The antimicrobial peptide derived from insulin-like growth factor-binding protein 5, AMP-IBP5, regulates keratinocyte functions through Mas-related gene X receptors

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Abbreviations:

AMP-IBP5: antimicrobial peptide derived from insulin-like growth factor-binding protein 5; **hBD:** human β-defensin; **HDP:** host defense peptide; **IGFBP:** insulin-like growth factor-binding protein; **IL:** interleukin; **MrgX:** Mas-related gene X; **TGF:** transforming growth factor; **VEGF:** vascular endothelial growth factor

Abstract

Background: In addition to their microbicidal properties, host defense peptides (HDPs) display various immunomodulatory functions, including keratinocyte production of cytokines/chemokines, proliferation, migration and wound healing. Recently, a novel HDP named AMP-IBP5 (antimicrobial peptide derived from insulin-like growth factor-binding protein 5) was shown to exhibit antimicrobial activity against numerous pathogens, even at concentrations comparable to those of human β-defensins and LL-37. However, the immunomodulatory role of AMP-IBP5 in cutaneous tissue remains unknown.

Objectives: To investigate whether AMP-IBP5 triggers keratinocyte activation and to clarify its mechanism.

Methods: Production of cytokines/chemokines and growth factors was determined by appropriate ELISA kits. Cell migration was assessed by *in vitro* wound closure assay, whereas cell proliferation was analyzed using BrdU incorporation assay complimented with XTT assay. MAPK and NF-κB activation was determined by Western blotting. Intracellular cAMP levels were assessed using cAMP enzyme immunoassay kit.

Results: Among various cytokines/chemokines and growth factors tested, AMP-IBP5 selectively increased the production of IL-8 and VEGF. Moreover, AMP-IBP5 markedly enhanced keratinocyte migration and proliferation. AMP-IBP5-induced keratinocyte activation was mediated by Mrg X1-X4 receptors with MAPK and NF-κB pathways working

downstream, as evidenced by the inhibitory effects of MrgX1-X4 siRNAs and ERK-, JNK-, p38- and NF-κB-specific inhibitors. We confirmed that AMP-IBP5 indeed induced MAPK and NF-κB activation. Furthermore, AMP-IBP5-induced VEGF but not IL-8 production correlated with an increase in intracellular cAMP.

Conclusions: Our findings suggest that in addition to its antimicrobial function, AMP-IBP5 might contribute to wound healing process through activation of keratinocytes.

Keywords: Antimicrobial (host defense) peptides, MrgX receptor, MAPK, NF-κB, Skin, Wound healing

Introduction

Skin derived-antimicrobial peptides, also known as host defense peptides (HDPs), provide the front-line mechanism against infection in innate immunity. In addition to their antimicrobial activity, HDPs are also involved in diverse biological processes, including chemotaxis, cytokine/chemokine production and modulation of apoptosis [1]. Moreover, HDPs display growth factor-like effects by enhancing the *in vitro* keratinocyte migration and proliferation and promoting angiogenesis and wound epithelialization [1]. HDPs interact with various cell surface receptors, including CC chemokine receptors, epidermal growth factor receptor (EGFR), Toll-like receptors and Mas-related gene X (MrgX) receptors [1-4]. MrgXs receptors (MrgX1 to X4) are mainly expressed in human neurons, but they are also detected in mast cells and keratinocytes, where they modulate pain, itchiness and various immune responses [4-6].

Recently, a novel HDP named antimicrobial peptide derived from insulin-like growth factor-binding protein 5 (AMP-IBP5) was discovered by the Osaki group [7]. AMP-IBP5 is a proteolytic product of the cleavage of insulin-like growth factor-binding protein 5 (IGFBP-5) by a defined set of processing proteases such as prohormone convertase (PC) 1/3, PC2 and carboxypeptidase, which are expressed in normal human skin [8,9]. IGFBPs comprise a family of six proteins (IGFBP-1 to -6) that all bind insulin-like growth factors (IGFs) with high affinity and specificity [10]. IGFBPs play an important role in many pathologic and

physiologic processes through not only regulating IGF action and bioavailability (IGF-dependent actions), but also mediating IGF-independent actions, including inhibition or enhancement of cell growth, migration, differentiation and induction of apoptosis [10]. IGFBP-5, the most conserved member of the IGFBP family, is found in large amounts in connective tissues, skin, extracellular matrix and in various cell types such as bone cells, fibroblasts, keratinocytes [10,11]. IGFBP-5 stimulates the growth of bone cells and prostate cancer cells, and is associated with the differentiation of epithelial cells and osteoblasts. On the contrary, IGFBP-5 inhibits cell proliferation and migration of a variety of cells, and induces apoptosis in many cancer cells [10,11]. In the skin, IGFBP-5 is reported to facilitate fibroblast and keratinocyte proliferation and migration and to promote tissue remodeling as well as wound healing [10,12]. These variable functions might be related to the presence of proteases, which degrade IGFBP-5 or interaction with the extracellular matrix [10,11].

AMP-IBP5 displays strong killing activities against a wide range of pathogens at the concentrations comparable or even lower than those of human β-defensins (hBDs) and cathelicidin LL-37, the two major families of skin-derived HDPs that are also implicated in immunomodulation [7]. However, to date, the immunomodulatory functions of AMP-IBP5 have not been reported. Because the parent protein of AMP-IBP5, IGFBP-5, and other skin-derived HDPs are implicated in the regulation of various keratinocyte functions [1,12], the present study was aimed to elucidate the possible role of AMP-IBP5 in activation of

human keratinocytes and to examine the plausible underlying mechanism. Here, we demonstrated that among the various cytokines/chemokines and growth factors tested, AMP-IBP5 selectively stimulated the production of interleukin (IL)-8 and vascular endothelial growth factor (VEGF), which are involved in the promotion of angiogenesis and the acceleration of the wound healing process [13-15]. AMP-IBP5 also accelerated keratinocyte migration and proliferation. Keratinocyte activation induced by AMP-IBP5 was shown to be mediated by the Mas-related gene (Mrg) X1-X4 receptors, which likely act upstream of the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling cascades. Taken together, these observations suggest that, in addition to its antimicrobial activity, AMP-IBP5 might also contribute to the regulation of cutaneous immunity through activation of keratinocytes. This HDP may be useful in situations where enhanced keratinocyte migration and proliferation is required, such as wound healing and skin regeneration.

Materials and methods

1. Reagents

AMP-IBP5 (AVYLPNCDRKGFYKRKQCKPSR-NH2) was purchased from the Peptide Institute (Osaka, Japan). Extracellular signal-regulated kinase (ERK) inhibitor (U0126), p38 inhibitor (SB203580), c-Jun N-terminal kinase (JNK) inhibitor II, and NF-κB activation inhibitor II were obtained from Calbiochem (La Jolla, CA). Rabbit polyclonal anti-phosphorylated ERK, JNK, p38 and IκB antibodies, as well as unphosphorylated ERK, JNK, p38 and IκB antibodies were obtained from Cell Signaling Technology (Beverly, MA). Human skin collagen I was purchased from Merck (Darmstadt, Germany). Forskolin, 3-isobutyl-1-methylxanthine (IBMX) and adenylyl cyclase inhibitor (SQ 22,536) were purchased from Sigma-Aldrich (St Louis, MO).

2. Culture and stimulation of human keratinocytes

Normal primary human keratinocytes (Kurabo Industries, Osaka, Japan) from neonatal foreskin were cultured in HuMedia-KG2 (Kurabo Industries), as reported previously [4]. Cells were serially passaged at 60 to 70% confluence, and all experiments were conducted using sub-confluent cells (60 to 80% confluence) cultured in 12-well plates and incubated with AMP-IBP5 in HuMedia without supplements.

3. Enzyme-linked immunosorbent assay (ELISA)

Cells were incubated with AMP-IBP5 at 37°C for indicated time periods, and the

cytokines/chemokines and growth factors released into the supernatants were measured with appropriate ELISA kits from R&D Systems (Minneapolis, MN), according to the manufacturer's instructions. In some experiments, keratinocytes were pretreated with various inhibitors 2 h before stimulation, and ELISA was performed as indicated above.

4. Cell migration

The migration of keratinocytes was analyzed by an *in vitro* wound closure assay, as previously described [4]. Cells were cultured in a collagen I-coated 24-well plate for 3 h, and a wound was made across the confluent keratinocyte monolayers with a p-200 pipette tip. After removing the cellular debris by washing with phosphate buffered saline, cells were stimulated for 24-72 h at 37°C, and the repopulation of wounded areas was photographed under the phase-contrast microscope (Keyence, Osaka, Japan).

5. Cell proliferation assays

Keratinocytes cultured onto Lab-Tek II eight-chamber glass slides (Nalge Nunc International, Naperville, IL) were stimulated with AMP-IBP5 for 24-96 h at 37°C and then incubated with 10 μM of bromodeoxyuridine (BrdU) for 1 h. Cell proliferation was assessed using a BrdU labeling and detection kit II (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instructions. The percentage of cells that had incorporated BrdU was calculated as the number of BrdU-positive cells / total number of cells × 100.

Cell proliferation was also assessed using an XTT assay kit (Roche, Mannheim, Germany),

according to the manufacturer's instructions. Keratinocytes cultured in a 96-well plate were treated with AMP-IBP5 for 24-96 h at 37°C, and XTT reagents were subsequently added into each well for 4 h to generate colorimetric formazan products. The amount of formazan dye was measured by absorbance at 450 nm with a microplate reader.

6. Western blot analysis

Cell lysates were obtained by lysing the cells in RIPA buffer (Cell Signaling Technology), and equal amounts of protein extracts were loaded onto a 12.5% SDS-PAGE gel for immunoblotting. Non-specific binding sites were blocked, and the membranes were incubated with appropriate antibodies, according to the manufacturer's instructions. The membranes were developed with the Luminata Forte Western HRP substrate (Millipore, Billerica, MA) and visualized using Fujifilm LAS-4000 Plus (Fujifilm, Tokyo, Japan).

7. RNA interference

Keratinocytes were cultured in 12-well plates until sub-confluent and then transfected with 500 nM of MrgX1-X4 small interfering RNAs (siRNAs) or control siRNA (Invitrogen, Carlsbad, CA) for 24 h at 37°C, according to the manufacturer's specifications. Gene silencing was conducted, and the efficacy of knockdown was confirmed by real-time PCR analysis (data not shown). Following transfection, the cells were incubated with AMP-IBP5 and tested for ELISA, cell proliferation, migration or Western blotting.

8. Intracellular cyclic adenosine monophosphate (cAMP) activation

Keratinocytes were pretreated with 1 mM IBMX for 30 min to inhibit phosphodiesterase activity to prevent the breakdown of cAMP within the cells. Cells were then stimulated at 37°C for indicated periods, and intracellular cAMP levels were measured using a cAMP enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI), according to the manufacturer's instructions.

9. Statistical analysis

The statistical analysis consisted of either ANOVA followed by the appropriate post-hoc test or Student's *t*-test using Prism GraphPad for Windows (Prism 6, GraphPad Software, San Diego, CA). $P < 0.05$ was considered significant. The results are presented as the mean values ± standard deviation.

Results

1. AMP-IBP5 increases the production of IL-8 and VEGF by keratinocytes

Because the parent protein of AMP-IBP5, IGFBP-5, is reported to activate keratinocytes [12], we hypothesized that AMP-IBP5 might also stimulate human keratinocytes. Among various cytokines/chemokines investigated, AMP-IBP5 selectively induced the production of IL-8. This effect was dose- and time-dependent, with a peak observed at 72 h post-stimulation (Fig. 1a). We observed that AMP-IBP5 failed to increase the production of IL-6, monocyte chemoattractant protein (MCP)-1 and -3, and macrophage inflammatory protein (MIP)-1α and -1β even at doses as high as 40 μM (data not shown), suggesting a specific effect of AMP-IBP5 on IL-8 production. Given that IGFBP-5 is involved in the regulation of angiogenic factors and the wound healing process [12,16], we examined whether AMP-IBP5 could also induce the production of angiogenic growth factors. AMP-IBP5 enhanced the production of VEGF in a dose- and time-dependent manner (Fig. 1b). However, AMP-IBP5 failed to increase the levels of other angiogenic growth factors, including epidermal growth factor, fibroblast growth factor and transforming growth factor (TGF)-β (data not shown). We confirmed that the doses of AMP-IBP5 used in this study were not toxic, as assessed by lactate dehydrogenase activity (data not shown). Together, the above observations suggest that AMP-IBP5 might be involved in modulation of inflammation and wound healing through its ability to induce IL-8 and VEGF production by keratinocytes.

2. AMP-IBP5 promotes keratinocyte migration and proliferation

Given that IGFBP-5 has been reported to promote keratinocyte migration and proliferation [12] and AMP-IBP5 induces the production of IL-8 and VEGF (Fig. 1), which are known to enhance keratinocyte proliferation and re-epithelialization [14,15], we predicted that AMP-IBP5 could also stimulate keratinocyte migration and proliferation. Cell migration, assessed by the *in vitro* wound closure assay, showed that keratinocytes stimulated with various doses of AMP-IBP5 migrated inwardly and almost covered the total area of the wounds (Fig. 2a). In preliminary experiments, AMP-IBP5-induced cell migration was first observed at 24 h and peaked at 48 h before decreasing at 72 h. The potency of AMP-IBP5 was comparable to that of TGF-α, a strong inducer of keratinocyte migration and proliferation [17].

We next examined the ability of AMP-IBP5 to induce keratinocyte proliferation using the BrdU incorporation assay complimented with the XTT assay. As pictured in Fig. 2b and 2c, when keratinocytes were treated with increasing concentrations of AMP-IBP5 for 48 h, significantly increased keratinocyte proliferation was observed (twofold increase, Fig. 2c). To further confirm the BrdU incorporation results, the XTT assay was performed and corroborated that AMP-IBP5 markedly increased keratinocyte proliferation (twofold increase), although this effect was not dose-dependent (Fig. 2d). Cell proliferation was first observed at 24 h and persisted up to 96 h post-stimulation (data not shown).

3. AMP-IBP5-induced keratinocyte activation is mediated through MrgX receptors

We recently reported that keratinocytes express MrgX1-X4 receptors and that a novel HDP with angiogenic activities, AG-30/5C, utilizes both MrgX3 and MrgX4 to activate keratinocytes [4]. Furthermore, hBDs and LL-37 have been shown to cause mast cell migration through MrgX2 [2,3]. Therefore, we examined the possible role of MrgXs in AMP-IBP5-induced keratinocyte activation by transfecting cells with MrgX1-X4 siRNAs to knock down their respective gene expression. Fig. 3a (left panel) shows that there was a significant, though not complete, inhibition of IL-8 production in MrgX1-X4 siRNA-transfected keratinocytes (36-52% inhibition). In contrast, MrgX1-X4 siRNAs showed a dramatic inhibition (60-77%) of AMP-IBP5-increased VEGF production (right panel). We also analyzed the effect of MrgX1-X4 siRNAs on AMP-IBP5-induced keratinocyte migration and proliferation and observed a great reduction in cell motility and proliferation in MrgX1-X4 siRNA-transfected keratinocytes (Fig. 3b and 3c). The above observations imply that activation of MrgX1-X4 is required for the AMP-IBP5-mediated keratinocyte activation.

4. AMP-IBP5 activates MAPK and NF-κB pathways, which are required for the production of IL-8 and VEGF

To further examine which signaling pathways act downstream of MrgXs, we focused on MAPK and NF-κB because these molecules are involved in diverse cellular activities, including cytokine/chemokine production [18]. Western blot analysis revealed that AMP-IBP5

induced phosphorylation of ERK, JNK and IκB as early as 5 min post-stimulation, and this activation of IκB persisted for 60 min. In contrast, phosphorylation of p38 was first observed at 15 min post-stimulation and peaked at 30 min before decreasing (Fig. 4a). We confirmed that the activation of MAPK and NF-κB pathways was indeed required for AMP-IBP5-mediated IL-8 and VEGF production. ERK and NF-κB inhibitors markedly suppressed IL-8 production (104% and 82% inhibition, respectively), while JNK and p38 inhibitors had no significant inhibitory effect (Fig. 4b, left panel). In contrast, as seen in Fig. 4b, right panel, all of the MAPK and NF-κB inhibitors markedly suppressed VEGF production (88-130% inhibition), suggesting that there might be different mechanism(s) between AMP-IBP5-induced IL-8 and VEGF production in keratinocytes.

Activation of MAPK and NF-κB pathways was linked to MrgX receptors because transfection of keratinocytes with MrgX1-X4 siRNAs noticeably reduced AMP-IBP5-mediated ERK, JNK, p38 and IκB phosphorylation (Fig. 5). This suggests that MAPK and NF-κB pathways act downstream of MrgX receptors.

5. AMP-IBP5-induced VEGF production is mediated via intracellular cAMP activation

Numerous studies have suggested that activation of intracellular cAMP is important for the regulation of cytokines/chemokines and growth factors, including IL-8 and VEGF [19-21]. In addition, previous studies have indicated that cAMP can regulate MAPK and NF-κB pathways [20,22]. Therefore, to further understand the mechanism by which AMP-IBP5

stimulates IL-8 and VEGF production, keratinocytes were treated for 1 h with a cAMP specific inhibitor, SQ 22,536, and then challenged with AMP-IBP5. This inhibitor did not affect IL-8 production, but it completely suppressed VEGF production (74-102% inhibition, Fig. 6a). We confirmed that AMP-IBP5 did indeed significantly enhance intracellular cAMP activation. AMP-IBP5 rapidly enhanced cAMP activation (threefold increase) at 2.5 and 5 min post-stimulation before activation decreased back to baseline (Fig. 6b). Forskolin, a strong inducer of intracellular cAMP [19], was used as a positive control. The above findings indicate that AMP-IBP5 likely stimulates IL-8 and VEGF production through different mechanisms.

Discussion

The present study demonstrated that a novel HDP, AMP-IBP5, has the ability to activate keratinocytes to generate IL-8 and VEGF production and to migrate and proliferate. AMP-IBP5-induced keratinocyte activation was mediated through MrgX1-X4 receptors, working upstream of MAPK and NF-κB pathways. Therefore, our findings provide evidence that AMP-IBP5 might promote wound healing through activation of keratinocytes in addition to its antimicrobial activities.

Skin-derived HDPs such as hBDs and LL-37 have been involved in the keratinocyte production of various cytokines/chemokines, including IL-6, IL-8, IL-10, IL-18, MCP-1, MIP-3α, and IFN-γ-inducible protein (IP-10) [1]. Here, we showed that among the various cytokines/chemokines tested, AMP-IBP5 selectively increased IL-8 production, which plays an important role not only in inflammation but also in angiogenesis and wound healing [13,15]. In addition to IL-8, AMP-IBP5 also induced the production of another potent angiogenic factor, VEGF, which is involved in wound healing, tissue remodeling and inflammation [23,24]. This production was also selective, as AMP-IBP5 had no effect on other angiogenic growth factors investigated (data not shown). The parent protein of AMP-IBP5, IGFBP-5, is reported to have controversial roles in inflammation and wound healing. For example, Yasuoka *et al*. have suggested a pro-inflammatory role of IGFBP-5 due to its ability to induce the infiltration of inflammatory and immune cells into lung tissue [25],

while Liu *et al*. have demonstrated that IGFBP-5 can act as an anti-inflammatory factor by downregulating the production of inflammatory cytokines, including IL-1β, IL-6, IL-8 and TNF-α in mesenchymal stem cells [26]. Furthermore, although IGFBP-5 activates angiogenic growth factors and promotes cell migration and proliferation, leading to wound healing [12,16], IGFBP-5 has also been shown to inhibit VEGF production, cell migration, proliferation, invasion and angiogenesis [27,28]. Together, these observations indicate that AMP-IBP5 and its parent protein display different biological functions. These differences are probably due to the differences in the molecular structures between IGFBP-5 and AMP-IBP5, the different cell types used in various studies, and the fact that IGFBP-5 mediates its effects in both IGF-dependent and IGF-independent pathways.

Because AMP-IBP5 induced the production of IL-8 and VEGF, which are both involved in the wound healing process [15,23], we predicted that AMP-IBP5 might accelerate wound healing by increasing keratinocyte migration and proliferation. As expected, AMP-IBP5 enhanced keratinocyte migration and proliferation, the two essential steps necessary for wound re-epithelialization [29]. IGFBP-5 also increases motility of mesangial cells [30], stimulates growth of intestinal muscle cells [31], and facilitates keratinocyte proliferation and migration [12]. Likewise, other HDPs such as hBDs, LL-37, S100A7 (psoriasin) and AG30/5C also contribute to cutaneous wound closure [1,4], suggesting an important role of HDPs in the wound healing process.

AMP-IBP5-induced keratinocyte activation was mediated by MrgX1-X4. These receptors were initially believed to be selectively expressed in human neurons, where they contribute to the regulation of pain and itchiness [5]. Later, MrgXs were detected in human mast cells (MrgX1 and X2) and keratinocytes (MrgX1-X4), where they are implicated in cytokine/chemokine production, cell migration and proliferation [4,6]. Since both mast cells and keratinocytes are involved in itching and wound healing [32,33], MrgXs expressed in these cells may be utilized not only in pruritus transmission but also in immunomodulation. MrgXs can bind to multiple ligands, including bovine adrenal medulla 8-22 peptide for MrgX1 [34], substance P, cortistatin, hBDs and LL-37 for MrgX2 [2,3,6], and AG-30/5C for MrgX3 and MrgX4 [4]. The finding that AMP-IBP5 activates keratinocytes through MrgX1-X4 adds this HDP to a long list of ligands for the MrgXs and further confirms the importance of these receptors in HDP-induced cell activation.

To further characterize the signaling mechanism by which AMP-IBP5 activates keratinocytes, we focused on the MAPK and NF-κB signal cascades, which are necessary for the keratinocyte production of various cytokines/chemokines and growth factors, including IL-8 and VEGF [21,35]. Similar to other HDPs such as hBDs, LL-37 and dermcidin that activate keratinocyte via the MAPK and NF-κB pathways [1,36], AMP-IBP5-induced IL-8 and VEGF production was mediated through the MAPK and NF-κB pathways, working downstream of MrgXs. ERK, JNK, p38 and NF-κB mediated VEGF production, whereas only

ERK and NF-κB facilitated IL-8 production, suggesting distinct regulatory mechanisms for IL-8 and VEGF induction by AMP-IBP5. Therefore, to gain a deeper understanding of this mechanism, the role of intracellular cAMP, a common regulator for both IL-8 and VEGF [19,37], was investigated. AMP-IBP5-enhanced intracellular cAMP activation was exclusively required for VEGF but not IL-8 production. Although numerous studies have demonstrated that cAMP inducers such as forskolin, prostaglandin E_2 and dibutyryl cAMP enhance IL-8 secretion [19,38], some studies have reported that depending on cell type and stimulant nature, cAMP may not be a prerequisite for IL-8 production [39,40]. AMP-IBP5-induced VEGF production was regulated through MAPKs and NF-κB, supporting the connection between cAMP/MAPK and cAMP/NF-κB previously reported [20,22].

The levels of AMP-IBP5 in the human body are not precisely known. In the current study, the minimum effective dose of AMP-IBP5 was 1.25 μM, which is in the range of the doses (0.3-10 μM) necessary for AMP-IBP5 antimicrobial activity [7]. Therefore, we assume that the concentrations of AMP-IBP5 used in this study are adequate for evaluating its physiological role in keratinocytes. In conclusion, this study provides novel evidence that, beyond its microbicidal activity, AMP-IBP5 may contribute to the regulation of skin immunity through activation of keratinocytes. Gaining an understanding of the role of AMP-IBP5 in keratinocyte migration and proliferation will help in the development of treatments for wound healing and other diseases in which modulation of cell migration and

proliferation are required.

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Figure legends

Figure 1. AMP-IBP5 induces the production of IL-8 and VEGF by keratinocytes

(a) and **(b)** Normal human keratinocytes were stimulated with 2.5-20 μM AMP-IBP5 for 24-72 h, and the amounts of IL-8 **(a)** and VEGF **(b)** released into the culture supernatants were determined by ELISA. Values are compared between stimulated and non-stimulated cells (Med). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n = 6.

Figure 2. AMP-IBP5 promotes keratinocyte migration and proliferation

(a) AMP-IBP5 stimulates keratinocyte migration. Wounded keratinocyte monolayers were stimulated with AMP-IBP5, 10 ng/ml TGF-α or medium (Med) for 48 h. Live cultures were recorded by phase-contrast microscopy. Data represent six separate experiments. Bar = 250 μm. **(b)-(d)** AMP-IBP5 promotes keratinocyte proliferation. Keratinocytes grown onto Lab-Tek II chamber slides were stimulated with AMP-IBP5 or TGF-α for 48 h, and BrdU-positive cells were counted under a microscope. Bar = 50 μm. Representative data **(b)** of five separate experiments are shown **(c)**. Bar = 50 μm. **(d)** Keratinocytes were stimulated for 48 h, and the XTT assay was performed. Values are compared between stimulated and non-stimulated cells (Med). **P* < 0.05, ***P* < 0.01, n = 5.

Figure 3. Effect of MrgX siRNAs on AMP-IBP5-activated keratinocytes

(a) Keratinocytes were transfected with 500 nM MrgX1-X4 siRNAs or control siRNA for 24

h and then stimulated with 20 μM AMP-IBP5 for 72 h. IL-8 and VEGF levels were measured by ELISA. **(b)** The scratching assay was performed across MrgX1-X4- or control siRNA-transfected keratinocytes, followed by incubation with 2.5 μM AMP-IBP5 for 48 h. Bar = 250 μm. **(c)** Transfected cells were also stimulated with 2.5 μM AMP-IBP5 for 24 h, and the BrdU incorporation assay was performed. Bar = 50 μm. Values obtained using the non-stimulated (Med) and stimulated cells (Ctrl siRNA) or with MrgX siRNA-transfected and control siRNA-transfected cells (Ctrl siRNA) were compared. ##$P < 0.01$, *$P < 0.05$, **$P < 0.01$, n = 4.

Figure 4. AMP-IBP5 activates keratinocytes through the MAPK and NF-κB pathways

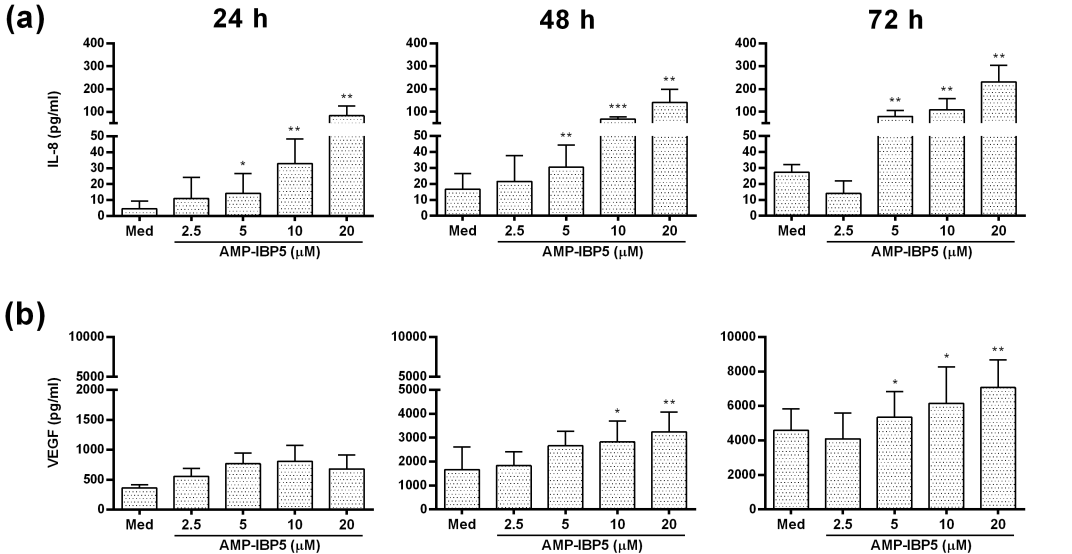
(a) Keratinocytes were stimulated with 20 μM AMP-IBP5 and subjected to SDS-PAGE using antibodies against phosphorylated or unphosphorylated ERK, JNK, p38 or IκB. Data are representative of six separate experiments. **(b)** Keratinocytes were pretreated with 20 μM U0126, JNK inhibitor II (JNK Inh II), SB203580 and NF-κB activation inhibitor II (NFκBAI II), or 0.1% DMSO (Med) for 2 h and exposed to 20 μM AMP-IBP5 for 72 h. IL-8 and VEGF levels were measured by ELISA. Values obtained using the non-stimulated (Medium containing 0.1% DMSO) and stimulated cells without inhibitor (AMP-IBP5) or with the presence or absence of each inhibitor were compared. ##$P < 0.01$, ###$P < 0.001$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n = 5.

Figure 5. Effect of MrgX siRNAs on AMP-IBP5-mediated MAPK and NF-κB activation

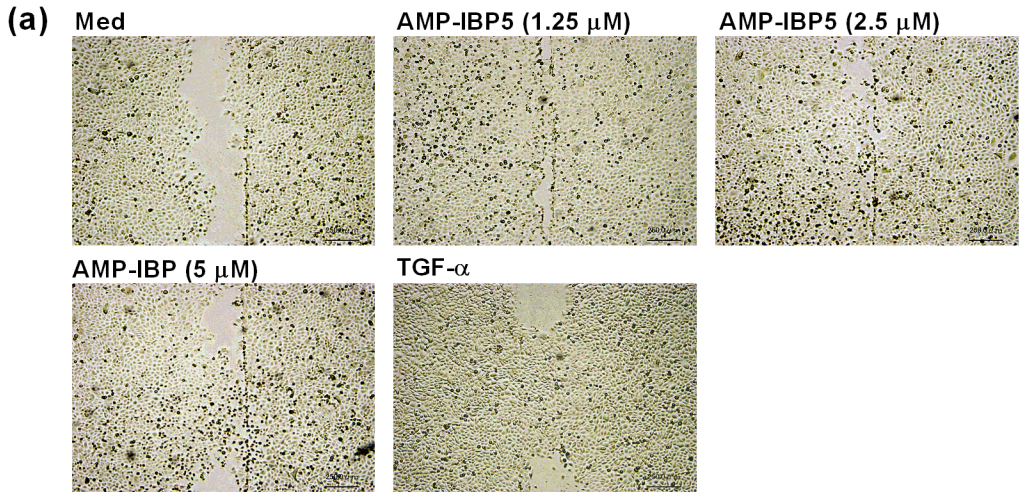
Keratinocytes were transfected with 500 nM MrgX1-X4 siRNAs or control siRNA for 24 h and then stimulated with 20 μM AMP-IBP5 for 15 min. The lysates were separated by SDS-PAGE using antibodies against phosphorylated or unphosphorylated ERK, JNK, p38, or IκB. Data are representative of four independent experiments with similar results.

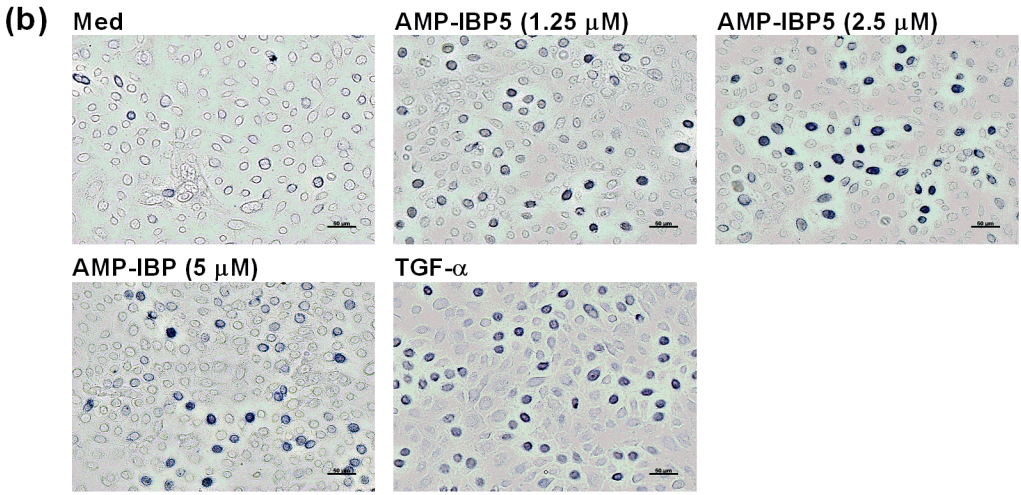
Figure 6. AMP-IBP5 increases intracellular cAMP levels necessary for VEGF production

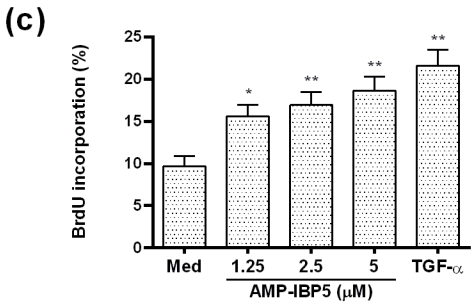
(a) Effect of SQ 22,536 on AMP-IBP5-induced IL-8 and VEGF production. Keratinocytes were pretreated with SQ 22,536 for 1 h and then challenged with 10 μM AMP-IBP5 for 72 h. IL-8 and VEGF levels were measured by ELISA. Values obtained using the non-stimulated (Medium containing 0.1% DMSO) and stimulated cells without inhibitor (AMP-IBP5) or with the presence or absence of inhibitor were compared. **(b)** AMP-IBP5 enhances intracellular cAMP levels. Keratinocytes were stimulated with 10 μM AMP-IBP5 (dots) and 20 μM forskolin (stripes) for 1-30 min. The cAMP levels in the supernatants were determined by the appropriate kit. Values were compared between stimulated and non-stimulated cells (0 min). #$P < 0.05$, *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, n = 4-5.

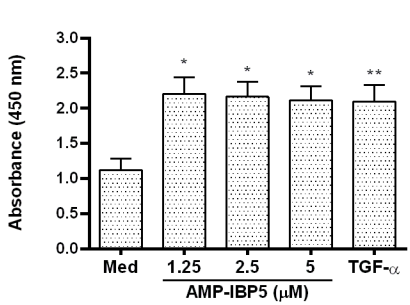


Chieosilapatham P. et al. Figure 1

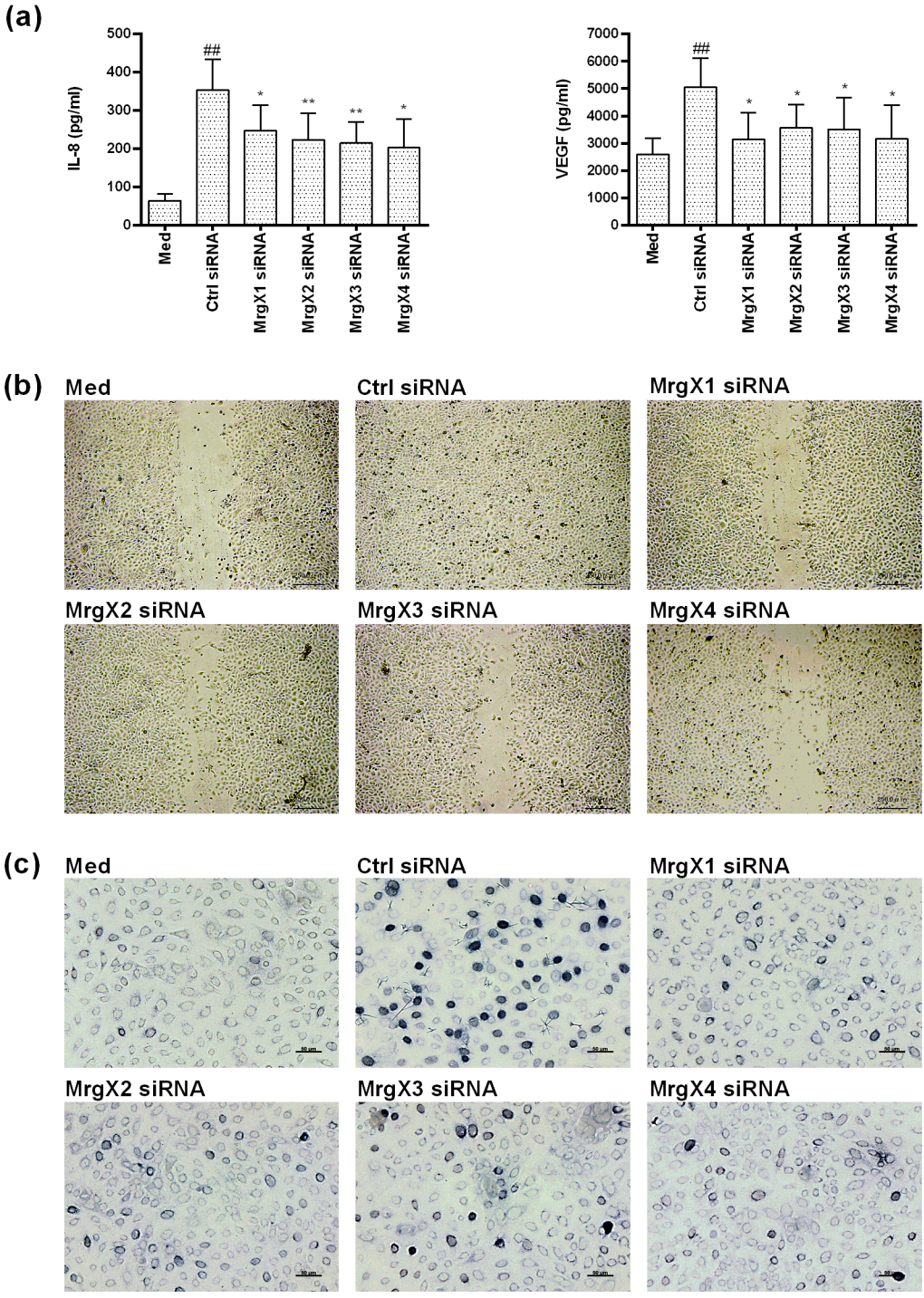




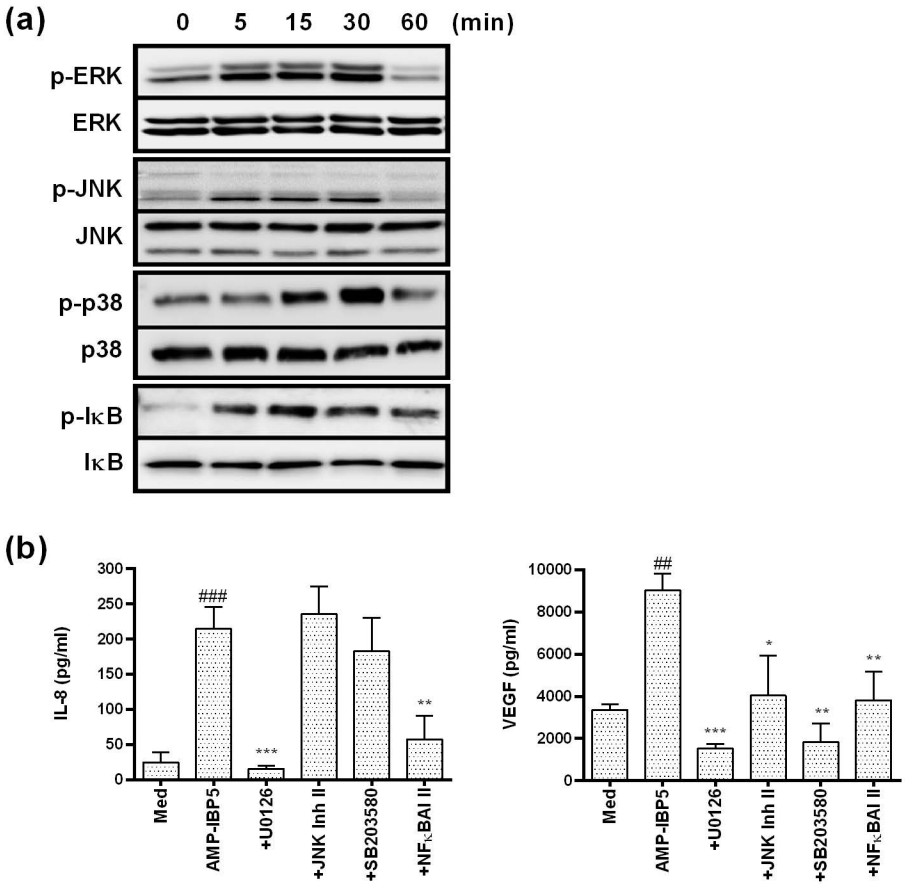




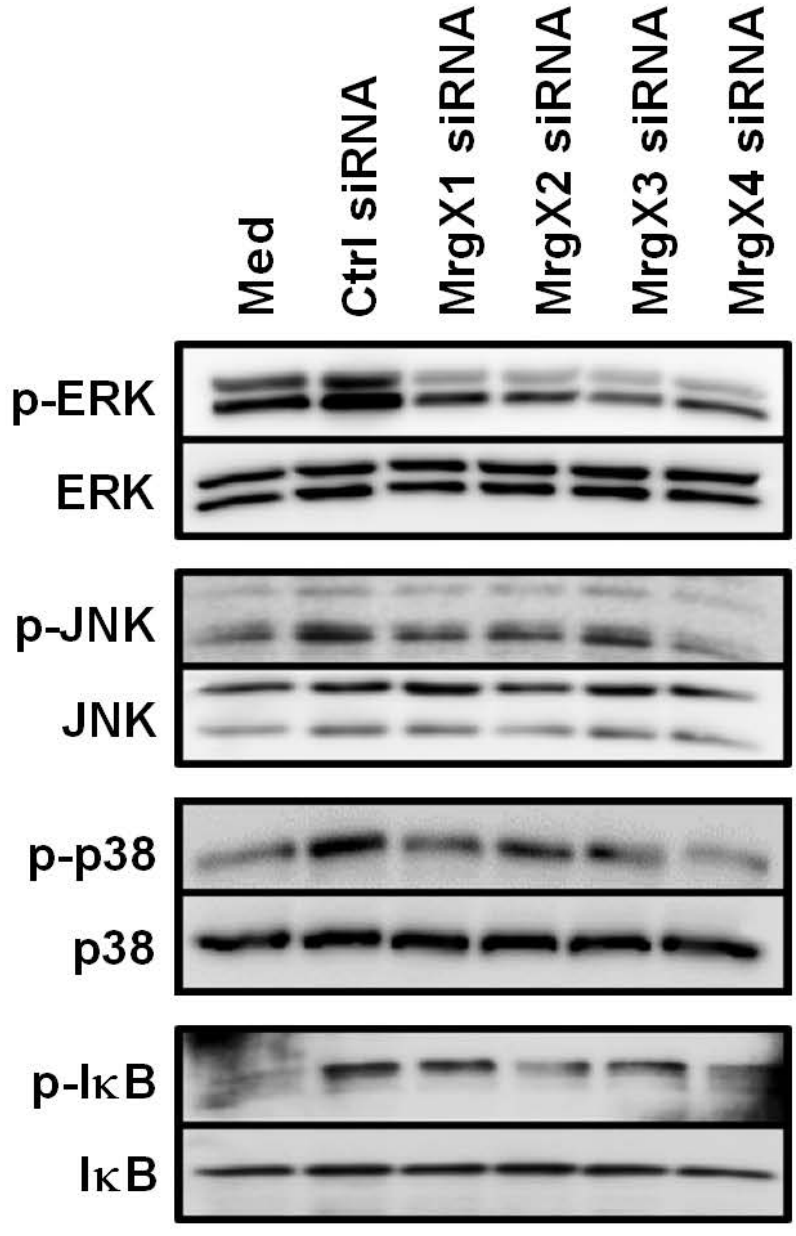
Chieosilapatham P. et al. Figure 2



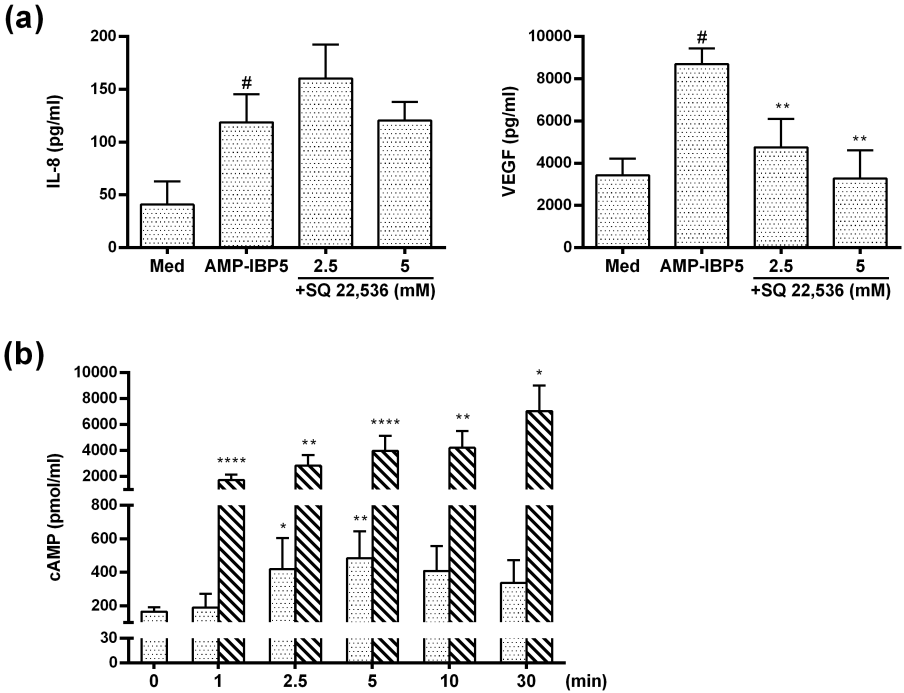
Chieosilapatham P. et al. Figure 3



Chieosilapatham P. et al. Figure 4



Chieosilapatham P. et al. Figure 5



Chieosilapatham P. et al. Figure 6