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Human catestatin enhances migration and proliferation of normal human epidermal keratinocytes

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A R T I C L E I N F O

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A B S T R A C T

Background: Skin-derived antimicrobial peptides, such as human β-defensins and cathelicidins, actively contribute to host defense by inactivating microorganisms. Catestatin, a neuroendocrine peptide that affects human autonomic functions, has recently been detected in keratinocytes upon injury/infection where it inhibits the growth of pathogens. Human catestatin exhibits three single nucleotide polymorphisms: Gly364Ser, Pro370Leu, and Arg374Gln.
Objective: To investigate the effects of human catestatin and its variants on keratinocyte migration and proliferation, and to elucidate the possible signaling mechanisms involved.
Methods: The migration of normal human keratinocytes was analyzed using Boyden microchamber assay and *in vitro* wound closure assay. Cell proliferation was evaluated by BrdU incorporation, cell count assay and cell cycle analysis. Intracellular Ca^{2+} mobilization was measured using a fluorescent calcium assay kit. The phosphorylation of epidermal growth factor receptor (EGFR), Akt, and MAPKs was determined by Western blotting.
Results: Catestatin and its variants dose-dependently enhanced keratinocyte migration and proliferation. Moreover, catestatin peptides increased intracellular Ca^{2+} mobilization and induced the phosphorylation of EGFR, Akt, extracellular signal-regulated kinase (ERK), and p38 in keratinocytes. The induction of keratinocyte migration and proliferation by catestatin peptides involved G-proteins, phospholipase C, EGFR, PI3-kinase, ERK, and p38, as evidenced by the specific inhibitory effects of pertussis toxin (G-protein inhibitor), U-73122 (phospholipase C inhibitor), AG1478 (EGFR inhibitor), anti-EGFR antibody, wortmannin (PI3-kinase inhibitor), U0126 (ERK inhibitor), and SB203580 (p38 inhibitor), respectively.
Conclusion: Besides inhibiting the growth of skin pathogens, catestatin peptides may also contribute to cutaneous wound closure by enhancing keratinocyte migration and proliferation at the wound site.
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1. Introduction

Antimicrobial peptides (AMPs), which are synthesized in the skin and other strategic tissues at the sites of potential microbial entry, provide a soluble barrier that impedes infection. In addition, these peptides modify the local inflammatory response by activating mechanisms of both the innate and adaptive arms of the immune system [1]. Human β-defensins (hBDs) and cathelicidins constitute the major families of well-studied skin-derived AMPs, although other AMPs have been demonstrated to display antimicrobial and immunomodulatory activities in the skin, including S100 proteins, dermcidin, and neuropeptides [2,3]. Upon infection or injury, the expression of AMPs in the skin is up-regulated due to increased synthesis by keratinocytes and deposition resulting from recruited neutrophil degranulation [2]. The levels of skin-derived AMPs, including hBDs and the cathelicidin LL-37, are considerably increased in epidermal keratinocytes and play pivotal roles in keratinocyte activities, such as in the production of cytokines and chemokines, cell migration, cell proliferation, and wound closure [1–3].

Recently, peptides derived from the chromogranin/secretogranin families of the neuroendocrine system have been reported to

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exhibit antimicrobial activities against various pathogens [4]. The proteolytic processing of the pro-hormone chromogranin A generates peptides that are released into the extracellular space upon stimulation [4]. Chromogranin A-derived peptides with antimicrobial activity include prochromacin, chromacin I and II, vasostatin-I, chromofungin, and catestatin [4,5]. Human catestatin (chromogranin $A_{352-372}$) exhibits antimicrobial activities against filamentous fungi, yeast and bacteria [6], although it was originally characterized as a catecholamine release-inhibitory peptide [7]. Human catestatin displays three natural polymorphisms (Gly364Ser, Pro370Leu and Arg374Gln), which are estimated to occur in ~4% of the population [8]. These polymorphisms show different potencies with regard to their inhibition of catecholamine secretion, with a rank order of Pro370Leu > wild-type catestatin > Gly364Ser > Arg374Gln [9].

Recently, the expression of catestatin in various cell types and its ability to activate cellular functions have been reported. For instance, catestatin is a strong chemotactic factor for human peripheral blood monocytes [10], and it induces migration, proliferation, and angiogenesis in endothelial cells [11]. Moreover, we have recently reported that catestatin peptides cause human mast cells to migrate, degranulate, and produce various cytokines and chemokines [12]. In human skin, the expression of catestatin has been detected in keratinocytes, and this expression was shown to increase in response to skin injury or infection in a mouse model [5]. Interestingly, we have recently shown that human catestatin can stimulate human keratinocytes in an autocrine fashion to release chemokines such as IL-8 [13]. Therefore, we hypothesized that catestatin peptides might also induce human keratinocyte migration and proliferation activities, which are among the first-essential steps in the process of cutaneous wound closure [14].

Here, we demonstrate that human catestatin and its naturally occurring variants increase keratinocyte migration and proliferation, and these activities are under the control of the following signaling proteins: G-proteins, phospholipase C (PLC), epidermal growth factor receptor (EGFR), Akt/PI3-kinase (PI3K), and the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and p38 pathways. This observation provides not only novel evidence of catestatin's contribution to cutaneous immuno-modulation but also highlights a new link between the neuroendocrine and cutaneous immune systems.

2. Materials and methods

2.1. Reagents

Human wild-type catestatin (SSMKLSFRARAYGFRGPGPQL), its variants Gly364Ser (SSMKLSFRARAYSFRGPGPQL), Pro370Leu (SSMKLSFRARAYGFRGPGLQL), and Arg374Gln (SSMKLSFRARAYGFRGPGPQLRQGWRPSSREDSLEAGLPLQVRGYPEE), and a scrambled form of catestatin sCst (MKLSSSFRAYARGFRGPGPQL) were synthesized using a solid-phase method on a peptide synthesizer (model PSSM-8; Shimadzu, Kyoto, Japan) by fluorenylmethoxycarbonyl (Fmoc) chemistry. The molecular masses of synthesized peptides were confirmed using mass spectrometry (model TSQ 700; Thermo Quest Finnigan, Manchester, UK). Synthetic hBD-3 was purchased from the Peptide Institute (Osaka, Japan). Transforming growth factor (TGF)-α, mitomycin C, U-73122, U-73343, wortmannin, staurosporine, U0126, SB203580, and SP600125 were purchased from Sigma–Aldrich (St. Louis, MO). Pertussis toxin (PTx), AG1478 and EGFR-neutralizing antibody (clone 225) were obtained from Calbiochem (La Jolla, CA). Rabbit polyclonal anti-phosphorylated EGFR (Tyr845), Akt (Ser473), ERK, p38, and c-Jun N-terminal kinase (JNK) antibodies and unphosphorylated EGFR, Akt, ERK, p38, and JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA).

2.2. Keratinocyte culture and stimulation

Normal human epidermal keratinocytes (Kurabo Industries, Osaka, Japan) were cultured in serum-free keratinocyte growth medium, HuMedia-KG2 (Kurabo Industries) supplemented with human epidermal growth factor (0.1 ng/ml), insulin (10 μg/ml), hydrocortisone (0.5 μg/ml), gentamycin (50 μg/ml), amphotericin B (50 ng/ml), and bovine brain pituitary extract (0.4% vol/vol). Cells were serially passaged at 60–70% confluence, and experiments were conducted at passage three with sub-confluent cells (60–80% confluence) in the proliferative phase, unless otherwise specified. For stimulation, keratinocytes were cultured in 6-well tissue culture plates, washed with phosphate-buffered saline, and incubated with peptides in HuMedia without supplements at the indicated periods.

Viability by trypan blue exclusion and lactate dehydrogenase activity were measured to evaluate possible cytotoxicity caused by the peptides or inhibitors to keratinocytes, as reported previously [15].

2.3. Cell migration assays

A modified Boyden chamber consisting of a 48-well chemotaxis microchamber (Neuro Probe, Cabin John, MD) was used to analyze the migration of keratinocytes. Peptides were pipetted into the lower wells of the microchamber, and an 8-μm pore-size polyvinylpyrrolidone-free polycarbonate membrane (Neuro Probe) was placed between the lower and upper wells. Keratinocytes resuspended in HuMedia without supplements were added into the upper wells of the chamber (2.5×10^3 cells/well) and allowed to migrate through the membrane for 3 h at 37 °C. Cells that migrated and adhered to the underside of the membrane were fixed and stained with DiffQuick (Kokusai Shiyaku, Kobe, Japan). The membrane was mounted onto slides, and cell migration was then quantified by counting under light microscopy in five high-power fields chosen randomly.

The migration of keratinocytes was also visualized by an *in vitro* wound closure assay, as reported previously [15] with some modifications. A confluent monolayer of keratinocytes was prepared in a 6-well culture plate by incubating the cells (6×10^5/well) for 3 h in the presence of mitomycin C (10 μg/ml) to prevent cell proliferation. The cell monolayer was wounded with a p-200 pipette tip to create a uniform cell-free zone in each well. After removing cellular debris by washing with phosphate-buffered saline, the wounded monolayers were cultured in complete HuMedia containing catestatin peptides in the presence of mitomycin C. After 24 h of incubation, repopulation of the wounded areas was observed under phase-contrast microscopy (Keyence, Osaka, Japan).

To analyze the inhibition of keratinocyte migration, the cells were pretreated with various inhibitors at the indicated concentrations for 2 h, and cell migration towards the catestatin peptides was determined using chemotaxis microchambers, as described above.

2.4. Cell proliferation assays

Proliferation of keratinocytes was evaluated by immunocytochemical detection of cells that permitted the incorporation of 5-bromo-2′-deoxy-uridine (BrdU) into cellular DNA. Keratinocytes were cultured at 40–50% confluence onto Lab-Tek II eight-chamber glass slides (Nalge Nunc International, Naperville, IL) and incubated with catestatin peptides at the indicated concentrations for 48 h at 37 °C. The stimulated cells were then incubated with 10 μM BrdU for 1 h at 37 °C, and cell proliferation was detected using a commercially available 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 number of BrdU-positive cells/total number of cells × 100.

Keratinocyte proliferation was also evaluated by a cell count assay. Cells were cultured at 40–50% confluence in 6-well plates and stimulated for 24–48 h with catestatin peptides in culture media without supplements. Cells were then exposed to trypsin and counted under a light microscope. To analyze the inhibition of cell proliferation, keratinocytes were pretreated with various inhibitors at the indicated concentrations for 2 h before stimulation with peptides, and cell proliferation was determined by the BrdU incorporation method, as described above.

2.5. Cell cycle analysis

Keratinocytes were incubated with catestatin peptides for 48 h, collected by trypsinization, and then fixed with 70% ethanol. Cells were incubated with 2 mg/ml RNase A (Sigma–Aldrich) for 30 min, followed by staining with 50 μg/ml propidium iodide (Sigma–Aldrich) for 20 min. DNA fluorescence was measured using a FACSAria flow cytometer (Becton-Dickinson, NJ). The percentages of cells in within G_0/G_1, S and G_2/M phases of the cell cycle were determined using FlowJo software.

2.6. Measurement of intracellular Ca^{2+} mobilization

Intracellular Ca^{2+} mobilization from keratinocytes was measured by a non-washing method using a FLIPR Calcium Assay Kit (Molecular Devices, Sunnyvale, CA). Keratinocytes (100 μl) were seeded at a density of 1×10^5 cells per well into a poly-D-lysine-coated 96-well black-walled clear bottom microtiter plate (Becton-Dickinson). The cells were then loaded for 1 h at 37 °C in an equivalent volume of Hank's balanced salt solution containing 20 mM HEPES, 2.5 mM probenecid (Sigma–Aldrich), and Calcium 3 Reagent (Molecular Devices, Menlo Park, CA), pH 7.4, prepared according to the manufacturer's instructions. To form a uniform monolayer of cells on the bottom of the wells, the microplate was gently centrifuged for 5 min with low acceleration and without using the break. The cell-containing plate was placed into a FlexStation II microplate reader (Molecular Devices), and a volume of 50 μl of peptide diluted in assay buffer was added to each well to achieve the final indicated concentration. The maximum change in fluorescence over the baseline was quantified using Softmax Pro (version 5) software (Molecular Devices).

2.7. Western blot analysis

Sub-confluent keratinocytes were stimulated with catestatin peptides for the indicated time periods. Cell lysates were obtained by treating the cells with lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.02% NaN_3, 0.1% SDS, and 1% Nonidet P-40) containing a protease inhibitor cocktail and Phosphatase Inhibitor Cocktail 1 and Cocktail 2 (Sigma–Aldrich) prepared following the manufacturer's instructions. The amount of total protein was determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL), and equal amounts of total protein were subjected to 12.5% SDS-PAGE. After the non-specific binding sites were blocked, the blots were incubated overnight with polyclonal antibodies against phosphorylated or unphosphorylated EGFR, Akt, ERK, p38, and JNK. The membranes were developed using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

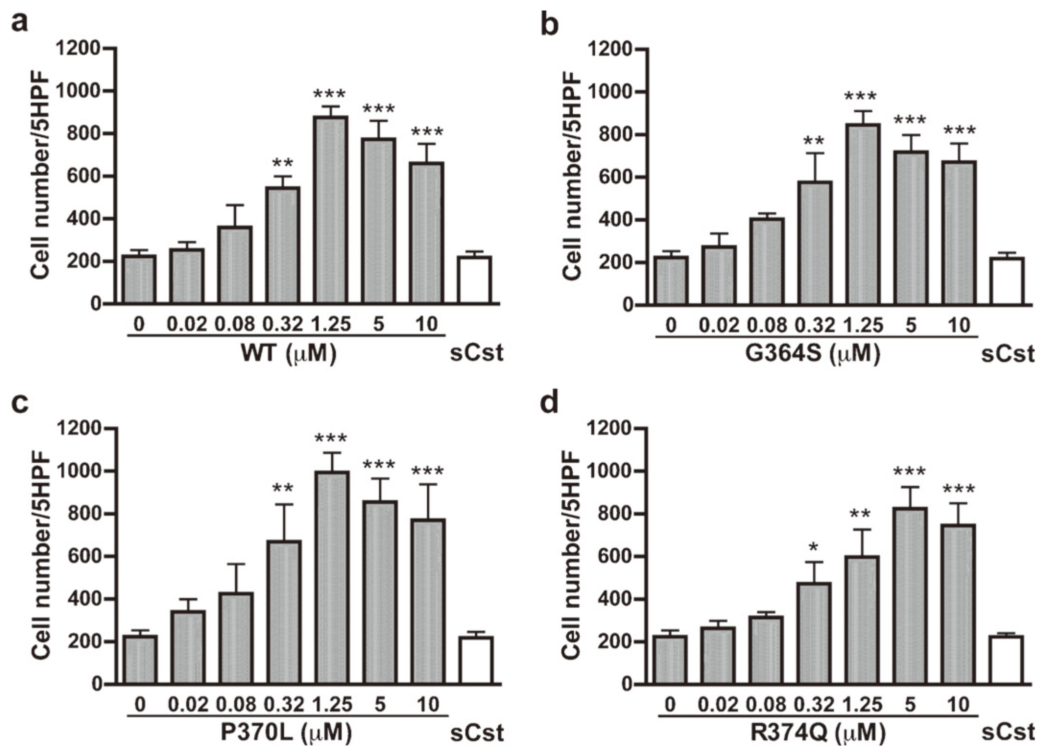

Fig. 1. Catestatin peptides induce keratinocyte migration (Boyden chamber assay. Keratinocytes were placed into the upper wells (2.5×10^3 cells/well) of a 48-well chemotaxis microchamber and allowed to migrate through an 8-μm pore-size polycarbonate membrane towards 0.02–10 μM of (a) wild-type catestatin (WT), (b) Gly364Ser (G364S), (c) Pro370Leu (P370L), and (d) Arg374Gln (R374Q), or scrambled catestatin (sCst, open bars) at the concentration of 1.25 μM in (a)–(c) and 5 μM in (d) in the lower wells for 3 h at 37 °C. The membrane was stained, and keratinocyte migration was assessed by counting the number of migrated cells under a light microscope in five randomly chosen high power fields (HPF). Each bar shows the mean ± SD of four to six separate experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ comparing stimulated and non-stimulated cells (treated with 0.01% acetic acid vehicle control).

2.8. Statistical analysis

Statistical analysis was performed using a one-way analysis of variance with a multiple comparison test or Student's *t*-test (Prism 4, GraphPad Software, San Diego, CA), and $P < 0.05$ was considered to be significant. The results are shown as mean ± SD.

3. Results

3.1. Catestatins stimulate the migration of human keratinocytes

Because human catestatin induces the migration of human monocytes [10], endothelial cells [11], and mast cells [12], we first evaluated the effects of catestatin and its variants on keratinocyte migration using a chemotaxis microchamber. Various concentrations of catestatins (0.02–10 μM) were applied to the lower section of the microchamber, and the migration of keratinocytes in the upper chamber was measured. Keratinocytes migrated significantly towards wild-type catestatin and the catestatin variants Gly364Ser, Pro370Leu and Arg374Gln (Fig. 1a–d) when compared to the control cells cultured with acetic acid vehicle control. The migratory effect of catestatin peptides was bell-shaped, reaching a peak at 1.25 μM for wild-type catestatin, Gly364Ser, and Pro370Leu or 5 μM for Arg374Gln. There was no increase in the migration of cells treated with the same optimal doses of the scrambled form of catestatin sCst, suggesting that the effect of catestatins on keratinocyte migration was specific.

Moreover, when catestatin peptides were tested in an *in vitro* wound closure assay, a noticeable induction of keratinocyte migration was observed. As shown in Fig. 2, keratinocytes

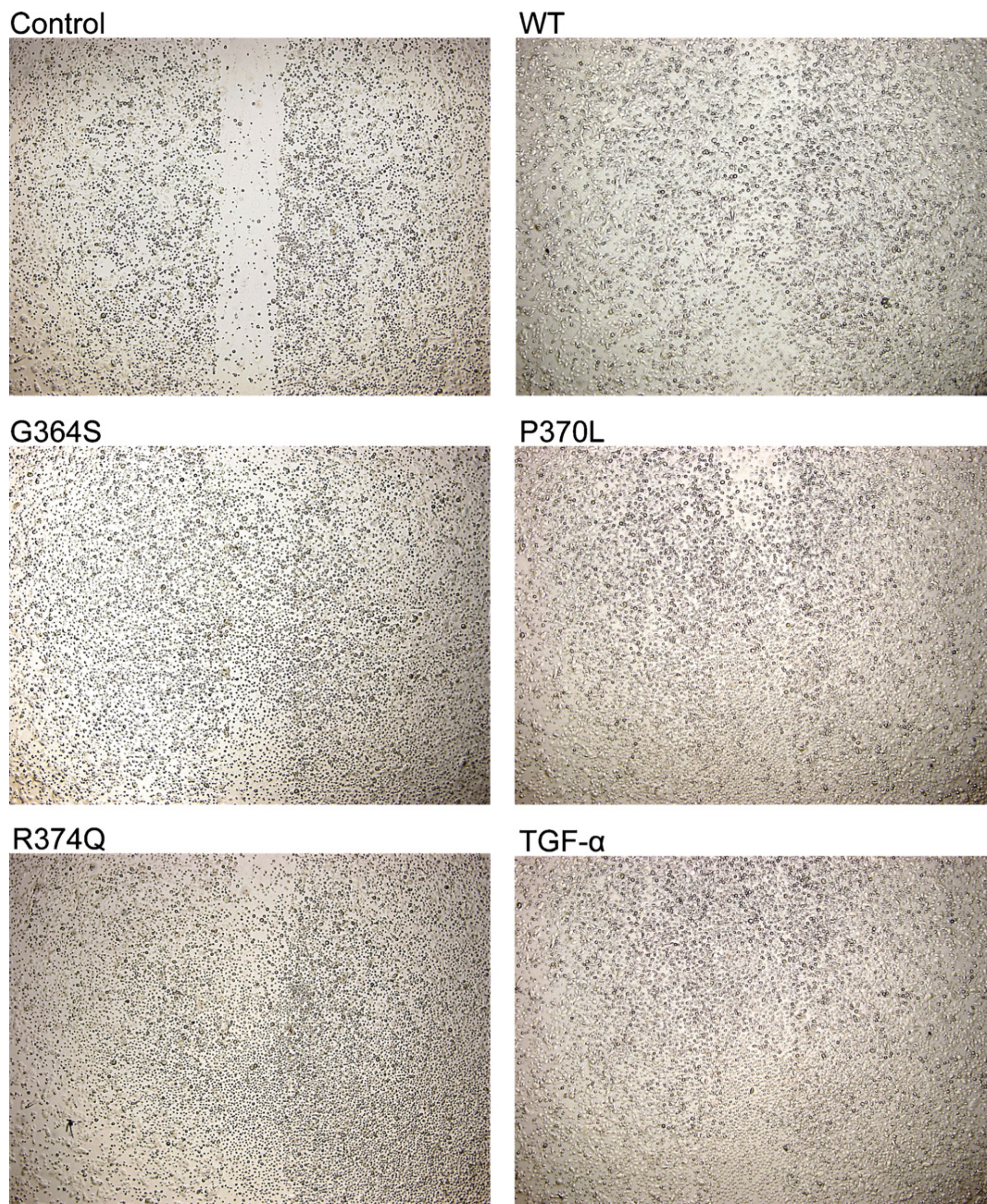

Fig. 2. Catestatins induce keratinocyte migration (*in vitro* wound closure assay). A cell-free zone was made in the confluent cultures of keratinocytes, as described in Section 2. The cells were incubated for 24 h with 1.25 μM wild-type catestatin (WT), Gly364Ser (G364S), or Pro370Leu (P370L), 5 μM Arg374Gln (R374Q), 10 ng/ml TGF-α, or 0.01% acetic acid (Control) in the presence of mitomycin C. Live cultures were photographed under phase-contrast microscopy. Data shown are representative of three independent experiments.

incubated for 24 h with optimal doses of catestatins (1.25 μM for wild-type catestatin, Gly364Ser and Pro370Leu, and 5 μM for Arg374Gln) migrated inwardly and almost completely covered the wound. In preliminary experiments, the migration of keratinocytes towards catestatin peptides was even observed at 3 h after the addition of peptides, although this migration was maximal at 24 h. TGF-α, a strong promoter of keratinocyte migration [16], also caused complete wound closure.

3.2. Catestatin peptides increase keratinocyte proliferation

Catestatin has been reported to promote cell proliferation and act as an angiogenic factor in endothelial cells [11]. Thus, we hypothesized that this peptide might also induce keratinocyte proliferation. First, keratinocytes were incubated with 5 μM of each catestatin peptide, and cell proliferation was analyzed by a cell count assay after 24 and 48 h. As shown in Fig. 3a, markedly increased cell numbers were observed after stimulation with wild-type catestatin, Gly364Ser, Pro370Leu or Arg374Gln, as compared to the control peptide or acetic acid vehicle. Stimulation with TGF-α was used as a positive control. Cell proliferation was further evaluated by BrdU incorporation. Following a 48-h incubation, stimulation with 0.32–10 μM of wild-type catestatin, Gly364Ser, Pro370Leu, or Arg374Gln dose-dependently increased BrdU uptake by keratinocytes, whereas the scrambled catestatin peptide had no significant effect on BrdU incorporation (Fig. 3b–f). Longer stimulation of keratinocytes with catestatin peptides (72–96 h) did not significantly increase cell proliferation above that observed at the 48 h time point (data not shown). Results of the BrdU incorporation studies indicate that catestatin peptides may induce G_1/S transition of cell cycle because BrdU is incorporated into newly synthesized DNA of S phase cells. Therefore, we performed a

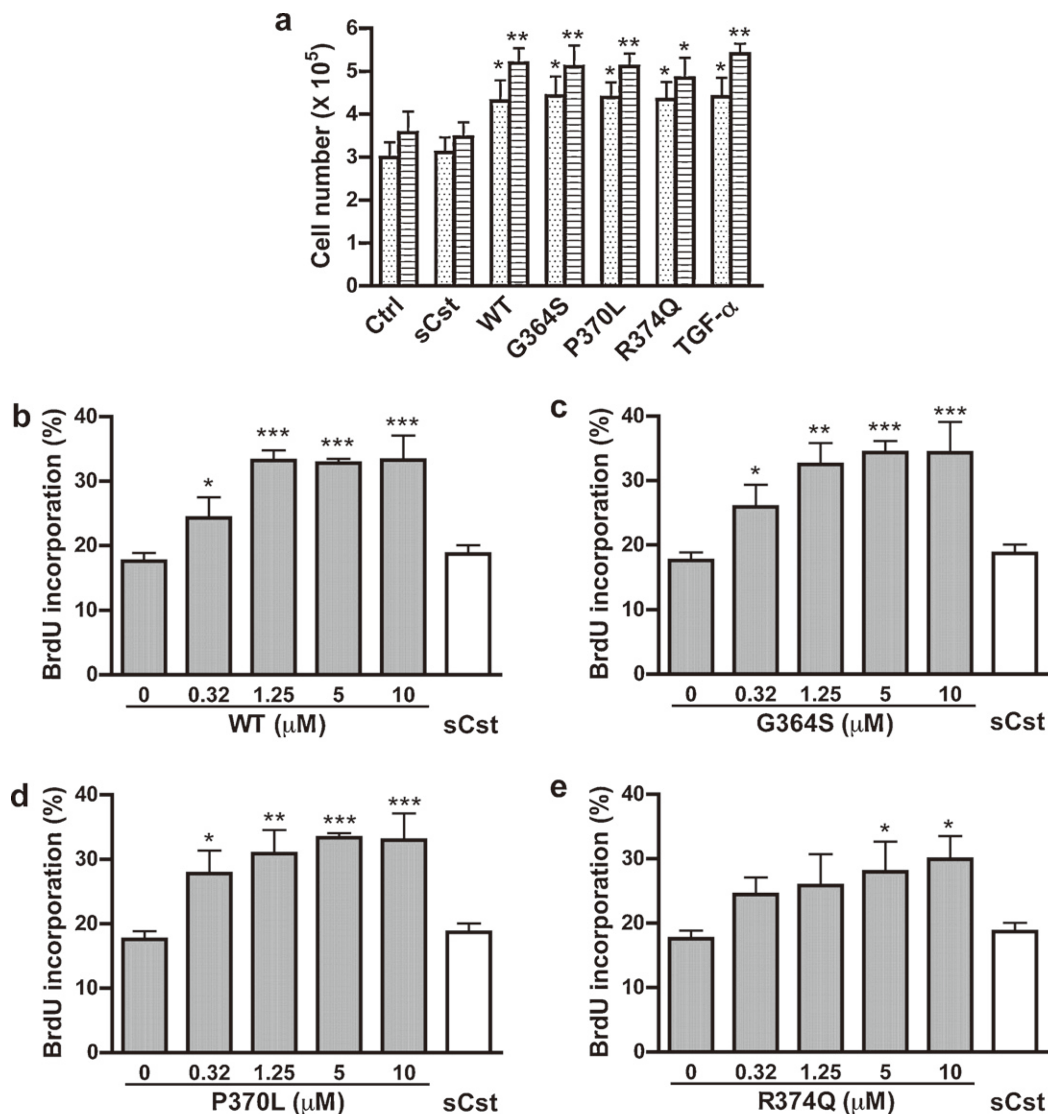

Fig. 3. Catestatin peptides induce keratinocyte proliferation. (a) Keratinocytes were cultured in 6-well plates at 40–50% confluence and stimulated with 5 μM wild-type catestatin (WT), Gly364Ser (G364S), Pro370Leu (P370L), Arg374Gln (R374Q), or scrambled catestatin (sCst), 10 ng/ml TGF-α, or 0.01% acetic acid (Ctrl) for 24 h (dots) or 48 h (stripes). Cells were then trypsinized and counted under a light microscope. Values are the mean ± SD of five separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, comparing stimulated and non-stimulated cells. Keratinocytes were also cultured onto Lab-Tek II eight-chamber glass slides at 40–50% confluence and incubated with 0.32–10 μM of (b) wild-type catestatin (WT), (c) Gly364Ser (G364S), (d) Pro370Leu (P370L), or (e) Arg374Gln (R374Q), 10 μM of scrambled catestatin (sCst, open bars), or 0.01% acetic acid for 48 h. After incubation with 10 μM BrdU for 1 h, the BrdU-positive cells were detected under a light microscope. Data are expressed as a percentage of BrdU-positive cells (BrdU incorporation). Values shown are the mean ± SD of five independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, comparing stimulated and non-stimulated cells. (f) Representative data of a phase-contrast image of BrdU-positive keratinocytes detected under a light microscope is shown. The arrow indicates BrdU-positive cells.

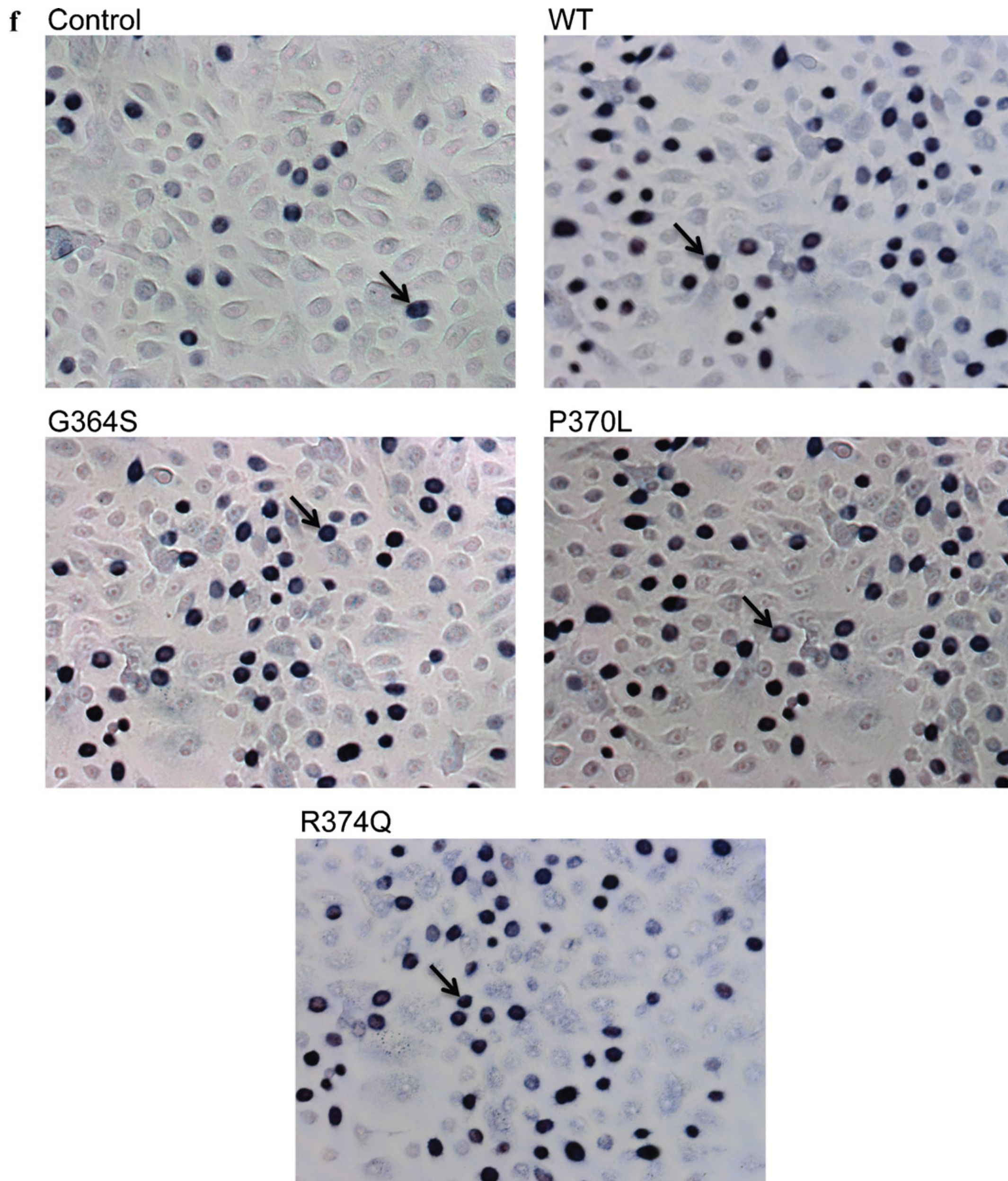

Fig. 3. (*Continued*).

cell cycle analysis and found that catestatin peptides decreased the proportion of cells in G_0/G_1 phase and increased the proportion of cells in S phase and G_2/M phase (Fig. 4).

3.3. Catestatins elicit intracellular Ca^{2+} mobilization in keratinocytes

AMPs induce a rapid Ca^{2+} entry across the plasma membrane and elevate Ca^{2+} in many cells types, including keratinocytes [15], and Ca^{2+} influx is involved in keratinocyte migration, proliferation, and differentiation [17]. Therefore, we next examined the ability of catestatin peptides to mobilize intracellular Ca^{2+} in keratinocytes. We observed that keratinocytes challenged with wild-type catestatin, Gly364Ser, Pro370Leu, or Arg374Gln noticeably increased intracellular Ca^{2+} concentrations, as compared to the control, and this increase was maximal following treatment with 20 μM of each peptide (Fig. 5). As a positive control, hBD-3 was used under the same experimental conditions and also markedly elevated intracellular Ca^{2+}concentrations in keratinocytes.

3.4. Catestatin-mediated keratinocyte migration and proliferation require G-protein and PLC activation

It has been reported that skin-derived AMPs such as hBDs and the cathelicidin LL-37, modulate various functions of several cell types via G-protein and PLC-mediated signaling pathways [15,18]. Moreover, catestatin has also been reported to activate monocytes [10] and mast cells [12] through the G-protein and/or PLC pathways. Thus, we evaluated the involvement of the G-protein and PLC pathways in catestatin-mediated keratinocyte migration and proliferation by using their specific inhibitors, PTx and U-73122, respectively. Treatment of keratinocytes with PTx and U-73122 markedly reduced the subsequent keratinocyte migration and proliferation induced by wild-type catestatin, Gly364Ser, Pro370Leu, or Arg374Gln (Fig. 6 a and b). U-73343, an inactive analog of U-73122, was used to substantiate the specific effects of U-73122 and did not affect cell migration or proliferation. Hence, these observations suggest that the G-protein and PLC pathways

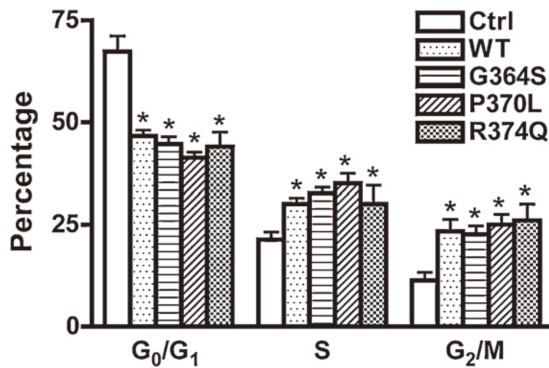

Fig. 4. Effects of catestatin peptides on cell cycle distribution. Keratinocytes were incubated with 10 μM wild-type catestatin (WT), Gly364Ser (G364S), Pro370Leu (P370L), or Arg374Gln (R374Q) or 0.01% acetic acid (Ctrl, control) for 48 h. The cell cycle distribution was analyzed by flow cytometric analysis of DNA content. Values shown are the mean ± SD of three independent experiments. $^{*}P < 0.05$, comparing stimulated and non-stimulated cells.

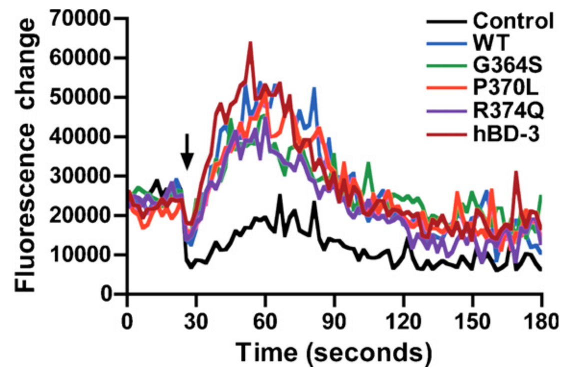

Fig. 5. Catestatins induce intracellular Ca^{2+} mobilization in keratinocytes. Keratinocytes (1×10^5 cells/well) were incubated for 1 h at 37 °C in Hank's balanced salt solution containing HEPES, probenecid and Calcium 3 Reagent, and the cells were stimulated with 20 μM wild-type catestatin (WT), Gly364Ser (G364S), Pro370Leu (P370L) or Arg374Gln (R374Q), 20 μg/ml human β-defensin (hBD)-3, or 0.01% acetic acid (Control) as described in Section 2. The data are shown as fluorescence change, and are representative of four independent experiments. The arrow indicates the addition of peptides or acetic acid.

are required for catestatin-mediated migration and proliferation of keratinocytes.

3.5. Activation of EGFR is necessary for catestatin-mediated keratinocyte migration and proliferation

Because hBDs and LL-37 have been reported to activate EGFR leading to keratinocyte migration and/or proliferation [15,19], we hypothesized that human catestatin could similarly stimulate keratinocytes via EGFR activation. As can be seen in Fig. 7 a, wild-type catestatin and its variants, Gly364Ser, Pro370Leu, and Arg374Gln, induced significant phosphorylation of EGFR in keratinocytes after 30 min of stimulation. We confirmed that this phosphorylation was necessary for catestatin-mediated keratinocyte migration and proliferation, as demonstrated by the noteworthy suppression of keratinocyte migration and proliferation following incubation with an EGFR-specific inhibitor, AG1478, or an anti-EGFR antibody (Fig. 7b and c). In contrast, control IgG antibody did not affect keratinocyte migration and proliferation.

3.6. Catestatin-mediated keratinocyte migration and proliferation involve the Akt/PI3K pathway

Akt is an important regulator of keratinocyte proliferation and survival, and its activation is mediated by PI3K [20]. Therefore, the effects of catestatin on Akt activation were examined. As shown in Fig. 8a, the phosphorylation of Akt in keratinocytes was significantly increased following 10 min of stimulation with wild-type catestatin, Gly364Ser, Pro370Leu or Arg374Gln. The association of PI3K or protein kinase C (PKC) was determined using their specific inhibitors, wortmannin and staurosporine, respectively. We observed that pre-treatment of keratinocytes with wortmannin markedly suppressed catestatin-induced migration and proliferation, whereas staurosporine did not exert considerable inhibitory effects (Fig. 8b and c). This observation implies the involvement of intracellular Akt/PI3K, but not the PKC pathway, in catestatin-mediated migration and proliferation of keratinocytes.

3.7. Catestatin peptides induce keratinocyte migration and proliferation via MAPK activation

We examined the roles of the MAPK pathways in catestatin-mediated keratinocyte migration and proliferation, as MAPKs participate in various cellular activities, including migration and proliferation [21]. Fig. 9 a clearly shows that the phosphorylation of ERK and p38, but not JNK, was markedly enhanced in keratinocytes within 5 min after stimulation with wild-type catestatin, or the catestatin variants Gly364Ser, Pro370Leu and Arg374Gln. Longer exposure of keratinocytes to the catestatin peptides (up to 2 h) did not lead to enhanced JNK phosphorylation. To evaluate the requirements of MAPKs in catestatin-induced keratinocyte activation, cells were pretreated with MAPK specific inhibitors. Both ERK and p38 inhibitors, U0126 and SB203580, respectively, caused significant inhibition of keratinocyte migration and proliferation,

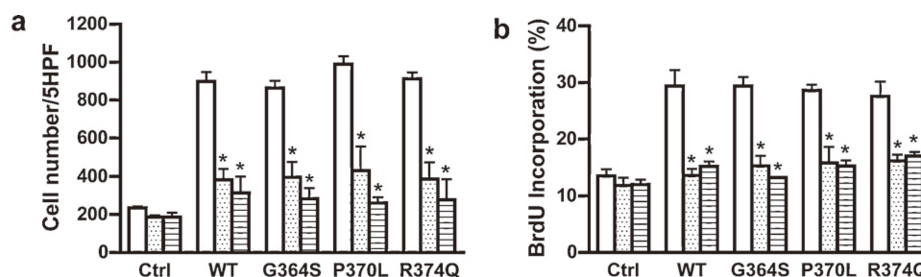

Fig. 6. Catestatin-induced keratinocyte migration and proliferation require the G-protein and PLC pathways. Keratinocytes were pre-treated with 1 μg/ml G-protein inhibitor pertussis toxin (dots), 30 μM PLC inhibitor U-73122 (stripes), 30 μM U-73343, an inactive analog of U-73122 (diagonal stripes), or 0.1% DMSO (open bars) for 2 h. (a) Cell migration towards 1.25 μM wild-type catestatin (WT), Gly364Ser (G364S), Pro370Leu (P370L), and 5 μM Arg374Gln (R374Q), or 0.01% acetic acid (Ctrl) was determined, as described in the legend of Fig. 1. $^{*}P < 0.05$, comparing cells in the presence of inhibitors to those without. Values shown are the mean ± SD of four to six separate experiments. (b) Pre-treated cells were also stimulated with 5 μM wild-type catestatin (WT), Gly364Ser (G364S), Pro370Leu (P370L), Arg374Gln (R374Q), or 0.01% acetic acid (Ctrl). A cell proliferation assay was performed, as described in the legend of Fig. 3. $^{*}P < 0.05$ comparing cells in the presence and absence of inhibitors. Values are the mean ± SD of four to six separate experiments.

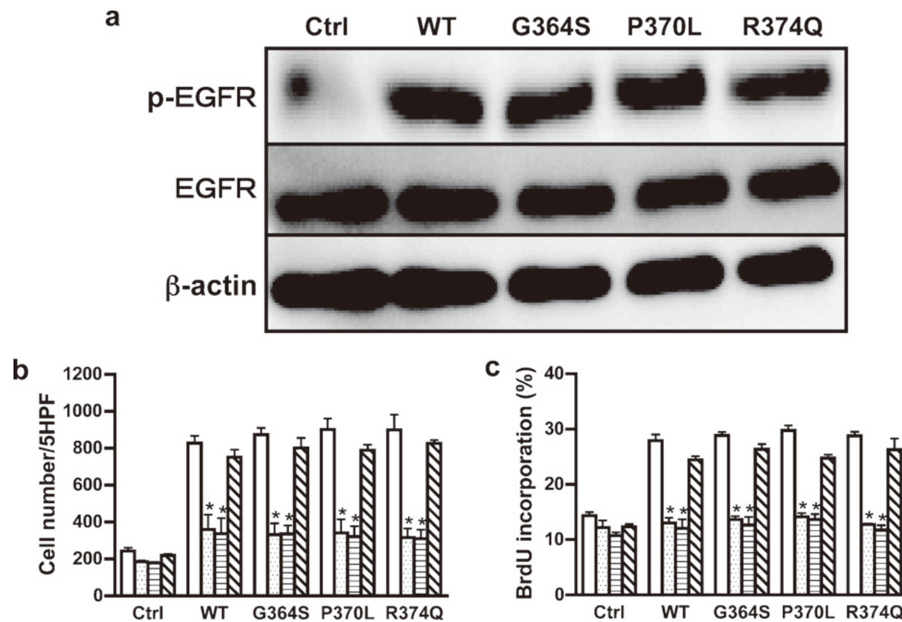

Fig. 7. Catestatin peptides induce phosphorylation of epidermal growth factor receptor (EGFR), which is required for keratinocyte migration and proliferation. (a) Keratinocytes were stimulated with 5 μM wild-type catestatin (WT), Gly364Ser (G364S), Pro370Leu (P370L), or Arg374Gln (R374Q), or 0.01% acetic acid (Ctrl) for 30 min, and phosphorylated EGFR (p-EGFR), unphosphorylated EGFR, and β-actin levels in cell lysates were determined by Western blot analysis. The data shown is one representative out of five independent experiments with similar results. (b) Keratinocytes were pre-treated with 50 nM of the EGFR inhibitor AG1478 (dots), 20 μg/ml anti-EGFR antibody (horizontal stripes), 20 μg/ml control IgG antibody (diagonal stripes), or 0.1% DMSO (open bars) for 2 h, and cell migration towards 1.25 μM wild-type catestatin (WT), Gly364Ser (G364S) or Pro370Leu (P370L), 5 μM Arg374Gln (R374Q) or 0.01% acetic acid (Ctrl) was determined, as described in the legend of Fig. 1. *$P < 0.05$, comparing cells in the presence and absence of inhibitor or blocking antibody. Values shown are the mean ± SD of four to six separate experiments. (c) Pre-treated cells were also stimulated with 5 μM wild-type catestatin (WT), Gly364Ser (G364S), Pro370Leu (P370L), or Arg374Gln (R374Q), or 0.01% acetic acid (Ctrl). The BrdU incorporation assay was performed, as described in the legend of Fig. 3b-e. *$P < 0.05$, comparing cells in the presence of inhibitor or antibody to those without. Values shown are the mean ± SD of four to six separate experiments.

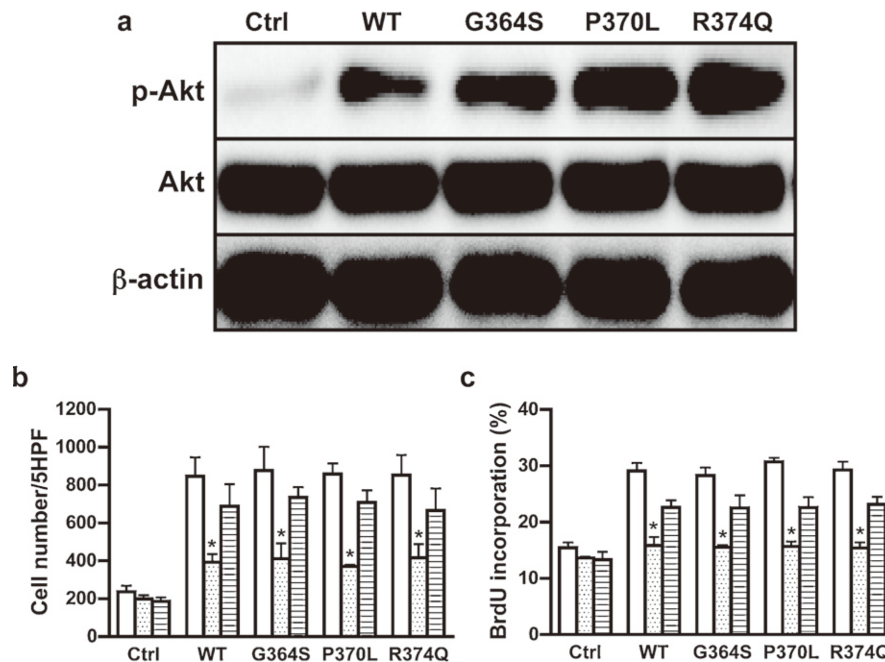

Fig. 8. Catestatin-induced keratinocyte migration and proliferation involve the Akt/PI3-kinase pathway. (a) Keratinocytes were stimulated with 5 μM wild-type catestatin (WT), Gly364Ser (G364S), Pro370Leu (P370L), or Arg374Gln (R374Q), or 0.01% acetic acid (Ctrl) for 10 min, and the phosphorylated Akt (p-Akt), unphosphorylated Akt and β-actin levels in the cell lysates were determined by Western blot analysis. One representative blot is shown out of five independent experiments with similar results. (b) Keratinocytes were pre-treated with 10 nM PI3-kinase inhibitor wortmannin (dots), 10 ng/ml protein kinase C inhibitor staurosporine (stripes), or 0.1% DMSO (open bars) for 2 h, and cell migration towards 1.25 μM wild-type catestatin (WT), Gly364Ser (G364S), or Pro370Leu (P370L), 5 μM Arg374Gln (R374Q) or 0.01% acetic acid (Ctrl) was determined, as described in the legend of Fig. 1. *$P < 0.05$ comparing cells in the presence of inhibitors to those without. Values shown are the mean ± SD of four to six separate experiments. (c) Pre-treated cells were also stimulated with 5 μM wild-type catestatin (WT), Gly364Ser (G364S), Pro370Leu (P370L), or Arg374Gln (R374Q), or treated with 0.01% acetic acid (Ctrl), and a cell proliferation assay was performed, as described in the legend of Fig. 3b–e. *$P < 0.05$ comparing cells in the presence and absence of inhibitors. Values are the mean ± SD of four to six separate experiments.

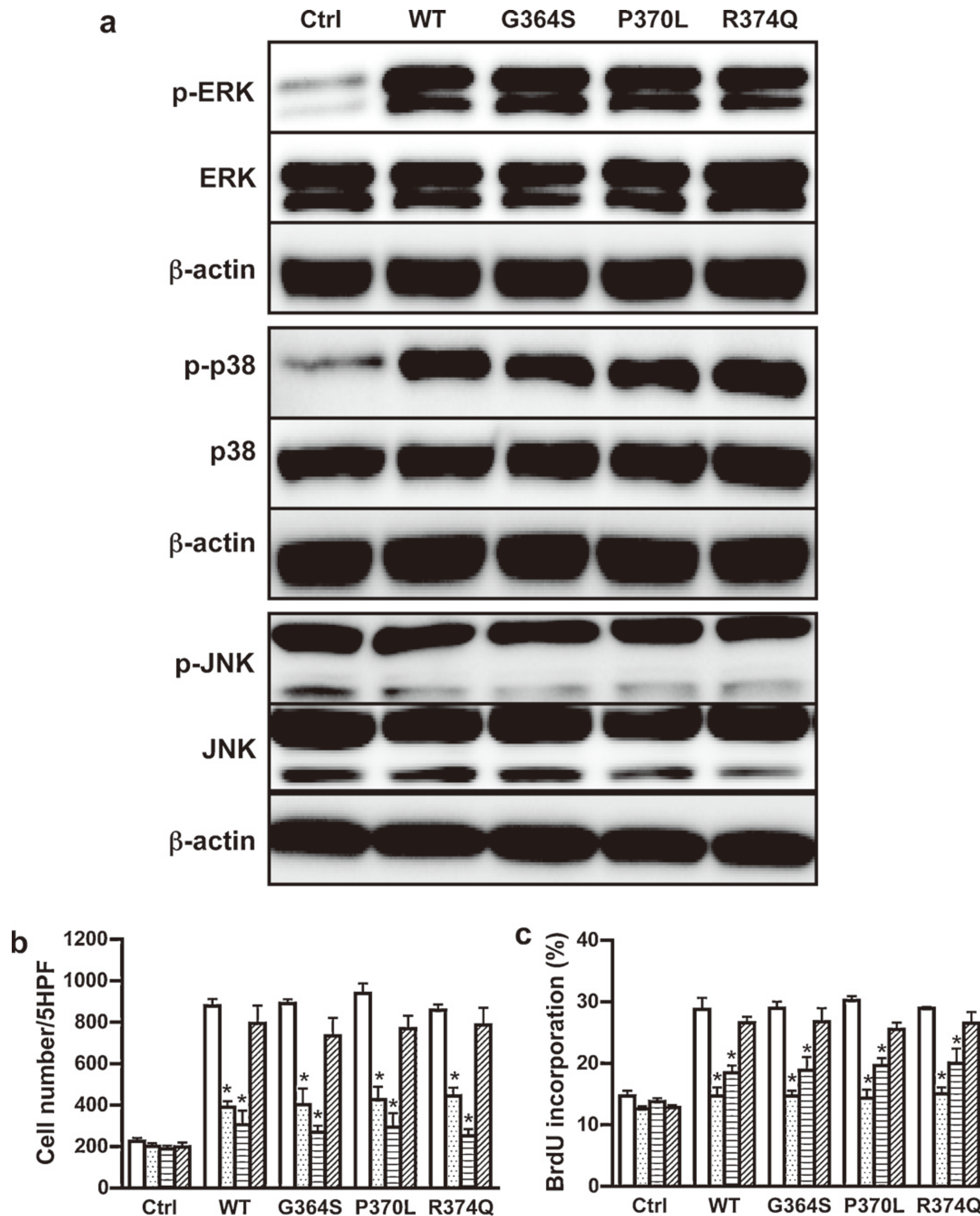

Fig. 9. Catestatins induce phosphorylation of extracellular signal-regulated kinase (ERK) and p38, which are required for keratinocyte migration and proliferation. (a) Keratinocytes were stimulated with 10 μM wild-type catestatin (WT), Gly364Ser (G364S), Pro370Leu (P370L), or Arg374Gln (R374Q), or 0.01% acetic acid (Ctrl) for 5 min. Phosphorylated ERK (p-ERK), p38 (p-p38), and c-Jun N-terminal kinase (JNK) (p-JNK) and unphosphorylated ERK, p38, JNK, and β-actin levels in cell lysates were determined by Western blot analysis. One representative blot is shown out of five independent experiments with similar results. (b) Keratinocytes were pre-treated with 10 μM ERK inhibitor U0126 (dots), 10 μM p38 inhibitor SB203580 (horizontal stripes), 10 μM JNK inhibitor SP600125 (diagonal stripes), or 0.1% DMSO (open bars) for 2 h, and cell migration towards 1.25 μM wild-type catestatin (WT), Gly364Ser (G364S), or Pro370Leu (P370L), 5 μM Arg374Gln (R374Q), or 0.01% acetic acid (Ctrl) was determined, as described in the legend of Fig. 1. *$P < 0.05$, comparing cells in the presence of inhibitors to those cultured without. Values shown are the mean ± SD of four to six separate experiments. (c) Pre-treated cells were also stimulated with 5 μM wild-type catestatin (WT), Gly364Ser (G364S), Pro370Leu (P370L), Arg374Gln (R374Q), or 0.01% acetic acid (Ctrl). A cell proliferation assay was performed, as described in the legend of Fig. 3b–e. *$P < 0.05$ comparing cells in the presence of inhibitors to those without. Values shown are the mean ± SD of four to six separate experiments.

whereas the JNK inhibitor SP600125 had no effect (Fig. 9b and c). These results suggest that the activation of ERK and p38 is essential for catestatin-mediated keratinocyte migration and proliferation.

4. Discussion

Studies of migration and proliferation by epidermal keratinocytes are focused in the pathogenesis of cutaneous wounds and various skin diseases, such as psoriasis, in which skin-derived AMPs (hBDs, the cathelicidin LL-37, S100 proteins, etc.) are highly expressed [1–3]. In the present study, we demonstrate that the neuroendocrine AMP, catestatin, and naturally occurring catestatin variants, induce keratinocyte migration and proliferation, and elevate intracellular Ca^{2+} mobilization in keratinocytes. Further, we show that catestatin-mediated keratinocyte activation involves the G-protein, PLC, EGFR, Akt/PI3K, and MAPK pathways.

A multilayered epidermis composed of keratinocytes separates the inner body from the outer environment and protects against invading pathogens. Hence, keratinocytes form an important component of cutaneous innate immunity, particularly during the

wound closure process. The current study revealed that catestatin peptides, which have been reported to be up-regulated at the skin wound site where they inhibit the growth of various pathogens [5], may participate in cutaneous wound closure through their ability to induce keratinocyte migration and proliferation. The specificity of catestatins was confirmed by the observation that a scrambled form of catestatin did not have any effect on keratinocyte activation.

Overall, wild-type catestatin and catestatin variants had almost equal potencies in inducing keratinocyte migration and proliferation, with one exception; Arg374Gln required relatively higher concentrations for an optimal chemotactic effect. This observation partially contradicts the literature relating to catestatins, where it has been shown that wild-type catestatin and its variants display differential potencies. For instance, the rank order of potency of catestatins in inhibiting catecholamine release and inducing monocyte migration was claimed to be Pro370Leu > wild-type catestatin > Gly364Ser > Arg374Gln [9,10]. However, both Pro370Leu and Gly364Ser have been shown to exhibit higher antimicrobial activity compared to wild-type catestatin against a number of skin pathogenic bacteria, fungi and yeast [5]. Of note, hydrophobicity analysis shows that the C-terminus of Pro370Leu is more hydrophobic than the C-terminus of wild-type catestatin [22]. In addition, circular dichroism spectrometry also reveals a clear disparity in the secondary structure between Pro370Leu and wild-type catestatin [22]. In the current study, our findings suggest that the potencies of catestatin peptides may vary not only between cell types, but also following the specific cellular activities induced by these peptides.

At the time of initial damage subsequent to an inflammation context, epithelial cells may communicate with each other through the fast propagation of intracellular Ca^{2+} waves, which may help neighboring cells to induce adhesion, migration and proliferation leading to wound repair [23,24]. This Ca^{2+} release can be mediated by the activation of PLC, which produces IP_3 (inositol-1,4,5-trisphosphate) and causes the release of Ca^{2+} from the endoplasmic reticulum. To date, three isoforms of PLC have been identified in mammalian cells (β, γ, and δ). However, only PLC-β isoforms are activated by agonists that bind to G-protein-coupled receptors [25]. In addition, the role of PLC-β isoforms in cell migration and proliferation has been clearly demonstrated [26,27]. In this report, catestatin peptides markedly increase intracellular Ca^{2+} release from keratinocytes, and notably cause cell migration and proliferation. The blockade of G-proteins and PLC by PTx and U-73122, respectively, significantly abrogates catestatin-mediated keratinocyte migration and proliferation. Thus, the activation of the G-protein-coupled PLC-β pathway likely regulates catestatin-elicited intracellular Ca^{2+} mobilization, leading to the migration and proliferation of keratinocytes. On the other hand, because high Ca^{2+} levels also induce keratinocyte differentiation and/or senescence, we cannot rule out the possibility that catestatin may enhance keratinocyte differentiation/senescence through its ability to elevate intracellular Ca^{2+} concentrations. Nevertheless, we do strongly believe that catestatin might increase keratinocyte proliferation, as evidenced by different cell proliferation assays, including BrdU incorporation, cell count and cell cycle analysis. Given that the activation of G-proteins implies the presence of functional receptors, further studies are necessary to explore the specific functional receptors for catestatins in keratinocytes.

To understand the receptor pathway by which catestatins activate keratinocyte migration and proliferation, a G-protein-coupled receptor, EGFR, was selected as a possible catestatin receptor, because it has been reported to regulate keratinocyte migration and proliferation [15,19,20,28,29]. We demonstrate that catestatin peptides induce phosphorylation of EGFR, and that the inhibition of EGFR activation by AG1478 and EGFR-neutralizing antibody results in the marked suppression of keratinocyte migration and proliferation. This observation strongly implies that the activation of EGFR might be required for catestatin-mediated keratinocyte activation. However, further studies are necessary to demonstrate whether EGFR activation by catestatin is direct or indirect. We also show that catestatins enhance phosphorylation of Akt in keratinocytes, which is consistent with previous reports showing that catestatin activates Akt in monocytes [10] and endothelial cells [11]. In fact, Akt activation plays an important role in diverse cellular processes, including cell migration, cell proliferation, and survival [30]. In epidermal keratinocytes, previous studies have reported that there is a correlation between Akt phosphorylation and keratinocyte proliferative potential, and that Akt phosphorylation is enhanced in cutaneous wounds [31]. Because PI3K is the upstream activator of Akt and operates downstream of EGFR [20], this prompted us to further evaluate catestatin signaling by inhibiting PI3K. In our study, inhibition of catestatin-induced migration and proliferation of keratinocytes by a PI3K inhibitor, wortmannin, suggests the involvement of the intracellular Akt/PI3K pathway in catestatin-mediated keratinocyte activation. In contrast, the inhibitor of PKC, staurosporine, does not exhibit significant inhibitory effect on catestatin-induced migration and proliferation, although the PKC superfamily displays high homology with Akt and is known to play key regulatory roles in cell migration and angiogenesis [32]. Of note, Akt/PI3K signaling has been involved in the chemotaxis and proliferation of various cells by chemokines and neuropeptides [11,20,33–35], and plays a crucial role in the regulation of keratinocyte migration, proliferation, survival and differentiation, suggesting that this enzyme is a key regulator of epidermal homeostasis and repair [31]. This has also been demonstrated in mice lacking PI3Kγ gene, which show a defect in macrophage and endothelial cell migration, proliferation, survival, and integration into endothelial networks [36–38].

The MAPKs are a well-documented family of serine/threonine kinases that include p38, ERK1/2 and JNK. These kinases have also been shown to mediate chemotactic and proliferative responses in various cell types, including epidermal keratinocytes [21]. We therefore studied the role of MAPKs in catestatin-induced keratinocyte activation. Catestatin peptides enhanced phosphorylation of ERK and p38, and a blockade of these MAPKs abolished catestatin-mediated keratinocyte migration and proliferation. Therefore, we concluded that MAPK activation also controls keratinocyte migration and proliferation induced by catestatins. This finding is also supported by our previous observation that catestatin induces IL-8 production by keratinocytes via MAPK activation [13]. Indeed, IL-8 is not only a potent chemoattractant, but also a strong pro-angiogenic factor, and an inducer of keratinocyte migration and proliferation. Furthermore, IL-8 stimulates wound healing [39,40]. Together, these data suggest that catestatin not only directly stimulates keratinocyte migration and proliferation, but may also indirectly augment these activities through the stimulation of IL-8 production.

Although the circulating plasma concentrations of catestatin are in the nanomolar range [41], catestatin concentrations in human skin tissue are not known. However, because the amount of catestatin in murine epidermis has been estimated to be approximately 20 μM [5], and because the sequence of catestatin is highly conserved between humans and mice, it is likely that the levels of catestatin in human skin are comparable to those in mice. In our study, the effective concentrations of catestatin peptides range from 0.32 to 20 μM, which is in the range of the effective doses reported for the antimicrobial activities of catestatin against skin pathogens (5 μM or greater concentrations) [5]. Hence, the catestatin concentrations used in this study are believed to be pathophysiologically relevant.

To our knowledge, this is the first study demonstrating the involvement of a neuroendocrine AMP, catestatin, in cutaneous wound closure via the stimulation of migration and proliferation of keratinocytes. This report provides novel evidence of the immunomodulatory roles of skin-derived AMPs in cutaneous immunity and highlights a new link between the endocrine and skin immune systems.

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