

Von Hippel-Lindau regulates interleukin-32 β stability in ovarian cancer cells

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Keywords: interleukin-32, von Hippel-Lindau, protein kinase C, hypoxia, apoptosis

Received: March 29, 2017

Accepted: June 12, 2017

Published: July 17, 2017

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ABSTRACT

Hypoxia-induced interleukin-32 β (IL-32 β) shifts the metabolic program to the enhanced glycolytic pathway. In the present study, the underlying mechanism by which hypoxia-induced IL-32 β stability is regulated was investigated in ovarian cancer cells. IL-32 β expression increased under hypoxic conditions in ovarian cancer cells as it did in breast cancer cells. The amount of IL-32 β was regulated by post-translational control rather than by transcriptional activation. Under normoxic conditions, IL-32 β was continuously eliminated through ubiquitin-dependent degradation by the von-Hippel Lindau (VHL) E3 ligase complex. Oxygen deficiency or reactive oxygen species (ROS) disrupted the interaction between IL-32 β and VHL, leading to the accumulation of the cytokine. The fact that IL-32 β is regulated by the energy-consuming ubiquitination system implies that it plays an important role in oxidative stress. We found that IL-32 β reduced protein kinase C δ (PKC δ)-induced apoptosis under oxidative stress. This implies that the hypoxia- and ROS-stabilized IL-32 β contributes to sustain survival against PKC δ -induced apoptosis.

INTRODUCTION

Interleukin-32 (IL-32) was originally identified as a secretory protein that enhances the production of pro-inflammatory cytokines, including IL-1, IL-6, and tumor necrosis factor α (TNF- α). Later, it was unveiled that IL-32 exists intracellularly in nonimmune cells such as epithelial and tumor cells [1–3]. IL-32 has nine isoforms that are generated by the alternative splicing of seven exons [4]. Functional studies of IL-32 in cancer cells have focused mainly on the IL-32 α , IL-32 β , and IL-32 γ isoforms because of their abundance [5]. IL-32 α and IL-32 β are

highly expressed in gastric, lung, pancreatic, breast, brain, and liver tumors [6–8], but they show context-dependent function in different tumors. For example, IL-32 α shows anti-proliferative effects in human colon cancer cells [9–11], whereas it enhances migration in human melanoma cells through the downregulation of E-cadherin [12] and promotes proliferation in pancreatic cells [6]. In addition, IL-32 β is positively correlated with tumor size, number of lymph node metastases and tumor stage in primary breast cancer tissues; increases migration and invasion through signal transducer and activator of transcription 3 (STAT3) activation; and enhances glycolysis in human

breast cancer cells [13, 14]. On the other hand, it shows anti-tumor activity through the activation of lymphocytes including cytotoxic T (CD8+) and natural killer (NK) cells, and inactivation of the nuclear factor- κ B (NF- κ B) and STAT3 signaling pathways [15].

The von Hippel-Lindau (VHL) tumor suppressor protein is an E3 ligase that ubiquitinates hypoxia-inducible factor-1 α (HIF-1 α) and causes its degradation by the proteasome [16, 17]. When oxygen is abundant, VHL binds to HIF-1 α by recognizing its two post-translationally hydroxylated proline residues (P402 and P564) [18]. On the other hand, a lack of oxygen due to the rapidly growing tumor mass cause the inactivation of prolyl hydroxylase (PHD) preventing VHL from recognizing and binding to HIF-1 α and thus leading to HIF-1 α accumulation, which induces the transcription of genes involved in adaptation to hypoxia [19, 20]. In addition to prolyl hydroxylation, asparaginyl hydroxylation has also been identified as an oxygen-regulated signal that determines the stability of HIF-1 α . Both reactions are catalyzed by members of the 2-oxoglutarate (2OG)-dependent oxygenase superfamily: HIF-1 α prolyl hydroxylation by PHD domains 1–3, and HIF-1 α asparaginyl hydroxylation by factor inhibiting HIF-1 α (FIH). Since hydroxylation is one of the important post-translational modifications, the assignment of molecular functions for all human 2OG oxygenases by combined biochemical and cellular approaches is required. As an effort, accumulating data on newly identified hydroxylated proteins are expanding [21, 22].

The protein kinase C (PKC) family comprises serine/threonine kinases that regulate a diverse set of cellular processes including proliferation, apoptosis, survival, and migration, and there is a substantial amount of evidence linking PKC to tumorigenesis [23]. According to their domain structure and respective activators, they have been divided into three major groups: the classical PKCs, including the α , β I, β II, and γ isoforms; the novel PKCs, including the θ , η , ϵ , and δ isoforms; and the atypical PKCs, including ζ and ν/λ [24]. Among various isoforms of PKC, PKC ϵ and PKC δ have opposing roles in regulating apoptosis, survival and proliferation. PKC ϵ promotes cell survival in many cell types, whereas PKC δ performs a growth inhibitory or pro-apoptotic role [23, 24]. When PKC δ is activated by oxidative and hypoxic stress, PKC δ forms complex with Abl in the endoplasmic reticulum (ER), and then PKC δ -Abl complex translocates from ER to the mitochondria, leading to caspase-3 cleavage and cytochrome c release to trigger apoptosis [24–26]. The functional connection between IL-32 β and PKC δ has recently been unveiled by the report that IL-32 β upregulates IL-10 production through its association with PKC δ around the nuclear membrane [27, 28].

In the present study, we investigated the function of IL-32 β in human ovarian cancer cells which is one of the representative female cancers. Our results revealed that IL-32 β is degraded by VHL-mediated ubiquitination,

a process that is prevented by hypoxia-induced reactive oxygen species (ROS) production. PKC δ forms trimers with IL-32 β and VHL. Under hypoxic conditions, the interaction between IL-32 β and PKC δ is sustained, whereas that with VHL is disrupted. In turn, the increased IL-32 β level reduces PKC δ -mediated apoptosis under oxidative stress.

RESULTS

IL-32 β level increases under hypoxic conditions in ovarian cancer cells

We had previously shown that IL-32 β level is increased and enhances glycolysis in breast cancer cells to survive under hypoxic conditions [14]. In the present study, we determined the molecular mechanism by which IL-32 β stability is increased under hypoxic conditions in ovarian cancer cells. The levels of IL-32 protein and mRNA were examined in three different human ovarian cancer cell lines: IGROV1, SKOV3, and OVCAR8 cells. Although IGROV1 cells expressed both IL-32 β and IL-32 γ mRNA, the proteins were not detected, even with longer exposure. SKOV3 cells showed lower levels of IL-32 β and IL-32 γ mRNA expression than OVCAR8 cells. On the other hand, the IL-32 β and IL-32 δ proteins were highly expressed in OVCAR8 cells (Figure 1A). To determine whether IL-32 β expression is increased by hypoxic conditions, the ovarian cancer cells were treated with CoCl₂, a hypoxia mimetic chemical. Whereas vascular endothelial growth factor (VEGF) mRNA expression (as a positive control) was markedly increased in all three cell lines, IL-32 β mRNA expression was only modestly increased in SKOV3 and OVCAR8 cells (Figure 1B). However, the IL-32 β protein was highly increased in SKOV3 and OVCAR8 cells 3 h after CoCl₂ treatment, but IL-32 β proteins were not detected even in the ROS-stimulated in IGROV1 cells (Figure 1C). One possibility is that IGROV1 cells may have epigenetically suppressed IL-32 β . Thus, we determined whether IL-32 β translation was increased. When cells were co-treated with cyclohexamide (CHX) and CoCl₂ for the indicated time intervals, the CoCl₂-induced increase in IL-32 β level was not observed (Figure 1D). If IL-32 β is regulated by proteasomal degradation, the pre-existing IL-32 β should be detected after the CHX treatment. However, if the steady state level of IL-32 β is very low, it is possible that IL-32 β level is low in the presence of CHX.

Hypoxic conditions inhibit IL-32 β degradation

There are two mechanisms to explain the increase of protein amount: increase of the translation rate, and inhibition of protein degradation. To examine the possibility of proteasomal degradation, SKOV3 and OVCAR8 cells were treated with MG-132 for the

indicated time intervals. IL-32 β was clearly increased 3 h after the treatment (Figure 2A). However, when cells were treated with pepstatin A, an inhibitor of lysosomal degradation, the increase in IL-32 β level was not observed (Figure 2B). These results imply that the translated IL-32 β had been continuously degraded by ubiquitination. In the previous study, we showed that the CoCl₂-induced increase in IL-32 β level was mediated by ROS production in breast cancer cells. The ovarian cancer cells also showed the CoCl₂-mediated increase in IL-32 β production, which was prevented by treatment with N-acetyl-cysteine (NAC), an anti-ROS reagent, in a dose-dependent manner (Figure 2C), and co-treatment of MG-132 with NAC reverse the latter's effect (Figure

2D). This implies that ROS stabilize IL-32 β levels through the inhibition of proteasomal degradation. To further confirm that IL-32 β is continuously degraded under normal conditions, we examined whether it is ubiquitinated. Cells were transfected with Myc-IL-32 β and Hemagglutinin (HA)-ubiquitin (Ub) and then treated with CoCl₂ or MG-132. IL-32 β was immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA antibody to examine whether IL-32 β is ubiquitinated. MG-132 treatment increased the level of ubiquitinated IL-32 β , whereas CoCl₂ treatment inhibited it (Figure 2E). Collectively, these results imply that hypoxia-induced ROS production increases IL-32 β levels by preventing its ubiquitination.

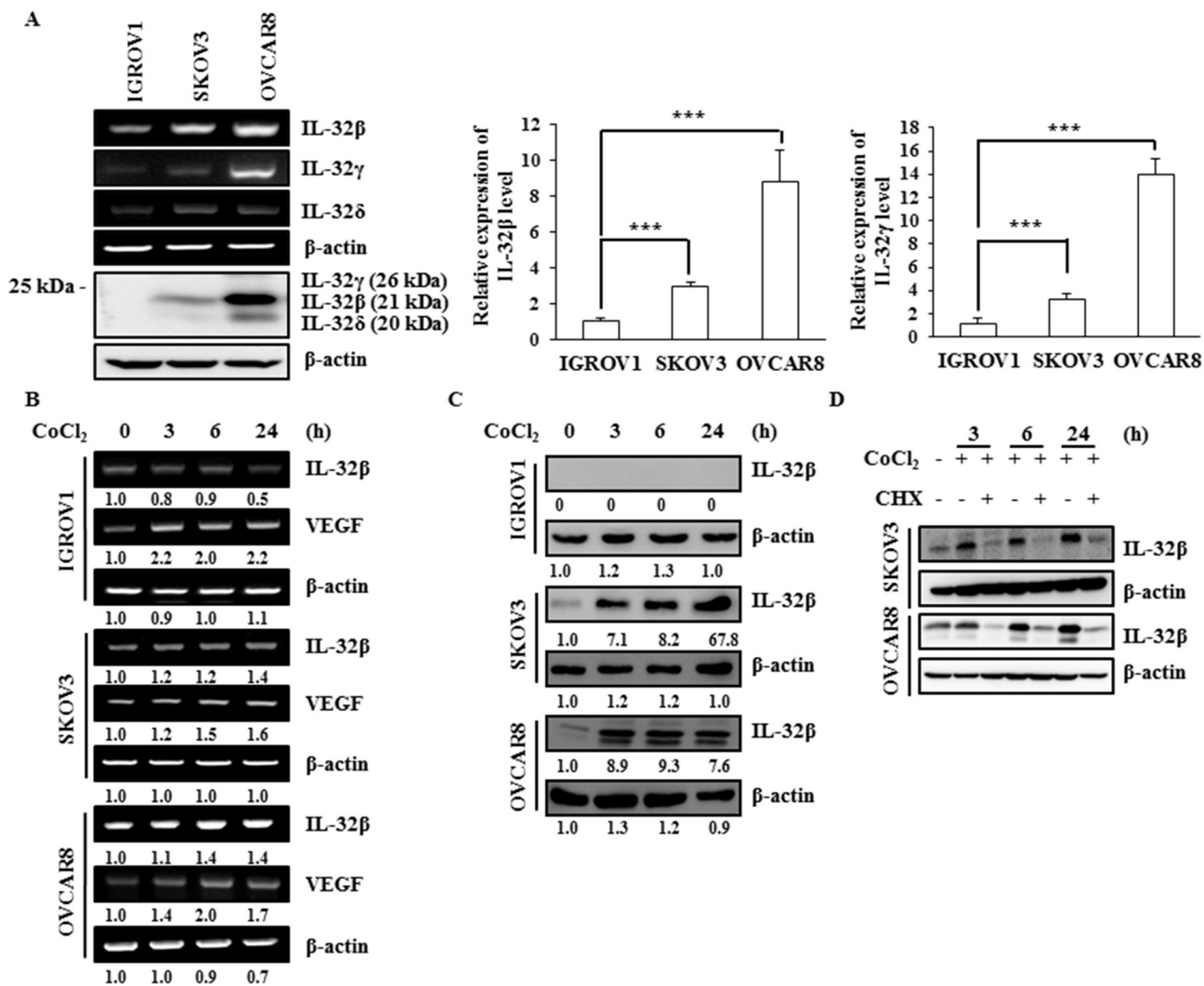


Figure 1: IL-32 β levels increase under hypoxic conditions in ovarian cancer cells. (A) The IL-32 levels in the human ovarian cancer cell lines IGROV1, SKOV3, and OVCAR8 were determined by RT-PCR, qRT-PCR, and immunoblot assays. The qRT-PCR results were normalized with the level of IL-32 β and IL-32 γ in IGROV1 cells, respectively. The data shown represent one of three independent experiments carried out in triplicate. (B, C) SKOV3 and OVCAR8 cells were treated with 150 μ M CoCl₂ and the IL-32 β levels were determined by RT-PCR and immunoblot assays at the indicated times. The bands were quantified using ImageJ software and the numbers indicate the comparison of each lane. (D) SKOV3 and OVCAR8 cells were treated with 150 μ M CoCl₂ and 5 μ g/mL CHX. The IL-32 β levels were determined by immunoblot assay at the indicated times.

The VHL E3 ligase complex is responsible for IL-32 β degradation

VHL is a well-known E3 ligase involved in HIF-1 α degradation under normoxic conditions. Kelch-like ECH-associated protein 1 (Keap1) is also an E3 ligase involved in nuclear factor erythroid 2-related factor 2 (Nrf-2) degradation in the absence of ROS, and Carboxyl terminus of HSP70-interacting protein (CHIP) is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein under ROS stress [29–32]. To find out which E3 ligase is responsible for IL-32 β ubiquitination,

SKOV3 and OVCAR8 cells were transfected with VHL small interfering RNA (siRNA), CHIP siRNA, and Keap1 siRNA. The IL-32 β was increased after VHL siRNA transfection (Figure 3A). To further confirm this, SKOV3 cells were induced to express IL-32 β and HA-VHL, whereupon the IL-32 β levels were decreased by HA-VHL in a dose-dependent manner (Figure 3B). Next, OVCAR8 cells highly expressing IL-32 β were transfected with increasing amounts of HA-VHL, whereupon the endogenous IL-32 β decreased (Figure 3C). These results imply that VHL is the E3 ligase responsible for IL-32 β ubiquitination.

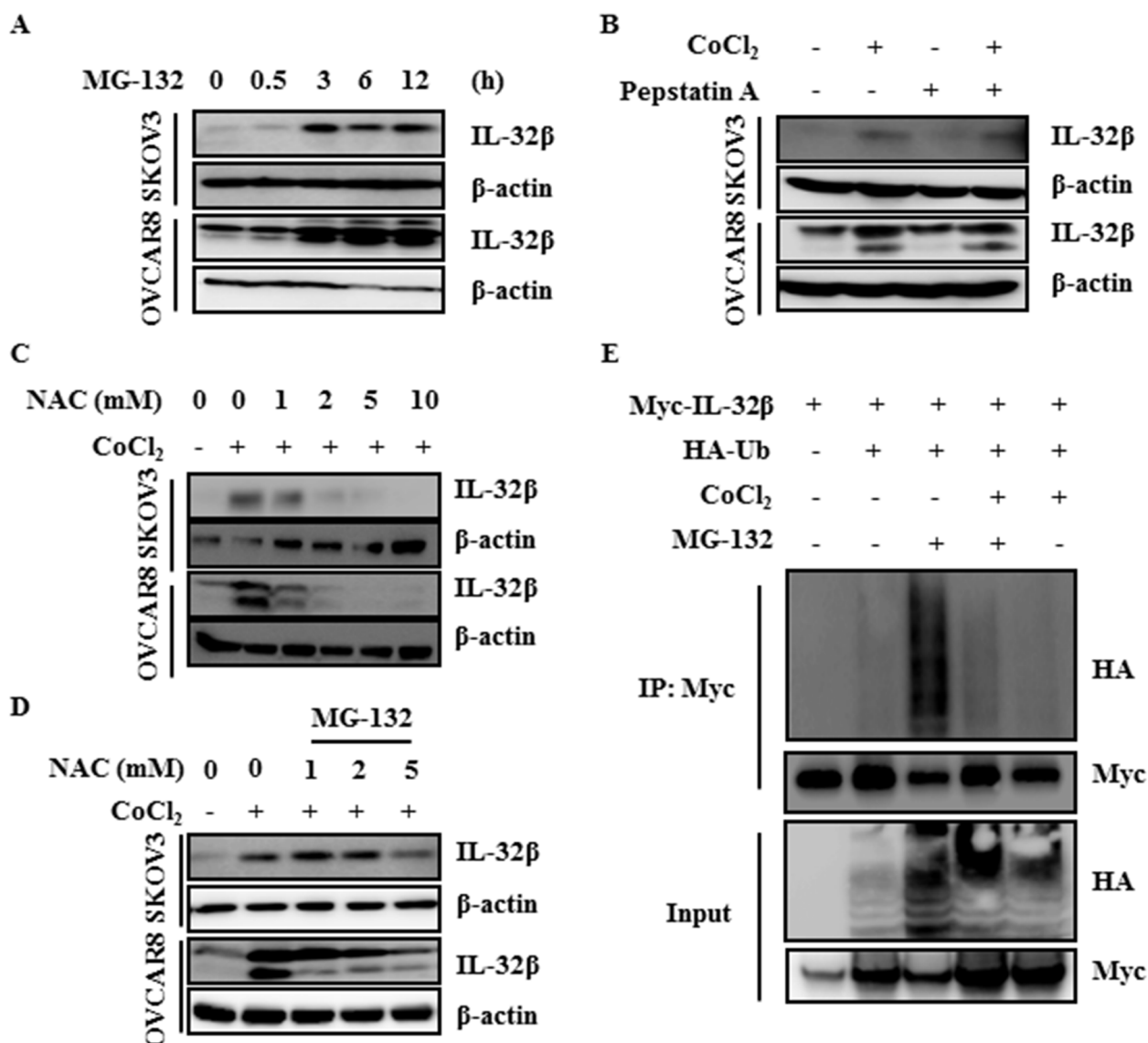


Figure 2: ROS inhibit ubiquitin-dependent IL-32 β degradation. (A) SKOV3 and OVCAR8 cells were treated with 5 μ M MG-132 and the IL-32 β levels were determined by immunoblot assay at the indicated times. (B) SKOV3 and OVCAR8 cells were pretreated with 1 μ g/mL pepstatin A for 1 h, and then treated with 150 μ M CoCl₂. (C) SKOV3 and OVCAR8 cells were pretreated with NAC for 1 h, and then treated with 150 μ M CoCl₂. (D) SKOV3 and OVCAR8 cells were pretreated with NAC for 1 h, and then treated with 150 μ M CoCl₂ and MG-132. (E) HEK 293T cells were transfected with Myc-IL-32 β and HA-Ub, and then treated with 150 μ M CoCl₂ or 5 μ M MG-132. The cell lysates were immunoprecipitated with anti-Myc antibody and the interaction was examined by immunoblot assay.

ROS disrupt the interaction between VHL and the exon 7 coding region of IL-32 β

To investigate the underlying molecular mechanisms by which IL-32 β is regulated by VHL E3 ligase, cells were induced to express Myc-IL-32 β and HA-VHL and then treated with H₂O₂. The immunoprecipitation assay revealed that the VHL and IL-32 β interaction was disrupted after H₂O₂ treatment (Figure 4A), implying that IL-32 β is degraded by VHL-mediated ubiquitination and this degradation is prevented by hypoxia-induced ROS production. To determine which domain of IL-32 binds to VHL, the interaction between different IL-32 isoforms and VHL was examined. All isoforms of IL-32 [33] bound to VHL (Figure 4B). Since only the exon 7-coded region is common to all isoforms of IL-32, it was the only regions shown to interact with VHL (Figure 4C). To determine which domain of VHL binds to IL-32 β , the interaction between IL-32 β and VHL domain using VHL19, which is absent in the acidic domain of first 53 amino acid residues [34], was examined. The interaction between IL-32 β and

VHL19 was increased after H₂O₂ treatment, implying that acidic domain of VHL may play an important role in disruption of the interaction between IL-32 β and VHL under hypoxic conditions (Figure 4D).

Next, we examined whether a proline residue of IL-32 β is hydroxylated by PHD, since VHL binds only to such proline residues in HIF-1 α [19, 20]. SKOV3 and OVCAR8 cells were treated with a PHD inhibitor, dimethylxaloylglycine (DMOG) or desferrioxamine (DFO), and IL-32 β expression was measured. Contrary to our expectation, DMOG and DFO did not affect the IL-32 β level in OVCAR8 cells, although SKOV3 cells showed increase in IL-32 β level (Figure 4E, 4F). In addition, SKOV3 and OVCAR8 cells were also transfected with PHD2 siRNA and the IL-32 β was examined. No difference was observed (Figure 4G). To further confirm this result, we examined the interaction of IL-32 β with VHL mutants that are not able to bind to hydroxyl proline residues of HIF-1 α [19, 34]. All VHL mutants still bound to IL-32 β (Figure 4H), implying that the interaction between IL-32 β and VHL is not dependent upon proline hydroxylation.

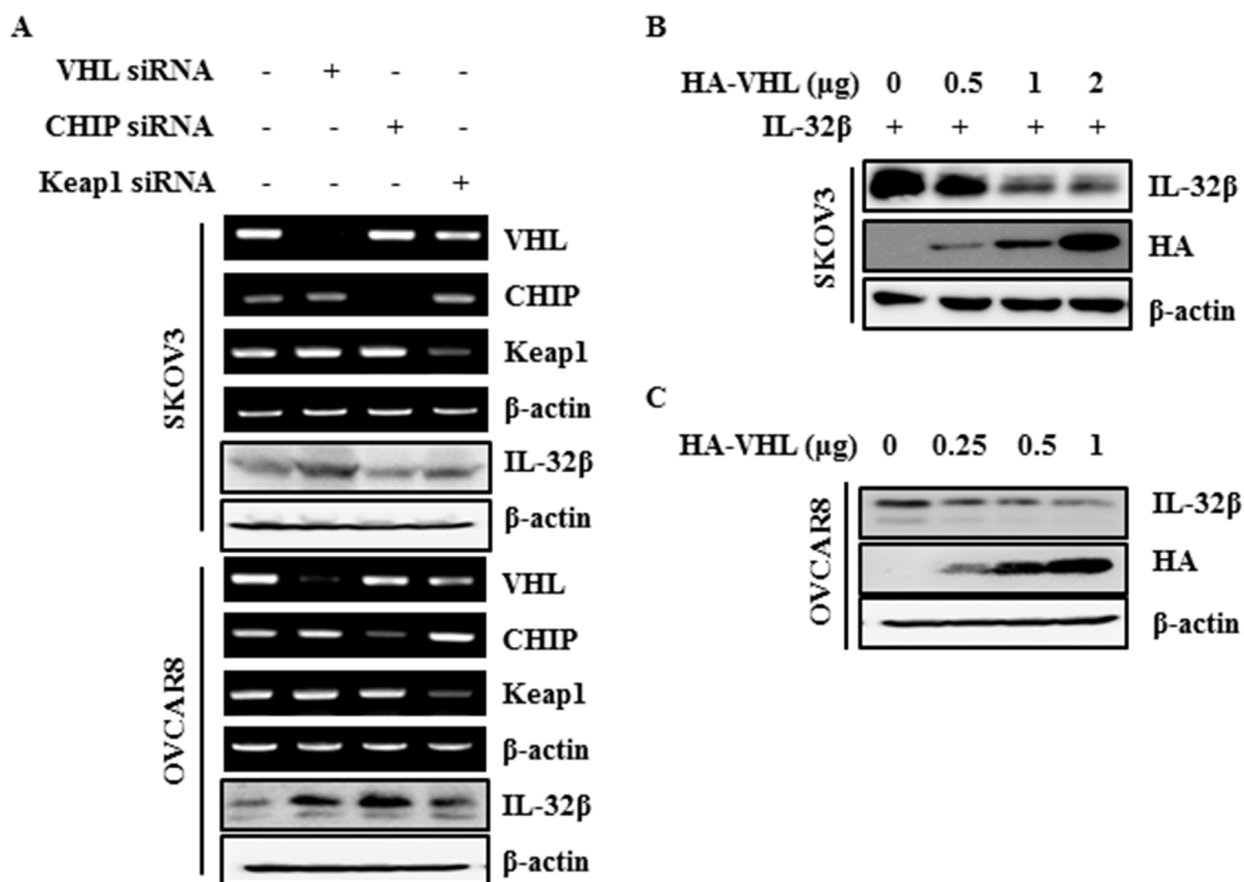


Figure 3: IL-32 β is regulated by E3 ligase VHL. (A) SKOV3 and OVCAR8 cells were transfected with VHL siRNA, CHIP siRNA, and Keap1 siRNA. The VHL, CHIP and Keap1 levels were determined by RT-PCR. The IL-32 β levels were determined by immunoblot assay. (B) SKOV3 cells were co-transfected with IL-32 β and HA-VHL-expressing plasmids. The IL-32 β and VHL levels were determined by immunoblot assay. (C) OVCAR8 cells were transfected with HA-VHL. The IL-32 β and VHL levels were determined by immunoblot assay.

PKC δ is associated with the interaction between IL-32 β and VHL

Since it is known that ROS activates PKC, which can interact with IL-32 and VHL [27, 35–37], PKC was examined for its possible association with the interaction between IL-32 β and VHL. Cells were induced to express HA-VHL, Myc-IL-32 β and Flag-PKC δ , and then immunoprecipitated with anti-Flag antibody. PKC δ precipitated with Myc-IL-32 β and HA-VHL (Figure 5A). In addition, when cells were placed in the hypoxic chamber, the co-immunoprecipitation of IL-32 β and

PKC δ with VHL was reduced (Figure 5B). These results imply that the interaction of VHL with either IL-32 β or PKC δ is reduced under hypoxia. However, the interaction between IL-32 β and PKC δ was constant regardless of the oxygen conditions (Figure 5C). To further confirm this interaction in human ovarian cancer cells, OVCAR8 cells were induced to express HA-VHL, Myc-IL-32 β and Flag-PKC δ , and then immunoprecipitated with anti-HA antibody. VHL precipitated with Myc-IL-32 β and Flag-PKC δ . In addition, when cells were treated with CoCl₂, the co-immunoprecipitation of IL-32 β and PKC δ with VHL was reduced (Figure 5D). These results collectively

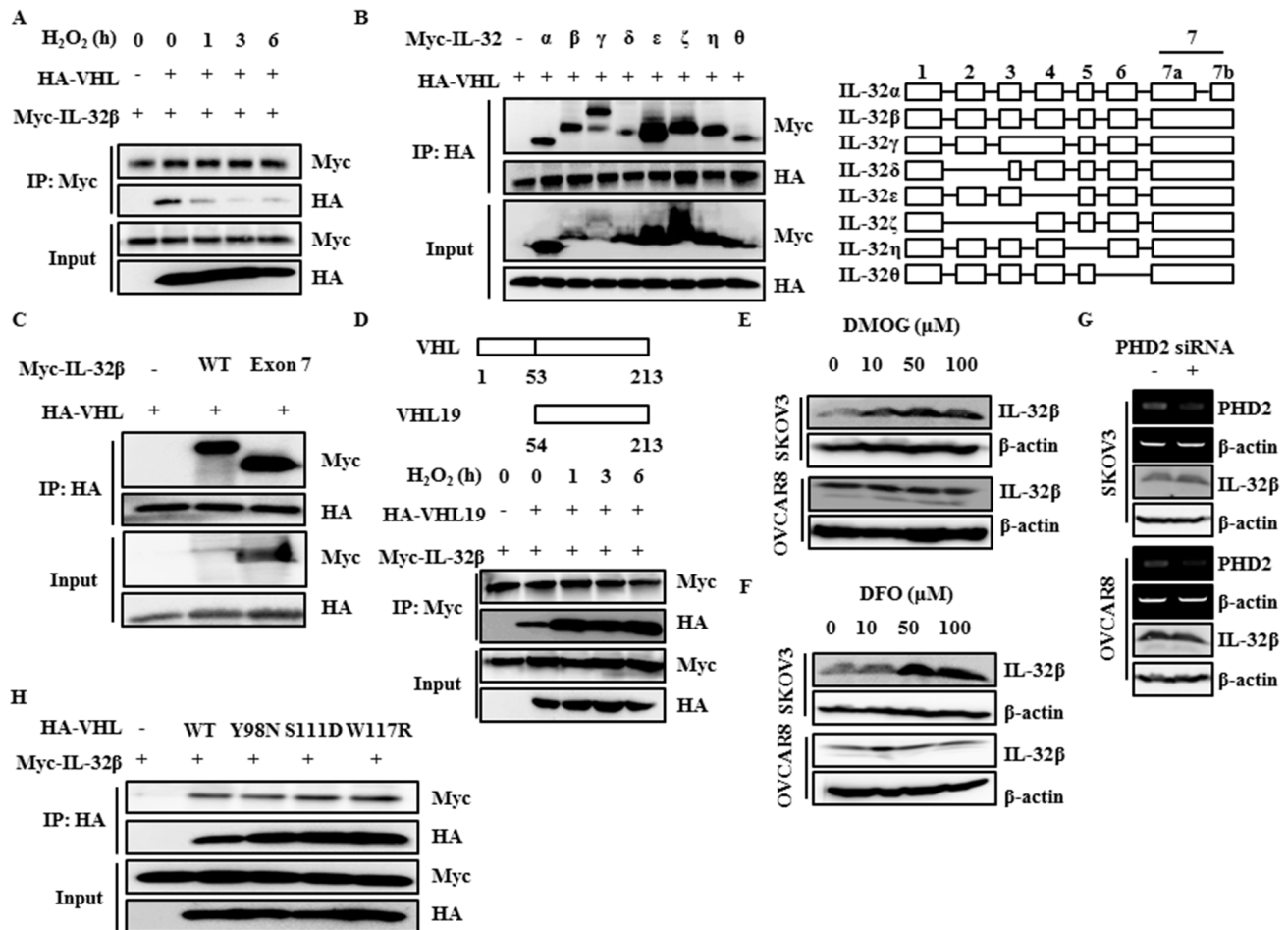


Figure 4: The interaction between IL-32 β and VHL is disrupted by ROS. (A) HEK 293T cells were transfected with Myc-IL-32 β and HA-VHL, and then treated with 100 μ M H₂O₂ for the indicated times. The cell lysates were immunoprecipitated with anti-Myc antibody and the interaction was examined by immunoblot assay. (B) HEK 293T cells were transfected with the Myc-IL-32 isoforms and HA-VHL. The cell lysates were immunoprecipitated with anti-HA antibody and the interaction was examined by immunoblot assay. (C) HEK 293T cells were transfected with Myc-IL-32 β , Myc-IL-32 exon 7, and HA-VHL. The cell lysates were immunoprecipitated with anti-HA antibody and the interaction was examined by immunoblot assay. (D) HEK 293T cells were transfected with Myc-IL-32 β and HA-VHL19, and then treated with 100 μ M H₂O₂ for the indicated times. The cell lysates were immunoprecipitated with anti-Myc antibody and the interaction was examined by immunoblot assay. (E) SKOV3 and OVCAR8 cells were treated with DMOG for 24 h. The IL-32 β levels were determined by immunoblot assay. (F) SKOV3 and OVCAR8 cells were treated with DFO for 24 h. The IL-32 β levels were determined by immunoblot assay. (G) SKOV3 and OVCAR8 cells were transfected with PHD2 siRNA. The PHD2 levels were determined by RT-PCR. The IL-32 β levels were determined by immunoblot assay. (H) HEK 293T cells were transfected with Myc-IL-32 β , HA-VHL wild type (WT), or HA-VHL mutant. The cell lysates were immunoprecipitated with anti-HA antibody and the interaction was examined by immunoblot assay.

suggest that IL-32 β , VHL, and PKC δ form a complex. Under the hypoxic condition, the interaction between VHL and IL-32 β or PKC δ is disrupted, not affecting the interaction between IL-32 β and PKC δ .

IL-32 β attenuates PKC δ -induced apoptosis in oxidative stress

To find out effect of IL-32 β and PKC δ co-expression on apoptosis, proliferation, and migration, SKOV3 cells

expressing a low level of IL-32 β were induced to express IL-32 β and PKC δ , and OVCAR8 cells expressing a high level of IL-32 β were transfected with IL-32 siRNA and PKC δ siRNA, and then cell apoptosis was examined since PKC δ is known to be involved in those functions [23]. PKC δ increased the level of cleaved-poly (ADP-ribose) polymerase (PARP) and the activity of caspase-3/7 after H₂O₂ treatment. However, co-expression of IL-32 β and PKC δ reduced the H₂O₂-induced apoptosis in the presence of PKC δ , implying that IL-32 β attenuates PKC δ -induced

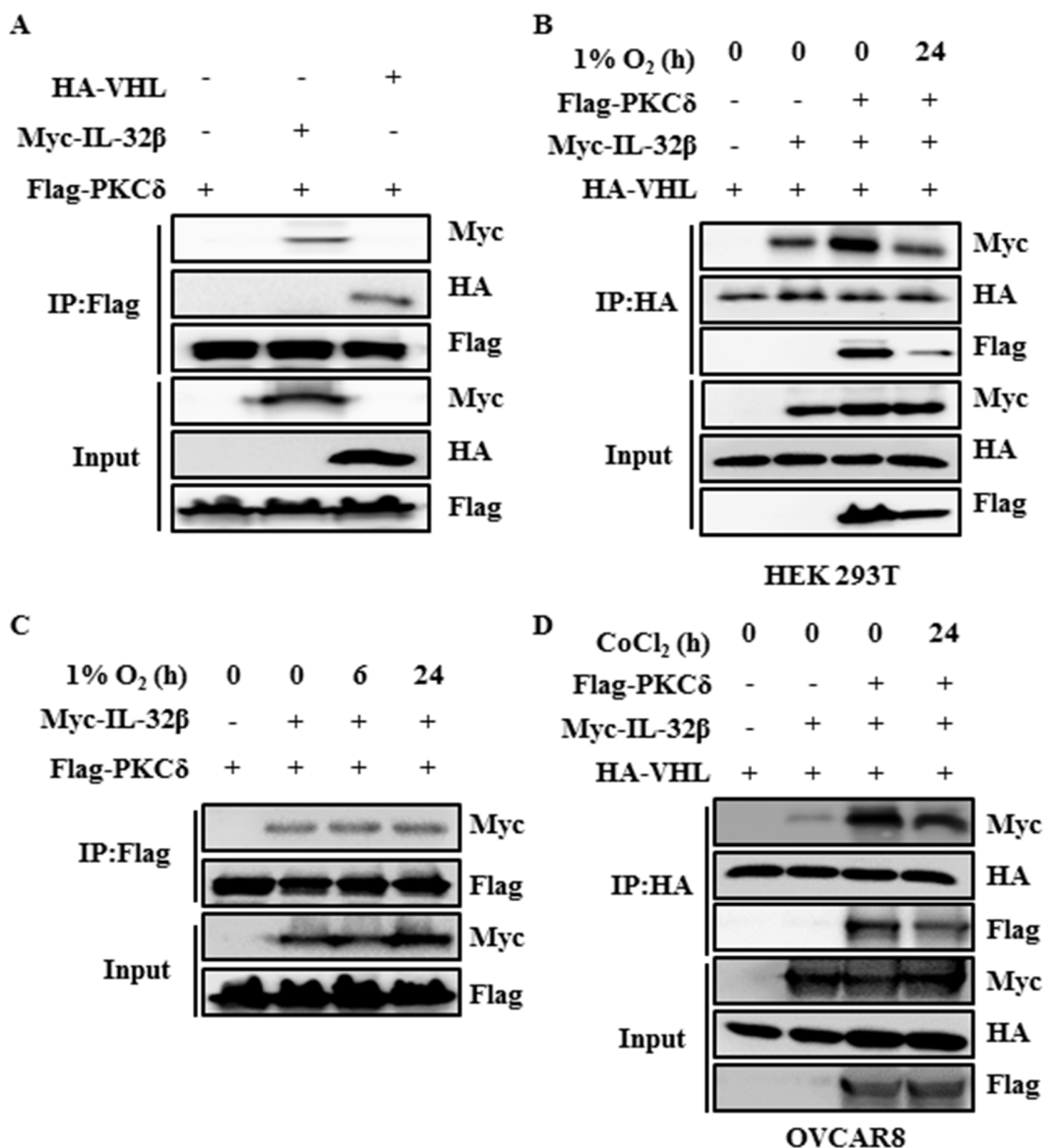


Figure 5: PKC δ forms a complex with IL-32 β and VHL. (A) HEK 293T cells were transfected with Myc-IL-32 β , HA-VHL, and Flag-PKC. The cell lysates were immunoprecipitated with anti-Flag antibody and the interaction was examined by immunoblot assay. (B) HEK 293T cells were transfected with Myc-IL-32 β , HA-VHL, and Flag-PKC δ , and then placed in a hypoxia chamber for 24 h. The cell lysates were immunoprecipitated with anti-HA antibody and the interaction was examined by immunoblot assay. (C) HEK 293T cells were transfected with Myc-IL-32 β and Flag-PKC δ , and then placed in a hypoxia chamber for 24 h. The cell lysates were immunoprecipitated with anti-Flag antibody and the interaction was examined by immunoblot assay. (D) OVCAR8 cells were transfected with Myc-IL-32 β , HA-VHL and Flag-PKC δ , and then treated with 150 μ M CoCl₂ for 24 h. The cell lysates were immunoprecipitated with anti-HA antibody and the interaction was examined by immunoblot assay.

apoptosis under oxidative stress (Figure 6A, 6B). Since PKC δ also has an anti-proliferative effect on cancer cells, its effect was examined in the presence of IL-32 β . The proliferation of SKOV3 and OVCAR8 cells was greatly reduced after CoCl $_2$ treatment, but this was not affected by the co-presence of IL-32 β and PKC δ (Figure 6C). Next, we examined effect of IL-32 β and PKC δ on migration under CoCl $_2$ -induced hypoxic conditions. SKOV3 and OVCAR8 cell migration was considerably decreased after CoCl $_2$ treatment, and this effect was not affected by the co-expression of IL-32 β and PKC δ (Figure 6D). These findings imply that the accumulated IL-32 β by hypoxia-induced ROS binds to PKC δ , leads to inhibit PKC δ -induced apoptosis under oxidative stress without affecting other functions, including proliferation and migration.

DISCUSSION

Hypoxia-stabilized HIF-1 α binds to hypoxia response elements, leading to the upregulation of several genes to promote survival under low-oxygen conditions. These genes code for enzymes that enhance glycolysis, which allows for ATP synthesis in an oxygen-independent manner [38, 39]. VEGF is a product of one of the HIF-1 α -induced genes, which promotes angiogenesis and supplies nutrients and oxygen to the tumor mass [40]. In the present study, we identified one more protein (i.e., IL-32 β) that is as urgently needed as HIF-1 α under hypoxic and ROS conditions. IL-32 β stability was regulated by the post-translational mechanism in SKOV3 and OVCAR8 human ovarian cancer cells. This cytokine was

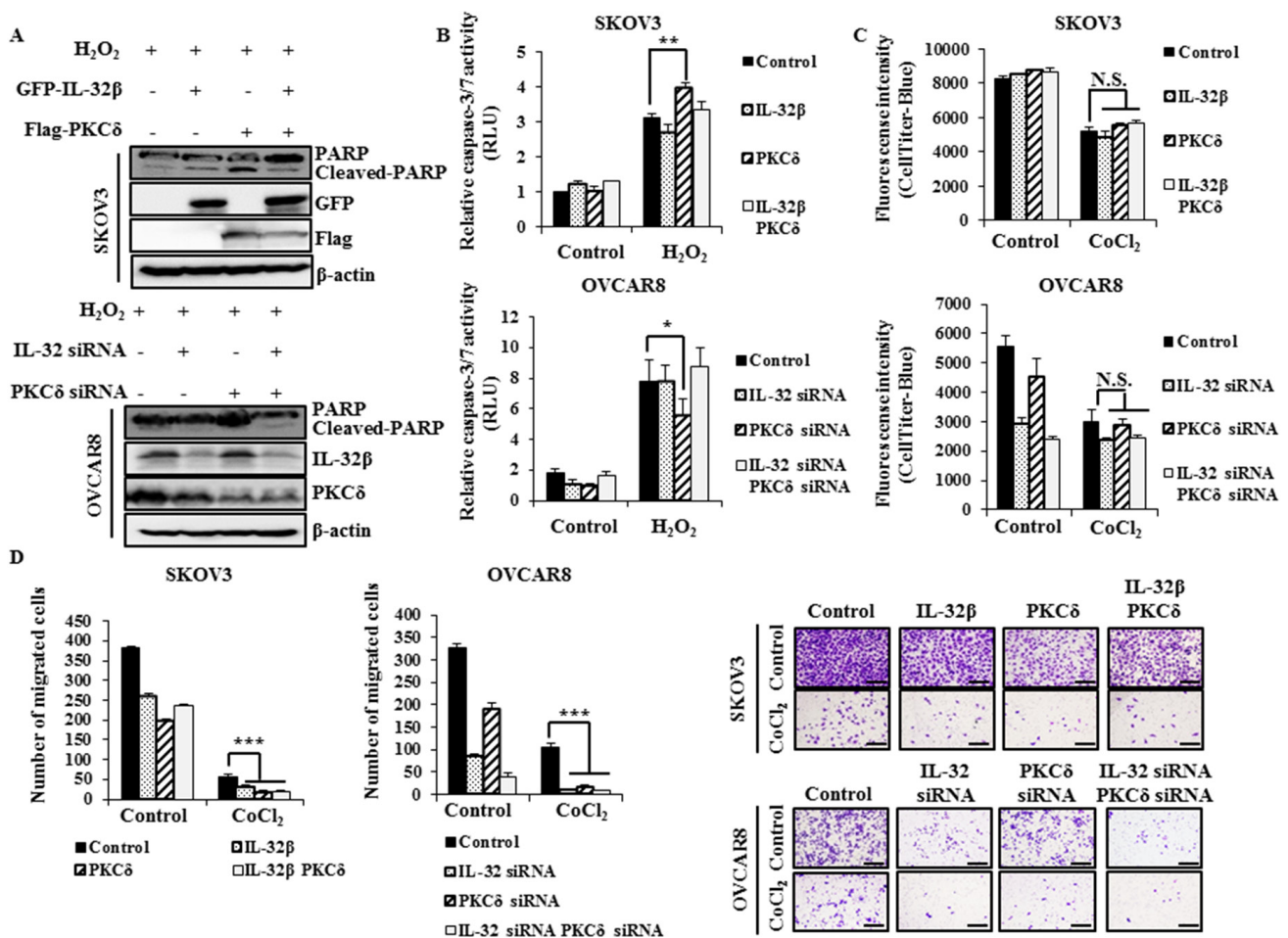


Figure 6: IL-32 β prevents PKC δ -induced apoptosis under oxidative stress. (A, B) SKOV3 cells were transfected with GFP-IL-32 β and Flag-PKC δ for 24 h, whereas OVCAR8 cells were transfected with IL-32 siRNA and PKC δ siRNA for 48 h. The transfected cells were treated with 100 μ M H $_2$ O $_2$ for 24 h. The caspase-3/7 activity was assessed using a luminescent assay. The cleaved-PARP levels were determined by immunoblot assay. (C) SKOV3 cells were transfected with GFP-IL-32 β and Flag-PKC δ . OVCAR8 cells were transfected with IL-32 siRNA and PKC δ siRNA, and then treated with 150 μ M CoCl $_2$ for 24 h. The proliferation was assessed using CellTiter-Blue. (D) SKOV3 cells were transfected with GFP-IL-32 β and Flag-PKC δ , whereas OVCAR8 cells were transfected with IL-32 siRNA and PKC δ siRNA. The transfected cells were seeded onto the upper part of a Transwell chamber in serum-free medium with or without 150 μ M CoCl $_2$. The migrated SKOV3 and OVCAR8 cells were counted after 24 or 48 h incubation, respectively. Scale bars = 10 μ m.

continuously synthesized and degraded by ubiquitination under normoxic conditions, but accumulated rapidly following exposure to low oxygen tension or ROS. Since the regulation of IL-32 β in some of the ovarian tumor cells is energy-consuming, we infer that tumor cells urgently need IL-32 β under the hypoxic and ROS conditions. We revealed that hypoxia- and ROS-stabilized IL-32 β prevents PKC δ -mediated apoptosis, and this would rescue tumor cells from sudden death caused by environmental changes (Figure 7).

Various isoforms of IL-32 are produced by alternative splicing mechanism and there are conflicting observations of either an oncogenic or a tumor suppressive role for IL-32 [12, 41]. These contradictory results might originate from differences in the IL-32 isoform expressed in tumors analyzed. It is reported that IL-32 γ and IL-32 β induce caspase-8-dependent cell death in HEK293 cells whereas IL-32 α does not, and that the treatment of alternative splicing inhibitor results in the predominant

expression of IL-32 γ splice variants and cell death in thyroid cancer cell lines [42]. On the other hand, IL-32 β itself shows either an oncogenic or a tumor suppressive role. In this study, ROS-induced IL-32 β reduced PKC δ -induced apoptosis, however, IL-32 β transgenic mice have the increased cytotoxic T lymphocytes and NK cells activity, leads to the inhibition to tumor growth [15]. A plausible explanation about contrary IL-32 β function could be that physiological environments such as ROS condition and change of immune system affects IL-32 β role.

To find out which E3 ligases are responsible for IL-32 β ubiquitination, the hypoxia- or ROS-related E3 ligases VHL, CHIP, and Keap1 [20, 29–32] were examined. Among these, only VHL could bind to and ubiquitinate IL-32 β . Whereas VHL binding to HIF-1 α is dependent on prolyl hydroxylation, the interaction between IL-32 β and VHL was not dependent on prolyl hydroxylation of IL-32 β . As with IL-32 β , it is known that Aurora kinase

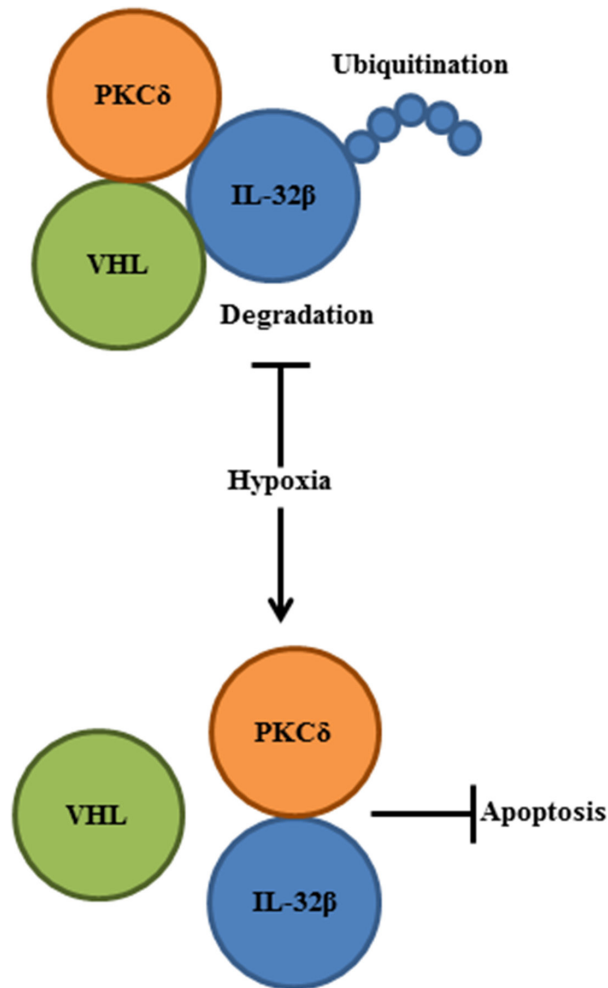


Figure 7: A schematic diagram summarizes IL-32 β function in human ovarian cancer cells. Hypoxia-induced ROS disrupted the interaction between IL-32 β and VHL, leading to the IL-32 β accumulation. The increased IL-32 β bound to PKC δ in the oxidative stress condition and PKC δ -mediated apoptosis was inhibited.

A is also regulated by VHL in a prolyl hydroxylation independent manner [43]. We have therefore provided one more protein that is a VHL target, but for which binding is hydroxylation independent. On the other hand, Stoehr et al. showed that the inhibition of prolyl hydroxylation by DMOG stabilizes many proteins that have potential function under the hypoxic conditions [44]. Since DMOG treatment stabilized IL-32 β (Figure 4D), the prolyl hydroxylation of IL-32 β itself could stimulate its degradation under normoxic conditions, but it would not depend on VHL interaction, since we showed that the interaction between VHL and IL-32 β was not dependent on IL-32 β prolyl hydroxylation. Therefore, we can infer that IL-32 β is degraded by two ways under normoxic conditions: prolyl hydroxylation-induced IL-32 β degradation, and VHL-mediated degradation in a prolyl hydroxylation-independent manner. On the other hand, since prolyl hydroxylation-induced IL-32 β degradation was not observed in OVCAR8 cells (Figure 4D, 4E), this process may need another factor that is absent in OVCAR8 cells.

It is known that PKC δ interacts with the β domain of VHL, but its overall protein levels are not affected by the interaction with VHL [45, 46]. The interaction of PKC δ with VHL retains PKC δ in the cytoplasm, which in turn impairs the association between PKC δ and the insulin-like growth factor 1 (IGF-I) receptor [37]. Therefore, besides the degradation of binding partners, another effect of the interaction with VHL is the limit of the localization of binding partners. In contrast to inactive PKCs, which are present mainly in the cytosol, activated PKCs are localized at the plasma membrane, the nucleus, and other subcellular compartments [47]. Hypoxic conditions in particular trigger the translocation of PKC δ to the endoplasmic reticulum or mitochondria, resulting in oxidative stress-induced apoptosis [24-26, 48]. Since the hypoxic stimulus translocates IL-32 β to the mitochondria in breast cancer cells [14], IL-32 β could inhibit PKC δ -mediated apoptosis. In turn, hypoxia- and ROS-stabilized IL-32 β contributes to cancer cell survival. On the other hand, IL-32 β interacts with PKC δ in the human monocytic cell line U-937, and its interaction shifts the immune reaction from an inflammatory to an anti-inflammatory one by enhancing anti-inflammatory cytokine IL-10 production and inhibiting the secretion of IL-1 β and TNF- α [27, 28]. These findings suggest that IL-32 β plays a favorable role in tumor cell survival by affecting the function of both tumor and immune cells.

In conclusion, hypoxia releases IL-32 β from ubiquitination-mediated degradation. Subsequently, the increased IL-32 β interacts with PKC δ and results in the inhibition of apoptosis, which can be induced by ROS-activated PKC δ . In turn, the hypoxia-induced IL-32 β is beneficial for tumor cell survival under hypoxic and ROS environments. The limitation of our study is that the effect of IL-32 β could not be validated using knockout

mice, because this cytokine is not expressed in rodents. Nonetheless, the functions of IL-32 β have been and continued to be unveiled with the use of various cancer models.

MATERIALS AND METHODS

Cell cultures and differentiation

The IGROV1, SKOV3 and OVCAR8 cells were maintained in RPMI-1640 medium (HyClone Laboratories, UT, USA) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories), whereas the HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (HyClone Laboratories) containing 10% heat-inactivated FBS. All cells were incubated at 37°C in a humidified 5% CO₂ incubator.

Plasmids

The green fluorescent protein-tagged IL-32 β (GFP-IL-32 β), Flag-PKC δ , and Myc-IL-32 isoforms and IL-32 β -expressing plasmids were obtained from Prof. Do-Young Yoon at Konkuk University. HA-Ub was obtained from Prof. Keun Il Kim at Sookmyung Women's University. HA-VHL and HA-VHL19 were obtained from Prof. Jae Whan Song at Yonsei University. The HA-VHL Y98N and W117R were purchased from Addgene (Cambridge, MA, USA). Truncated mutants of IL-32 were prepared by PCR and subcloned into the *EcoRI-SalI* sites of pEGFPN2, or the *EcoRI-XhoI* sites of the Myc-pcDNA3.1 mammalian expression vector. VHL S111D was prepared by PCR and subcloned into the *EcoRI-XhoI* sites of the HA-pcDNA3 mammalian expression vector.

Antibodies

The following antibodies were used: anti-IL-32 antibody (provided by Prof. Do-Young Yoon), anti- β -actin antibody, anti-HA antibody, anti-GFP antibody, anti-PKC δ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) anti-Flag antibody (Sigma Aldrich, St. Louis, MO, USA), anti-Myc-Tag antibody, and anti-PARP antibody (Cell Signaling Technology, Danvers, MA, USA).

Chemicals

The following chemicals were obtained from commercial sources: CoCl₂, CHX, MG-132, NAC, pepstatin A, H₂O₂, DMOG, and DFO (Sigma Aldrich).

RNA interference and transfection

For the RNA interference assay, IL-32- and VHL-specific siRNA oligonucleotides were purchased from Samchully Pharmaceuticals (Seoul, Korea). The following sequences were used for the

construction of the siRNAs: IL-32 siRNA forward 5'-GCUCUCUGUCAGAGCUCUU-3' and reverse 5'-AAGAGCUCUGACAGAGAGC-3'; and VHL siRNA forward 5'-CCACAGCUACCGAGUGUAUTT-3' and reverse 5'-AUACACUCGGUAGCUGUGGTT-3'. CHIP siRNA, Keap1 siRNA and PHD2 siRNA were purchased from Santa Cruz Biotechnology. PKC δ siRNA was purchased from Bioneer (Seoul, Korea). The cells were transfected with 10 nM of siRNA using RNAiMAX (Invitrogen, Carlsbad, CA, USA).

Immunoblot analyses

The cell lysates were prepared after transfection with plasmids or siRNA and mixed with 5 \times sodium dodecyl sulfate (SDS) sample buffer. The mixed samples were heated at 99°C for 10 min and separated electrophoretically on a SDS-polyacrylamide gel. Subsequently, the proteins were transferred onto a 0.45- μ m nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) for 2 h. The membrane was blocked for 30 min at room temperature with 5% skim milk (Invitrogen) and subsequently incubated with primary antibody overnight at 4°C. Then, the membrane was incubated with an anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Assay Designs, Ann Arbor, MI, USA) at room temperature for 2 h. The proteins were visualized using an enhanced chemiluminescent substrate (Thermo Fisher Scientific, Logan, MA, USA) and analyzed using the LAS3000 luminescent image analyzer (Fuji Film, Tokyo, Japan). The protein bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunoprecipitation assay

The HEK 293T and OVCAR8 cells were transfected with plasmids for 24 h and then treated with chemicals. The cell lysates were prepared using cell immunoprecipitation lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 250 mM phenylmethylsulfonyl fluoride, 1% NP-40) containing a proteinase inhibitor cocktail. The cell lysates were incubated with antibody for 2 h at room temperature, and then protein-G agarose beads were added. The entire lysates were centrifuged after 2 h incubation, and then washed four times with washing buffer.

RT-PCR

Total RNA samples were prepared from SKOV3 and OVCAR8 cells, using the extraction reagent RNAiso Plus (TaKaRa, Tokyo, Japan). The prepared total RNAs were reverse-transcribed using RevertAid reverse transcriptase (Thermo Fisher Scientific) at 42°C for 1 h. PCR was performed to amplify the mRNAs that encode IL-32 isoforms, β -actin, VEGF, VHL, CHIP, Keap1,

and PHD2 using the following appropriate primer pairs: IL-32 forward 5'-ATGTGCTTCCCGAAGGTCCTC-3' and reverse 5'-TCATTTTGAGGATTGGGGTTC-3'; β -actin forward 5'-GTGGGGCGCCCCAGGCACCA-3' and reverse 5'-CTCCTTAATGTCACGCACGAT-3'; VEGF forward 5'-ATGAACTTCTGCTGTCTTG-3' and reverse 5'-CCGCCTCGGCTTGTACATCTGC-3'; VHL forward 5'-GAGGTCACCTTTGGCTCTTCA-3' and reverse 5'-CCAGATCTTCGTAGAGCGACC-3'; CHIP forward 5'-GTGATCACCCGGAACCCG-3' and reverse 5'-GCGCTTCTTCTCGCGATTC-3'; Keap1 forward 5'-CATCCACCCTAAGGTCATGGA-3' and reverse 5'-GACAGGTTGAAGAACTCCTCC-3' and PHD2 forward 5'-GAAGGCAAAGCCCAGTTTGTCT-3' and reverse 5'-CGTGCTCTCTCATCTGCATCAA-3'. Real-time PCR was performed with Maxima SYBR Green/ROX qPCR master mix (Thermo Fisher Scientific) to amplify the mRNAs that encode IL-32 β , IL-32 γ , and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using the following appropriate primer pairs: IL-32 β forward 5'-CAGGGGAGATACCATGATCG-3' and reverse 5'-ACGGACTAATACGGCAACAGA-3'; IL-32 γ forward 5'-TGACATGAAGAAGCTGAAGGC-3' and reverse 5'-CATGACCTTGTCACAAAAGCTC-3'; and GAPDH forward 5'-G TGTTCCTACCCCAATGTGT-3' and reverse 5'-ATTGTCATACCAGGAAATGAGCTT-3'.

Activity assay

The Caspase-Glo 3/7 assay (Promega, Madison, WI, USA) was carried out according to the manufacturer's instruction. The assay is based on the release of aminoluciferin as a result of active caspase-3/7 cleaving the luminogenic substrate containing the DEVD amino acid sequence.

Proliferation assay

Cell proliferation was evaluated using the CellTiter-Blue assay (Promega). Cells were seeded on 48-well plates and incubated for 24 h. Then, the cells were treated with CoCl $_2$ for 24 h. After washing with PBS, diluted CellTiter-Blue reagent was added directly to each well and the plates were incubated at 37°C for 1 h. The reagent was transferred to 96-well plates and, the fluorescence was measured at 560/590 nm.

In vitro migration assay

Cell migration was assessed in a 24-well plate Transwell system (Corning, Corning, MD, USA). Each 8- μ m Transwell insert was seeded with 100 μ L of SKOV3 or OVCAR8 cells in serum-free RPMI-1640 medium with or without CoCl $_2$, and the lower chamber was filled with 500 μ L RPMI-1640 medium containing 10% FBS with or without CoCl $_2$. The cells were incubated for 24 or 48 h, respectively. Images of the membrane were taken in

three random fields per chamber, and the total number of migrated cells was counted.

Statistical analyses

Statistical analyses were performed by a paired Student's t-test and one-way factorial analysis of variance. The Scheffe and Bonferroni tests were also performed. Values of * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered benchmarks of significant differences. Data are presented as the means \pm standard deviation.

Abbreviations

2OG, 2-oxoglutarate; CC-RCC, clear cell renal cell carcinoma; CHIP, carboxyl terminus of HSP70-interacting protein; CHX, cyclohexamide; CoCl₂, cobalt chloride II; DAG, diacylglycerol; DFO, desferrioxamine; DMEM, Dulbecco's modified Eagle's medium; DMOG, dimethylxaloylglycine; FBS, fetal bovine serum; FIH, factor inhibiting HIF; H₂O₂, hydrogen peroxide; HIF-1 α , hypoxia-inducible factor-1alpha; HRE, hypoxia-response element; IGF-I, insulin-like growth factor 1; IL, interleukin; IP, Immunoprecipitation; Keap1, Kelch-like ECH-associated protein 1; NAC, N-acetyl-cysteine; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; NK cells, natural killer cells; Nrf-2, nuclear factor erythroid 2-related factor 2; PHD, prolyl hydroxylase; PKC, protein kinase C; ROS, reactive oxygen species; SD, standard deviations; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA; STAT3, signal transducer and activator of transcription 3; TNF α , tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FUNDING

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIP) [NRF-2016R1A2B2011683, NRF-2015M3A9B6027818 2016R1A5A1011974].

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