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Capacity of human β-defensin expression in gene-transduced and cytokine-induced cells ☆

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Abstract

The purpose of this study was to determine the capacity of cells transduced with human β-defensins (HBDs) to express antimicrobial peptides, since sufficient expression level is required for effective antimicrobial activity. Retroviral vector pBabeNeo and lentiviral vector SIN18cPPTRhMLV (SIN18) carrying HBDs were utilized to transduce non-HBD-expressing cells such as fibroblasts or HBD-producing oral epithelial cells. We found that HBD-3 gene transfer to fibroblasts was possible not via retrovirus but by direct vector transfection. SIN18 had high transduction efficiencies (80.9–99.9%) and transduced cells expressed higher amounts of HBD-2 than those by pBabeNeo. Primary human gingival epithelial cells (HGECs) expressed greater amounts of HBD-2 than primary fibroblasts after lentiviral transduction. Additionally, HBD-2 secretion from transduced HGECs cells was further increased when stimulated with IL-1 or TNFα. Our data indicate that while HBD-2 expression is limited in primary fibroblasts, its expression in HGECs may be maximized by gene transduction plus cytokine induction.

Keywords: HBD-2; HBD-3; Lentiviral vectors; Retroviral vectors; IL-1; TNFα; Gingival epithelial cells

Natural antimicrobial peptides have been considered as an alternative option for infection control due to the increasing resistance of microbes to conventional antibiotics. Because some potent antimicrobial peptides are encoded by single genes, the possibility of artificially delivering these genes into tissues/organs to augment innate immunity against infection or creating transgenic animals to produce antimicrobial peptides has been explored [1–5]. Subsequently, the term antimicrobial gene therapy has emerged [6] and engineered skin models have been used to test the concept of antimicrobial gene therapy both in vitro and in vivo [7,8]. Human β-defensins (HBDs or DEFB) and cathelicidin LL37 are being actively used as study models for their potent ability to kill a broad spectrum of bacteria and fungi [6,7,9–13], as well as to inhibit viral infections [14,15]. HBDs are expressed mostly in epithelia [11,12,16–24]. Besides the cloned, sequenced, and well-characterized HBD-1, -2, -3, and -4, there appears to be at least 28 more HBDs existing based on the genomic surveys using computational search strategy [25–27]. Fourteen HBDs or DEFB gene transcripts (DEFB-1, -4, and DEFB-103 to -114) were investigated using reverse transcription-polymerase chain reaction (RT-PCR) to detect their expression in gingival

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keratinocytes and a selected few have been classified as constitutive or inducible [27].

HBDs are considered to play an important role in epithelial defense mechanism and the regulation of their expression in diseased conditions is being actively investigated. Bacteria and cytokines IL-1 and TNFα upregulate HBD-2 expression in lung and skin epithelial cells [10,28] and induction of HBDs by these stimuli is differentially regulated involving multiple mechanisms [29]. Expression of HBDs in oral tissues has also been extensively studied [23,30–40]. In gingival epithelium, variable expression levels of HBD-1, -2 or -3 are found in either healthy or diseased gingival tissues [41,42]. Cultured oral epithelial cells increase HBD-1 and -3 stimulated with IFNγ, while HBD-2 is induced by IL-1β and TNFα [43]. Periodontal bacteria *Fusobacterim nucleatum* and *Actinobacillus actinomycetemcomitans* also induce HBD-2 and HBD-3, respectively, in oral epithelial cells [44–46]. HBD-2 effectively kills Gram-positive bacteria in vitro such as *Staphylococcus epidermidis* and *Streptococcus agalactiae*, as well as *Streptococcus mutans* and *Lactobacillus acidophilus* that are associated with dental caries [10,47–49]. Periodontal bacteria *A. actinomycetemcomitans* and *Porphyromonas gingivalis* are also sensitive to HBD-2 [48].

Previously, we transduced a number of non-HBD-2-expressing cell lines to produce HBD-2 with the notion that these cells may become part of the innate defense mechanisms against infection [6,49]. However, it is not known whether the transgene expression level in non-HBD-2-expressing cells is comparable to or higher than that in HBD-2 naturally producing cells such as oral epithelial cells. In the context of effective antimicrobial gene therapy, it is important to know the maximal capacity of HBD production in transduced cells. In the present study, our objective was to compare the amount of HBDs produced by HBD-transduced non-HBD-expressing cells (fibroblasts) and HBD-producing cells (human gingival epithelial cells, HGECs) and to ascertain the capacity of HBD production by HBD-transduced HGECs. To achieve the goal, we first compared the effectiveness of HBD transduction of various types of human and mouse cells between a retroviral vector and an HIV-based vector before measuring the capacity of HBD production levels in cells after gene transduction as well as combining gene transduction and cytokine induction.

Materials and methods

Cell cultures

Cell lines. The following cell cultures and cell lines were utilized: PA317 (ATCC CRL-9078); NIH3T3 (ATCC CRL-1658); HT-1080, human fibrosarcoma cell line (ATCC CCL-121); HSG, human submandibular salivary gland cell line [50], from Dr. L. Bobek, SUNY, Buffalo, NY; A375, human malignant melanoma (ATCC CRL-1619), from Dr. M. Kolodney, UCLA, Los Angeles, CA; SCA-9, mouse submandibular gland cells (ATCC CRL-1734); and HOK-18A, immortalized oral epithelial cells derived from primary HGECs, provided by Dr. N.-H. Park, UCLA [51]. PA317, NIH3T3, A375, SCA-9 were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies/Gibco-BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum (FBS); HT-1080 in minimum essential medium–alpha medium with 10% FBS; HSG in DMEM/F12 (1:1 mixture, with glutaMAX I, Life Technologies) with 10% FBS; and HOK-18A in DMEM/F12 (3:1, v/v) supplemented with 10% FBS, 5 ng/ml human epidermal growth factor, 5 μg/ml bovine insulin, 0.4 μg/ml hydrocortisone, 0.1 nM choleratoxin, 5 μg/ml transferrin, and 2 nM 3,3′,5-triiodo-L-thyronine. All cell culture media contained 100 U/ml penicillin-G, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (Gemini Bio-Products, Woodland, CA).

Primary cell cultures. Primary HGECs were cultured from gingival tissue as described previously [52]. Briefly, gingival tissues were prepared by digestion with dispase/collagenase to separate the epithelial layer from the underlying mesenchyme, followed by trypsin-treatment of the epithelial sheets. The dissociated single epithelial cells were grown in keratinocyte growth medium (KGM-2 with BulletKit) supplemented with 30 μg/ml bovine pituitary extract, 0.1 ng/ml human epidermal growth factor, 5 μg/ml bovine insulin, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamicin, and 50 ng/ml amphotericin-B (Clonetics, San Diego, CA). Human gingival or pulp tissues were obtained from healthy patients receiving routine periodontal surgeries or extraction of third molars at UCLA School of Dentistry, Periodontal Clinic or Section of Oral Surgery, respectively. Pulp tissue and gingival tissue with epithelial layer removed were fragmented and placed in culture dish containing DMEM supplemented with 10% FBS. Cells outgrown from the tissue explants showing fibroblast-like phenotype were named human gingival fibroblasts (HGF) or human pulp cells (HPC), as described previously [6,49,53]. These cells were allowed to reach confluence, passed at 1:2 ratio, and passages 3–8 were used for experiments. Primary mouse skin fibroblasts (MSkF) were outgrown from explants of skin tissues of C57BL/6NTac mice (Taconic, Germantown, NY) in culture medium the same for HPC and HGF. Procedures involving the human tissue sample collection followed a protocol approved by the UCLA Medical Institutional Review Board. The experimentation and handling of mice followed a protocol approved by the UCLA Animal Research Committee.

Construction and production of viral vectors

pBabeNeo vectors. The retroviral vector pBabeNeoHBD-2 was constructed previously [6] and transfected into the packaging cells PA317 using a lipofectin method. The supernatant of one stably transfected clone PA317-HBD-2 was used to infect target cells. In parallel, pBabeNeo plasmid [54] alone was used as a control to produce PA317-pBabe and the supernatant. HBD-3 cDNA previously cloned into a baculoviral vector (from Dr. T. Ganz, UCLA, Los Angeles, CA) was released with *Kpn*I and *Eco*RI restriction enzymes and inserted into the *Bam*HI and *Eco*RI multiple cloning sites of pBabeNeo yielding pBabeNeoHBD-3 (Fig. 1A). The *Kpn*I end of the released HBD-3 cDNA and the *Bam*HI site of the plasmid were blunt-ended to allow insertion. These vectors are abbreviated as pBabe-HBD-2 or -3 hereafter. PA317-pBabe or PA317-HBD-2/-3 cells were grown to confluence. Medium was refreshed one day after the confluence. One to two days later, the supernatant was collected and used to infect the target cells.

Lentiviral vectors. A lentiviral vector SIN18cPPTRhMLV-E (abbreviated as SIN18-E hereafter), the packaging plasmid pCMVR8.2DVPR [55,56], and the vesicular stomatitis virus G protein expression plasmid (pHCMV-G) [57] were obtained from Dr. I. S.Y. Chen, UCLA, Los Angeles [58]. The SIN18-E contains the enhanced green fluorescent protein (EGFP) reporter cDNA which was released with *Bam*HI and *Sal*I restriction enzyme digestion and inserted with HBD-2 cDNA into the same sites yielding SIN18cPPTRhMLV-HBD-2 (abbr. as SIN18-HBD-2 hereafter) (Fig. 1B).

Packaging cells 293T (2×10^7) were co-transfected with 12.5 μg SIN18-HBD-2 or SIN18-E, 12.5 μg pCMVR8.2DVPR, and 5 μg pHCMV-G plasmids using a calcium phosphate precipitation method [59]. The viral supernatant was collected on days 2 and 3 after transfection, filtered (0.45 μm), and concentrated 100-fold by ultracentrifugation as described

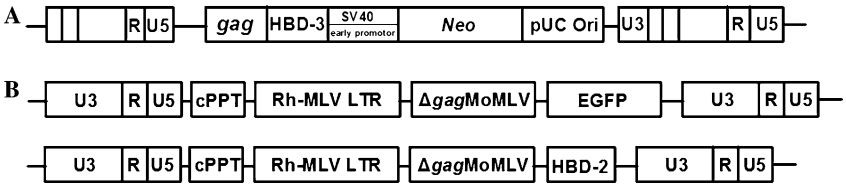

Fig. 1. Constructs of (A) pBabeNeo-HBD-3 (abbr., pBabe-HBD-3) and (B) lentiviral vector SIN18cPPTRhMLV-HBD-2 from SIN18cPPTRhMLV-E (abbr., SIN18-HBD-2 and SIN18-E).

[58]. Viral concentration was measured against known concentrations of viral protein p24 and virus titration was performed using 293T cells with various dilutions of the viral stock and analyzed for EGFP expression by fluorescence microscopy and flow cytometry on day 3 postinfection. The titers of vectors were routinely 10^8 infectious units/ml. Expression of HBD-2 was analyzed by ELISA described below.

Infection of target cells

pBabeNeo vectors. The infection procedures were described previously [6]. In brief, supernatants from the packaging cells were added into target cell cultures in the presence of 8 μg/ml polybrene (Sigma, St. Louis, MO) for 3 h followed by adding more medium to dilute the polybrene to 2 μg/ml and the culture was incubated for 3 days. After which, cells were split into selection conditions using G418 and 7–10 days later selected cells were pooled and subcultured in 12-well plates. Expression of HBD-2/-3 was detected in the supernatants by ELISA or immune dot blot; or detected in cells by Northern blot or RT-PCR described below.

SIN18 vectors. Produced virus from the packaging cell line 293T was used to infect HT-1080, A-375, HPC, HGF, HGECs. HSG, or SCA-9 cells. Cells (5×10^5) were exposed to 400 ng of virus at 37 °C in the presence of 5 μg/ml polybrene. After overnight incubation, cell cultures were replaced with fresh medium and incubated for 3 days. Expression of EGFP (transduced by SIN18-E) was analyzed by fluorescence microscopy and secreted HBD-2 was detected by ELISA.

Reverse transcription-polymerase chain reaction

To detect HBD-2/-3 expression, RT-for-PCR kit from Clontech laboratories (Palo Alto, CA) was used to synthesize cDNA from 1 μg of total RNA isolated from cells. Appropriate amounts of the cDNAs, specific primers for HBD-2/-3, and *pfu* DNA polymerase (Stratagene, La Jolla, CA) were then used for PCR: amplification was performed at 95 °C for 1 min followed by 35 cycles of 94 °C for 45 s, 55 °C for 30 s, 72 °C for 1.5 min, and 72 °C for 7 min as the final step. The sequences of the primer set for HBD-2 were: 5′-CCAGCCATCAGCCATGAGGGT-3′ (sense), 5′-GGAGCCCT TTCTGAATCCGCA-3′ (antisense) with an expected targeting size of 255 bp; for HBD-3 were: 5′-CTTCTGTTTGCTTTGCTCTTCC-3′ (sense), 5′-CACTTGCCGATCTGTTCCTC-3′ (antisense) with an expected targeting size of 149 bp; PCR products were size fractionated on a 1% agarose gel for visualization. The quality of the RNA was first determined in agarose gels and by RT-PCR using human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers along with human placenta RNA provided in the kit as controls before HBD-2/-3 RT-PCR was performed.

Immune dot blot analysis

Experimental procedures followed protocols described previously [6,29]. In brief, supernatants were purified with Macro-Prep CM support system (Bio-Rad Laboratories, Hercules, CA) and further purified/desalted with ZipTipC18 chromatography system (Millipore, Bedford, MA). Purified samples were dotted onto a nitrocellulose membrane and fixed in 0.05% glutaraldehyde in Tris-buffered saline (TBS) for 30 min followed by blocking with 0.75% skimmed milk in phosphate-buffered saline (PBS). The blots were then incubated overnight with the primary antibody rabbit anti-human HBD-3, washed, and incubated with alkaline phosphatase-conjugated polyclonal goat anti-rabbit secondary antibodies (Pierce, Rockford, IL). After wash, blots were visualized with a developing solution containing nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) as a chromogenic substrate.

Northern blot analysis

Cellular RNA was isolated using TRIzol (Invitrogen/Life Technologies, Carlsbad, CA). Ten micrograms of total RNA was size fractionated on 1.5% formaldehyde-agarose gels, transferred to Hybond nylon membranes (Amersham Pharmacia Biotech, UK), and probed with a ^{32}P-labeled cDNA fragment specific for HBD-2. A ^{32}P-labeled cDNA fragment of GAPDH was used as the control probe to verify an equal amount of RNA loaded per lane for each analysis. Signals were visualized by a PhosphorImager System (Molecular Dynamics, Sunnyvale CA) and images of specific bands were quantitated by an ImageQuant software program (Molecular Dynamics). The GAPDH message level was used as reference for adjusting levels of HBD-2 message.

mRNA half-life determination

RNA from HT-1080 transduced with pBabe-HBD-2 and SIN18-HBD-2 were treated with Actinomycin D (ActD, 10 μg/ml) to block transcription. At different time points following ActD treatment, RNA was isolated and subjected to Northern blot analysis. The signals were visualized by autoradiography as described above. The half-lives of HBD-2 transcripts were measured by comparison with the mRNA level at time 0 (100%) as described [61].

Enzyme-linked immunosorbent assay

The amount of HBD-2 secreted into the supernatant was determined by enzyme-linked immunosorbent assay (ELISA) as described previously [6] using monoclonal anti-human HBD-2 antibodies (1:5000 dilution) as anchoring antibodies, polyclonal rabbit anti-human HBD-2 antibodies (1:2000 dilution) as detecting antibodies, and horseradish peroxidase (HRPO)-labeled polyclonal goat anti-rabbit immuno-globulin G (Pierce, Rockford, IL) as a second-step antibody. Bound HRPO was visualized with fresh developing buffer containing substrate of optimal concentrations of *o*-phenylenediamine dihydrochloride (OPD, Sigma), 0.01% H_2O_2, and 20 mM sodium citrate, pH 4.0 [60]. The developing reaction was stopped with the addition of 2.5 M sulfuric acid. Absorbance was determined at 490 nm with a microplate reader (Bio-Tek Instrument, Laguna Hills, CA) and the concentrations were determined with Delta Soft III software. HPLC-purified recombinant HBD-2 produced from baculovirus-transfected insect cells was used as a standard. All the antibodies and recombinant HBD-2 were kindly provided by Dr. T. Ganz (UCLA).

Immunohistochemistry

Cells cultured in chamber slides (Nalge Nunc Int., IL) or in 48-well plates were fixed in 3.7% formaldehyde in PBS for 10 min. Permeabilized cells were washed with TBS for 5 min and incubated with rabbit-anti-HBD-2 primary antibody (1:1000 dilution). After overnight incubation, the specimens were washed with TBS/0.05% Tween 20 twice for 20 min each and incubated overnight with 1:2000 dilution of AP-conjugated goat

anti-rabbit IgG (Pierce). The specimens were then developed in Fast Red TR/Naphthol AS-MX with levamisole (Sigma) followed by counter-staining in Harris hematoxylin for 30 s and stopped by running through tap water. Slides were air-dried and coverslipped with crystal mount. All steps were performed at room temperature. TBS washes were made of 500 mM NaCl and 20 mM Tris–HCl, pH 7.5. Antibody dilution buffers included the addition of 1% gelatin (Sigma), 0.05% Tween 20, and 0.01% thimerosal.

Results

Gene transfer to cultured cells with pBabe-HBD-3 retroviral vectors

Previously, we transduced cultured human and mouse cells of various cell types using pBabe-HBD-2 vectors [6,49]. In the present study, we first used pBabe-HBD-3 to transduce cultured cells with the same approach used for HBD-2 transduction. Stable PA317 transfectants were obtained by G418 selection. Expression of HBD-3 mRNA was verified by RT-PCR. The conditioned medium of one clone of PA317 cells bearing HBD-3 was used to infect NIH 3T3, HT-1080, SCA-9, and primary HPC, followed by G418 selection. Selected target cells were tested for HBD-3 expression using RT-PCR and the supernatants of HBD-3 mRNA positive cells were concentrated/desalted for immune dot blot analysis. The supernatant of PA317-HBD-3 did not transduce any of the target cells used. Lipofectin transfection of NIH 3T3 and HT-1080 cells with pBabe-HBD-3 vectors was then performed and followed by G418 treatment. Only HT-1080 cells were stably transfected by the Lipofectin method. The supernatants from HT-1080 contained secreted HBD-3 approximately 100 ng/5×10^7 cells/2 days using a semi-quantitative analysis by comparing to the known concentrations of standard HBD-3 (Fig. 2) (note: the potential loss of HBD-3 during concentration/desalting was not assessed). This is the first evidence showing the production of HBD-3 in non-HBD-3-expressing cells after transfection.

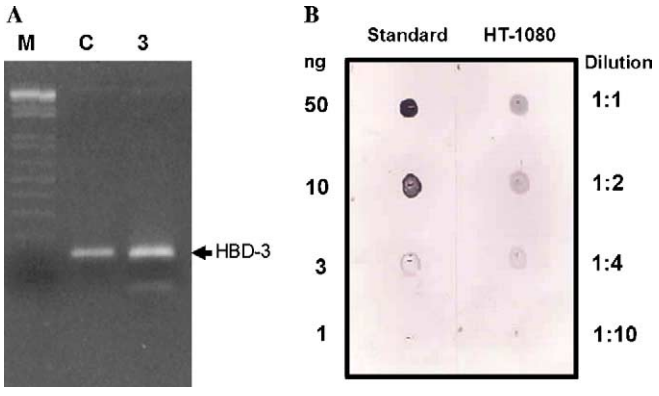

Fig. 2. HBD-3 expression in HT-1080 transfected with pBabe-HBD-3. (A) RT-PCR detection of HBD-3 mRNA of an HT-1080 clone #3. M, 1 kb DNA ladder; C, positive control plasmid containing an HBD-3 insert. (B) Immune dot blot analysis of secreted HBD-3 peptides. Cells were grown to confluence and fresh medium was added to allow accumulation of HBD-3 for 2 days before collection of supernatant for desalting/condensation and dot blot analysis. Standard, synthetic HBD-3 of known amounts.

Transduction of cells with lentiviral vectors carrying EGFP

Retroviral vector pBabeNeo did not provide effective transduction (<10%) and required selection to attain sufficient number of cells expressing the transgenes. Lentiviral vectors, in contrast, yielded higher transduction rates of 80.9–99.9% as presented in Fig. 3A showing the mean fluorescence intensity (MFI) of cells transduced with SIN18-E measured by flow cytometry analysis. Fig. 3B shows the images of EGFP expression by these transduced cells 3 days after the viral infection. Among the cells transduced, we observed that, while the transduction rates were similar between human and mouse cells, human cells appeared to have a higher transgene expression levels than mouse cells except HSG (human salivary gland cells) which had low expression levels similar to the mouse counter part–submandibular gland cells SCA-9. However, among multiple independent experiments EGFP expression was on average lower in SCA-9 than in HSG cells as indicated in Fig. 3B. Similar results were obtained using concentrated virus to transduce above-mentioned cells (data not shown). Additionally, repeated infection with the virus of those cells having low-level transgene expression did not result in noticeable increase of expression.

HT-1080 transduced by SIN18-HBD-2 expressed higher HBD-2 levels than by pBabe-HBD-2

While lentiviral vectors had a much higher transduction rate than the retroviral pBabeNeo vectors, it is not known whether the transgene expression level is also higher. HT-1080 cells were transduced by pBabe-HBD-2 and selected by G418, or by SIN18-HBD-2, before harvested for Northern blot analysis. As shown in Fig. 4A, SIN18-HBD-2 transduced HT-1080 cells expressed approximately 9- to 16-fold higher levels of HBD-2 mRNA than those transduced with pBabe-HBD-2. We then compared HBD-2 mRNA half-lives between HT-1080 cells transduced by SIN18-HBD-2 and those by pBabe-HBD-2. As shown in Fig. 4B, there is approximately 2.2-fold greater in mRNA half-life of HT-1080 cells transduced by SIN18-HBD-2 than by pBabe-HBD-2. Supernatants from non-transduced and the SIN18-HBD-2 transduced HT-1080 cells were collected and analyzed by ELISA. The relative levels of secreted HBD-2 from transduced cells indicated in Fig. 4C were in parallel with those message levels.

Secretion of HBD-2 from HGECs transduced with SIN18-HBD-2

Our previous findings showed that pBabe-HBD-2 is able to transduce various cell types [6,49], however, among those tested, only fibroblast type of cells secreted consider-

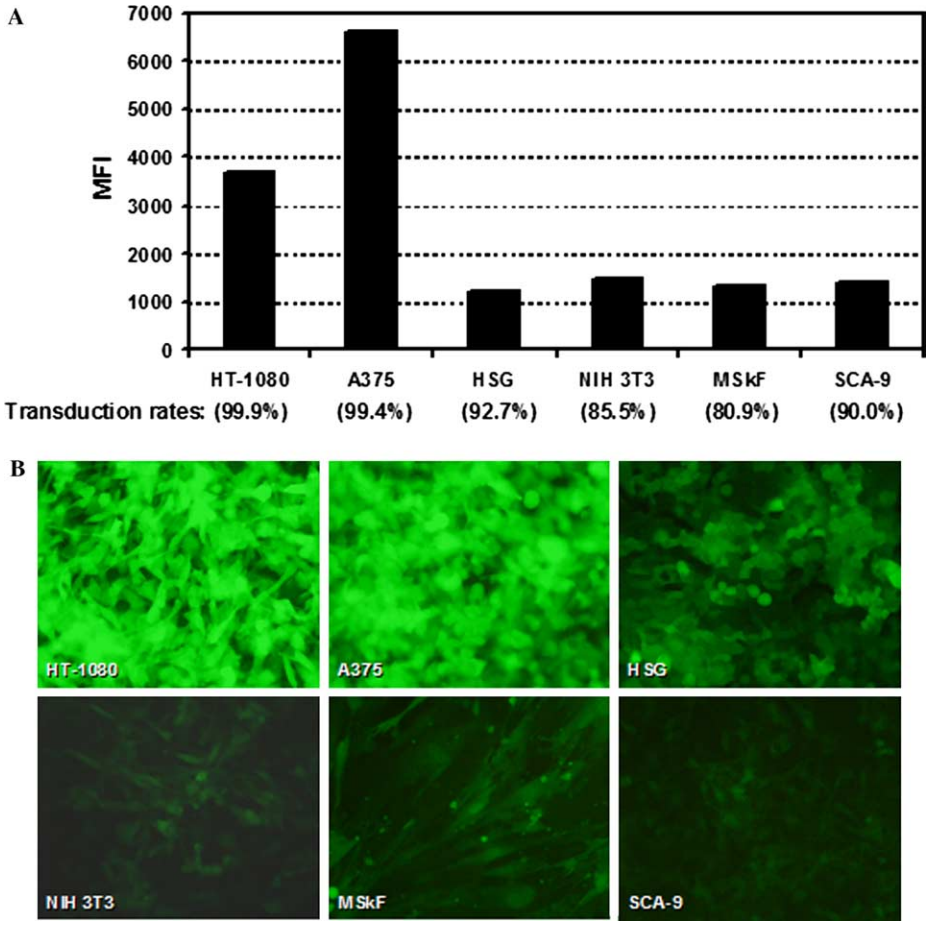

Fig. 3. EGFP expression of cells in vitro transduced by SIN18-E. (A) Relative EGFP expression in various cells from an independent experiment. MFI, mean fluorescence intensity. The transduction rate of each cell line or primary cells is indicated in the bottom of the diagram in parentheses. Non-transduced control cells not presented here all had very low relative MFIs. (B) Representative images of fluorescence microscopy of EGFP gene expression in cell lines transduced with the lentiviral vectors. Same amount of virus was added to each culture (original magnification, 200×).

able amount of HBD-2. Lentiviral vector, while demonstrated superior transduction efficiency and appeared to allow higher levels of transgene expression based on the findings above, it is not known whether gingival epithelial cells secrete a higher amount of HBD-2 than fibroblasts after transduction because HBD-2 in nature is expressed inducibly in skin and oral epithelial cells. We first tested the HBD-2 expression of several selected non-HBD-2-expressing cell lines and primary HPC before and after transduction. Fig. 5A demonstrates the below-detection levels of HBD-2 in non-transduced cells vs a clear HBD-2 expression after SIN18-HBD-2 transduction.

Primary HGECs and the gingival epithelial cell line HOK-18A, along with HSG, SCA-9, and primary HGF and HPC were transduced with SIN18-HBD-2. As presented in Fig. 5B, gingival epithelial cells, especially HOK-18A, secreted higher amounts of HBD-2 than other cells except HT-1080. Among primary cells, HGECs produced higher amounts of HBD-2 than HGF and HPC. Minimal secretion of HBD-2 was detected in transduced HSG and SCA-9 cells, which suggests that salivary gland cells, although secretory cells, do not express sufficient amount of HBD-2 after transduction.

Pro-inflammatory cytokines further induced HBD-2 secretion by HGECs transduced with SIN18-HBD-2

Since HBD-2 is induced in skin and gingival epithelial cells by cytokines IL-1 and TNFα [10,43], we then asked if stimulation of the HBD-2-transduced HGECs with cytokines can further increase HBD-2 production. First we determined whether HBD-2-transduced epithelial cells secrete comparable amount of HBD-2 to cytokine-induced epithelial cells. The results demonstrated in Fig. 6 show that cytokine-induced HGECs secreted higher or similar amount of HBD-2 to transduced but not induced cells. To determine whether HBD-2 has reached the maximal secretion capacity after cytokine stimulation or transduction, we further stimulated the HBD-2-transduced cells with cytokines. The data revealed that the transduced HGECs further secreted more HBD-2 upon cytokine stimulation.

Immunohistochemical analysis of HBD-2 expression in SIN18-HBD-2-transduced cells

In addition to measuring secreted HBD-2 in supernatants, we also performed immnuohistochemistry to detect

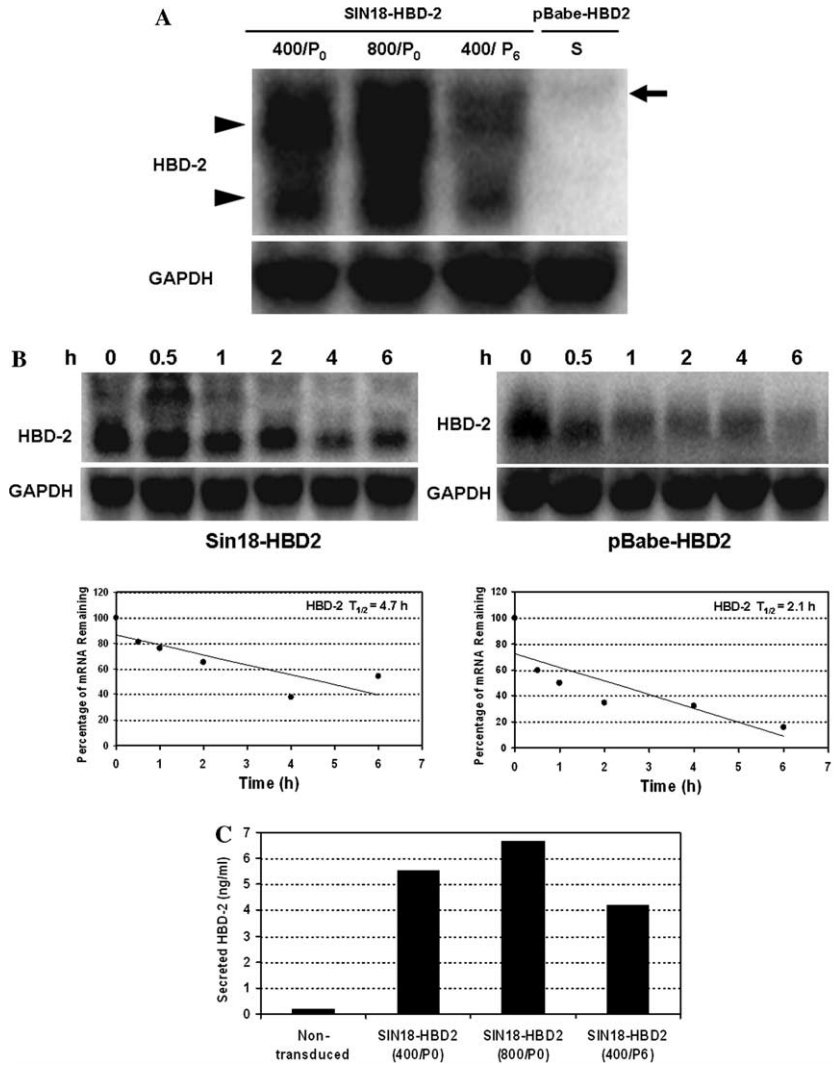

Fig. 4. Northern blot analysis of HBD-2 expression in transduced HT-1080. (A) HT-1080 cells (5×10^5) were plated into 6-well plates and exposed to 400 or 800 ng SIN18-HBD2 virus in the presence of 5 μg/ml polybrene. After overnight incubation, 2 ml fresh medium was added to replace the previous medium. HT-1080 cells (5×10^5) transduced with 400 ng Sin18-HBD2 per 5×10^5 cells beforehand and maintained to passage 6 (P_6) were plated at the same time, pBabe-HBD-2-transduced HT-1080 cells (5×10^5) subcultured after G418 selection were also plated. After 3 days, cell cultures were harvested, total RNA was isolated, and 10 μg RNA per lane subjected to Northern blot analysis. P_0, passage 0; S, G418 selected cells. Arrowheads or arrow indicate the different sizes of HBD-2 mRNA resulting from differential processing of mRNAs by the two types of vectors. (B) HBD-2 mRNA half-lives were analyzed using actinomycin D (ActD) transcription inhibition approach. HT-1080 cells (5×10^5) infected with Sin18-HBD2 (400 ng virus) for 24 h or pBabe-HBD-2-transduced HT-1080 seeded in 6-well plates were treated with ActD. At different time points (0, 0.5, 1, 2, 4, and 6 h) following the treatment, total RNA was isolated and subjected to Northern blot analysis. (C) ELISA for secreted HBD-2 by SIN18-HBD-2-transduced HT-1080 cells. Culture supernatants were collected at above-mentioned time described in (A).

HBD-2 expression in cells. In non-transduced HGF or HPC, HBD-2 protein was not detected in cells, whereas in most SIN18-HBD-2-transduced cells, HBD-2 staining was seen in cell cytoplasm (Fig. 7A). Primary non-stimulated, non-transduced (control) HGECs demonstrated HBD-2 staining and more so in SIN18-HBD-2-transduced HGECs. The staining was particularly observed in large, more differentiated cells (Fig. 7B). With IL-1α or IL-1β stimulation, similar to the SIN18-HBD2-transduced cells, the number of HBD-2 positive cells and the intensity of staining greatly increased. Also, most HBD-2 heavily stained cells showed enlarged cell size characteristic of more differentiated keratinocytes. This observation correlates to the reports by Liu et al. [10] in dermal tissue and by Abiko et al. [33] in oral mucosal tissue that HBD-2 mainly expressed in upper layers of more differentiated epithelial cells.

Discussion

Utilizing antimicrobial gene therapy approach to control tissue infection may potentially be a useful clinical application. Cells that do not express antimicrobial gene products may be transferred with these genes and become part of innate defense mechanisms. We previously tested the possibility of transducing non-HBD-2-expressing cells

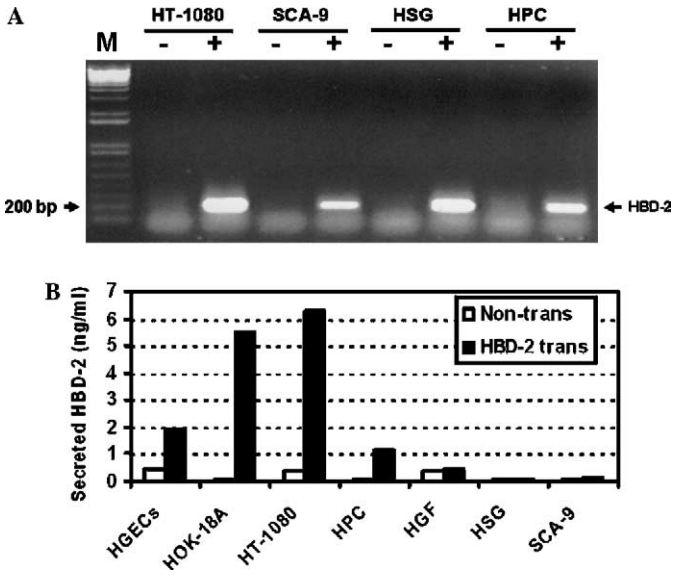

Fig. 5. HBD-2 from SIN18-HBD-2-transduced cells detected by RT-PCR (A) and ELISA (B). Cultured cells (10^5/well) were plated in triplicate into 24-well plates and exposed to 100 ng SIN18-HBD-2 virus in the presence of 5 μg polybrene. After overnight incubation, cultures were replaced with fresh medium and continued for 3 days. The cells were harvested for RNA isolation and RT-PCR, and culture supernatants were collected for ELISA. Data indicate a representative experiment. RT-PCR products do not represent relative levels of HBD-2 among tested cell types. −, non-transduced; +, SIN18-HBD-2-transduced.

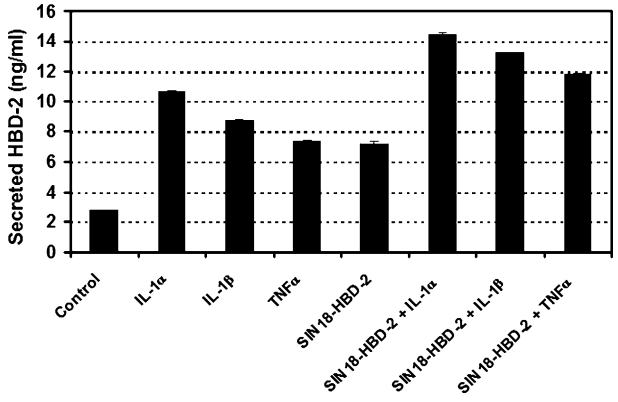

Fig. 6. HBD-2 from SIN18-HBD-2-transduced HGECs detected by ELISA. Non-transduced or transduced HGECs (10^5/well) seeded in duplicate into 24-well plates were stimulated with 100 ng/ml IL-1α, IL-1β or 10 ng/ml TNFα for 24 h and then maintained with 0.5 ml fresh medium for another 48 h. Accumulated cell culture supernatants were then collected for ELISA. For cell transduction, the same procedures were performed as described in Fig. 5 before cytokine stimulation. Control, unstimulated. Data indicate a representative experiment. Error bars are standard deviation.

to secrete HBD-2 using the retroviral vector pBabeNeo. We found that fibroblasts appear to produce higher amounts of HBD-2 compared to other cell types tested [6,49], and the secreted HBD-2 from selected transduced cells exert antimicrobial activities [6]. In these reports, the HBD-2 expression by non-transduced and transduced cells were detected using various detecting methods including Northern blot, RT-PCR, ELISA, and Western blot. HBD-2 expression was only detected after, but not before transduction. In the present study, we attempted to test HBD-3 transduction using the same vector system. However, none of the target cells tested was transduced by the packaged virus but only by direct transfection with the vectors using a Lipofectin method. Also, the amount of HBD-3 secretion was relatively low. The failure of HBD-3 transduction using virus may have been that HBD-3 somehow interfered the viral infection of the target cells because the packaging cells PA317-HBD-3 produced both the virus and HBD-3 peptides into the supernatants. To date, no report has shown that non-HBD-3-expressing cells (mammalian system) produce HBD-3 after gene transduction. Only HBD-3-expressing cells (skin epithelial cell line HaCaT) have been transduced with HBD-3 and secrete functional HBD-3 reported by Carretero et al. [7] and Sawamura et al. [8]. However, it is not clear in those reports what the levels of HBD-3 production per unit number of cells per time period are. Nonetheless, HBD-3 antimicrobial activity was demonstrated in the bioengineered skin in the reports, indicating that the secreted HBD-3 from transduced skin epithelial cells has reached effective concentrations.

Although antimicrobial activity was noted in our previous in vivo studies using a fibrosarcoma cell line HT-1080, the amount of HBD-2 secreted by transduced primary fibroblasts may be insufficient to exert microbiocidal effect as the human gingival and dental pulp fibroblasts produced much lower HBD-2 than HT-1080 and NIH 3T3 cell lines after transduction. In addition, the use of pBabNeo retroviral vector had a low transduction rate such that a selection process needed to take place. Lentiviral vectors are known for their high transduction efficiency, therefore, we used SIN18cPPTRhMLV that has a strong promoter to test whether more efficient transduction and expression of HBD-2 can be accomplished. First, we used the lentiviral vector carrying the reporter gene EGFP to transduce various cells for its convenience in observing the transduction results. Our data showed that the transduction rate using the lentiviral vector was far superior than pBabe vector. Based on the cell types tested in our studies, it appeared that the transgene expression levels were lower in mouse cells than in human cells. In addition, transgene expression levels in salivary gland cells were lower than those in other cell types (Fig. 3), implicating that utilizing salivary glands as a target organ for lentiviral vectors may not procure sufficient levels of desired gene expression. Shai et al. [62] compared the transduction efficiency of different viral vectors targeting mouse salivary glands and found that the efficiency of lentiviral vector is low compared to adenoviral, vaccinia, and herpes simplex type 1 vectors. Later, the research group found that feline immunodeficiency virus (FIV)-based lentiviral vectors appear to provide satisfactory long-term expression of the transgene in mouse salivary glands [63], suggesting a potential future use of this vector for mouse salivary gland gene transduction.

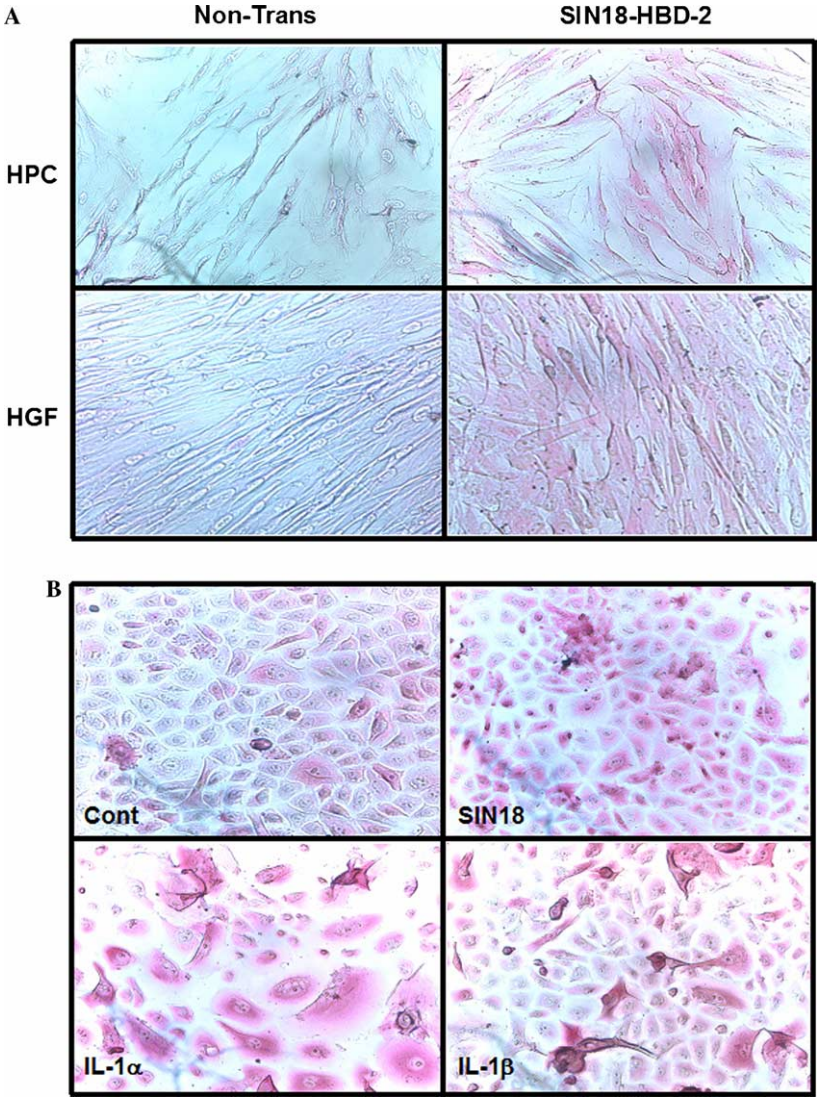

Fig. 7. Immunohistochemical analysis of HBD-2 in cells transduced with SIN18-HBD-2. (A) HPC (top images) and HGF (bottom images) non-transduced (left) or transduced with 800 ng/10^6 cells SIN18-HBD-2 (right), and then grown in chamber slides for immunohistochemical staining (red deposits). (B) Primary HGECs grown in a 48-well plate displaying HBD-2 immunostaining. Cont, unstimulated control; SIN18, cells transduced with SIN18-HBD-2 (800 ng/10^6 cells); IL-1α or IL-1β, cells stimulated with 100 ng/ml of each cytokine for 48 h (Original magnification, 200×).

When comparing HBD-2 mRNA expression in transduced HT-1080 cells by pBabe and SIN18 vectors, HBD-2 expression level is higher in cells transduced by SIN18. Our mRNA half-life studies suggest that the higher amount of HBD-2 mRNA is the result of greater transcript stability and more so, transcription. Possibly, either SIN18 has a stronger promoter or a greater number of gene copies were introduced into the cells. Together with having a higher transduction rate, lentiviral vectors appear to be superior to pBabe vectors to transduce human cells. However, HBD-2 production level is still relatively low when transducing primary fibroblasts such as HPC and HGF using SIN18. We inferred that maybe primary fibroblasts are not naturally programmed to produce HBD-2, therefore, they lack cellular machinery necessary to produce/secrete sufficient amount of HBD-2. A simple approach was to determine whether HBD-2 secretion by HBD-2-producing cells, HGECs, after transduction produce more HBD-2 than fibroblasts. Our results indicate that the gingival epithelial cell line HOK-18 secreted comparable amount of HBD-2 to but not higher than the fibrosarcoma line HT-1080. Nonetheless, although primary HGECs secreted lower amount of HBD-2 than the cell lines, they did produce more HBD-2 than primary HPC and HGF. Also, the baseline level of HBD-2 of the cultured HGECs was already higher than those of HPC and HGF. The findings were further supported by our immunohistochemical analysis (Fig. 7).

More interestingly, HBD-2 was further induced in transduced HGECs by cytokines which finding addresses an important question if antimicrobial gene therapy were to be used by transducing HGECs to make a high quantity of HBD-2 for infection control. As mentioned earlier, although HBD-2 has shown antimicrobial effect on many

oral and periodontal bacteria, those that play a critical role in periodontal diseases are relatively resistant to HBD-2 or require a very high concentration of HBD-2 to be killed, such as certain strains of *A. actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis* (MICs ranged from 6.5 to >250 μg/ml HBD-2) [64]. Sawaki et al. [37] measured the HBD-2 concentration in normal oral epithelium to be ~40 μg/ml and as high as ~3.85 mg/ml in the oral squamous cell carcinoma samples. HBD-2 levels increased in oral epithelial cells from samples having oral candidiasis [36]. HBD-2 expression levels in skin epithelium stimulated by IL-1α were ~10 μg/ml studied by using epidermal cultures [10,65]. While under normal conditions, oral epithelium may not express HBD-2 to a level sufficient to inhibit some periodontal bacteria mentioned above, they may have the capacity to express higher levels of HBD-2 after stimulation. Carretero et al. [7] and Sawamura et al. [8] demonstrated that skin epithelial cells transduced with antimicrobial peptide genes in an engineered dermal setting exert antimicrobial capacity against some bacteria that are often involved in skin infection. While it remains to be verified whether a similar approach also works for oral mucosal infection control against oral or periodontal bacteria, our data suggest a possibility that by enhancing the HBD-2-expressing cells (epithelial cells) to produce more HBD-2 using both gene transduction and cytokine stimulation may strengthen the tissue innate defense in addition to other immune mechanisms. Transduced primary fibroblasts did not produce relatively high amount of HBD-2, they may at best play a supportive role beneath the epithelium to help fight microbial infection. Taken together, antimicrobial gene transduction of epithelial cells, along with natural production of cytokines during early inflammatory responses, may enhance local innate immunity against infection for the integumentary tissues, including skin and oral mucosa. Furthermore, in the context of tissue engineering for wound repair, introducing antimicrobial genes into cells in the engineered tissues may strengthen the protection of the grafts from infection during healing, especially for immuno-compromised patients.

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