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ORIGINAL ARTICLE

In vitro and *In vivo* Wound Healing-Promoting Activities of Human Cathelicidin LL-37

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The human antimicrobial peptide LL-37 plays an important role in host defense against infection. In addition to its antimicrobial action, other activities have been described in eukaryotic cells that may contribute to the healing response. In this study, we demonstrated that *in vitro* human cathelicidin activates migration of the human keratinocyte cell line HaCaT, involving phenotypic changes related to actin dynamics and associated to augmented tyrosine phosphorylation of proteins involved in focal adhesion complexes, such as focal adhesion kinase and paxillin. Other events involved in the LL-37 response were the induction of the Snail and Slug transcription factors, activation of matrix metalloproteinases and activation of the mitogen-activated protein kinase , and phosphoinositide 3-kinase/Akt signaling pathways. These signaling events could be mediated not only through the transactivation of EGFR but also through the induction of G-protein-coupled receptor FPRL-1 expression in these cells. Finally, by *in vivo* adenoviral transfer of the antimicrobial peptide to excisional wounds in ob/ob mice, we demonstrated that LL-37 significantly improved re-epithelialization and granulation tissue formation. The protective and regenerative activities of LL-37 support its therapeutic potential to promote wound healing.

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INTRODUCTION

Antimicrobial peptides have been described as mediators of the body's innate defense response. Among the different families, cathelicidins include the human LL-37 peptide, which is expressed in neutrophils and keratinocytes of inflamed skin. Besides the role in host defense against infection, other activities that appear to be receptor-mediated have been reported for this antimicrobial peptide. LL-37 induces chemotaxis of immune cells (Agerberth *et al.*, 2000; Niyonsaba *et al.*, 2002) and dendritic cell differentiation (Davidson *et al.*, 2004). It was previously shown that LL-37 induced the expression of chemokines and chemokine receptors in macrophages, thus contributing to the immune response against infection by indirectly promoting the migration of immune cells (Scott *et al.*, 2002). It also increases the production of cytokines and chemokines such as IL-6, IL-8, tumor necrosis factor-α, GM-CSF, IL-10, interferon-inducible protein-10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-3 alpha (MIP3α) and regulated upon activation, normal T cell expressed and secreted (RANTES) by normal human keratinocytes (Braff *et al.*, 2005; Niyonsaba *et al.*, 2007). LL-37 stimulates IL-18 protein release in keratinocytes through activation of p38 and extracellular signal regulated kinase 1 (ERK1)/2 pathways, either alone or synergistically with antimicrobial peptides of the β defensin family (Niyonsaba *et al.*, 2005).

The chemotactic receptor expressed in monocytes, neutrophils and subsets of T cells that respond to LL-37 is formyl peptide receptor-like 1 (FPRL-1), a seven transmembrane pertussis toxin-sensitive G-protein-coupled receptor defined as a low-affinity fMLF receptor. Apart from the chemotactic response, the activation of this receptor leads to the initiation of signaling events leading to enhanced phagocytosis, release of oxygen intermediates, augmented bacterial killing and increased adhesion (De *et al.*, 2000). Mast cell chemotaxis induced by LL-37 is also mediated through a G-protein-

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Abbreviations: DAPI, 4′, 6′-diamidino-2-phenylindole; ERK1, extracellular signal regulated kinase 1; EMT, epithelial-mesenchymal transition; FAK, focal adhesion kinase; FPRL-1, formyl peptide receptor-like 1; GFP, green fluorescent protein; GSK-3, glycogen synthase kinase 3; HB-EGF, heparin-binding epidermal growth factor; ILK, integrin-linked kinase; IP-10, interferon-inducible protein-10; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; MDCK, Madin-Darby Canine kidney epithelial cells; MIP3α, macrophage inflammatory protein-3 alpha; MMP-2, matrix metalloproteinase-2; PI-3K, phosphoinositide 3-kinase; PLA2, phospholipase A2; PY, phosphotyrosine; RANTES, regulated upon activation, normal T cell expressed and secreted; TRITC, tetramethyl rhodamine isothiocyanate; uPAR, urokinase-type plasminogen activator receptor; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide

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coupled receptor, but distinct from FPRL-1 (Niyonsaba *et al.*, 2002). Endothelial cells also express functional FPRL-1, and it has been demonstrated that LL-37 mediates angiogenic activity through the activation of this receptor (Koczulla *et al.*, 2003).

Cutaneous injury induces the expression of cathelicidins in both human and mouse epidermis and these peptides have been found in the wound fluid (Dorschner *et al.*, 2001). Other inflammatory stimuli have been shown to induce LL-37 expression (Frohm *et al.*, 1997). Moreover, we have also demonstrated that the expression of LL-37 might be involved in the induction of other antimicrobial peptides, such as human β defensins (Carretero *et al.*, 2004). The role of LL-37 antimicrobial peptide in the re-epithelialization process was previously suggested by using blocking antibodies against this peptide in wounded skin equivalents (Heilborn *et al.*, 2003). It has also been demonstrated previously that LL-37 induces the healing of airway epithelial wounds by stimulating proliferation and chemotaxis of epithelial cells (Shaykhiev *et al.*, 2005). Evidence for a possible activity of LL-37 in wound healing, mediated in part through induction of keratinocyte migration via EGFR transactivation, was recently provided (Tokumaru *et al.*, 2005). Thus, therapeutic opportunities with LL-37 in the field of cutaneous repair may not only rely on its antimicrobial activity but also on its putative epidermal healing promoting effect. In a previous study, we explored the idea of using LL-37, through gene therapy, as an aid against infection during skin engraftment (Carretero *et al.*, 2004). In this study, we provide new supporting data for a bonafide role of LL-37 in re-epithelialization and wound closure processes. We demonstrate that LL-37 gene-transferred HaCaT keratinocytes undergo cytoskeleton reorganization leading to the acquisition of a migratory-like phenotype and activate specific signaling pathways and transcription factors that could influence this phenotypic conversion. We also found that FPRL-1 induction in HaCaT keratinocytes might contribute to the signaling events leading to the activation of migration. In addition to the *in vitro* data, we provide *in vivo* evidence, through adenoviral-mediated local administration of the antimicrobial peptide to excisional wounds in ob/ob diabetic mice, that LL-37 significantly enhances wound healing. Our findings point to LL-37 as a pleiotropic factor helping skin wound closure through differential but cooperative responses.

RESULTS

Recombinant or gene-transferred LL-37 induced a migratory behavior in HaCaT keratinocytes

Proliferation and migration of human keratinocytes are critical factors that contribute to efficient healing. Several growth factors and cytokines have been shown to influence these processes (Martin, 1997; Falanga, 2005). We have observed previously that proliferation appeared to be unaffected in HaCaT cells transduced with an adenoviral vector encoding LL-37 (Carretero *et al.*, 2004; Figure 1). Moreover, increasing concentrations of the LL-37 synthetic peptide ranging from 25 to 500 ng/ml did not appear to affect HaCaT cell proliferation significantly (data not shown).

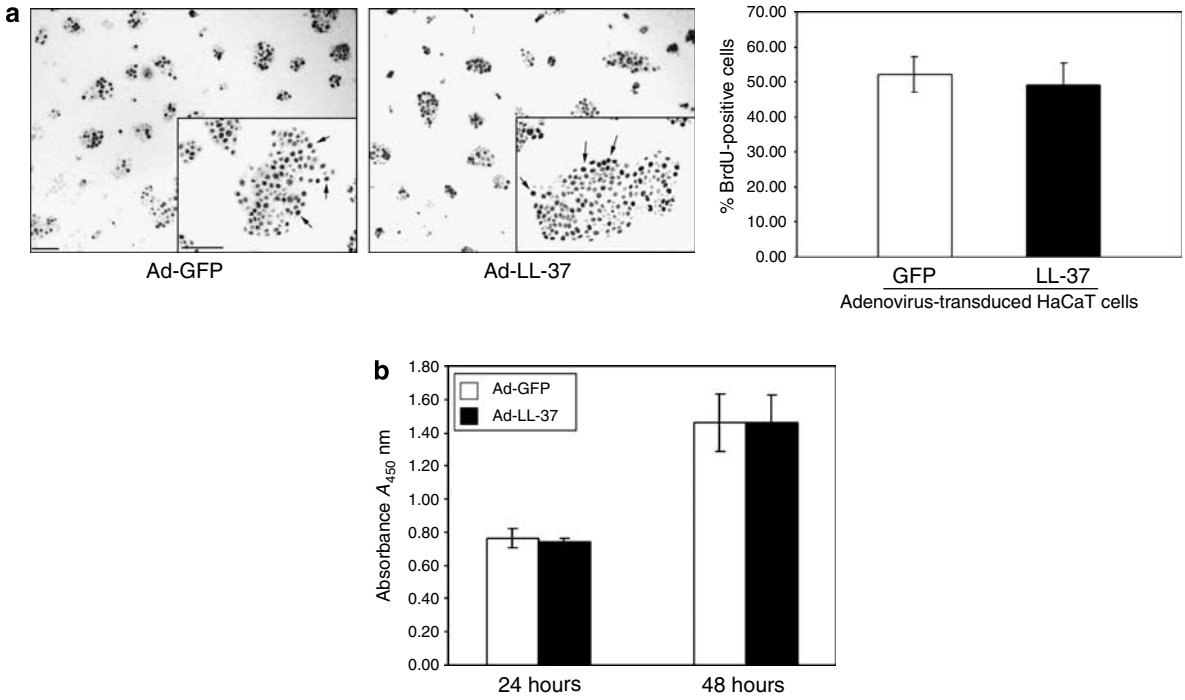

Figure 1. Proliferation of HaCaT cells in the presence of the LL-37 antimicrobial peptide. (**a**) Phase-contrast photographs of adenoviral transduced HaCaT cells after BrdU immunocytochemistry. Arrows point to BrdU-labeled cells 24 hours after infection. Quantification of BrdU-positive cells is shown. For each condition, 10 different fields of 100–200 cells were counted and the mean values ± SD were calculated. Bar = 50 μm. (**b**) HaCaT cell proliferation was quantified 24 and 48 hours after adenoviral infection using an XTT assay. Data are means ± SD of each condition determined in triplicates. GFP, green fluorescent protein.

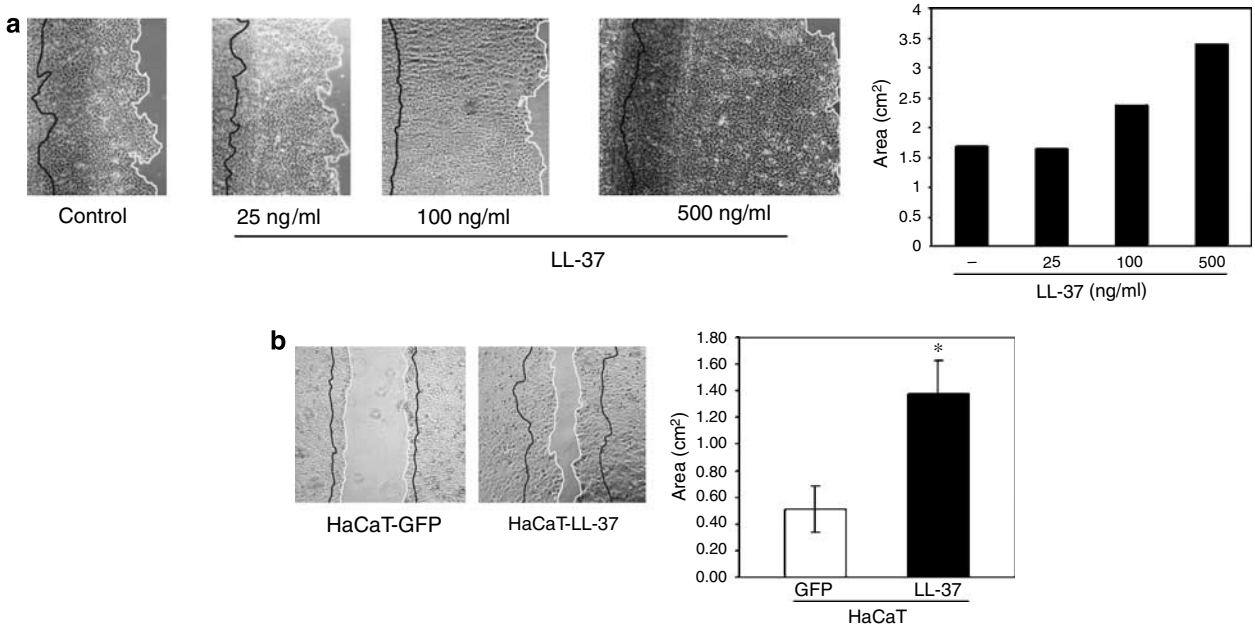

Figure 2. LL-37-induced migration of HaCaT cells in an *in vitro* wound model. (**a**) HaCaT cells were grown to confluence on fibronectin-coated six-well tissue culture plates. Cells were serum-starved for 12 hours. Different concentrations of the synthetic LL-37 antimicrobial peptide ranging from 25 to 500 ng/ml were used in an *in vitro* wound assay, where half of each well was denuded from cells and then re-coated with fibronectin. Migration was monitored for up to 72 hours. Same fields were photographed immediately after wounding (wound margin depicted as black line) and 72 hours later (white line) and pictures were superposed using Adobe Photoshop. Areas were measured using Scion Image. A representative experiment is shown. (**b**) Retrovirus-transduced HaCaT cells were used in a similar assay. Cells were grown to confluence on collagen I-coated 24-well tissue culture plates. Cells were treated with mitomycin C to inhibit cell proliferation and then an *in vitro* wound was produced using a sterile pipette tip. Wound margin at $t = 0$ hour is depicted as black line. Cell migration was monitored for up to 48 hours (wound margin depicted as white line) and quantified by image analysis. The data represent the mean $\pm$ SD of three experiments. *Statistical significance ($P < 0.05$). GFP, green fluorescent protein.

However, when the LL-37 peptide was tested in an *in vitro* wound-healing assay, a clear induction of migration either on fibronectin or collagen type I was observed. A dose-response analysis showed that LL-37 induced keratinocyte migration on fibronectin at concentrations up to 500 ng/ml (Figure 2a). We also tested the migratory activity of HaCaT cells that were genetically modified using a retroviral vector to express permanently the LL-37 antimicrobial peptide. These cells showed an enhanced migratory activity either on collagen type I (Figure 2b) or fibronectin (data not shown).

In addition to enhanced antimicrobial activity (Carretero *et al.*, 2004), Ad-LL-37-transduced HaCaT cells exhibited cytoplasmic protrusions at the free edge of colonies (Figure 3a). We thus decided to study the changes associated with the migratory phenotype in these cells. Phalloidin staining revealed that LL-37 induced actin reorganization in these cells. In contrast, a typical stationary phenotype associated with the formation of marginal bundles of actin filaments was observed at the edge of HaCaT cell colonies transduced with control Ad-green fluorescent protein (GFP). The transition to a motile phenotype observed in Ad-LL-37 transduced HaCaT cells involved loss of marginal actin bundles and development of a large leading lamella and formation of filopodial extensions (Figure 3b, upper panels). The same phenotypic conversion was observed in retrovirus-transduced HaCaT cells and in HaCaT cells treated with different concentrations of the synthetic antimicrobial peptide (Figure 3b, middle and lower panels).

LL-37 induced changes in focal adhesions

Immunofluorescence analysis using an anti- focal adhesion kinase (FAK) antibody revealed increased levels of focal adhesion kinase (p125FAK) at sites on the edge of the HaCaT cell colonies expressing LL-37, where new focal contacts are being formed (Figure 4a, upper panels). p125FAK is a non-receptor tyrosine kinase that regulates the assembly of focal adhesions promoting cell spreading on the extracellular matrix. It can recruit p60c-Src to phosphorylate paxillin, a critical step in focal adhesion assembly (Richardson *et al.*, 1997). Whole-cell phosphotyrosine (PY) immunofluorescence analysis of Ad-LL-37 transduced HaCaT cells demonstrated a general increase in tyrosine phosphorylation that was also apparent at the edge of colonies, at similar location as FAK (Figure 4a, lower panels). Phosphotyrosine immunoprecipitation followed by Western blot analysis using specific antibodies showed that both p125FAK and paxillin presented increased levels of tyrosine phosphorylation in the presence of the LL-37 antimicrobial peptide, either in adenovirus- or retrovirus-transduced HaCaT cells (Figure 4b). Very likely, the augmented phosphorylation of FAK and paxillin accounts for the general cellular PY increase observed.

LL-37 induced Snail and Slug transcription factors in HaCaT keratinocytes

Considering that epidermal migratory leading edges during skin wound healing are characterized by transient epithelial-

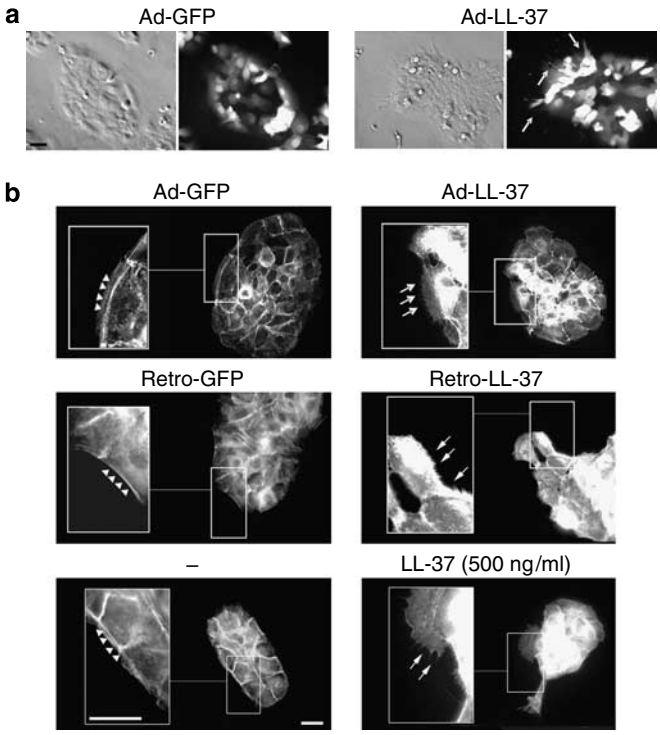

Figure 3. LL-37 induced a migratory-like phenotype in HaCaT cells. (**a**) HaCaT cells were transduced using either an adenoviral vector containing the human LL-37 antimicrobial peptide together with an Ires-GFP expression cassette or a control Ad-GFP. After 48 hours, HaCaT cells transduced with Ad-LL-37 showed the formation of cytoplasmic protrusions (white arrows) at the free edge of colonies by direct visualization under UV light (365 nm). (**b**) After fixation, cells were stained for actin using phalloidin-TRITC. The majority of edge-colony control cells showed a typical stationary phenotype with marginal actin bundles (white arrowheads) that is lost in Ad-LL-37-transduced HaCaT cells showing the formation of filopodial extensions (white arrows). The same was observed in both retrovirus-transduced HaCaT cells and in cells treated with the synthetic antimicrobial peptide. Bar = 10 μm. GFP, green fluorescent protein.

mesenchymal transition (EMT) events and invasive phenotypes (Escamez *et al.*, 2004; Thiery and Sleeman, 2006), we sought to determine whether LL-37 was also able to modulate these phenomena.

We examined different signaling cascades acting in the EMT process that might be, to some extent activated, by the LL-37 antimicrobial peptide. The Snail zinc finger transcription factor is known to be involved in EMT during embryonic development. It was demonstrated previously that stable expression of Snail in Madin-Darby Canine kidney epithelial cells (MDCK) epithelial cells induced a conversion to a mesenchymal phenotype through loss of E-cadherin and the acquisition of an invasive behavior. Interestingly, the morphology of Snail-transfected cells showed abundant membrane extensions and long filaments resembling filopodia (Cano *et al.*, 2000). Reverse transcriptase (RT)-PCR analysis demonstrated that LL-37 was capable of inducing Snail mRNA in cells permanently expressing LL-37 (Figure 5a) and also in adenovirus-transduced HaCaT cells at 24 and 48 hours after infection as well as in HaCaT cells treated with different concentrations of the LL-37 synthetic antimicrobial peptide (data not shown). LL-37 was also shown to induce the expression of Slug mRNA (Figure 5a), a member of the Snail family of transcription factors that was reported to be upregulated in keratinocytes during wound healing (Savagner et al., 2005; Turner *et al.*, 2006). Immunofluorescence analysis revealed increased Snail protein expression preferentially in cells at the edge of the colonies (Figure 5b, middle panels). As predicted, all cells expressed Snail when HaCaT cells were modified using a snail-encoding retrovirus (Figure 5b, lower panels). Conversely to other Snail overexpressing epithelial cells, the LL-37-driven Snail increase appeared to be insufficient for the downregulation of E-cadherin expression as assessed by either semi-quantitative RT-PCR or Western blot analysis of total cellular lysates (data not shown). However, in spite of this apparent lack of molecular response to Snail induction, cell-cell contacts appeared to be discontinuous at the edge of colonies expressing the LL-37 peptide as assessed by immunofluorescence analysis using anti-E-cadherin and anti-Desmoplakin 1/2 antibodies (Figure 5c, upper and middle panels). These results indicate that the upregulation of Snail transcription factor is taking place at cells that undergo the phenotypic conversion. In this regard, by double immunofluorescence analysis, we observed that in cells located at the edge of the LL-37 expressing colonies marginal bundles of actin disappeared (phalloidin labeled in red), and microtubules extended to the cell periphery (tubulin labeled in green) as it was observed in HaCaT cell colonies expressing Snail by retroviral transfer (Figure 5c, lower panels).

In addition to phenotypic changes related to actin/tubulin filament redistribution, retrovirus-transduced HaCaT cells constitutively expressing Snail exhibited an augmented migratory activity as compared with control cells (Figure 5d), suggesting that LL-37-mediated migratory activity may be associated with Snail induction.

LL-37 induced the mitogen-activated protein kinase and phosphoinositide 3-kinase /Akt signaling pathways in HaCaT keratinocytes

Several kinases have been shown to be involved in the EMT process. The mitogen-activated protein kinase (MAPK) pathway mediates the induction of Snail transcription factor by transforming growth factor-β in epithelial cell lines (Peinado *et al.*, 2003) and glycogen synthase kinase 3 (GSK-3) has been shown to maintain the epithelial phenotype, as it inhibits the transcription of Snail (Bachelder *et al.*, 2005). Other kinases promote the EMT, such as Akt (Grille *et al.*, 2003) and integrin-linked kinase (ILK) (Oloumi *et al.*, 2004). Phosphoinositide 3-kinase (PI-3K) also promotes EMT by suppressing GSK-3 activity (Woodgett, 2001). Western blot analysis demonstrated that LL-37 activated both the MAPK and the PI-3K/Akt signaling pathways in both adenovirus- and retrovirus-transduced HaCaT cells (Figure 6).

LL-37 enhanced proteolytic activities in HaCaT cells

As a consequence of an enhanced migratory phenotype, cells may vary their expression of genes coding for cell adhesion

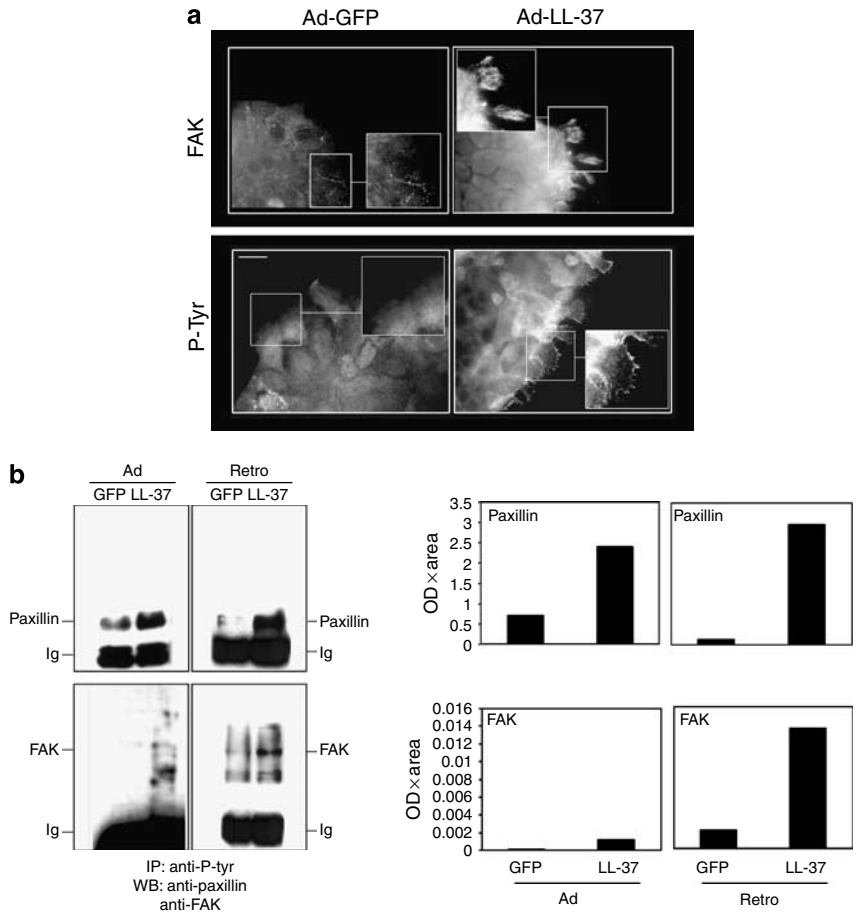

Figure 4. LL-37 induced changes in focal adhesions in HaCaT keratinocytes. (**a**) Immunofluorescence analysis showed increased focal adhesion kinase (FAK) and PY-20 staining at protrusive regions of edge-colony Ad-LL-37 transduced HaCaT cells. (**b**) PY-20 immunoprecipitates from 24 hours transduced HaCaT cell lysates were subjected to Western blot analysis with anti-paxillin and FAK antibodies. The relative band intensity was assessed by densitometric analysis of digitalized autoradiographic images using Scion Image software. GFP, green fluorescent protein.

molecules and intermediate filaments and, in some cases, may synthesize extracellular matrix molecules and proteolytic enzymes involved in matrix degradation (Boyer *et al.*, 2000). Zymography using 24 hours conditioned media from HaCaT cells, plated either on fibronectin or collagen type I and treated with increasing concentrations of the LL-37 antimicrobial peptide, revealed a dose-response induction of gelatinolytic activity at ~72 and ~92 kDa, consistent with the latent forms of matrix metalloproteinase-2 (MMP-2) and MMP-9, respectively (Figure 7a). These results were also confirmed by semiquantitative RT-PCR analysis using specific oligonucleotides for MMP-2 (data not shown). In addition to MMPs, the urokinase-type plasminogen activator receptor (uPAR) signaling has been shown to modulate the migratory activity of keratinocytes (Blasi and Carmeliet, 2002). Although keratinocytes do not normally express uPAR in skin, it has been reported that the protein is induced in the epithelial leading edge during wound healing (Romer *et al.*, 1994). Semiquantitative RT-PCR analysis of both LL-37 adenovirus- and retrovirus-transduced HaCaT cells showed increased uPAR mRNA levels, whereas uPA mRNA remained unaffected (Figure 7b).

LL-37 upregulates the expression of its putative receptor FPRL-1 in HaCaT keratinocytes

Both cell motility responses and the possible underlying activation of signaling pathways such as those described here will likely depend on receptor-mediated signals. FPRL-1 has been postulated as the natural receptor for LL-37, at least in neutrophils. The expression of FPRL-1 was determined in both control and Ad-LL-37 or retro-LL-37 transduced HaCaT cells. Semiquantitative RT-PCR analysis revealed that FPRL-1 mRNA was clearly upregulated in HaCaT cells expressing the LL-37 peptide (Figure 8a). To investigate the functional implication of this receptor in the LL-37-mediated activation of keratinocyte migration, we took advantage of the use of chemical inhibitors of specific signaling pathways in the above-mentioned *in vitro* wound-healing assays. As shown in Figure 8b, the G_i protein-coupled signaling inhibitor pertussis toxin inhibited the LL-37-mediated HaCaT cell migration to collagen type I. In contrast, the specific EGFR tyrosine kinase inhibitor AG1478 did not affect the LL-37-mediated cell migration. Although we cannot rule out a general involvement of G protein-coupled receptor signaling in HaCaT cell migration (Figure 8b, retro-GFP + pertussis toxin), our results

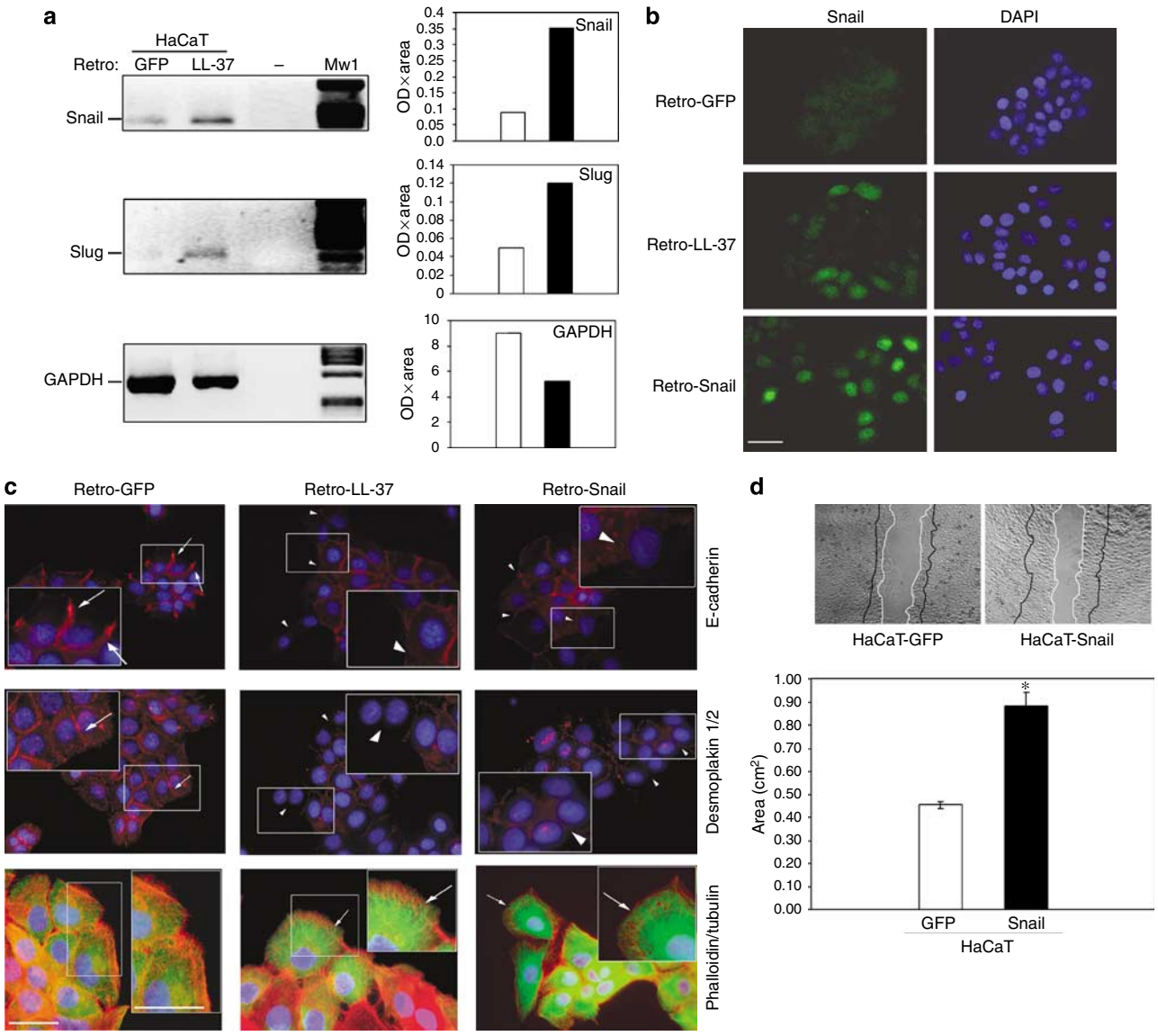

Figure 5. Expression of Snail, Slug, E-cadherin, and Desmoplakin in HaCaT cells expressing the LL-37 antimicrobial peptide. (**a**) mRNA expression was examined by semiquantitative RT-PCR in retrovirus-transduced HaCaT cells. The expression of GAPDH served as a control for the amount of cDNA in each sample. Molecular weight marker 1: ϕX174 DNA cleaved with *Hae*III. The relative band intensity was assessed by densitometric analysis. (**b**) Retrovirus-transduced HaCaT cells were analyzed by immunofluorescence assay with anti-Snail antibody. Cells were stained with DAPI to localize nuclei. HaCaT cells transduced with a retroviral vector containing human Snail were used as control. Bar = 10 μm. (**c**) Retrovirus-transduced HaCaT cells were processed for immunofluorescence using antibodies to E-cadherin (Adherens junctions) and Desmoplakin I + II (Desmosomes). Control cells presented well-formed adherens junctions and desmosomes at the edge of colonies (white arrows) that appeared disrupted in HaCaT cells transduced with retroviral vectors for human LL-37 and Snail (white arrowheads). Lower panels show double immunofluorescence analysis for actin (red) and tubulin (green). Microtubules extended to the cell periphery in edge cells presenting disrupted marginal actin bundles (white arrows). Bar = 10 μm. (**d**) *In vitro* wound healing assay using the retrovirus-transduced HaCaT cells expressing human Snail. Cells were grown to confluence on collagen I-coated 24-well tissue culture plates and were then treated with mitomycin C to inhibit cell proliferation. An *in vitro* wound was produced using a sterile pipette tip and and wells were then re-coated with collagen I. Same fields were photographed immediately after wounding (wound margin depicted as black line) and 24 hours later (white line) and pictures were superposed using Adobe Photoshop. Areas were quantified by image analysis using Scion Image software. The data represent the mean ± SD of three experiments. *Statistical significance ($P < 0.05$). GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

suggest that the LL-37-mediated activation of HaCaT keratinocyte cell migration involves signaling events distinct to the transactivation of the EGFR.

LL-37 enhanced wound healing in diabetic mice

To address whether LL-37 could serve as a beneficial tool in gene therapy protocols for wound repair, we performed excisional wound-healing experiments in the healing impaired ob/ob mouse model. We performed adenovirus-mediated LL-37 gene transfer by dermal injection around 4-mm flank wounds that was confirmed by detection of green fluorescence after 3 and 6 days (Figure 9a). The effect of Ad-LL-37 treatment was assessed by histologic and immunohistochemical examination of epithelial gap closure

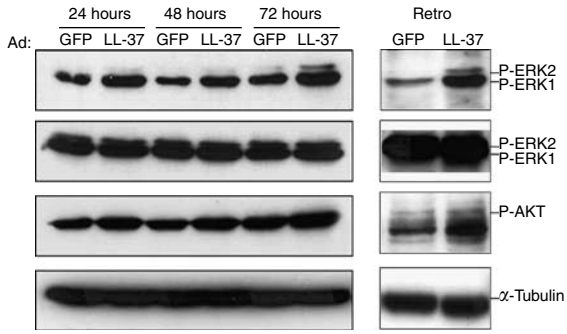

Figure 6. LL-37 induced activation of the MAPK and PI-3K signaling pathways in HaCaT keratinocytes. Whole cell extracts from either adenovirus or retrovirus-transduced HaCaT cells expressing the LL-37 antimicrobial peptide were obtained and analyzed in Western blot for total protein levels and phosphorylated forms of ERK1/2 and Akt. α-Tubulin served as a control for the amount of protein in each sample. ERK1, extracellular signal regulated kinase 1; GFP, green fluorescent protein.

and granulation tissue formation. The measurement of the distance between epithelial tongues revealed a clear enhancement of the re-epithelialization process in most of the LL-37-treated wounds at days 3 and 6 post-wounding (Figure 9d). The percentage of re-epithelialization was improved by $19.72 \pm 7.04\%$ after 3 days, and by $20.50 \pm 8.67\%$ after 6 days of treatment with Ad-LL-37. At day 3, an increase in the lengths of the tongues of new migrating epithelium was clearly observed in LL-37-treated wounds. At day 6, a similar increase was observed, as in most cases the epithelial tongues had converged to completely cover the wound when the Ad-LL-37 was applied, whereas in the corresponding Ad-GFP control wounds the epithelial gap was still evident (Figure 9b). An immunohistochemical analysis of GFP expression revealed the presence of GFP-positive dermal cells with fibroblast morphology, not only at the site of the injection but also in the center of the wound area in Ad-LL-37-treated 3-day wounds (Figure 9c). In contrast, GFP-positive cells remained close to the edge in Ad-GFP- treated wounds, indicating that a more active migratory process occurred in the Ad-LL-37-treated wounds. Granulation tissue developed more slowly in control wounds, as the cell density in the central area of the 3-day LL-37-treated wounds was higher than that of control wounds (Figure 9b). At day-15 post-wounding, although the number of myeloperoxidase-positive inflammatory cells appeared to be augmented in some of the LL-37-treated wounds, the collagen deposition and remodeling was similar to control wounds as assessed by picrosirius red staining and visualization under polarized light (data not shown).

DISCUSSION

We have demonstrated previously the feasibility of using an adenoviral vector to overexpress LL-37 in a skin equivalent to prevent bacterial infection (Carretero *et al.*, 2004). Others (Jacobsen *et al.*, 2005) have also demonstrated the efficacy of a transient cutaneous adenoviral delivery of this antimicrobial peptide in the infected rat burn model. In addition to its antimicrobial activity, several reports indicated that LL-37 is also able to mediate cellular responses that contribute to the immune response and might enhance the wound-healing process (Bals and Wilson, 2003; Bardan *et al.*, 2004).

Migration of keratinocytes into the wound bed is one of the main factors contributing to epidermal wound healing. Several soluble factors have been shown to influence keratinocyte migration, as in the case of TGF-β and cytokines (GM-CSF, IL-1β and IL-6) (Martin, 1997). LL-37 was shown previously to induce migration of epidermal cells by transactivating the EGFR (Tokumaru *et al.*, 2005). It was also shown to stimulate bronchial epithelial cell migration and proliferation (Shaykhiev *et al.*, 2005). It has been shown previously that re-epithelialization of organ-cultured skin wounds is inhibited by antibodies against LL-37 in a dose-dependent mode (Heilborn *et al.*, 2003). That study suggested that LL-37 might affect proliferation of epithelial cells, based on the lack of Ki67-immunoreactive cells in the epithelium of the wound samples treated with LL-37 antiserum. Although we have been unable to demonstrate the involvement of the LL-37 antimicrobial peptide in the proliferation of the human keratinocyte cell line HaCaT, this peptide clearly induced a conversion of HaCaT cells to a migratory phenotype showing numerous filopodia-like protrusions of the plasma membrane, which resulted in an enhancement of cell migration onto different matrices. The interaction with extracellular matrix proteins is essential in the wound-healing response. After wound healing, keratinocytes migrate onto a provisional matrix that contains several extracellular matrix proteins, the more abundant being type I collagen and fibronectin. We have demonstrated that the LL-37 peptide induced cellular migration when both substrates were used.

Cell migration is a complex process that involves the continuous formation (at the leading edge of protrusions) and disassembly (at the cell rear and at the base of protrusions) of adhesions. Several kinases and adaptor molecules, including FAK, Src, p130CAS, paxillin, ERK, and myosin light chain kinase have been shown to be critical for adhesion turnover at the cell front, a process central to migration (Webb *et al.*, 2004). We observed a general increase in tyrosine phosphorylation in HaCaT cell colonies expressing the LL-37 antimicrobial peptide. In particular, the cathelicidin induced an increase in the tyrosine phosphorylation of FAK and paxillin in these cells. Increased FAK tyrosine phosphorylation was previously shown to correlate with the level of human keratinocyte motility (Yurko *et al.*, 2001). Cell invasion is linked to transient accumulation of FAK at lamellipodia, which can activate different signaling pathways (Hsia *et al.*, 2003). We have shown that FAK expression is increased at the new protrusions formed in edge colony cells in the presence of the peptide. It has been demonstrated that a cross-talk between integrin and growth factor signaling pathways may be coordinated through the actin cytoskeleton, and may converge at FAK (Boudreau and Jones, 1999).

Some of the events involved in the EMT process are observed in the presence of the LL-37 antimicrobial peptide, such as induction of the Snail transcription factor. Snail was previously shown to induce the EMT process in epithelial

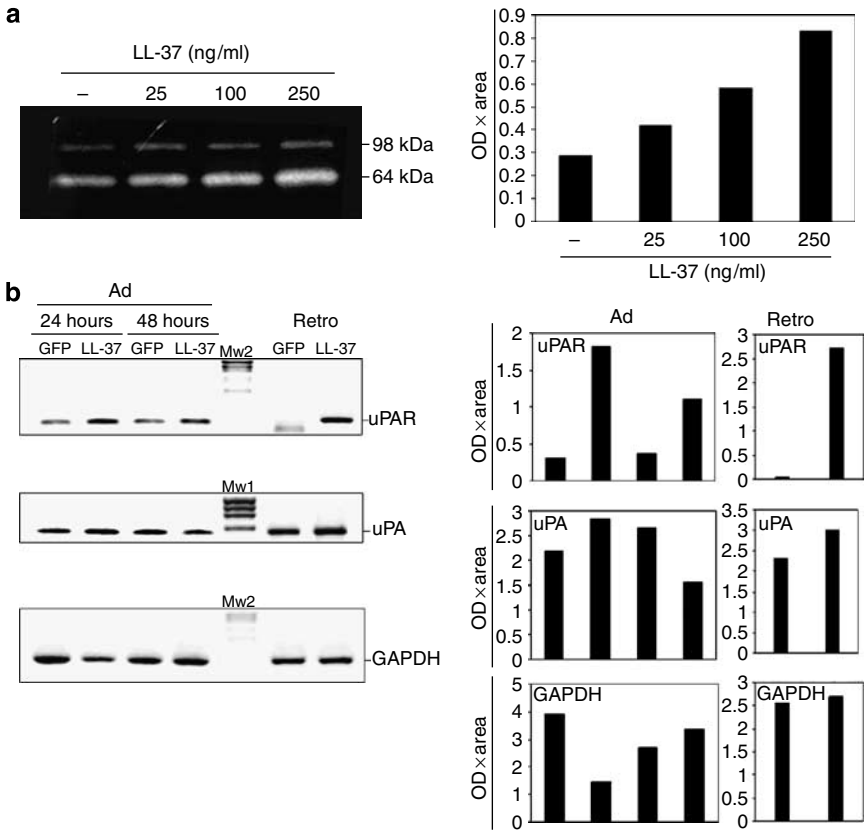

Figure 7. LL-37 induced the activity of matrix metalloproteinases and uPAR expression in HaCaT keratinocytes. (**a**) Gelatin zymography was performed using 24 hours conditioned medium from HaCaT cells treated with increasing concentrations of LL-37 synthetic peptide. The relative band intensity of the lower band was assessed by densitometric analysis of digitalized autoradiographic images using Scion Image software. (**b**) uPA and uPAR mRNA expression was examined by semiquantitative RT-PCR in LL-37 genetically modified HaCaT cells. Time points post-adenoviral infection are indicated. The expression of GAPDH served as a control for the amount of cDNA in each sample. Molecular weight marker 1: ϕX174 DNA cleaved with *Hae*III. Molecular weight marker 2: λDNA cleaved with *Bst*EII. GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; uPAR, urokinase-type plasminogen activator receptor.

cells, leading to the loss of E-cadherin expression, acquisition of a fibroblast-like morphology, and upregulation of vimentin expression. Snail represses the E-cadherin promoter activity by binding to the E-box sequences (Cano *et al.*, 2000). We could not observe a downregulation of E-cadherin expression in the presence of the LL-37 peptide possibly due to a dilution effect of using whole-colony mRNA and protein. However, by immunofluorescence analysis using antibodies against E-cadherin (adherens junctions) and Desmoplakin1/2 (desmosomes) cell-cell contacts appeared to be discontinuous at the edge of colonies expressing the LL-37 peptide. It is clear that LL-37 did not lead to a complete EMT conversion in HaCaT keratinocytes, as the Snail induction and cell-cell contact disruption is only evident at the edge of the colonies. There are other cell movement processes in which Snail is involved and that do not require a full EMT, such a mesoderm formation in anamniote vertebrate embryos or gastrulation in *Drosophila*. Snail participates in these processes in which cells maintain contact with each other as they move (reviewed in Barrallo-Gimeno and Nieto, 2005). Alternatively, in some cell systems, several growth factors have been reported to require cooperation with H-Ras activation to induce a complete EMT process (Gotzmann *et al.*, 2002; Janda *et al.*, 2002).

Snail may also act as a transcriptional activator affecting the expression of genes involved in the control of motility and migration. Among them, MMPs are augmented in the EMT process and are expressed during the wound-healing response (Mott and Werb, 2004; Thiery and Sleeman, 2006). It has been demonstrated previously that Snail induces MMP-2 expression in squamous-cell carcinoma cells (Yokoyama *et al.*, 2003) and MMP-9 expression in the MDCK epithelial cell line (Jorda *et al.*, 2005). Here we showed that MMP-2 (gelatinase A) and MMP-9 (gelatinase B) appeared to be augmented in the presence of the LL-37 peptide. It has been shown that MMP-2 plays an important role during granulation and early phases of the wound-healing process, and localizes mainly in fibroblasts and endothelial cells. MMP-9 is stimulated in response to injury and is expressed in migrating keratinocytes (Salo *et al.*, 1994). Makela *et al.* (1999) have shown the role of MMP-2 produced by oral mucosal and skin keratinocytes in activating cell migration.

Another protease-mediated signaling system, uPAR, is induced by LL-37 in HaCaT cells. It participates in the control

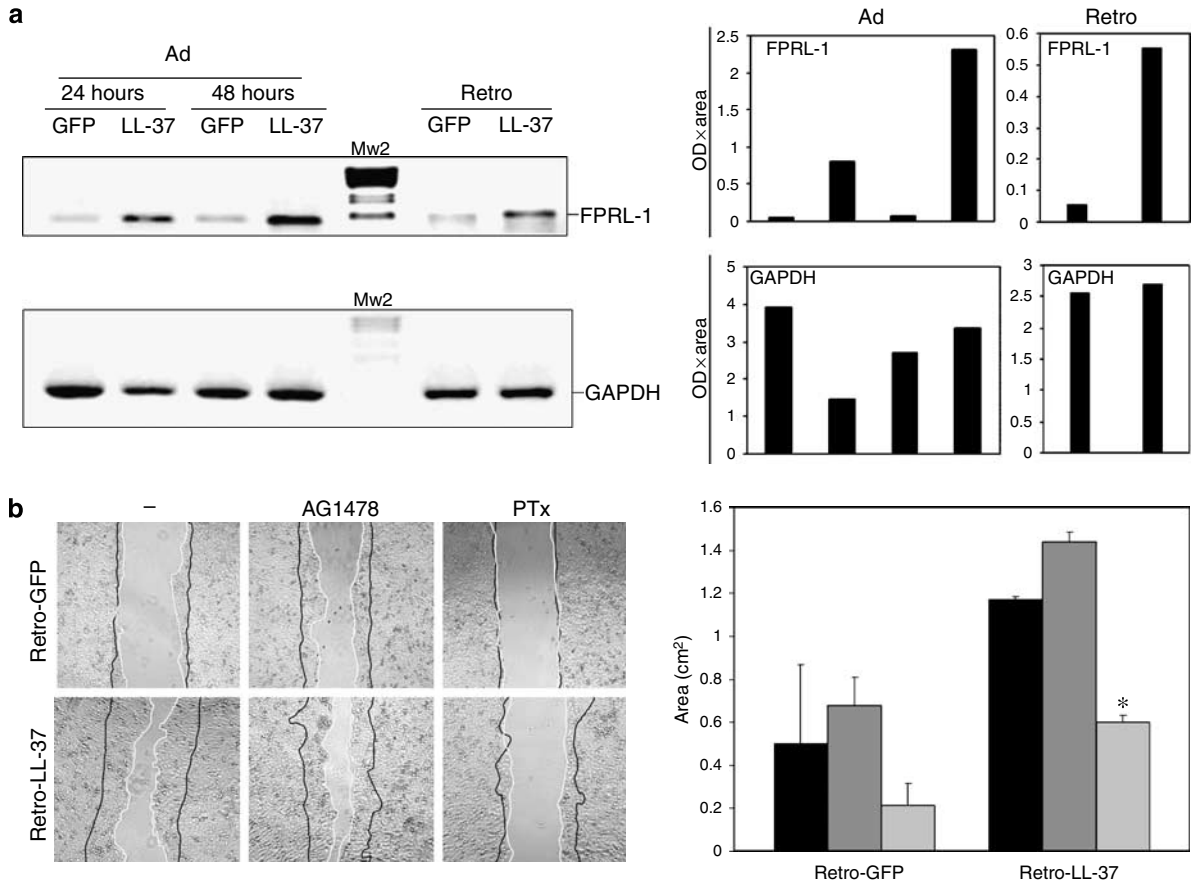

Figure 8. LL-37 upregulates FPRL-1 mRNA expression in HaCaT keratinocytes. (**a**) FPRL-1 mRNA expression was examined by semiquantitative RT-PCR in LL-37 genetically modified HaCaT cells. Time points post-adenoviral infection are indicated. The expression of GAPDH served as a control for the amount of cDNA in each sample. Molecular weight marker 1: ϕX174 DNA cleaved with *Hae*III. Molecular weight marker 2: λDNA cleaved with *Bst*EII. The relative band intensity was assessed by densitometric analysis. (**b**) Retroviral-transduced HaCaT cells were grown to confluence on collagen I-coated 24-well tissue culture plates. Cells were treated with mitomycin C to inhibit cell proliferation. Where indicated, cells were pretreated with either the G protein inhibitor pertussis toxin (200 ng/ml) or the EGFR tyrosine kinase inhibitor AG1478 (1 μM) for 2 hours. Then, an *in vitro* wound was produced using a sterile pipette tip and wells were re-coated with collagen I. Wound margin at $t=0$ hour is depicted as black line. Cell migration was monitored for up to 48 hours (wound margin depicted as white line) and quantified by image analysis. The data represent the mean $\pm$ SD of three experiments. *Statistical significance ($P<0.05$). GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

of cell motility through the activation of the proteolytic cascade initiated by the conversion of plasminogen into plasmin. It has been shown to be highly expressed in migrating keratinocytes during wound repair (Solberg *et al.*, 2001). Because uPAR lacks an intracellular domain, several transmembrane mediators have been identified as mediators of the signaling response to uPA, such as integrins and FPRL-1. They are involved in uPA-mediated regulation of cell adhesion and migration through the activation of several signaling pathways (Blasi and Carmeliet, 2002).

Herein we demonstrated the involvement of both MAPK, and PI-3K signaling pathways in the LL-37-mediated signal transduction in HaCaT cells. The MAPK cascade has been shown to participate in cell migration by inducing phosphorylation of the myosin light chain kinase (Klemke *et al.*, 1997) or by the activation of calpain (Glading *et al.*, 2000). PI-3K has also been shown to regulate actin cytoskeletal reorganization through the small GTPase Rac (Nobes and Hall, 1995; Reif *et al.*, 1996). It has been demonstrated previously that LL-37-mediated EGFR transactivation in airway epithelial cells leads to activation of the MAPK signaling pathway (Tjabringa *et al.*, 2003). Thus, the observed LL-37-mediated activation of MAPK in HaCaT cells would conceivably occur by a mechanism involving the shedding of heparin-binding epidermal growth factor (HB-EGF) that was described previously in keratinocytes (Tokumaru *et al.*, 2005). It remains to be determined whether EGFR transactivation is involved in the different signaling events analyzed here as we have also demonstrated that LL-37 was able to induce the expression of the FPRL-1 receptor. In this regard, the LL-37-mediated HaCaT cell migration appears to involve a G protein-coupled receptor but not the activation of the EGFR, as deduced for the use of specific chemical inhibitors in *in vitro* wound-healing assays. The FPRL-1 receptor shows high level of homology to FPR, which has been shown to activate protein kinase C, phospholipase A2 (PLA2),

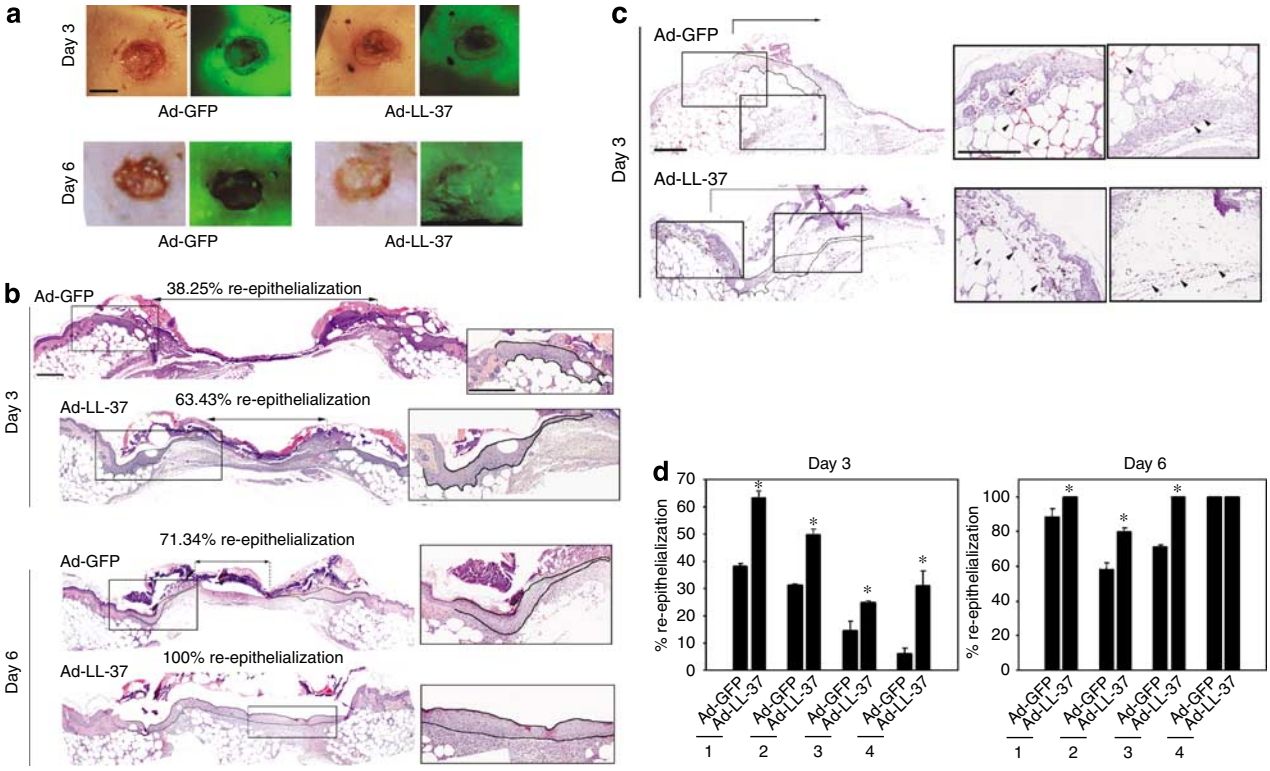

Figure 9. Adenovirus-mediated LL-37 gene transfer promotes wound healing in diabetic ob/ob mice. (**a**) Macroscopic examination at days 3 and 6 post-wounding. Correct adenoviral gene transfer around the wounds was confirmed by illuminating the animal with blue light under a fluorescence stereomicroscope (right panels). Bar = 3 mm (**b**) Composite pictures of microphotographs at the center of the wounds showing hematoxylin and eosin staining. Dermo-epidermal boundaries are delineated with a solid black line. Arrows indicate epidermal gap, which determine the percentage of re-epithelialization. A higher magnification of boxed area in (**a**) containing the epithelial tongue is shown. Bar = 300 μm. (**c**) GFP expression was detected by immunostaining in serial sections of the same 3-day wounds. Left panels show one half of the wound, and middle and right panels are higher magnifications of boxed areas. Bar = 300 μm. (**d**) Percent re-epithelialization from the original wound was evaluated microscopically using 2–3 tissue sections stained with H&E and containing the center of each wound. Four paired wounds were analyzed 3 and 6 days post-wounding. The % re-epithelialization was calculated for each wound; data were analyzed using the Student's *t*-test and represented graphically (*$P<0.01$ related to control wounds). GFP, green fluorescent protein.

D, MAPK and PI-3K in neutrophils (Selvatici *et al.*, 2006). It was demonstrated previously that fibroblasts express FPRs (FPR and FPRL-1), and that fMLP induces actin polymerization in these cells as well as calcium mobilization, adhesion and directed migration (VanCompernolle *et al.*, 2003). The human lung epithelial cell line A549 also expresses functional FPR (Rescher *et al.*, 2002). Thus, the LL-37-mediated activation of the MAPK and/or PI-3K pathway in HaCaT cells might be at least in part a consequence of the signaling through FPRL-1. Moreover, preliminary evidence suggests that FPRL-1 expression might be activated in wound edge keratinocytes (M Carretero, unpublished results), suggesting that LL-37 could signal through this receptor in migrating keratinocytes. The *in vivo* functional relevance of this activation for tissue repair remains to be determined.

In a previous study (Carretero *et al.*, 2004), we demonstrated that a genetically modified cultured skin equivalent expressing the LL-37 human antimicrobial peptide in the epidermal component exhibited microbicidal activity against pathogens frequently isolated from burn wounds. We proposed LL-37 as a good candidate for a transient antimicrobial skin gene therapy in severe burn patients, not only to prevent infection but also in the definitive coverage. Herein, we provide early evidence that *in vivo* delivery of an adenoviral vector containing the human LL-37 antimicrobial peptide improves wound healing by increasing the re-epithelialization rate and granulation tissue formation in diabetic wounds. In normal wound healing, the resolution of inflammation is crucial for the progression of the wound to matrix deposition and remodeling phases. As LL-37 is known to exert a potent proinflammatory activity by recruitment of granulocytes (De *et al.*, 2000), it would be expected that a sustained activity of the antimicrobial peptide would hamper the final phases of the wound-healing process. However, this is not the case as inflammatory cells present in the 15-day LL-37-treated wounds did not impair collagen deposition and remodeling.

Overall, our results shed new light on the pleiotropic responses elicited by the LL-37 cathelicidin and further support its potential therapeutic applications for skin regeneration.

MATERIALS AND METHODS

Cells

The human keratinocyte cell line HaCaT was grown in DMEM with Glutamax (Gibco-BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum.

Animals

Female C57BL/6J-*ob/ob* mice were purchased from Janvier Laboratories (Le Genest, St Isle, France) and maintained under a 12 hours light-dark cycle at 22°C until they were 8–10 weeks of age. Mice were housed for the duration of the experiment at the CIEMAT Laboratory Animals Facility (Spanish registration no. 28079-21 A) in pathogen-free conditions using micro isolators type IIL (16 air changes per hour) and 10 kGy γ-irradiated soft wood pellets as bedding. All handling was carried out according to European and Spanish laws and regulations (European Convention ETS 1 2 3, about the use and protection of vertebrate mammals used in experimentation and other scientific purposes and Spanish R.D. 1201/2005 about the protection and use of animals in scientific research). Procedures were approved by our Animal Experimentation Ethical Committee according to all external and internal biosafety and bioethics guidelines.

Antibodies and chemical compounds

Anti-pTyr PY-20 monoclonal antibody, anti-Akt1/2, anti-ERK1/2, anti-pERK and anti-Snail rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-FAK, anti-paxillin, and anti-E-cadherin monoclonal antibodies were from BD Transduction Laboratories Inc. (Franklin Lake, NJ) and anti-pAkt (Ser 473) rabbit monoclonal antibody and anti-Desmoplakin1/2 monoclonal antibody were purchased from Cell Signaling Technology Inc. (Beverly, MA) and Chemicon International (Temecula, CA), respectively. Anti-α-tubulin monoclonal antibody and phalloidin-tetramethyl rhodamine isothiocyanate (TRITC) was from Sigma (St Louis, MO). The rabbit polyclonal antibody was against GFP from Molecular Probes (Eugene, OR). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). The EGFR tyrosine kinase inhibitor AG1478 and pertussis toxin were purchased from Sigma.

Adenoviral vectors

Adenoviral vector containing the human LL-37 antimicrobial peptide together with an Ires-GFP expression cassette was described previously (Carretero *et al.*, 2004). Control vector Ad-GFP was kindly provided by Dr David T. Curiel (University of Alabama, Birmingham, AL). Adenoviruses were propagated in 293 cells, purified by double CsCl density centrifugation and stored at −70°C in 10 mM Tris-HCl pH 7.8/1 mM $MgCl_2$, containing 10% glycerol. Viral titers were determined by end-point dilution assay by applying serial dilutions of the purified viruses to 293 cells. The tissue culture infectious dose 50 (TCID 50) was calculated by evaluating the cytopathic effect after 14 days (violet crystal). For *in vitro* studies, cells were infected at a multiplicity of infection of 100 pfu/cell. *In vivo* adenoviral transfer was performed via intradermal injection of a volume of 50 μl containing 10^9 pfu.

Retroviral-mediated gene transfer to HaCaT cells

The packaging cell line PA317, obtained from ATCC, was used to generate stable cell lines to produce amphotropic retroviral particles containing either the pLZR-ires-EGFP, the pLZR-LL-37-ires-EGFP, or the pLZR-Snail-ires-EGFP sequence. HaCaT cells were genetically modified by retroviral gene transfer following a procedure that was previously applied successfully to human primary keratinocytes (Del Rio *et al.*, 2002). Cells were analyzed for EGFP expression and selected by fluorescence-activated cell sorting on a FACStar PLUS flow cytometer (Becton Dickinson, San Jose, CA).

Detection of HaCaT cell proliferation by BrdU

For BrdU incorporation analysis, 10^5 cells were seeded in 24-well plates. After 24 hours, cells were transduced with adenoviral vectors for 6 hours at 37°C in a humid atmosphere containing 5% CO_2. Adenovirus-containing supernatants were then discarded, and fresh medium was added to each well. After 24 hours medium was changed and after additional 24 hours BrdU incorporation analysis was performed. BrdU (10 μM) was added 2 hours before fixation in cold methanol for 3 minutes. Cells were then processed for BrdU immunocytochemistry and subsequent quantification. For each condition, 10 different fields of 100–200 cells were counted and the mean ± SD were calculated.

2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide colorimetric assay

Different numbers of HaCaT cells were plated in 96-well tissue culture plates. After 24 hours, cells were transduced with adenoviral vectors for 6 hours at 37°C in a humid atmosphere containing 5% CO_2. Adenovirus-containing supernatants were then discarded, and 100 μl fresh medium was added to each well. At 24 and 48 hours post-infection, cells were incubated with 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide (XTT) (Boehringer Mannheim GmbH, Mannheim, Germany) and absorbance at 450 nm (cell number) was measured. Triplicate experiments were performed.

In vitro wound assay

For wound assays in the presence of LL-37 synthetic peptide, HaCaT cells were seeded to confluence onto fibronectin-coated six-well tissue culture plates in DMEM supplemented with 10% fetal bovine serum. Cells were serum starved for 12 hours and then half of each well was denuded from cells by using a sterile rubber policeman. The cultures were washed twice with phosphate-buffered saline, and re-coated with fibronectin (10 μg/ml) for 1 hour at 37°C. At this time point ($t = 0$ hour) wound margins were photographed. Then, cells were treated with different concentrations of the synthetic LL-37 antimicrobial peptide for up to 72 hours at 37°C and 5% CO_2 in the absence of serum, and the same fields of the wound margin were photographed at different time points. Pictures were superposed using Photoshop (Adobe) and areas were measured using Scion Image (Scion Corporation, Frederick, MD) software.

For wound assays using retrovirus-transduced HaCaT cells, cells were seeded to confluence onto collagen type I-coated 24-well

tissue culture plates in DMEM supplemented with 10% fetal bovine serum. Cells were treated with mitomycin C (5 μg/ml) to inhibit cell proliferation for 2 hours at 37°C and 5% CO_2 and then an *in vitro* wound was produced using a sterile pipette tip. Then wells were re-coated with collagen type I (10 μg/ml). At this time point ($t = 0$ hour) wound margins were photographed and migration was monitored for up to 48 hours. Same fields of the wound margin were photographed at different time points. Pictures were superposed using Photoshop (Adobe) and areas were measured using Scion Image software. For wound assays in the presence of inhibitors, cells were pretreated with the indicated compound for 2 hours before wounding.

In vivo wound-healing experiments

The backs of mice were shaved, and four full-thickness wounds were created on each animal using 4-mm biopsy punches (Stiefel Laboratories, Offenbach, Germany) after 1% mepivacaine local anesthetic injection. *In vivo* adenoviral transfer was performed via intradermal injection of a volume of 50 μl containing 10^9 pfu of either Ad-GFP or Ad-LL-37-iresGFP applied via three separate injections around the wound edge. Gene transfer was confirmed by detection of green fluorescence by illuminating the animal with blue light under a fluorescence stereomicroscope (Olympus, Barcelona, Spain).

Histological and immunohistochemical analyses

At days 3, 6 and 15 after wounding, mice were killed (four animals in each group, two paired wounds per animal), and rectangular samples of skin containing complete wounds were harvested, fixed in 3.7% formaldehyde and paraffin embedded. To determine the center of the wound and adequately monitor the healing process, the whole sample was serially cross-sectioned (4 μm) and 1 of every 10 sections was stained with hematoxylin-eosin (Gill-2 hematoxylin and alcoholic eosin Y; Thermo Shandon GmbH, Darmstadt, Germany). The percentage of re-epithelialization was calculated by measuring the linear distance between epithelial tongues and dividing it by the distance between the original wound edges. Studies were performed in informative wounds (~50%). Paraffin sections were dewaxed by melting for 30–60 minutes at 60°C, cleared in xylene three times for 5 minutes, and re-hydrated in water solutions containing decreasing percentages of ethanol. Collagen fibers were stained with picrosirius red and viewed under polarized light. Immunohistochemistry was performed following standard procedures and using specific antibodies against GFP (Molecular Probes, Eugene, OR). Immunoperoxidase staining was developed using the Vectastain ABC kit (Vector, Burlingame, CA). Images were taken using an Olympus Bx41 microscope and digital camera.

Fluorescence microscopy

Cells were plated on coverslips to 40% confluence, and where indicated either transduced with adenoviral vectors for 6–12 hours at 37°C in a humid atmosphere containing 5% CO_2 or treated with different concentrations of synthetic LL-37 antimicrobial peptide. Cells were fixed at 24 hours post-adenovirus infection or peptide treatment, using either 4% paraformaldehyde in phosphate-buffered saline for 10 minutes at room temperature (for actin staining, PY-20 and Snail immunofluorescence) or methanol for 3 minutes at −20°C (for FAK immunofluorescence). After fixation with paraformaldehyde, cells were permeabilized with 0.1% Triton X-100 for 5 minutes. The cells were mounted on Mowiol (Calbiochem) containing 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma) and the preparations were visualized with an Axioplan 2 imaging fluorescence microscope (Zeiss, Jena, Germany).

Western blot

Cells were lysed in lysis buffer (1.5% Triton X-100, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinine, 1 μg/ml leupeptine, 20 mM NaF, 10 mM sodium PPi, 200 μM sodium orthovanadate). Total protein was measured using Bradford's reagent (Bio-Rad, Richmond, CA). For total lysate analyses, each sample (30 μg) was denatured at 100°C for 5 minutes and separated by 10% SDS-PAGE. For immunoprecipitation studies, 5×10^6 cells were lysed using 500 μl of 1.5% Triton X-100 lysis buffer, and incubated with 2 μg of PY-20 anti-phosphotyrosine antibody for 4 hours at 4°C. Then, complexes were recovered using protA/G agarose (Santa Cruz Biotechnology). Immunoprecipitates were separated by 8% SDS-PAGE. Transfer to a nitrocellulose membrane (Bio-Rad Laboratories Inc., Hercules, CA) was performed at 100 mA for 90 minutes in transfer buffer containing 20% methanol. Membranes were blocked in 10% non-fat dry milk in TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) for 2 hours, and then incubated with primary antibodies diluted in 1% non-fat dry milk in TBS-T (0.1% Tween-20/TBS) either for 2 hours at room temperature or at 4°C overnight. Membranes were washed three times with TBS-T and incubated for 45 minutes at room temperature with horseradish peroxidase-conjugated secondary antibodies diluted in TBS-T. After washing with TBS-T, bound antibodies were visualized by using an enhanced chemoluminescence SuperSignal Solution (Pierce Biotechnology Inc., Rockford, IL) and exposure to X-ray films. Densitometric analysis of bands was performed using Scion Image software.

Semiquantitative RT-PCR

Total RNA was isolated using TRIzol (Gibco-BRL) and incubated with Turbo DNase (Roche Applied Sciences, Indianapolis, IN). Total RNA (1 μg) was reverse-transcribed using random hexamers and SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA) in a $V_f = 20$ μl. Then, 1 μl of the RT reaction was employed in PCR using the following pairs of specific primers: Snail (forward primer: 5′-CACTATGCCGCGCTCTTTC-3′; reverse primer: 5′-GGTCGTAGGG CTGCTGGAA-3′), Slug (forward primer: 5′-ATGAGGAATCTGGCT GCTGT-3′; reverse primer: 5′-CAGGAGAAAATGCCTTTGGA-3′), uPA (forward primer: 5′-GACTCCAAAGGCAGCAATG-3′; reverse primer: 5′-CGATGGTGGTGAATTCTCC-3′), uPAR (forward primer: 5′-ATTGCCGTGTGGAAGAGTG-3′; reverse primer: 5′-GGTGTCGT TGTTGTGGAAAC-3′), FPRL-1 (forward primer: 5′-GAGAAAAATGG CCTTTTGGCTG-3′; reverse primer: 5′-CATTGCCTGTAACTCAGTC TCTGC-3′), GAPDH (forward primer: 5-ATCTTCCAGGAGCGAG ATCC-3; reverse primer: 5-ACCACTGACACGTTGGCAGT-3). PCR products were subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Densitometric analysis of bands was performed using Scion Image software.

Zymography

HaCaT cells were grown to 40–60% confluence on either fibronectin (10 μg/ml) or collagen type I (10 μg/ml) coated plates. Cells were

treated with different concentrations of the LL-37 synthetic antimicrobial peptide for 24 hours in the absence of fetal bovine serum. Conditioned medium was collected and concentrated using Microcon YM-10 devices (Millipore, Billerica, MA) following the recommendations of the manufacturer. An aliquot of each sample was mixed with Tris-glycine SDS sample buffer and separated on 10% SDS-PAGE co-polymerized 0.1% gelatin. Following electrophoresis, gels were washed with 2.5% Triton X-100 for 1 hour and incubated for 48 hours in enzyme assay buffer (50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 0.1% Triton X-100, 0.02% Na_3N) for the development of enzyme activity bands. After incubation, the gels were stained with 0.25% Coomassie brilliant blue and destained in 4% methanol with 8% acetic acid. Densitometric analysis of lytic bands at ~72 kDa was performed using Scion Image software.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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