   +   −   +

100
75
(kDa)

IP & IB: αHSP90β

C

Relative expression of BAZF mRNA to GAPDH

VEGF-A   −   −   +   +
Pu-H71   −   +   −   +

D

Relative expression of BAZF mRNA to GAPDH

VEGF-A   −   −   +   +
17AAG   −   +   −   +

E

Percentage of remaining BAZF mRNA (%)

control=1.72 h

Pu-H71=0.74 h

■ control
○ Pu-H71

Incubation time with Actinomycin D (h)

F

Fold induction of luciferase activity

□ control
□ Pu-H71
□ PMA
■ PMA+Pu-H71

pCMV-luc   pCMV-luc-BAZF3' UTR

G

Fold induction of luciferase activity

□ DMSO
■ Pu-H71

pCMV-luc   pCMV-luc-BAZF3' UTR   pCMV-luc   pCMV-luc-BAZF3' UTR

mAG1   CA PRKD2

H

pI 5   6

100   KD PRKD2

100   CA PRKD2

(kDa)   IB: αHSP90β

KD PRKD2

CA PRKD2

IB: αHSP27

Total length (mm)

AdGFP   +   +   −
AdBAZF   −   −   +
DMSO   +   −   −
Pu-H71   −   +   +

I

Pu-H71   −   +   +

AdGFP   AdGFP   AdBAZF

◄ **Fig. 5** PRKD2 activation induces interaction of HSP 90β and *BAZF* mRNA. **a** HUVECs were treated with PMA for 2 h, and the lysate was precipitated with biotinylated *BAZF* mRNA. The precipitated proteins were separated by SDS-PAGE and identified by mass spectrometry. The *arrow* indicates HSP90β. **b** The cell lysate from HUVECs with the indicated treatments was incubated with anti-HSP90β. RNA was isolated from the immunoprecipitates, and *β2-microgloblin* (*β2M*) or *BAZF* mRNA was detected by RT-qPCR. The mRNA abundance was normalized by the HSP90β protein level which was analyzed by ImageJ software. The *bottom panel* shows the protein level of immunoprecipitated HSP90β in each cell lysate. **c, d** HUVECs were pretreated with Pu-H71 (1 μM) (**c**) or 17AAD (1 μM) (**d**). HSP90β-inhibitor-treated HUVECs were stimulated with VEGF-A for 2 h. **e** The half-life of *BAZF* mRNA was measured by incubating HUVECs with actinomycin D. Total RNA was collected at each of the indicated times after actinomycin D treatment, and *BAZF* mRNA levels were measured by RT-qPCR. Thirty minutes after actinomycin D pretreatment, HUVECs were treated with Pu-H71. Data were normalized to the *18S* rRNA and represented as a percentage of the mRNA levels before actinomycin D treatment. (F) HUVECs transfected with reporter vectors were pretreated with Pu-H71 for 30 min and then exposed to 10 nM PMA for 6 h. Luciferase activity was assayed at 18 h post-transfection and was normalized as described above. Data were presented as the fold induction relative to the luciferase activity of the control. **g** mAG1 or CA PRKD2-expressing HUVECs were transfected with each reporter vector. HUVECs were treated with Pu-H71 at 6 h after transfection, and luciferase activity was measured at 18 h post-transfection. **h** Cell lysates from KD and CA PRKD2-expressing HUVECs were subjected to 2-dimensional gel electrophoresis. HSP90β and HSP27 were detected by appropriate antibodies. **i** HUVECs were infected with green fluorescence protein (GFP)- or BAZF-expressing adenoviruses at 250 m.o.i. Transfected cells were seeded on Matrigel with or without Pu-H71 treatment. The total length of each network was measured and calculated in Image-Pro Plus software (Roper). The experiments were independently performed at least 3 times. * $p < 0.05$; ** $p < 0.01$

study, we demonstrated that the HSP90 inhibitors 17-AAG and Pu-H71 inhibited PRKD2-mediated stabilization of *BAZF* mRNA. 17-AAD binds to the ATP-binding pocket of the N-terminal region in HSP90 to associate with adenosine

di- or triphosphate [67, 68]. On the other hand, purine scaffolds, such as Pu-H71, also interact with the ATP-recognition site in the N-terminal region of HSP90 [68]. Nucleotides such as mRNA could also interact with the N-terminal region of HSP90β, and these HSP90 inhibitors may compete with mRNA to bind to HSP90β.

A recent study revealed that HSP70 regulates the stability of *Bim* mRNA in a cytokine-dependent manner [69]. HSP70 binds to AREs in the 3′UTR of specific mRNAs and enhances the stability. Another report also indicated that HSP90 can stabilize *BCL-6* mRNA and protein in lymphoma cells [28]. The 3′UTR of *BCL-6* also has several AREs. These studies suggest that HSP family proteins work not only as protein chaperones, but also as mRNA stabilizers.

Phosphorylation of serine and/or threonine residues on HSP90 has been shown to regulate HSP90 activity to induce the conformational maturation of oncogenic proteins such as ErbB2, c-Src, Akt, and Raf-1 [70, 71]. HSP90β has several potential PRKD phosphorylation sites. However, 2-dimensional gel electrophoresis revealed that the phosphorylation state of HSP90β did not change with either CA or KD PRKD2 overexpression (Fig. 5h). HSP90β may be indirectly regulated by PRKD2 activation. As a next step to understanding PRKD2-HSP90β-regulated mRNA stabilization, we need to identify all of the components complexed with HSP90β after PRKD2 activation.

In this report, we found that pro-angiogenic *BAZF* mRNA stability was regulated by a PRKD2-HSP90β-dependent pathway (Fig. 6). The current study suggested that activated ECs could quickly enter into an angiogenic state from angiogenic quiescence through stabilization of mRNA coding for pro-angiogenic genes, such as *BAZF*. Further studies on the molecular mechanisms of this mRNA stabilization

Fig. 6 Scheme of the molecular mechanism. VEGF-A stimulation induces activation of PRKD2 through VEGFR activation. Activation of PRKD2 leads to recruitment of several molecules, including HSP90β, to the 3′UTR of *BAZF* mRNA, inhibiting the decay of mRNA. As a result, increased *BAZF* translation promotes angiogenesis progression
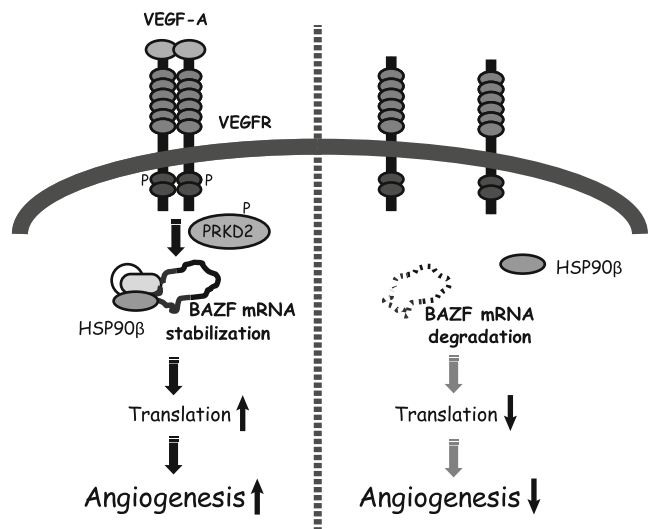
system may help to identify new targets for the treatment of angiogenesis-associated diseases.

Conflict of interest The authors declare that there are no conflicts of interest that would prejudice the impartiality of this scientific work.

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