ORIGINAL RESEARCH

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Vascular endothelial growth factor production is induced by histone deacetylase 1 and suppressed by von Hippel-Lindau protein in HaCaT cells

Abstract

Purpose: In hypoxic tumoral tissues, vascular endothelial growth factor (VEGF) expression is positively regulated by histone deacetylase 1 (HDAC1) and negatively regulated by the tumour suppressor protein von Hippel-Lindau (VHL) via hypoxia induced factor-1 alpha (HIF-1alpha). It has been reported that VEGF, HDAC1 and LL-37, but not VHL, are over-expressed in psoriatic skin. Although HIF-1alpha is constitutively expressed in normal keratinocytes, it is not known if HDAC1 and VHL can regulate VEGF production in these cells.

Methods: The participation of HDAC1 and VHL in the regulation of VEGF expression in HDAC1-, VHL- and LL-37-transfected HaCaT cells, and in HaCaT cells treated with HDAC1 inhibitors, was studied.

Results: The production of VEGF was increased in HDAC1- and LL-37-transfected HaCaT cells and maintained in VHL-transfected cells under hypoxic conditions; meanwhile, VEGF production decreased in HaCaT cells treated with TSA, in cells transfected with HDAC1-siRNA, in cells co-transfected with HIF-1alpha-siRNA and pHDAC-1 and in VHL-transfected HaCaT cells. The levels of cytoplasmic HIF-1alpha were high in pLL37-transfected cells and low in pVHL- and pHDAC1-transfected cells; however, HIF-1alpha was detected in the nucleus of the HDAC1-transfected cells. The expression of VEGF was high in cells co-transfected with pHDAC1- and pLL-37, and the expression decreased when pVHL was present.

Conclusions: These data demonstrate that HDAC1, LL-37 and VHL can modulate the production of VEGF via HIF-1alpha in HaCaT cells.

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Angiogenesis is a complex process for the formation of new blood vessels from pre-existing vessels in tissues. This process is essential for healing wounds, but angiogenesis also plays an important role in the exacerbation of some diseases, such as cancer, arthritis and psoriasis. The angiogenic process can be triggered by several molecules produced by epithelial cells, fibroblasts, endothelial cells and tumoral cells, and vascular endothelial growth factor (VEGF) is the principal pro-angiogenic factor released by these cells.[1]

In hypoxic tumoral cells, hypoxia induced factor 1 (HIF-1) is the main transcription factor involved in the expression of VEGF;[2] histone deacetylase 1 (HDAC1)[3] and the tumour suppressor protein von Hippel-Lindau (VHL)[4] regulate the levels of the HIF-1alpha subunit in the cellular cytoplasm. In keratinocytes, factors such as transforming growth factor-alpha (TGF-alpha), epidermal growth factor, and insulin-like growth factor I and II(IGF-I/II) are involved in the induction of VEGF production.[5] Although HIF-1alpha is constitutively expressed in normal keratinocytes,[6-7] it is not known if HDAC1 and VHL can regulate the levels of HIF-1alpha in keratinocytes to alter VEGF production.

HIF-1 is a heterodimeric transcription factor activated by hypoxia, and it is composed of the HIF-1alpha and HIF-1beta subunits.[8] The HIF-1beta subunit is expressed constitutively, but the HIF-1alpha subunit is regulated based on oxygen conditions. In normoxic conditions, HIF-1alpha is hydroxylated at the proline residues 402 and/or 564 by prolyl hydroxylases (PHD)[9-10] and is acetylated at lysine-532 by the ARD1 acetyltransferase.[11] In normoxic conditions, the tumour suppressor protein von Hippel-Lindau (VHL) is also expressed; VHL forms a complex with Elongin-C, which in turn, then recruits Elongin-B and other subunits. This complex conduces the ubiquitination of acetylated and hydroxylated HIF-1alpha to be degraded in the proteasome, which leads to the downregulation of VEGF.[12] The lack of VHL is associated with a variety of tumours, including haemangioblastomas of the retina and tumours of the central nervous system.[13-14] In hypoxic endothelial cells, VHL is downregulated by histone deacetylases (HDACs).[3]

The HDACs are expressed ubiquitously, are localised predominantly in the nucleus, and display high enzymatic activity towards histone substrates.[15] The global inhibition of HDAC activity inhibits angiogenesis and tumour growth by reducing the expression of proangiogenic factors.[16-18] In particular, the levels of HDAC1 are increased in hypoxic conditions, which leads to the decrease of VHL and p53, allowing an accumulation of HIF-1alpha and an increase in VEGF production in human endothelial cells.[3] The porcine catheli-

cidin, PR39, is another molecule with angiogenic activity. PR39 inhibits proteasome activity, avoiding HIF-1alpha degradation.[19] Human cathelicidin LL-37 augments VEGF mRNA expression in HaCaT cells [20].

In the psoriatic-skin lesions, HDAC1, HIF-1alpha, LL-37 and VEGF, but not VHL, are upregulated,[21] which suggests that these molecules are involved in psoriatic angiogenesis. It is well known that keratinocytes produce VEGF in psoriatic lesions; however, the regulation of VEGF expression in these cells is poorly understood. In this study, HaCaT cells were transfected with the cDNA coding sequences of HDAC1, LL-37 and VHL to observe the participation of these molecules in the regulation of VEGF expression.

Materials and Methods

Cloning

Gene-specific primers were used for RT-PCR to amplify the DNA coding sequences of the HDAC1 and LL-37 of HaCaT cells and the VHL of healthy skin biopsies.[21] Cloning was performed using the pcDNA3.1-TOPO vector (Invitrogen, Carlsbad CA, USA). The generated plasmids were named pHDAC1, pLL-37 and pVHL, which contained the genes *hdac1*, *ll37* and *vhl*, respectively.

Transfection assays

HaCaT cells were cultured in DMEM medium supplemented with 5% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in 6-well plates until 80% confluence was reached. The cells were transfected with the pHDAC1, pLL-37 and pVHL plasmids using Lipofectamine 2000 (Invitrogen) as per by the supplier's directions. Supernatants were obtained from cellular cultures and were stored at -70°C until use. Transfected cells were treated with Trizol (Invitrogen) to extract RNA for mRNA expression analysis. To transfect HaCaT cells for the small interference RNA (siRNA) assays, 250, 500 and 1000 ng/mL of HDAC1-siRNA, 1000 ng/mL of HIF-1alpha-siRNA or 500 ng/mL of control siRNA (c-siRNA; Santa Cruz, Santa Cruz CA, USA) were used. pVHL-transfected HaCaT cells were cultured in the presence of 10 mM cobalt chloride to generate hypoxia-mimetic conditions, and the amount of VEGF was evaluated in the supernatant.

RNA isolation and RT-PCR analysis

Total RNA extraction was performed with TRIzol (Invitrogen). For the Reverse Transcriptase (RT) reaction, total RNA (3 µg) with 0.5 µg of oligo-(dT)₁₅₋₁₈ (Invitrogen) was dena-

tured at 70°C for 10 min. The master mix was prepared with 1X single strand buffer, 0.5 mM DTT, 500 µM of each dNTP (Invitrogen) and 200U of MMLV reverse transcriptase (Invitrogen). The RT reactions were performed at 42°C for 1 h. The PCR reactions were performed using 1U of Taq DNA polymerase (Invitrogen), 1µl cDNA, 1X buffer, 1 mM MgCl₂, 200 µM of each dNTP and 0.2 µM of each corresponding specific primer.[21] The optimal PCR conditions were 30 cycles of 30 s at 92°C, 30 s at 60°C and 30 s at 72°C.

Inhibition of HDAC1 activity in HaCaT cells with Trichostatin A (TSA)

HaCaT cells were cultured in 6-well plates with supplemented DMEM until they reached 85% confluence. The cells were treated with 30, 90, 150, 240 or 480 ng/mL of TSA dissolved in ethanol (Sigma, St. Louis, MO, USA) for 24 hours to inhibit the HDAC1 activity. The supernatants were kept to measure VEGF production by ELISA.

ELISA

ELISA was performed to quantify the amount of VEGF (Human VEGF CytoSet™ kit, Invitrogen BioSource) in the supernatants of transfected and non-transfected cells. Briefly, polystyrene wells were coated with anti-human VEGF monoclonal antibody diluted in carbonate buffer and incubated at 4°C overnight. The wells were blocked with 3% fat-free milk, then 100 µL of the supernatant of transfected cultures was added to each well and incubated for 1 hour. After washing, the wells were incubated with the biotin-conjugated rabbit anti-human-VEGF secondary antibody. Streptavidin-conjugated peroxidase was used to develop the colour with H₂O₂ and o-phenylenediamine as the substrates. Absorbance was measured at 492 nm.

Immunofluorescence analysis

HaCaT cells were cultured on cover slides. The cells were fixed with cold absolute methanol for 10 minutes and washed with TBS. The cover slides were treated with blocking solution (bovine serum albumin 3%) for 1 hour. The cells were first incubated with an HIF-1α-, VEGF-, VHL-specific antibody or rabbit IgG as control (Santa Cruz) for 1 hour at 37°C. Goat anti-Rabbit IgG FITC-conjugated (Invitrogen) secondary antibodies were used to observe the expression of the proteins by fluorescence microscopy.

Western blot analysis.

HaCaT cells transfected with pHDAC1 and pcDNA3.1 were lysed and cytoplasmic and nuclear proteins were obtained using a nuclear extraction kit (IMGENEX Corporation; San Diego CA, USA) according with the supplier. In both extracts HIF-1α was analyzed using anti-HIF-1α antibody.

Results

HDAC1 positively regulates VEGF expression in HaCaT cells.

To analyse if HDAC1 regulates VEGF expression in HaCaT cells, cells were transfected with pHDAC1. The cells transfected with pHDAC1 expressed higher levels of HDAC1 mRNA compared with cells transfected with the empty vector pcDNA3.1-TOPO (Fig. 1a). When VEGF production was analysed in the supernatants of the pHDAC1-transfected HaCaT cells, an over-production of VEGF was detected in a time-dependent manner. The highest VEGF production occurred at 72 hours post-transfection, but the over-production of VEGF was abolished in HaCaT cells co-transfected with HIF-1α-siRNA and pHDAC1 ($p < 0.05$; Fig. 1b). High levels of VEGF were detected by immunofluorescence analysis in the cytoplasms of the pHDAC1-transfected HaCaT cells at 72 hours post-transfection (Fig. 1c). To corroborate the role that HDAC1 plays in VEGF expression, trichostatin A (TSA) and HDAC1-siRNA were used to inhibit the activity of HDACs. HaCaT cells treated with TSA showed a decrease in VEGF production in a dose-dependent manner ($p < 0.05$; Fig. 1d). The HaCaT cells treated with HDAC1 siRNA (250-1000 nM) showed lower levels of HDAC1 mRNA than the cells treated with c-siRNA (Fig. 1e). As expected, and as seen in TSA-treated cells, HDAC1-siRNA-transfected HaCaT cells produced low quantities of VEGF, when compared with levels seen in c-siRNA-treated cells ($p < 0.05$; Fig. 1f). These results indicate that HDAC1 positively regulates VEGF expression in keratinocytes.

The expression of VHL decreases VEGF production in HaCaT cells

The pVHL-transfected HaCaT cells showed higher levels of VHL mRNA expression than those transfected with the control vector (Fig. 2a). Analysing the expression of VHL by immunofluorescence, a high level of VHL expression was detected in the cytoplasm of the pVHL-transfected cells and the expression of VHL was seldom observed in the control cells (Fig. 2b). When the production of VEGF was analysed in the supernatants of pVHL-transfected HaCaT cells, the levels of

FIGURE 1. HDAC1 increases VEGF production in HaCaT cells. (a) HaCaT cells were transfected with 0.4 μ g of control vector (pcDNA3.1) or with 0.4 μ g of pHDAC1, and RT-PCR was performed to analyse HDAC1 mRNA expression; GAPDH expression was used as a loading control. C= non-transfected cells. (b) Extracellular VEGF production was quantified by ELISA in the supernatants of transfected cells with 0.4 μ g pHDAC1 with different times of transfection. The levels of VEGF in non-transfected cells and in pcDNA3.1-transfected cells were measured at 72 h of culture and of post-transfection, respectively. Co-transfection was also performed with HIF-1 α siRNA (1000 ng/mL), and pHDAC1 and VEGF were quantified at 72 h post-co-transfection. (c) The immunofluorescence of HaCaT-transfected cells using 0.4 μ g plasmid and cultured for 72 hours. Rabbit IgG anti-VEGF was used as the primary antibody and stained with fluorescein-conjugated goat IgG anti-rabbit-IgG as the secondary antibody. Rabbit IgG-antibody was used as a control. Propidium iodide was used to stain the nuclei. (d) The quantification of VEGF from the supernatants of HaCaT cells treated with different concentrations of TSA/Ethanol was performed by ELISA. C= non treated cells. The analysis of HDAC1 mRNA expression by RT-PCR (e) and the quantification of VEGF by ELISA (f) in cells transfected with different concentrations of HDAC1 siRNA (HDAC1-siRNA) and of control (c-siRNA). Tukey's test was performed. (*) indicates a significant difference between non-treated (C) and treated cells ($p < 0.05$) with $n = 3$.

FIGURE 2. VHL decreases VEGF expression in HaCaT cells. (a) HaCaT cells were transfected with 0.4 μ g of control vector (pcDNA3.1) or with 0.4 μ g of pVHL, and RT-PCR was performed to analyse VHL mRNA expression; GAPDH expression was used as a loading control. C= non-transfected cells. (b) Immunofluorescence of HaCaT-transfected cells using 0.4 μ g of plasmid, rabbit IgG anti-VHL as the primary antibody and fluorescein-conjugated goat IgG anti-rabbit-IgG as the secondary antibody. Rabbit IgG was used as a control. Propidium iodide was used to stain the nuclei. (c) The quantification of VEGF in supernatants of HaCaT cells transfected with 0.4 μ g of pVHL or with 0.4 μ g of control plasmid (pcDNA3.1) at different time points post-transfection was performed by ELISA. C= non-transfected cells. The levels of VEGF in non-transfected cells and in pcDNA3.1-transfected cells were measured at 72 h of culture and of post-transfection, respectively. VEGF was also determined in supernatants of pVHL-transfected HaCaT cells cultures in the presence of 10mM cobalt chloride (hypoxia-mimetic condition). (d) Similar to (b), but the antibodies used were rabbit IgG anti-VEGF as the primary antibody and fluorescein-conjugated goat IgG anti-rabbit-IgG as the secondary antibody. Tukey's test was performed; (*) indicates a significant difference between non-transfected (C) and transfected cells ($p < 0.05$) with $n=3$.

VEGF decreased in a time-dependant manner. After 72 hours post-transfection, the production of VEGF was reduced approximately five fold; however, this decrease in VEGF production was not observed in pVHL-transfected HaCaT cells cultured in hypoxia-mimetic conditions (Fig. 2c). To confirm the decrease of VEGF in the VHL-transfected HaCaT cells, intracellular VEGF was assayed by immunofluorescence, and it was found to be decreased in the cytoplasm (Fig. 2d). These results

demonstrate that VEGF expression is negatively regulated by VHL in keratinocytes.

LL-37 increases the production of VEGF in HaCaT cells

High levels of VEGF were found in the supernatants of LL-37-transfected HaCaT cells, and this increase from control levels occurred in a time-dependent manner (Fig. 3). These results suggest that LL-37 can positively regulate the expression of VEGF in keratinocytes.

FIGURE 3. LL-37 increases VEGF expression in HaCat cells. The quantification of VEGF from the supernatants of HaCaT cells transfected with 0.4 μ g of pLL-37 or with 0.4 μ g of control plasmid (pcDNA3.1) at different time points post-transfection was performed by ELISA. C= non-transfected cells. The levels of VEGF in non-transfected cells and in pcDNA3.1-transfected cells were measured at 72 h of culture and of post-transfection, respectively. Tukey's test was performed; (*) indicates a significant difference between non-transfected (C) and transfected cells ($p < 0.05$) with $n = 3$.

Co-participation of HDAC1, LL-37 and VHL in the regulation of VEGF production in HaCaT cells

Co-transfection assays were performed in HaCaT cells to analyse how VEGF production is affected when HDAC1, LL-37 and VHL interact. To study the effect of VHL on HDAC1 activity in VEGF production, HaCaT cells were co-transfected with a constant amount of pHDAC1 (0.4 μ g) and with different quantities of pVHL (0.1 to 1.6 μ g). VEGF production decreased as pVHL was augmented, overcoming the inductive activity of HDAC1 ($p < 0.05$; Fig. 4a). When the reverse analysis was performed using a constant quantity of pVHL and a variable amount of pHDAC1, a recovery in the production of VEGF was observed as HDAC1 was augmented ($p < 0.05$; Fig. 4b). These results show that HDAC1 and VHL have opposite effects on the production of VEGF in keratinocytes.

The effect of LL-37 on HDAC1 activity in VEGF production was also studied. HaCaT cells, co-transfected with a constant amount of pHDAC1 (0.4 μ g) and variable amounts of pLL-37, showed an increase in VEGF expression as pLL-37 was increased ($p < 0.05$; Fig. 4c). Similar results were obtained

when the cells were co-transfected with a constant amount of pLL-37 and different amounts of pHDAC1 ($p < 0.05$; Fig. 4d). These results suggest that both molecules have a positive effect on the regulation of VEGF expression in keratinocytes. The positive effect of LL-37 was also observed in co-transfected cells with constant amounts of pVHL and increasing amounts of pLL-37: the production of VEGF was recovered as LL-37 was augmented with a constant expression of VHL (Fig. 4e). However, the opposite effect was observed in the cells co-transfected with constant amounts of pLL-37 and variable pVHL: the expression of VEGF decreased as VHL was augmented ($p < 0.05$; Fig. 4f). These results show that LL-37 has a positive effect on VEGF expression and that VHL has the opposite effect of LL-37 and HDAC1 in keratinocytes.

Effect of the overexpression of HDAC1, LL-37 and VHL on the cytoplasmic levels of HIF-1alpha in HaCaT cells

The expression and localisation of HIF-1alpha in HDAC1-, LL-37- and VHL-transfected HaCaT cells was studied. Non-transfected cells and pcDNA3.1-transfected HaCaT cells have a basal expression of HIF-1alpha in the cytoplasm (Fig. 5a). In pHDAC1-transfected HaCaT cells, the translocation of HIF-1alpha into the nucleus was visualised by immunofluorescence and then verified through western blot analysis (Fig. 5a and 5b, respectively). In pLL-37-transfected HaCaT cells, there were increased levels of HIF-1alpha in the cytoplasm. Low levels of HIF-1alpha were detected in the cytoplasm of pVHL-transfected HaCaT cells (Fig. 5a).

Discussion

HDACs have been related with angiogenesis and hyperproliferation processes because tumour cells treated with HDAC inhibitors, such as TSA[22] and suberoylanilide hydroxamic acid (SAHA),[23] disrupted these processes.[15] In this study, in keratinocytes treated with TSA and with HDAC1-siRNA, the VEGF production was found to decrease, suggesting that HDAC1 plays an important role in the production of VEGF in keratinocytes.

It is known that HDAC1 regulates the expression of VHL; for instance, human endothelial cells transfected with HDAC1 have a low expression of VHL.[3] This study demonstrated that HDAC1 can act as an antagonist of the VHL-related effect on VEGF production when HaCaT cells were co-transfected with pHDAC1 and pVHL. This study also showed that when keratinocytes were transfected with HDAC1, HIF-1alpha was translocated into the nucleus and the production of VEGF was upregulated; the knockdown of HIF1-alpha de-

FIGURE 4. Co-activity of HDAC1/VHL, HDAC1/LL-37 and VHL/LL-37 on the VEGF expression in HaCaT cells. The quantification of VEGF in the supernatants of HaCaT cells co-transfected with pHDAC1/pVHL, pHDAC1/pLL-37 or pVHL/pLL-37 after 72 hours post-transfection was performed by ELISA. Tukey's test was performed; (*) indicates a significant difference between the non-transfected cells and co-transfected cells ($p < 0.05$) with $n = 3$.

FIGURE 5. The expression and localization of HIF-1alpha in HaCaT cells transfected with HDAC1, VHL or LL-37. (a) The immunofluorescence analysis in HaCaT cells transfected with 0.4 µg of pHDAC1, pVHL or pLL-37. (b) The nuclear and cytoplasmic protein extract of HaCaT cells transfected with 0.4 µg of pHDAC1 was analysed by Western blot with anti-HIF-1alpha antibody.

creased the production of VEGF (Fig. 5a). This result suggests that HDAC-1 regulates VEGF production via HIF-1alpha.

The antagonistic effect of HDAC1 on VHL might occur in keratinocytes because acetylated-HIF-1alpha, an HDAC1 substrate, shows low affinity to VHL when deacetylated.[24] In breast cancer cell lines, the deacetylation of HIF-1alpha is mediated by the nucleosome remodelling histone deacetylation (NuRD) complex.[25] This complex is composed of HDAC1 or HDAC2 and the metastasis-associated protein 1 (MTA1). MTA1,[26] HDAC1 and HDAC2[27-28] are expressed in human epidermis and HaCaT cells. Thus, the NuRD complex could be deacetylating HIF-1alpha to avoid its degradation via VHL in pHDAC1-transfected HaCaT cells, leading to the accumulation of VEGF in the cell.

It has been well established in tumour cells that under normoxic conditions, VHL protein is part of the ubiquitin E3 ligase complex that ubiquitinates the hydroxylated-HIF-1alpha and that ubiquitinated HIF-1alpha is susceptible to proteasomal degradation. One consequence of low levels of HIF-1alpha is the decrease of VEGF production.[12] In this work, the production of VEGF was also observed to be negatively controlled by the activity of VHL in keratinocytes (Fig. 2). These data are consistent with observations in psoriatic skin biopsies (highly vascularised tissues) because the absence of the VHL expression and the presence of HDAC1[21] could explain the high production of VEGF. Additionally, mice with a *vhl*^{-/-} epidermal deletion show an increase in erythropoietin and in skin vascularisation;[13] *vhl*^{-/-} keratinocytes over-express HIF-1alpha and HIF-2alpha,[4] which causes the increase of the

expression of HIF target genes such as VEGF, PGK, GLUT1, INOS mRNAs and erythropoietin.[29] Studying the opposite biological phenomenon in keratinocytes, the upregulation of VHL decreased the basal levels of HIF-1alpha in the cytoplasm and decreased VEGF production (Fig. 5a); in hypoxic-mimetic cells transfected with pVHL, which produce high levels of HIF-1alpha, VEGF production was not decreased. These results suggest that, as in tumour tissues, VHL negatively regulates VEGF expression via HIF-1alpha in keratinocytes.

Recently, LL-37-transfected keratinocytes were seen to produce high quantities of mRNA of VEGF[20] and peptidoglycan from *Staphylococcus aureus* was seen to induce the production of LL-37 and VEGF in this cell type.[30] While evaluating keratinocytes for the regulatory activity of LL-37 on VEGF production, LL-37 and HDAC1 were observed to increase HIF-1alpha and VEGF expression in transfected cells. These results suggest that LL-37 could have an important role in the angiogenesis of psoriasis. In psoriatic lesions, this peptide is highly expressed with other molecules associated with pro-angiogenic activity, such as HIF-1alpha and HDAC1.[21] LL-37 has recently been suggested to be a possible trigger in the etiopathogenesis of psoriasis. Lande et al. reported that stressed keratinocytes release DNA that forms complexes with the LL-37, which, in turn, activates plasmacytoid dendritic cells (pDCs) to produce interferon-alpha (IFNalpha)[31]. Dermal DCs activated by IFN-alpha (produced by pDCs) lead to the production of Th1 and Th17 cytokines, which, in turn, induce the proliferation of keratinocytes.[31] It has recently been reported that the HIF-1alpha and HDAC-1 system may play an important role in T-cell differentiation to Th17 in psoriasis patients.[32-33]

These results, when combined with what occurs in psoriatic lesions, suggest that the hyperproliferation of keratinocytes leads to hypoxic conditions.[29] [34] [30] In this environment, psoriatic keratinocytes produce high quantities of VEGF via HIF-1alpha that is not ubiquitinated by VHL due to the intervention of HDAC1 and LL-37. The same phenomenon could be occurring in squamous cell carcinoma (SCC) as HDAC1 overexpression has been observed in this cancer[35] and because in oral squamous cell carcinoma, VEGF is over-expressed and is regulated by HIF-1alpha under hypoxic conditions.[36-37] In the same way, the dysfunction of VHL is also associated with the development of tongue cancer [39] as well as in psoriasis.[21]

Thus, new compounds that inhibit the activity of HDAC1 and LL-37 or that activate VHL could be used as targets for the control of the angiogenesis of psoriasis.

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