p85α mediates NFAT3-dependent VEGF induction in the cellular UVB response

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Summary

Exposure to solar ultraviolet B (UVB) radiation is known to induce several pathological reactions in the skin. In these processes, upregulation of VEGF expression has been demonstrated to be important in angiogenesis-associated photodamage and even skin cancers. However, the signaling events that are responsible for VEGF induction under UVB exposure have not been fully defined. Here, we demonstrate that the regulatory subunit of the phosphoinositide 3-kinase (PI3K), p85α, plays a role in mediating UVB-induced VEGF expression in mouse embryonic fibroblasts (MEFs) and mouse epithermal cells, the effect of which is unrelated to the PI3K activity. The transcriptional factor NFAT3 functions as a downstream target of p85α to mediate the induction of VEGF expression in the UVB response. Although lacking NFAT3-binding ability, p85α is required for the recruitment of NFAT3 to the NFAT-response element within the *vegf* promoter. Furthermore, by identifying the adjacent NFAT- and AP-1-binding sites within the *vegf* promoter, we also found an induced interaction between NFAT3 and one of the AP-1 components, c-Fos, after UVB irradiation. Without the aid of c-Fos, NFAT3 lost its *vegf*-promoter-binding ability. Taken together, our results reveal a novel PI3K-independent role for p85α in controlling VEGF induction during the cellular UVB response by regulating NFAT3 activity. Targeting p85α might be helpful for preventing UVB-induced angiogenesis and the associated photodamage.

Key words: UVB, p85α, VEGF, NFAT3, c-Fos

Introduction

Exposure to solar ultraviolet B (UVB) radiation (280–320 nm wavelengths), a ubiquitous environmental stress factor, is known to induce a variety of pathologies. Besides causing the permanent genetic changes in the DNA damage response, UVB irradiation induces the production and secretion of several cytokines that can subsequently mediate the inflammatory and angiogenic responses (Fisher et al., 2002; Ichihashi et al., 2003; Lee et al., 2005; Marrot and Meunier, 2008; Yaar and Gilchrest, 2007). There is increasing evidence that angiogenesis, mainly in the generation of new capillary blood vessels, contributes largely to the onset of skin malignancy and tumor expansion in the cellular UVB response. Among a variety of the angiogenic growth factors, vascular endothelial growth factor (VEGF) expression appears to be highly susceptible to UVB irrradiation, which plays a predominant role in mediating angiogenesis-associated photodamage and carcinogenesis (Clydesdale et al., 2001; Hirakawa et al., 2005; Li et al., 2006). However, the molecular mechanisms related to UVB-induced VEGF expression are still largely unknown.

Class IA phosphoinositide 3-kinase (PI3K) is a central component in the transduction of the growth factor signals and comprises a p110 catalytic subunit and a regulatory subunit. At least five isoforms of the regulatory subunit of PI3K, derived from three distinct genes, have been identified in the mammalian cells: p85α and its two truncated splicing versions, p55α and p50α, p85β and p55γ. Of these isoforms, p85α is predominantly and ubiquitously expressed in most tissues and is known to play a major role in response to most stimuli (Geering et al., 2007; Luo and Cantley, 2005). Apart from forming a complex with the p110 catalytic subunit and regulating PI3K activity, p85α also serves as a crucial mediator in various physiological processes via PI3K-independent mechanisms (Brachmann et al., 2005; Luo and Cantley, 2005; Luo et al., 2005; Taniguchi et al., 2007; Ueki et al., 2002; Ueki et al., 2003; Yin et al., 1998). However, how p85α functions in a PI3K-independent manner under stress conditions is still elusive.

Our group is interested in clarifying the PI3K-independent role of p85α in the cellular UVB response. Therefore, primary mouse embryonic fibroblasts (MEFs) isolated from specific p85α gene knockout mice and the corresponding wild-type mice with an otherwise identical genetic background were used in our studies (Song et al., 2011; Song et al., 2007). We have demonstrated that only p85α expression is specifically abolished in the $p85\alpha^{-/-}$ MEFs as a result of a single deletion of exon 1A (containing the initiation codon for the p85α gene, *Pik3r1*), but the levels of other isoforms of PI3K regulatory subunit (p50α, p55α and p85β) are comparable in the wild-type (WT) and $p85\alpha^{-/-}$ MEFs. Most importantly, p85α deficiency does not affect the activation status of the PI3K-dependent pathway in the UVB-treated $p85\alpha^{-/-}$ MEFs, probably due to the compensatory effect of other PI3K regulatory subunit isoforms (Song et al., 2007). Therefore, we believe that $p85\alpha^{-/-}$ MEFs are an ideal model to investigate the

PI3K-independent function of p85α. According to our previous results, p85α is involved in both UVB-induced DNA damage and inflammatory responses, the effects of which are unrelated to PI3K activity but are elicited by regulating the transactivation of p53 and the induction of TNFα expression, respectively (Song et al., 2011; Song et al., 2007). In the current study, we define a novel and an additional role for p85α in mediating UVB-induced VEGF expression by modulating NFAT3 activity. The established function of p85α in mediating the angiogenic response under UVB might provide a new approach for the prevention of UVB-induced photodamage.

Results and Discussion

p85α is required for UVB-induced VEGF expression in the MEFs

We demonstrated in our recent study that UVB exposure induces a significant increase of VEGF expression in the mouse fibroblasts, which is mediated by IKKα-dependent AP-1 transactivation (Dong et al., 2012). Since p85α has been proved to play a crucial role in regulating p53 transactivation and TNFα expression, and therefore mediate both DNA damage and inflammatory injury under UVB irradiation (Song et al., 2011; Song et al., 2007), we next wanted to address whether p85α also contributes to VEGF expression and therefore participates in the angiogenesis-associated photodamage.

To this end, a VEGF luciferase reporter plasmid containing ~3.0 kb of the *vegf* promoter was transfected into p85α$^{+/+}$ and p85α$^{-/-}$ MEFs. When these cells were exposed to different doses of UVB, we repeatedly found a dose-dependent increase of *vegf*-promoter-dependent luciferase activities in the wild-type MEFs, but this effect was almost totally blocked in the p85α$^{-/-}$ MEFs under the same UVB exposure conditions (Fig. 1A). To further confirm this result, enzyme-linked immunosorbent assay (ELISA) was performed to detect the VEGF protein levels in the supernatants of p85α$^{+/+}$ and p85α$^{-/-}$ MEFs irradiated by a single dose of UVB (0.5 kJ/m^2). As shown in Fig. 1B, UVB exposure induced a significant time-dependent VEGF production in the WT MEFs, whereas this response was dramatically suppressed by *p85α* deficiency. Moreover, when the p85α gene was re-introduced into the p85α$^{-/-}$ MEFs, UVB-induced VEGF expression was restored upon p85α reconstitution (Fig. 1C). These results together indicate that p85α is required for UVB-induced VEGF expression in the MEFs.

To further address whether p85α mediates VEGF induction via a PI3K-dependent or -independent pathway, WT MEFs were pre-treated with PI3K inhibitor, LY294002, followed by UVB exposure. As shown in Fig. 1D, LY294002 pre-treatment did not affect the induction of VEGF expression in the WT MEFs. Furthermore, reconstitution of p85α$^{-/-}$ MEFs with Δp85α, the p85α mutant lacking p110-binding ability, rescued UVB-induced VEGF expression in the p85α$^{-/-}$ MEFs to the similar level as the wild-type p85α did (Fig. 1E). Therefore, we believe that role of p85α in regulating VEGF expression is unrelated to the PI3K-dependent pathway.

To clarify whether p85α can exert a general effect on the regulation of VEGF expression under UVB irradiation, we next detected the levels of VEGF induction in the UVB-treated mouse epidermal cells, Cl41, in the absence or presence of p85α expression. We found that knockdown of p85α expression in the Cl41 cells significantly inhibited VEGF induction after UVB exposure (Fig. 1F). These data suggest that the role for p85α in regulating VEGF induction might be a general effect in the UVB response.

NFAT3 functions as the downstream target of p85α responsible for VEGF induction in the UVB response

In the following study, we tried to reveal how p85α is involved in VEGF induction in response to UVB irradiation. In the attempt to find the downstream target of p85α in the cellular UVB response, we compared the activities of a variety of protein kinases and transcriptional factors in UVB-treated WT and p85α-null cells in our previous study and obtained evidence of some p85α-regulated signaling molecules including NFAT3 (Song et al., 2007). We found a putative NFAT-responsive element (−2908 to −2903) located adjacent to one of the AP-1-binding sites (−2900 to −2892) within the *vegf* promoter (Fig. 2A). Therefore, we thought that NFAT3 might function as the downstream target of p85α in the regulation of UVB-induced VEGF expression. To address this possibility, WT MEFs stably transfected with the NFAT luciferase reporter plasmid were exposed to different doses of UVB irradiation and then NFAT transactivation was detected. As shown in Fig. 2B, UVB irradiation could induce a dose-dependent increase of NFAT transactivity in the WT MEFs. Because NFAT3 is the predominant NFAT family member expressed in MEFs (Song et al., 2007), we next tested whether NFAT3 activation contributes to *vegf* transcription upon UVB treatment. For this purpose, a NFAT3 shRNA expression plasmid was transfected into WT MEFs to establish the stable transfectants with extremely low NFAT3 expression (Fig. 2C). We found that UVB-induced VEGF expression was significantly inhibited in the NFAT3 shRNA transfectants compared with that in the control shRNA-transfected cells (Fig. 2D). These data indicate that NFAT3 functions as the downstream target of p85α to mediate VEGF induction in the UVB response.

p85α does not interact with NFAT3, but is required for the recruitment of NFAT3 to the *vegf* promoter in the UVB response

In our previous study, we proved that p85α is critical for the nuclear translocation of NFAT3, one of the key steps for the activation of this transcription factor, upon UVB irradiation (Song et al., 2007). Since binding partners are known to be important for the nuclear accumulation of NFAT family members (Macián et al., 2001; Müller and Rao, 2010) we considered that p85α might interact with NFAT3 and facilitate its nuclear distribution in the UVB response. To address this possibility, FLAG-NFAT3 and GFP-p85α expression plasmids were co-transfected into the WT MEFs and the possible interaction between NFAT3 and p85α was detected by immunoprecipitation. As shown in Fig. 3A, although these two proteins were expressed at high levels in the WT cells, no p85α–NFAT3 binding signals were observed either in the untreated or UVB-irradiated cells. No endogenous p85α–NFAT3 complex formation was obtained in WT MEFs, either (Fig. 3B). These results indicate that p85α does not interact with NFAT3 directly, but can facilitate NFAT3 nuclear translocation via a currently unidentified mechanism in the UVB response.

Recruitment to the target promoter DNA is another crucial step for NFAT to mediate the expression of NFAT-responsive genes (Macián et al., 2001; Müller and Rao, 2010). Therefore, a chromatin immunoprecipitation (ChIP) assay was performed to test whether p85α regulates the promoter-binding ability of

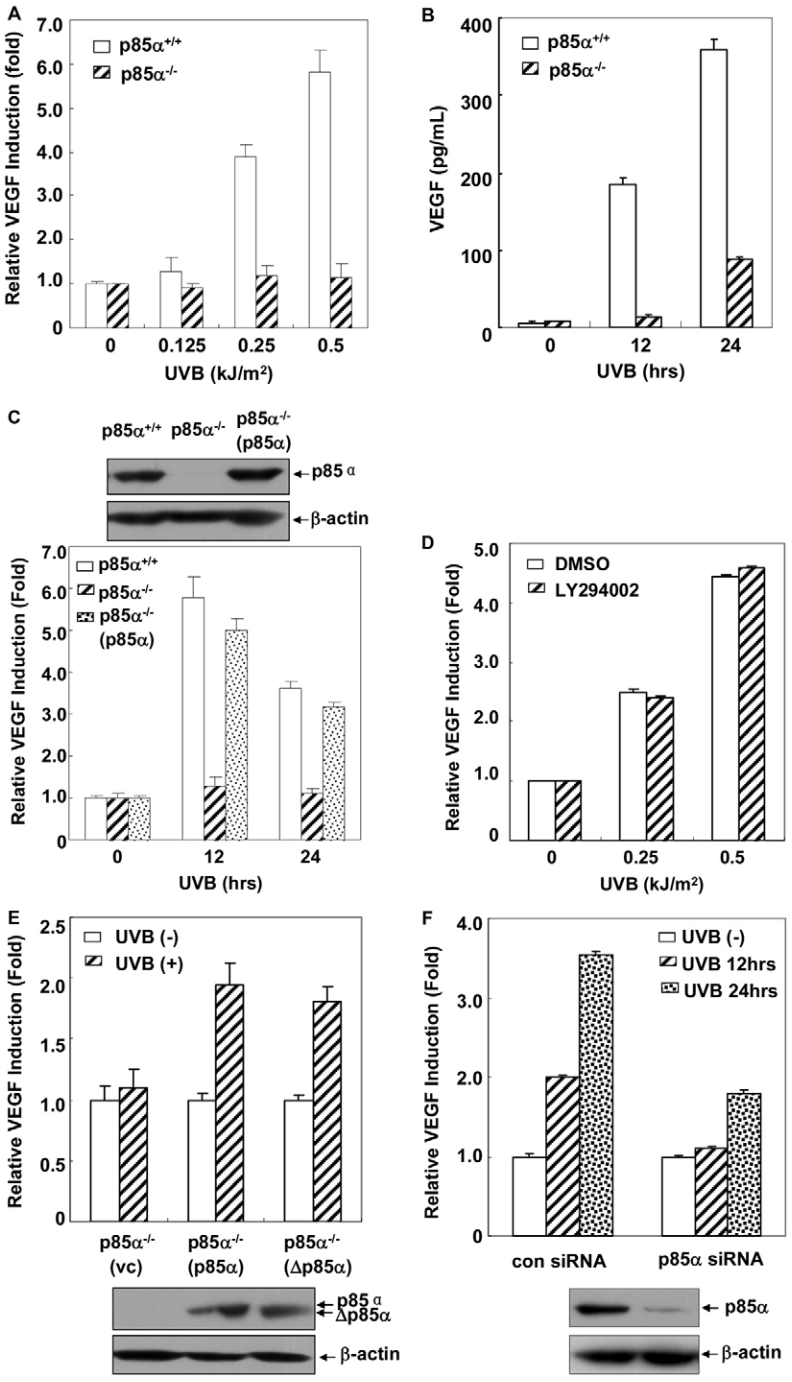

Fig. 1. p85α is required for UVB-induced VEGF expression in MEFs. (**A**) $p85\alpha^{+/+}$ and $p85\alpha^{-/-}$ MEFs were transfected with a VEGF-promoter-driven luciferase reporter plasmid and then exposed to different doses of UVB. The VEGF luciferase activities were detected 12 hours after UVB exposure. The results are expressed as the relative VEGF induction normalized to the luciferase activity of the cells without any treatment. (**B**) $p85\alpha^{+/+}$ and $p85\alpha^{-/-}$ MEFs were exposed to UVB (0.5 kJ/m^2) and then the VEGF in the culture medium 24 hours later was detected by ELISA. (**C**) $p85\alpha^{+/+}$, $p85\alpha^{-/-}$ and reconstituted $p85\alpha^{-/-}$(p85α) MEFs were transfected with the VEGF-promoter-driven luciferase reporter plasmid and then exposed to UVB (0.5 kJ/m^2) 36 hours after transfection. VEGF luciferase activities were detected at the indicated time points after UVB exposure. (**D**) $p85\alpha^{+/+}$ MEFs were transfected with a VEGF-promoter-driven luciferase reporter plasmid and then exposed to the PI3K inhibitor, LY294002 (20 μM), for 1 hour followed by treating with different doses of UVB. The VEGF luciferase activities were detected at 12 hours after UVB exposure. (**E**) $p85\alpha^{-/-}$ MEFs were transfected with the wild-type p85α or Δp85α expression plasmid in combination with the VEGF-promoter-driven luciferase reporter plasmid. The cells were exposed to UVB 36 hours after transfection and the luciferase activities were detected 12 hours after UVB exposure. (**F**) Cl41 cells were transfected with p85α siRNA or its control siRNA in combination with the VEGF-promoter-driven luciferase reporter plasmid and then exposed to UVB (0.5 kJ/m^2) 36 hours after transfection. The VEGF luciferase activities were detected at 12 hours after UVB exposure.

NFAT3. As shown in Fig. 3C, a significant enrichment of *vegf*-promoter-bound NFAT3 was observed in the UVB-treated WT MEFs, indicating the inducible recruitment of NFAT3 to the endogenous *vegf* promoter during the UVB response. No signal was observed in the control-IgG-immunoprecipitated samples, supporting the specificity of this assay. Under the same UVB exposure conditions, the enrichment of NFAT3 at the target promoter DNA was impaired in the p85α-null cells and obviously rescued in the p85α-reconstituted p85α-null cells (Fig. 3C). These data together indicate that the presence of p85α is required for the recruitment of NFAT3 to the *vegf* promoter upon UVB irradiation.

Interaction with c-Fos is essential for NFAT3 recruitment to the *vegf* promoter under UVB irradiation

Cooperation between NFAT and AP-1 is crucial for the induced transcription of diverse genes during the inflammatory response (Macián et al., 2001; Müller and Rao, 2010). By identifying the adjacent NFAT- and AP-1-binding sites within the *vegf* promoter and the roles of both in VEGF induction in the UVB response, we next asked whether concomitant activation of NFAT and AP-1 is required for VEGF expression upon UVB exposure. To this end, WT MEFs were transfected with FLAG-NFAT3 expression plasmid and then left untreated or exposed to UVB radiation. When the cell lysates were immunoprecipitated with the

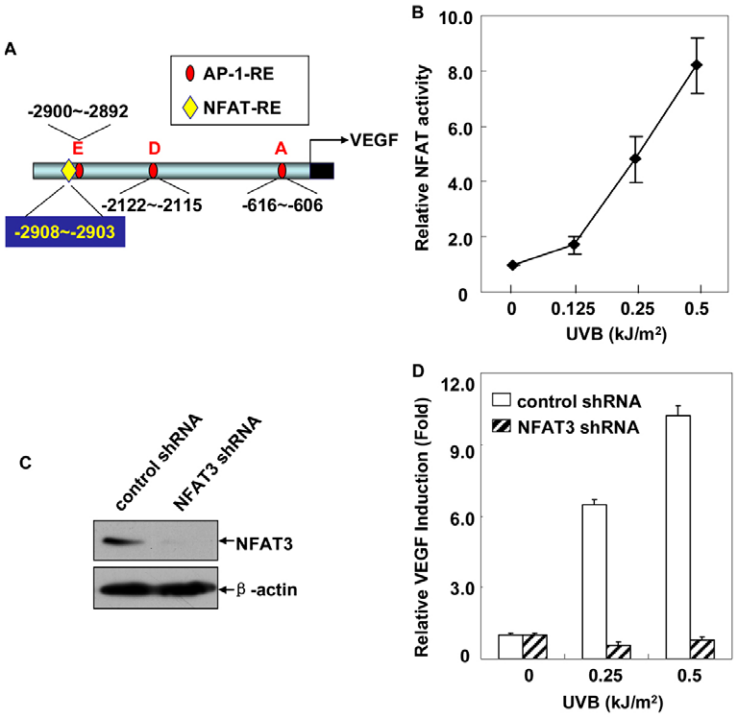

Fig. 2. NFAT3 is involved in UVB-induced VEGF expression in MEFs. (**A**) Identification of a putative NFAT-responsive element (−2908 to −2903) located adjacent to one of the AP-1-binding sites (−2900 to −2892) within the *vegf* promoter. (**B**) An NFAT-dependent luciferase reporter plasmid was introduced into the WT MEFs and then stable transfectants were established. The cells were exposed to different doses of UVB and then the induction of NFAT-dependent luciferase activities was detected 12 hours after UVB exposure. (**C**) WT MEFs were transfected with the NFAT3 shRNA or control shRNA expression plasmids and then stable transfectants were established. The efficiency of the NFAT3 shRNA was determined by western blot assay. (**D**) WT/control shRNA and WT/NFAT3 shRNA cells were transfected with the VEGF luciferase reporter plasmid and then exposed to different doses of UVB. The VEGF luciferase activities were determined 12 hours after UVB exposure.

anti-FLAG antibody, a significant binding of c-Fos, one of the AP-1 components, with the exogenous FLAG–NFAT3 was readily observed in the UVB-treated samples (Fig. 4A). We also found interaction of c-Fos with endogenous NFAT3 in the UVB-treated WT MEFs (Fig. 4B). These data indicate that NFAT3 interacts with c-Fos upon UVB irradiation and therefore might regulate VEGF expression in a c-Fos/AP-1-dependent manner. To address this possibility, WT MEFs were stably transfected with c-Fos

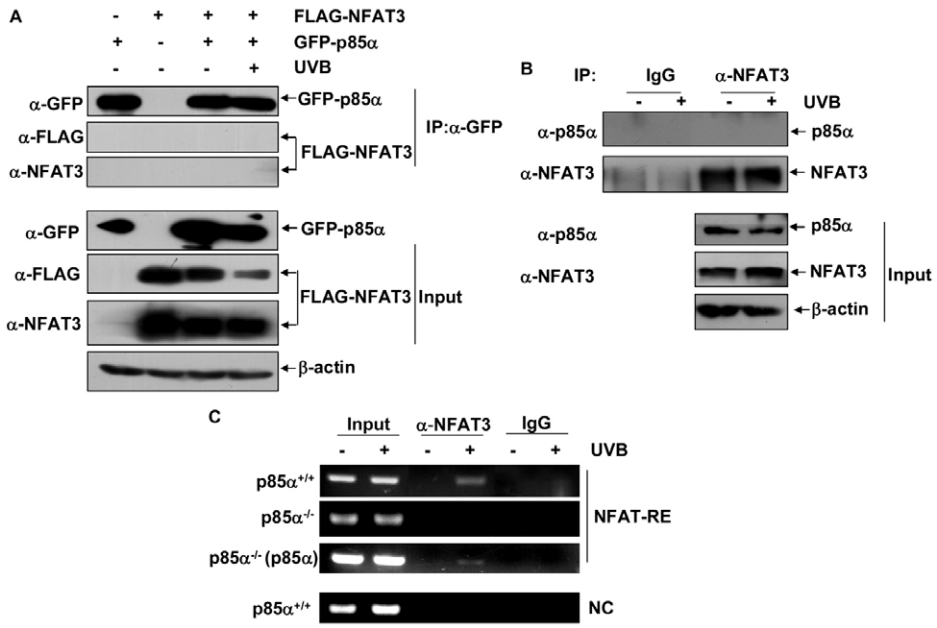

Fig. 3. p85α does not interact with NFAT3, but is required for the recruitment of NFAT3 to the *vegf* promoter. (**A**) WT MEFs were transfected with FLAG-NFAT3 or GFP-p85α expression plasmids separately or together and then left untreated or exposed to UVB (0.5 kJ/m^2). The cell lysates were immunoprecipitated with anti-GFP antibody and then the possible interaction between NFAT3 and p85α was determined. (**B**) WT MEFs were left untreated or exposed to UVB (0.5 kJ/m^2). The cell lysates were immunoprecipitated with control IgG or anti-NFAT3 antibody and then the possible interaction between endogenous NFAT3 and p85α was detected. (**C**) p85α$^{+/+}$, p85α$^{-/-}$ and the reconstituted p85α$^{-/-}$ (p85α) MEFs were exposed to UVB radiation (0.5 kJ/m^2) for 10 hours. Then the soluble chromatin was prepared and immunoprecipitated with an anti-NFAT3 antibody or control IgG. The DNA extractions were amplified with primers that covered the putative NFAT- and AP-1-binding sites within the *vegf* promoter or the coding sequence in the third exon of the *vegf* gene, which was used as the negative control (NC).

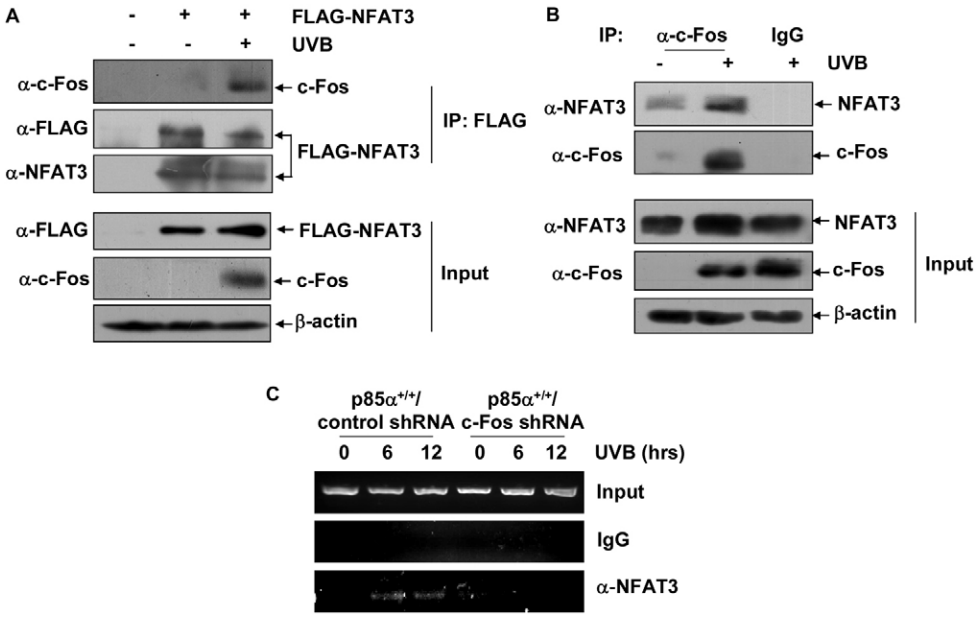

Fig. 4. NFAT3 interacts with c-Fos and is recruited to the *vegf* promoter to regulate VEGF induction in a c-Fos-dependent manner. (**A**) WT MEFs were transfected with FLAG-NFAT3 expression plasmid and then left untreated or exposed to UVB (0.5 kJ/m^2) for 8 hours. Then the cell lysates were immunoprecipitated with anti-FLAG antibody and the interaction of the exogenous NFAT3 with the endogenous c-Fos under UVB irradiation was detected. (**B**) WT MEFs were either untreated or exposed to UVB (0.5 kJ/m^2) for 8 hours. Then the cell lysates were immunoprecipitated with anti-c-Fos antibody or control IgG and the interaction of endogenous NFAT3 with c-Fos under UVB irradiation was detected. (**C**) WT cells were transfected with control shRNA or c-Fos shRNA and then stable transfectants were established. The cells were subjected to UVB irradiation (0.5 kJ/m^2) for 10 hours and then a ChIP assay was performed as described in Fig. 3C.

shRNA or control shRNA and then subjected to a ChIP assay to compare the interaction of NFAT3 with the *vegf* promoter in the absence or presence of c-Fos expression. As shown in Fig. 4C, NFAT3 almost lost its *vegf*-promoter-binding ability when c-Fos induction was blocked by its specific shRNA. These data indicate that interaction with c-Fos is required for NFAT3 to associate with the *vegf* promoter and exert its regulatory role in VEGF induction under UVB irradiation.

In summary, this study has revealed a novel signaling cascade controlling VEGF expression upon UVB exposure, which is mediated by p85α-dependent NFAT3 activation. Moreover, taken together with our previous study (Dong et al., 2012), the induced interaction between NFAT3 and c-Fos, indicates a synergistic effect and convergence of IKKα–AP-1 and p85α–NFAT3 signaling pathways on UVB-induced VEGF expression. Therefore, our findings contribute to a greater understand of the signaling events that regulate skin angiogenesis following UVB irradiation and might provide a new approach for the prevention of angiogenesis-related skin damage by targeting p85α or IKKα.

Materials and Methods

Cells, plasmids, siRNA and antibodies

The p85α$^{+/+}$, p85α$^{-/-}$ and the reconstituted p85α$^{-/-}$(p85α) MEFs, Cl41 cells, and the VEGF– and NFAT–luciferase reporter plasmids have been described in our previous studies (Song et al., 2011; Song et al., 2007). The FLAG-NFAT3 expression plasmid was provided by Dr Qinong Ye (Beijing Institute of Biotechnology, China). NFAT3 and c-Fos shRNA expression plasmid were described in our previous studies (Dong et al., 2012; Song et al., 2007). GFP-p85α expression plasmid was described in our previous study. Δp85α expression plasmid was constructed by deleting the p110-binding domain (amino acids 479–513) in the wild-type p85α as described previously (Hara et al., 1994). Mouse p85α siRNA (no. 6912) and the antibodies against p85α and GFP were purchased from Cell Signaling Technology (Beverly, MA). The antibodies against c-Fos, NFAT3 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG antibody was obtained from Sigma (St Louis, MO).

Cell culture and transfection

All MEFs were maintained in DMEM supplemented with 10% FBS. Cl41 cells were maintained in MEM supplemented with 5% FBS. The transfections were performed with LipofectAMINE or iMAX (Life Technologies, Inc., Rockville, MD) according to the manufacturer's instructions. To establish the stable transfections, cultures were subjected to either G418 or hygromycin B selection.

Luciferase reporter assay

Cells were transiently or stably transfected with the luciferase reporter constructs. The luciferase activities were tested as described previously (Dong et al., 2012).

Enzyme-linked immunosorbent assay

VEGF production in the cell culture supernatants was quantified using a mouse VEGF immunoassay kit (R&D Systems, Inc., Minneapolis, MN) as described previously (Dong et al., 2012).

Immunoprecipitation and western blot assay

WT MEFs were left untreated or transfected with FLAG-NFAT3 expression plasmid, and then exposed to UVB for 8 hours. Cell lysates were immunoprecipitated with either anti-c-Fos or anti-FLAG antibody and then immunoblotted with anti-NFAT3 or anti-c-Fos antibodies to detect the interaction of c-Fos with the endogenous or exogenous NFAT3. For the co-immunoprecipitation experiment, FLAG-NFAT3 and GFP-p85α expression plasmids were co-transfected into HEK-293T cells, which were then exposed to UVB for 8 hours. Cell lysates were immunoprecipitated with an anti-GFP antibody and then the immunoprecipitants were subjected to a western blot assay with an anti-FLAG and anti-NFAT3 antibodies. The signals were detected as described in our previous reports (Dong et al., 2012; Song et al., 2011).

ChIP assay

The ChIP assay was performed using an EZ ChIP kit (Upstate Biotechnology) as described previously (Dong et al., 2012; Song et al., 2011; Song et al., 2007). Owing to adjacent NFAT (−2908 to −2903) and AP-1 (−2900 to −2892)-responsive elements within the mouse *vegf* promoter, the PCR primers (−3079 to −2783) previously used to specifically amplify the regions containing AP-1 binding sites were also subjected to the detection of the DNA fragments containing NFAT3-binding sites. The primers to amplify the region covering the third exon of the *vegf* gene were used as a negative control (NC) in this ChIP experiment (Dong et al., 2012).

Author contributions

W.D., Y.L., X.L., M.H. and A. performed the experiments; L. Sun, N.G. and S.Y. provided technical support. L. Song designed the experiments and wrote the manuscript.

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