

## Nuclear Localization of HBD-1 in Human Keratinocytes

*Roger J. Bick,<sup>a</sup> Brian J. Poindexter,<sup>a</sup> L. Maximilian Buja,<sup>a</sup> Carl H. Lawyer,<sup>b</sup> Stephen M. Milner,<sup>b,c</sup> and Satyanarayan Bhat<sup>b,c</sup>*

<sup>a</sup>Department of Pathology and Laboratory Medicine, University of Texas Medical School at Houston, Houston; <sup>b</sup>The Institute for Plastic and Reconstructive Surgery, Southern Illinois University School of Medicine, Springfield; and <sup>c</sup>Johns Hopkins Burn Center, Johns Hopkins University, Baltimore, MD.

Correspondence: sbhat3@jhmi.edu

Published August 24, 2007

---

**Objective:** Human defensins and cathelicidins are a family of cationic antimicrobial peptides (AMPs), which play multiple roles in both innate and adaptive immune systems. They have direct antimicrobial activity against several microorganisms including burn pathogens. The majority of components of innate and adaptive immunity either express naturally occurring defensins or are otherwise chemoattracted or functionally affected by them. They also enhance adaptive immunity and wound healing and alter antibody production. All mechanisms to explain multiple functions of AMPs are not clearly understood. Prior studies to localize defensins in normal and burned skin using deconvolution fluorescence scanning microscopy indicate localization of defensins in the nucleus, perinuclear regions, and cytoplasm. The objective of this study is to further confirm the identification of HBD-1 in the nucleus by deconvolution microscopic studies involving image reconstruction and wire frame modeling. **Results:** Our study demonstrated the presence of intranuclear HBD-1 in keratinocytes throughout the stratum spinosum by costaining with the nuclear probe DAPI. In addition, HBD-1 sequence does show some homology with known cationic nuclear localization signal sequences. **Conclusion:** To our knowledge, this is the first report to localize HBD-1 in the nuclear region, suggesting a role for this peptide in gene expression and providing new data that may help determine mechanisms of defensin functions.

---

Antimicrobial peptides (AMPs), including both defensins and cathelicidins, are part of the innate immune system, which provides the first line of defense against a wide spectrum of common burn and wound pathogens.<sup>1-5</sup> In the past few years, multiple functions of AMPs have been identified.<sup>6-9</sup> They are chemotactic for neutrophils, monocytes, immature dendritic cells, memory T cells, and mast cells, and play a role in recruiting them. They also alter transcriptional responses in macrophages, induce degranulation of mast cells, and stimulate wound vascularization and re-epithelialization in wound healing, as well as altering antibody production, thus linking innate and adaptive immunity. In addition, LL-37, a human cathelicidin, possesses the ability to bind and neutralize gram-negative

lipopolysaccharide (LPS), decrease nitric oxide/inducible nitric oxide synthase (NO/iNOS) production, and reduce septic shock.<sup>10</sup> The mechanisms by which AMPs are able to exhibit such multiple functions are not fully understood.

Human defensins are cationic antimicrobial peptides with molecular weights of 4 to 5 kDa, containing a conserved motif of 6 cysteines linked by 3 disulfide bonds. On the basis of their size and pattern of disulfide bonding, human defensins are classified into  $\alpha$  and  $\beta$  categories, and are expressed in various cell types.<sup>11–20</sup> Out of 6  $\alpha$ -defensins, human neutrophils express 4 of them, viz, human neutrophil peptides (HNP1–4), whereas the other 2  $\alpha$ -defensins (HD-5 and HD-6) are abundantly expressed in Paneth cells of the small intestine and epithelial cells of the female urogenital tract. The human  $\beta$ -defensins (HBDs; HBD-1–4) are widely expressed in epithelial cells of multiple organs including skin. The  $\beta$ -defensins are less abundant, and their production by epithelial cells is subject to regulation by a variety of inflammatory and microbial stimuli.<sup>11–20</sup>

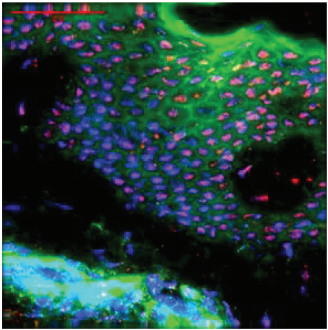
In our previous work, we have localized AMPs in burned and normal skin to specific cell types and areas of the epidermis and dermis, using deconvolution fluorescence microscopy.<sup>21,22</sup> During these current studies, we observed evidence of an intranuclear localization of HBD-1. A recent study conducted by Bandholtz et al<sup>23</sup> demonstrated uptake of LL-37 by monocyte-derived dendritic cells and subsequent localization to both cytoplasmic and nuclear elements. Also, LL-37 was shown to mediate uptake of DNA plasmids into cytoplasmic and nuclear location of mammalian cells.<sup>24</sup> Since LL-37 and HBD-1 share some similar properties, we have extended our work in the current study to focus on nuclear localizations of HBD-1 in normal skin. This new finding may have implications in gene expression and shed light on the multiple roles of defensins.

## MATERIALS AND METHODS

Patients admitted to the Regional Burn Center at Memorial Medical Center, Springfield, IL, or the Johns Hopkins Burn Center with 10% to 40% total body surface area burns were included in the study. Normal skin samples were obtained from remnants of split thickness skin autografts harvested during skin grafting procedures and were frozen in sucrose-based embedding media (O.C.T. Compound Tissue-Tek, Torrance, Calif). Ten-micrometer sections were cut in a microtome, floated onto 18-mm glass cover slips that had been coated with poly-L-lysine, and fixed with 3.7% paraformaldehyde. This was followed by 60-min incubation in goat serum and staining with appropriate probes and antibodies as previously described.<sup>22</sup>

### Deconvolution Microscopy

The stained sections were placed under Elvanol (an antifade, DuPont), and covered with a cover slip. The samples were scanned with an Applied Precision Delta Vision Scanning Microscope (Issaquah, Wash), fitted with an Olympus 1×70 microscope (Olympus America, Melville, NY). Image scans were acquired in a series at a step-size thickness of 0.2  $\mu$ m by a Photometries (Tucson, Ariz) PXL CCD camera. Image analysis was performed by transferring each data set to a Silicon Graphics workstation using SoftWoRx™ software (Applied Precision, Issaquah, Wash). All data sets were subjected to 5 deconvolution iterations and



**Figure 1.** Localization of HBD-1 in normal skin by deconvolution fluorescence microscopy. HBD-1 was found homogeneous throughout the epidermis, in the nuclei of keratinocytes. (magnification bar = 50  $\mu\text{m}$ ; blue = DAPI (nuclei), green = actin, and red = texas red tagged peptide antibody.)

subsequently used for image reconstructions and modeling. Stacking each of the individual sections produces a 3-dimensional (3D) image on a 2-dimensional background, resulting in an image projection.<sup>22</sup> Wire framing of the images is carried out as described previously to identify distinct colors of defined wavelength patterns for HBD-1 and DAPI.<sup>25</sup>

## RESULTS

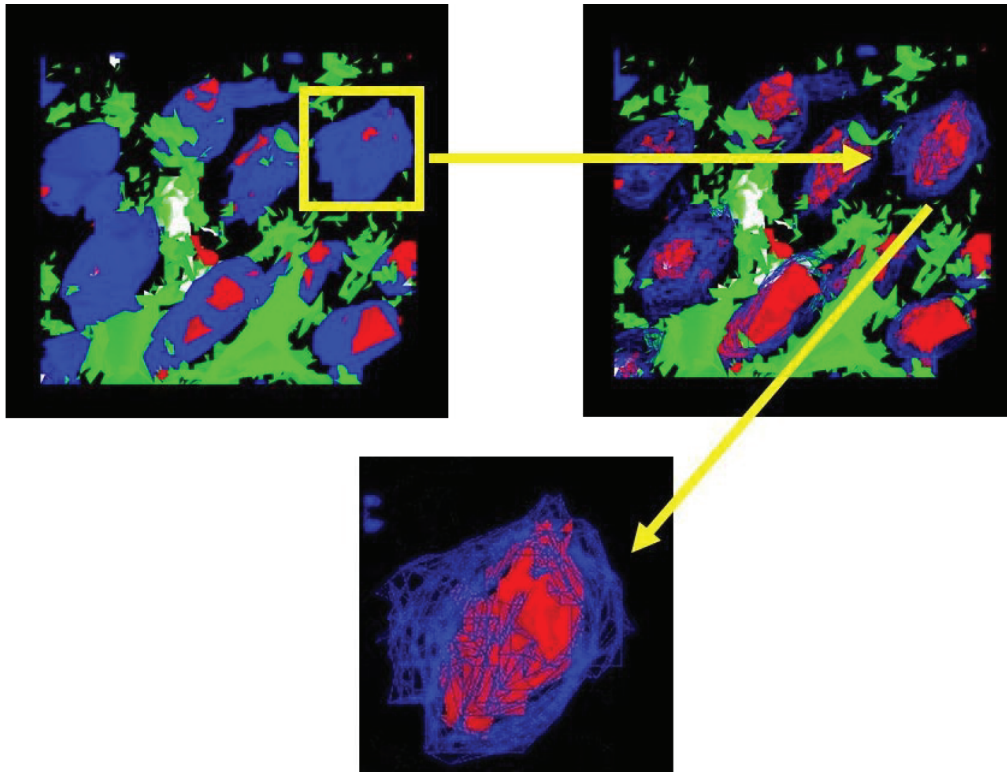
The Figure 1 illustrates localization of HBD-1 in normal skin in which its presence was found homogeneously throughout the epidermis, contained in keratinocytes. By using the fluorescent nuclear probe DAPI, we noticed that HBD-1 appeared to be colocalized with the nuclei so; to further characterize this apparent nuclear, we stacked multiple deconvoluted (5 iterations) to generate an image representing some thickness of skin, and then modeled these stacked renditions to produce a 3D model that permitted rotation (Fig 2).

The extraction of a number of cells and nuclei from these 3D models and the application of fish-net mapping permitted us to visualize specific probes as distinct areas with no color-mixing (colocalization) and single color/channel imaging. It is apparent (Fig 2) that the red HBD-1 is within the nucleus, being encompassed by the blue of the DAPI. This finding is further confirmed in Figure 3, using a wire frame procedure, splitting the images into the 2-component probe channels, and applying color assignments.

We attempted to detect nuclear targeting sequences in HBD-1. This was achieved using a database search engine (<http://walnut.bioc.columbia.edu/srs71bin/cgi-bin/wgetz>). HBD-1 show some homology with the nuclear targeting sequences (KRX(10-12)KRRK) of the known nuclear translocation proteins, monopartite and bipartite (Figure 4). This may indicate that defensins may be transported into the nucleus, possibly through the nuclear pore.

## DISCUSSION

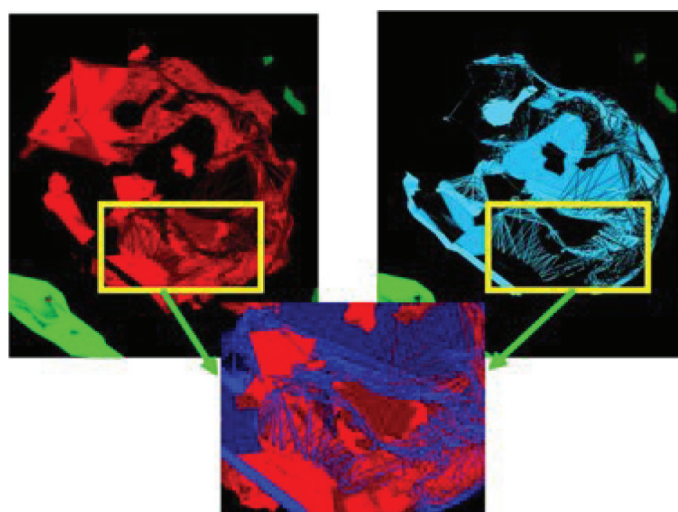
This study demonstrates nuclear localization of HBD-1. Considering that both defensins and cathelicidins display multiple functions, we hope that current findings may help explain mechanisms for some of these functions. AMPs are able to differentially recognize and disrupt negatively charged bacterial cell membranes,<sup>1-4</sup> while also being able to recruit many components of the innate and adaptive immune systems such as macrophages, neutrophils,



**Figure 2.** Deconvoluted, 3-dimensional rendition of multiple-stacked acquisitions of HBD-1 in the epidermis. A nucleus from the volume rendered image is then extracted and wire framed, allowing visualization of the HBD-1 within the nucleus, shown at a high magnification in the final panel (magnification  $\times 4000$ ).

antigen presenting dendritic cells, NK-cells, and T- and B-cells, by chemo attraction.<sup>6-8</sup> It has been shown that the mechanisms of chemo-attraction and direct killing are independent of each other,<sup>9</sup> the upregulation or release of AMPs by other cells at the site of injury helping recruit other innate immune components, while some components of both innate and adaptive immunity also produce defensins and cathelicidins. AMPs are implicated in wound healing, angiogenesis, gene expression, cell differentiation and migration, antigen presentation, and antibody production. AMPs affect intracellular signaling by diverse kinase pathways, alter chemokine and chemokine receptor gene expression, and also mediate and modulate cytokine release.<sup>26-33</sup> Even though some of the mechanisms for these multiple functions have already been defined, we still need to know the other pathways involved in the actions of AMPs, and future studies may reveal these mechanisms, particularly as defensins have been shown to alter the expression of cytokines through an, as yet, undefined pathway.

Currently, there are 2 AMPs which have been localized to the nucleus, this study localizing HBD-1 in the nucleus of keratinocytes in skin, and the recent study by Bandholtz et al,<sup>23</sup> localizing the LL-37 to the nuclei of monocyte-derived dendritic cells (MDDC). LL-37 also promotes nuclear transport of plasmid DNA in mammalian cells,<sup>24</sup> and so taking



**Figure 3.** Three-dimensional volume rendition to demonstrate the 2-color channels (DAPI and Texas Red), and show intranuclear localization of HBD-1. The blue of the nucleus overlying the red of the antimicrobial peptide further confirms the intranuclear localization of HBD-1.

these findings together, it is reasonable to hypothesize that AMPs enter the nucleus, possibly playing a role in the modulation of gene expression and/or protein synthesis.

The mechanism by which AMPs enter the nucleus has not been studied and is subject to speculation, although several possibilities exist. In eukaryotic cells, signal-mediated macromolecular transport between the nucleus and the cytoplasm is an integral part of many processes, such as gene expression, signal transduction, and cell-cycle progression. Even though molecules can enter the nucleus of actively dividing cells through nuclear membrane breaks during mitosis, the only portal of entry into the nucleus of nondividing cells is the nuclear pore complex (NPC). NPCs allow passive exchange of ions, small molecules, and small proteins (<20 kDa), but restrict the passage of macromolecules to only those bearing appropriate nuclear localizing signal (NLS). Considering the small size of AMPs (4–5 kDa for defensins), the question arises as to whether NPCs do allow the passive transport of AMPs into the nucleus.



**Figure 4.** The sequences of HBD-1 is compared with nuclear targeting sequences of monopartite and bipartite proteins which is known to contain reported nuclear targeting sequence (KRx(10-12)KRRK). Yellow color indicates homology.

Another mechanism by which AMPs could enter the nucleus is based on AMP cationic sequence, a sequence that could constitute a nuclear localization and recognition signal and promote nuclear transportation. The directional transport of thousands of proteins and RNAs through the NPC in the course of normal cellular metabolism is mediated by a family of more than 20 proteins, the karyopherins.<sup>34–37</sup> The import of proteins carrying a cationic NLS involves an adaptor protein karyopherin- $\alpha$  (Kap $\alpha$ ), which in turn binds to karyopherin- $\beta$  and interacts with the NPC for translocation into the nucleus. Karyopherin- $\beta$  can also directly bind proteins for nuclear import. The sequence motifs for direct karyopherin- $\beta$  translocation are not well defined, and are not necessarily cationic. HBD-1 shares some homology with known nuclear translocation signals of cationic monopartite and bipartite molecules. Kap $\alpha$  recognizes a variety of classical NLSs, such as the basic monopartite SV40 T antigen NLS (PKKKRKV), the more hydrophobic monopartite c-Myc NLS (PAAKRVKLD) and the bipartite nucleoplasmin NLS (VKRPAATKKAGQAKKKKLD).<sup>38</sup> Although lacking a specific consensus sequence, the classical NLSs contain either 1 or 2 clusters of basic residues.

Polymers of basic amino acids [i.e poly (Lys)<sub>16</sub> and poly (Arg)<sub>16</sub>] promote translocation of plasmid DNA into the nucleus. Many cationic peptides, other than those known to function as nuclear localizing motifs, appear to have a nuclear translocation ability regarding DNA plasmids. The ability of LL-37 to deliver plasmid DNA into mammalian cells has been demonstrated.<sup>24</sup> The physiologically relevant concentrations of LL-37, widely expressed in bone marrow and epithelial cells, protects plasmid DNA against serum nuclease degradation and efficiently targets DNA to the nuclear compartment of mammalian cells. Furthermore, mechanistic data indicate that LL-37-DNA complexes enter mammalian cells via endocytosis that involves noncaveolar lipid raft domains, as well as cell surface peptidoglycon.<sup>24</sup> Currently, however, there have been no data demonstrating the ability of HBD-1 and other defensins to deliver plasmid DNAs.

One possible functional importance of nuclear localization of AMPs may be coupled to affecting gene expression.<sup>26</sup> Even though there is no direct link that HBD-1 participates in gene expression, sufficient evidence exists to demonstrate that other AMPs, for example, HBD 2-4 and LL-37, alter gene expression. It has now been clearly demonstrated that AMPs alter antibody production in response to exposure to antigen.<sup>6–8</sup> LL-37 triggers a transformation from monocytes to MDDC, which involves alteration in gene expression. It has also been reported that immature MDDCs (iMDDC) take up LL-37, which is predominantly localized not only to cytoplasmic locales but also to some extent to the nucleus. LL-37 also alters MDDC phenotype with increased expression of the antigen-presenting molecule HLA-DR and the costimulatory molecule CD86.<sup>34</sup> MDDCs is known to express HBD-1. As it is known that AMPs acts as an adjuvant, it is quite reasonable to hypothesize that AMPs could influence gene expression responsible for overall alteration of antibody patterns. It is also interesting to note that B-cells are a site of defensin production and are chemo attracted by AMPs.<sup>35</sup> Previous suggestions have been made that the action of AMP may be similar to that of lactoferrin, that has been shown to undergo translocation into lymphocyte nuclei, in order to bind DNA and activate transcription.<sup>37,41</sup> In addition, these AMPs could alter gene expression indirectly, without getting translocated to nucleus by other mechanisms involving cell surface receptor binding.

In summary, our observation, when taken together with other previously reported findings of the actions and mechanisms of AMPs, suggest possibilities for new roles for

AMPs in the modulation of gene expression and/or protein synthesis. Further studies are required to elucidate these possibilities.

## REFERENCES

1. Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. *Clin Microbiol Rev*. 2006;19:491–511.
2. Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol*. 2003;3:710–720.
3. Pazgier M, Hoover DM, Yang D, Lu W, Lubkowski J. Human beta-defensins. *Cell Mol Life Sci*. 2006;63:1294–1313.
4. Hancock RE, Lehrer R. Cationic peptides: a new source of antibiotics. *Trends Biotechnol*. 1998;16:82–88.
5. Niyonsaba F, Ogawa H. Protective roles of the skin against infection: implication of naturally occurring human antimicrobial agents beta-defensins, cathelicidin LL-37 and lysozyme. *J Dermatol Sci*. 2005;40:157–168.
6. Bowdish DM, Davidson DJ, Hancock RE. Immunomodulatory properties of defensins and cathelicidins. *Curr Top Microbiol Immunol*. 2006;306:27–66.
7. Yang D, Biragyn A, Kwak LW, Oppenheim JJ. Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol*. 2002;23:291–296.
8. Yang D, Chertov O, Oppenheim JJ. The role of mammalian antimicrobial peptides and proteins in awakening of innate host defenses and adaptive immunity. *Cell Mol Life Sci*. 2001;58:978–989.
9. Kluver E, Adermann K, Schulz A. Synthesis and structure-activity relationship of beta-defensins, multifunctional peptides of the immune system. *J Pept Sci*. 2006;12:243–257.
10. Ciornei CD, Egesten A, Bodelsson M. Effects of human cathelicidin antimicrobial peptide LL-37 on lipopolysaccharide-induced nitric oxide release from rat aorta in vitro. *Acta Anaesthesiol Scand*. 2003;47:213–220.
11. Ganz T, Lehrer RI. Antimicrobial peptides of vertebrates. *Curr Opin Immunol*. 1998;10:41–44.
12. Quayle AJ, Porter EM, Nussbaum AA, et al. Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *Am J Pathol*. 1998;152:1247–1258.
13. Jones DE, Bevins CL. Paneth cells of the human small intestine express an antimicrobial peptide gene. *J Biol Chem*. 1992;267:23216–23225.
14. Selsted ME, Miller SI, Henschen AH, Ouellette AJ. Enteric defensins: antibiotic peptide components of intestinal host defense. *J Cell Biol*. 1992;118:929–936.
15. O’Neil DA, Cole SP, Martin-Porter E, et al. Regulation of human beta-defensins by gastric epithelial cells in response to infection with *Helicobacter pylori* or stimulation with interleukin-1. *Infect Immun*. 2000;68:5412–5415.
16. Van Wetering S, Sterk PJ, Rabe KF, Hiemstra PS. Defensins: key players or bystanders in infection, injury, and repair in the lung? *J Allergy Clin Immunol*. 1999;104:1131–1138.
17. Wada A, Mori N, Oishi K, et al. Induction of human beta-defensin-2 mRNA expression by *Helicobacter pylori* in human gastric cell line MKN45 cells on cag pathogenicity island. *Biochem Biophys Res Commun*. 1999;263:770–774.
18. Wada A, Ogushi K, Kimura T, et al. *Helicobacter pylori*-mediated transcriptional regulation of the human beta-defensin 2 gene requires NF-kappaB. *Cell Microbiol*. 2001;3:115–123.
19. Hamanaka Y, Nakashima M, Wada A, et al. Expression of human beta-defensin 2 (hBD-2) in *Helicobacter pylori* induced gastritis: antibacterial effect of hBD-2 against *Helicobacter pylori*. *Gut*. 2001;49:481–487.
20. Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human beta-defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem*. 2001;276:5707–5713.
21. Poindexter BJ, Bhat S, Buja LM, Bick RJ, Milner SM. Localization of antimicrobial peptides in normal and burned skin. *Burns*. 2006;32:402–407.
22. Milner SM, Bhat S, Buja M, Gulati S, Poindexter BJ, Bick RJ. Expression of human beta defensin 2 in thermal injury. *Burns*. 2004;30:649–654.
23. Bandholtz L, Ekman GJ, Vilhelmsson M, et al. Antimicrobial peptide LL-37 internalized by immature human dendritic cells alters their phenotype. *Scand J Immunol*. 2006;63:410–419.

24. Sandgren S, Witttrup A, Cheng F, et al. The human antimicrobial peptide LL-37 transfers extracellular DNA plasmid to the nuclear compartment of mammalian cells via lipid rafts and proteoglycan-dependent endocytosis. *J Biol Chem.* 2004;279:17951–17956.
25. Merritt TM, Bick R, Poindexter BJ, Hecht JT. Unique matrix structure in the rER cisternae of pseudoachondroplasia chondrocytes. *Amer J Pathol.* 2007;170:293–300.
26. Scott MG, Davidson DJ, Gold MR, Bowdish D, Hancock RE. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J Immunol.* 2002;169:3883–3891.
27. Niyonsaba F, Ushio H, Nakano N, et al. Antimicrobial peptides human beta-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. *J Invest Dermatol.* 2007;127:594–604.
28. Davidson DJ, Currie AJ, Reid GS, et al. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. *J Immunol.* 2004;172:1146–1156.
29. Agerberth B, Charo J, Werr J, et al. The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. *Blood.* 2000;96:3086–3093.
30. Yang D, Chen Q, Schmidt AP, et al. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med.* 2000;192:1069–1074.
31. Tjabringa GS, Aarbiou J, Ninaber DK, et al. The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. *J Immunol.* 2003;171:6690–6696.
32. Bowdish DM, Davidson DJ, Speert DP, Hancock RE. The human cationic peptide LL-37 induces activation of the extracellular signal-regulated kinase and p38 kinase pathways in primary human monocytes. *J Immunol.* 2004;172:3758–3765.
33. Niyonsaba F, Iwabuchi K, Someya A, et al. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology.* 2002;106:20–26.
34. Richard JP, Melikov K, Vives E, et al. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J Biol Chem.* 2003;278:585–590.
35. Pemberton LF, Paschal BM. Mechanisms of receptor-mediated nuclear import and nuclear export. *Traffic.* 2005;6:187–198.
36. Fabre JW, Collins L. Synthetic peptides as non-viral DNA vectors. *Curr Gene Ther.* 2006;6:459–480.
37. Penco S, Scarfi S, Giovine M, et al. Identification of an import signal for, and the nuclear localization of, human lactoferrin. *Biotechnol Appl Biochem.* 2001;34:151–159.
38. Son KN, Park J, Chung CK, et al. Human lactoferrin activates transcription of IL-1beta gene in mammalian cells. *Biochem Biophys Res Commun.* 2002;290:236–241.
39. Masjedi K, Jakobson E, Boman HG, Karlberg AT, Scheynius A. The antimicrobial peptide LL-37 upregulates HLA-DR molecules on dendritic cells. *11th International Congress of Immunology. Scand J Immunol.* 2001;54(suppl 1):74.
40. Wah J, Wellek A, Frankenberger M, Unterberger P, Welsch U, Bals R. Antimicrobial peptides are present in immune and host defense cells of the human respiratory and gastrointestinal tracts. *Cell Tissue Res.* 2006;324:449–456.
41. Conti E, Kuriyan J. Crystallographic analysis of the specific yet versatile recognition of distinct nuclear localization signals by karyopherin alpha. *Structure Fold Des.* 2000;8:329–338.