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Transgenic Expression and Antimicrobial Potential of CAMA-Syn in A549 Cells

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Abstract: CAMA-syn, a hybrid composed of N-terminal α-helical segment of CA (amino acid 1-8) and N-terminal α-helical segment of MA (amino acid 1-12) is a novel small peptide with the potent antibacterial and synergistic activity without cytotoxicity. In order to test the antibacterial function of CAMA-syn produced specially in lung epitheliuml cells, several vectors containing the synthesized CAMA-syn DNA fragment initiated by CMV and Surfactant Protein C (SP-C) promoter were constructed and transfected into recipient cells. The results showed that CAMA-syn fusion to Green Fluorescent Protein (GFP) or to Hemagglutinin epitope (HA) tag was expressed in both 293 and A549 cells. The antibacterial assays of CAMA-syn were conducted against both Gram positive and negative bacteria including *S. abortusovis*, *P. anatis*, *S. hyicus* and *S. suis*. The results of colony forming efficiency and cell growth curves proved that the *in vitro* expressed CAMA-syn could inhibit the growth of bacterial, demonstrating that lung epithelial cell-specific expression of antimicrobial peptide CAMA-syn could have antibacterial activity.

Key words: CAMA-syn, lung epithelium, antibacterial assay, antimicrobial peptide, N-terminal, China

INTRODUCTION

Antimicrobial Peptides (AMPs) are recognized as an important component of the nonspecific host defense system and innate immunity of insects, amphibians and mammals. Cecropin A (CA), a cationic 37 amino acid antimicrobial peptide was isolated from the hemolymph of giant silk moth (Lehrer and Ganz, 1999; Miyasaki and Lehrer, 1998). Magainin 2 (MA), a 23 amino acid antimicrobial peptide was discovered in the skin of the African clawed frog (Soballe *et al*., 1995; Zasloff, 1987). CA and MA display powerful lytic activity against both gram positive and negative bacteria but have a less cytotoxic effect against human erythrocytes and other eukaryotic cells. Cecropin A-magainin 2 (CAMA) hybrid peptides comprising the N-terminal amphipathic basic region of CA and the N-terminal hydrophobic region of MA also exhibited higher antibacterial and antitumor activities yet showed no hemolytic activity at 100 μg mL^{-1} (Oh *et al*., 1999, 2000). It was reported that the hybrid peptide analogue CAMA-syn with substitutions of Ile^{10} and Ser^{16} with Lys in CAMA led to the increasing of total positive charge, a better antibacterial activity and lower cytotoxicity (Jeong *et al*., 2009). CAMA-syn had antimicrobial activity against a broad range of microorganisms such as *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis* and *E. faecalis* (Jeaong *et al*., 2009). Thus, this hybrid peptide possesses potent therapeutic potential against bacterial infections in transgenic animals.

Disease resistance could be enhanced by engineering endogenous production of antimicrobial compounds in transgenic animals. The ideal system for transgenic animals is expressing an antimicrobial in the blood (Reed *et al*., 1997; Wen *et al*., 2009) and respiratory epithelial cells. However, the expression of CAMA-syn in lung epithelium to inhibit bacterial growth has not been reported yet. The purpose of this study was to express CAMA-syn peptide in lung epithelial A549 cells and to determine whether cell-specially expressed CAMA-syn has the antibacterial potential.

MATERIALS AND METHODS

Bacterial strains: *Escherichia coli* DH5α strain grown in Luria-Bertani (LB) broth or on LB agar plates was used for the recombinant DNA cloning, plasmid preparation and transformation. Gram negative bacterial strains (*Salmonella abortusovis* and *Pasteurella anatis*) and gram-positive bacterial strains (*Staphylococcus hyicus* and *Streptococcus suis*) were routinely cultured in LB plates. Colony forming efficiency was calculated by the number of Colony Forming Units (CFU) of the organisms

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determined on LB agar plates. To determine the antibacterial activity of CAMA-syn peptide, bacterial strains were inoculated in 3 mL LB broth supplemented with 300 uL cell lysates or 3 mL cell-free culture supernatants from the transfected 293 and A549 cells. The bacteria were incubated in the culture shaker (230 rpm) at 37°C and the growth curve of bacteria was plotted in line with OD600 value recorded per hour. The assays were repeated for three times and the means were used to draw bacterial growth curves.

Mammalian cell lines: Cell lines of 293 and A549 were grown in DMEM medium (12100-046, Invitrogen, USA) supplemented with 10% fetal bovine serum (Gibco BRL, USA) at 37°C in 5% CO_2. To perform the cell transfection, vectors (0.5 ug mL^{-1}) were transfected into 293 and A549 cells using Lipofectamine™ 2000 (Invitrogen, USA) in serum-free medium. After 6 h incubation, the fresh medium was replaced. In 48 h after transfection, cell pellets were used for the experiment of immunofluorescence and Western blotting and cell-free culture supernatants were collected for antibacterial assays. The control vector pEGFP-N1 was used to confirm the transfection efficiency.

Immunoassays: To perform the immunocytochemistry analysis, A549cells transfected with pSPC-CAMA-HA were grown on the cover slip for 2 days and fixed with 4% paraformaldehyde for 10-20 min then blocked with 5% BSA for 2 h. Samples were incubated with rabbit anti-HA antibody (1:500, Signalway Antibody Co., USA) at 4°C overnight and then incubated with goat anti rabbit antibody FITC-conjugated (1:100 in PBS) for 1 h at 37°C. Nuclei were stained with Hoechst 33342 (Sigma-Aldrich, USA) and viewed under the fluorescence microscope (Leica, Heidelberg, Germany). For negative control, the parallel experiments were performed with cells using pre-immune goat or rabbit serum.

To detect CAMC-syn fusion protein expression, the transfected cells were collected in EP tubule, washed with PBS solution and lysed by RIPA lysis buffer (50 mmol L^{-1} Tris-HCl pH 7.4, 150 mmol L^{-1} NaCl, 1 mmol L^{-1} PMSF, 1 mmol L^{-1} EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) on ice for 30 min. The cell lysate was collected by centrifuge in 4000g for 10 min at 4°C and then stored in -80°C. The equal amount of protein samples were separated on 12% PAGE-SDS gel under 80 V for 2 h. Proteins were blotted onto the nitrocellulose membrane under 15 V for 60 min. The membrane was then blocked with 5% BSA for 2 h and incubated with anti-GFP antibodies (1:1000 dilution) for overnight at 4°C and then incubated with HRP-conjugated secondary antibody (1:1000 dilution) for 1 h. Immunoreactive bands were detected by the enhanced chemiluminescence kit.

Antimicrobial activity assay: Cell lysates or cell-free culture supernatants from 35 mm dish were spread on LB agar plates and dried in the fume hood for 1 h. Four bacterial strains, *S. abortusovis*, *P. anatis*, *S. hyicus* and *S. suis* were inoculated on the LB agar plates and colony-forming efficiency was counted after the overnight incubation. The tests were repeated three times and the means were used to calculate the colony forming efficiency.

Statistical analysis: All values were expressed as mean±standard error of means. To compare the difference between two groups, a two-tailed Student's t-test subsequent Analysis of Variance (ANOVA) was used. A $p<0.05$ was considered significant difference.

RESULTS AND DISCUSSION

Construction of eukaryotic expression vectors: In order to express CAMA-syn in mammalian cells, the vectors with different promoters and tags were constructed (Fig. 1a). The peptide sequence of CAMA-syn (KWKLFKKIGKGKFLHKAKKF) was encoded according to the codon usage in mammalian. The synthesized nucleotide sequence is AAGTGGAAGCTGTTCAAGA AGATCGGCAAGGGCAAGTTCCTGCACAAGGCCAA GAAGTTC. Since, small peptide tag is easy to be detected and less influence the biology function of fusion protein, a HA tag was infused at the 3' end of CAMA-syn sequence. The bovine IFN gamma signal peptide was added in upstream of CAMA-syn-tag sequence to enable secretion of CAMA-syn into the culture medium. Two promoters, CMV promoter for global expression and SP-C promoter for lung epithelium specific expression were used to drive CAMA-syn expression in different cell types. The constructed vectors have been confirmed by DNA sequencing.

CAMA-syn fusion protein expression in mammalian cells: To confirm the expression of CAMA-syn in mammalian cells, the vectors of pCMV-CAMA-GFP and pSPC-CAMA-GFP were transfected into 293 and A549 cells, respectively. In 24 h, cells were examined under a fluorescence microscope. The results demonstrated that CAMA-GFP fusion protein was strongly expressed in A549 cells (Fig. 1, b1-2) and 293 (Fig. 1, b3-4). Meanwhile, the immunofluorescence analysis confirmed the CAMA-HA expression in A549 cells (Fig. 1c). The Western blotting showed that CAMA-GFP was strongly expressed

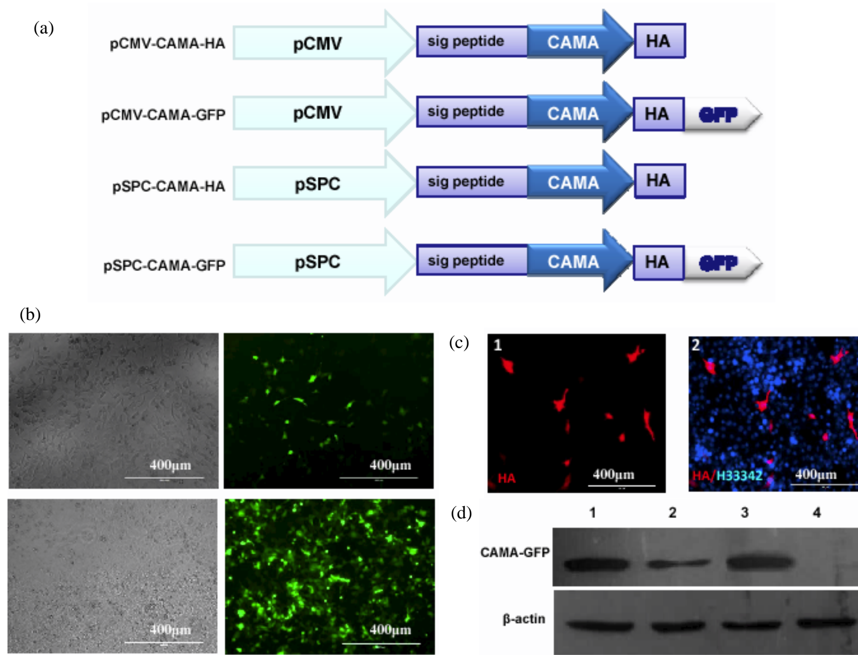

Fig. 1: Expression of CAMA-syn in 293 and A549 cells. Several CAMA-syn expression vectors with different promoters and tags were constructed and transfected into 293 and A549 cells, respectively; a) Schematic diagram of the vector constructs used in this study. pCMV, CMV promoter; pSPC, promoter of surfactant protein C promoter; sig peptide, bovine IFN gamma signal peptide; HA, the HA tag; b) The constructs of pCMV-CAMA-GFP and pSPC-CAMA-GFP were transfected into A549 (1-2) and 293 (3-4) cells, respectively and GFP was detected by fluorescence microscope; c) The construct of pSPC-CAMA-HA was transfected into A549 cells and detected by anti-HA antibody through the immunofluorescence assay (red). Cell nuclei were stained by Hoechst 33342 (blue); d) Western blotting analysis of CAMA-syn/GFP fusion protein in A549 cells (1-3) and 293 cells (4). Lane 1, pEGFP-N1 as the control; Lane 2, pSPC-CAMA-GFP; Lane 3, pCMV-CAMA-GFP; Lane 4, pSPC-CAMA-GFP

in 293 with pCMV-CAMA-GFP. On the other hand, SP-C promoter directed CAMA-GFP was only expressed in A549 cells but not in 293 cells suggesting that SP-C promoter has the tissue specificity and can be tightly regulated in lung epithelium (Fig. 1d).

Functional analysis of CAMA-syn antibacterial activity: To investigate that transgenic cells were capable of producing biologically active CAMA-syn, the antibacterial activity of cell lysates and cell-free culture supernatants were assessed and found to be greater than those found in control group. The results of colony-forming efficiency showed that both cell lysates with CAMA-syn had higher antibacterial activity than control groups (Fig. 2a). The differences of bacterial colony-forming efficiency in 293 (*S. abortusovis*, 75.7%) versus A549 (*S. abortusovis*, 68.5%) were not statistically significant. However, the observation of antibacterial activity slightly higher in 293 than in A549 might be due to the various potent promoters between CMV and SP-C.

To further evaluate the antibacterial activity, the time-dependent analysis was conducted to detect the inhibition activity of CAMA-syn. The results of bacteria growth curve showed that CAMA-syn treatments could significantly inhibit the bacterial growth (Fig. 2b). In 4-6 h incubation, the control bacteria were in the log phase while the CAMA-syn treated bacteria reached the plateau phase suggesting that CAMA-syn could prevent the bacterial proliferation and have the antibacterial activity *in vitro*.

Since, the constructs contain the bovine IFN gamma signal peptide, the expressed CAMA-syn can be secreted into culture media. To test whether expressed CAMA-syn peptide secreted from cells have biological function, the cell-free culture supernatants from 293 and A549 cells were used to examine the antibacterial activity. The results of colony-forming efficiency and growth curve experiments showed that CAMA-syn expressed in both 293 and A549 cells could be secreted into the media which had the potent suppression of bacterial proliferation

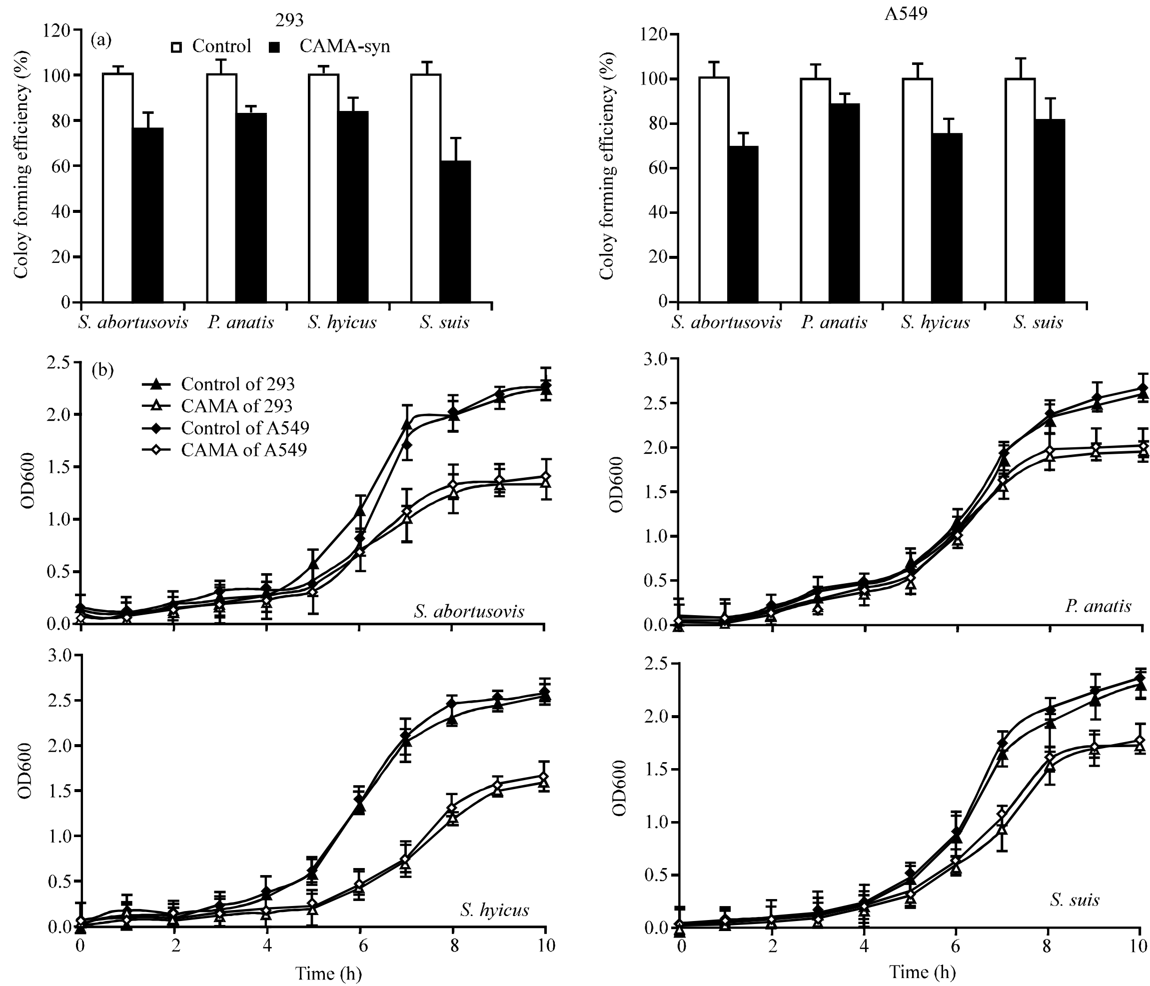

Fig. 2: Antimicrobial activity assay of 293 and A549 cell lysates. a) 293 cells were transfected by pCMV-CAMA-HA and A549 cells by pSPC-CAMA-HA. The cell lysates containing CAMA-syn peptide were prepared and added onto LB plates which four bacterial strains were inoculated, respectively. After 24 h incubation, the colony forming efficiency was counted. *$p<0.05$. n = 3; b) Growth curves of four bacterial strains inoculated in LB medium with cell lysates containing CAMA-syn. CAMA in 293, cell lysate of 293 transfected by pCMV-CAMA-HA; control in 293, cell lysate of 293 transfected by pEGFP-N1; CAMA in A549, cell lysate of A549 transfected by pSPC-CAMA-HA; control in A549, cell lysate of A549 transfected by pSPC-EGFP-N1

(Fig. 3a). Overall, the observation of cell-free culture supernatants showed higher antimicrobial activity to both of gram-positive and gram-negative bacterial than cell lysates. For instance, the colony-forming efficiency of *P. anatis* was 87.7% in A549 cell lasystes while it reached 80.4% in A549 cell-free supernatants. The results of bacteria growth curve also showed that CAMA-syn treatments could significantly inhibit the bacterial growth (Fig. 3b). There are several biological expression systems which have been used to produce antimicrobial peptides including yeasts and bacteria (Verma *et al*., 1998). Attempts have been made to produce AMPs in transgenic eukaryotic expression system. This system should accelerate the implementation of the peptide for use as antimicrobial agent and medicinal settings (Yarus *et al*., 1996). For example, humanβ-defensin 2 was used as a model for *in vivo* antimicrobial gene therapy (Huang *et al*., 2002). Also, human host defense peptide LL-37 was effective for the treatment of burn wound infections (Jacobsen *et al*., 2005). Transient expression systems have various advantages because they do not require a DNA integration procedure or a selection step and they can be prepared more rapidly are flexible and are unaffected by chromosomal positional effects (Yi and Li,

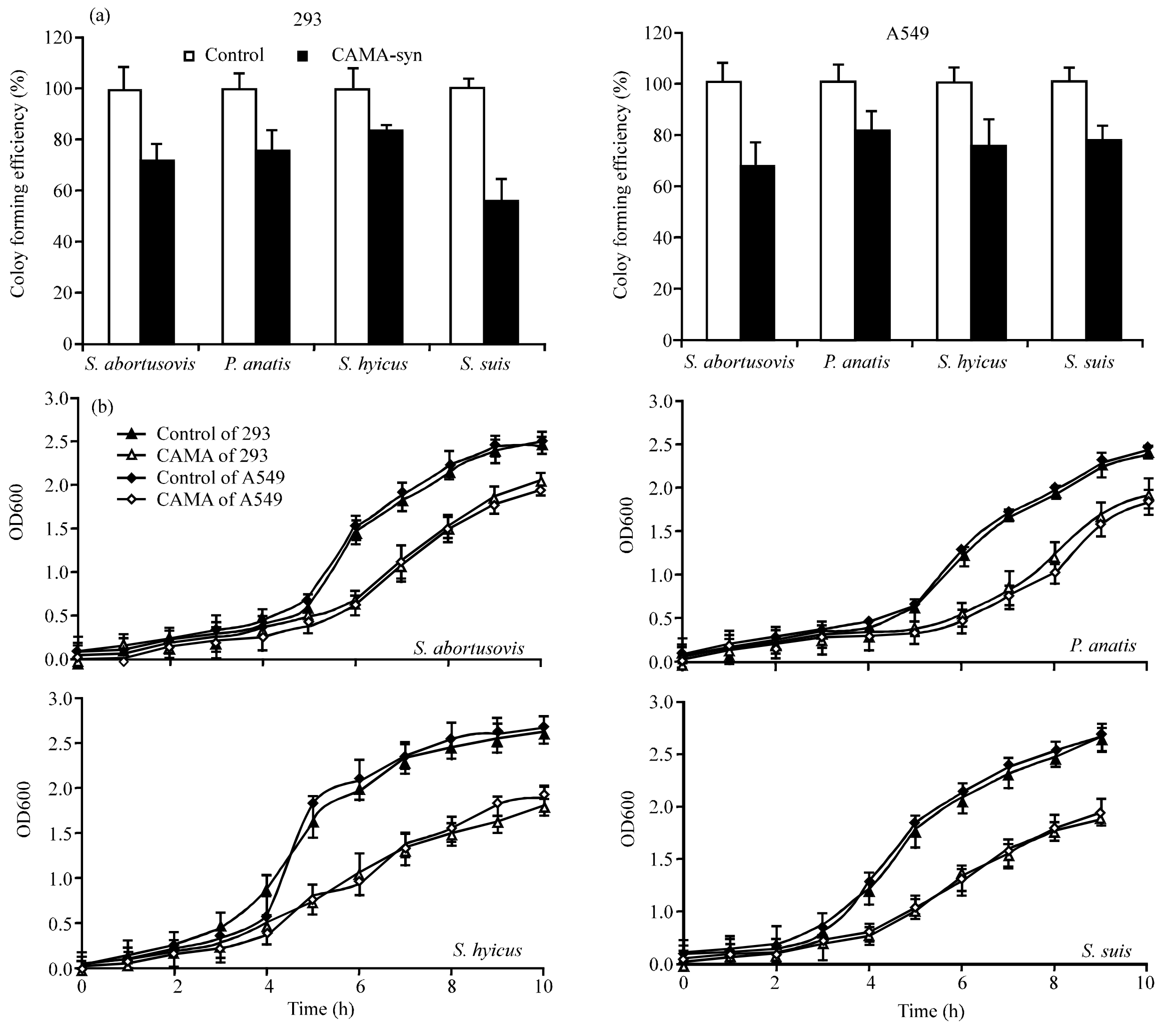

Fig. 3: Antimicrobial activity assay of cell-free culture supernatants; a) 293 cells were transfected by pCMV-CAMA-HA and A549 cells by pSPC-CAMA-HA. The cell-free culture supernatants containing CAMA-syn peptide were prepared and added onto LB plates which four bacterial strains were inoculated, respectively. After 24 h incubation, the colony forming efficiency was counted. *$p < 0.05$. $n = 3$; b) Growth curves of four bacterial strains inoculated in LB medium with cell-free culture supernatants containing CAMA-syn. CAMA of 293, cell-free culture supernatant of 293 transfected by pCMV-CAMA-HA; control of 293, cell-free culture supernatant of 293 transfected by pEGFP-N1; CAMA of A549, cell-free culture supernatant of A549 transfected by pSPC-CAMA-HA; control of A549, cell-free culture supernatant of A549 transfected by pCMV-EGFP-N1

2010). Production of recombinant proteins by transient gene expression in mammalian cells has been described such as the cell lines of COS (Corsi *et al*., 1998), HEK293 (Liu *et al*., 2010) and BHK (Wurm and Bernard, 1999) to produced the recombinant proteins. In this study, constructed several vectors to express AMPs in 293 and A549 cells. The constructs comprised of three basic components. The first is the lung epithelium Special Promoter (SP-C) and the second is signal peptide of an endogenous lymphokine, bovine INF gamma, produced predominantly by Natural Killer (NK) and Natural Killer T (NKT) cells (Schoenborn and Wilson, 2007). The first 23 amino acids translated as a signal peptide for secretion of INF gamma. The amino acids sequence is highly conserved among species as diverse as the human, mouse, bovine and sheep. The third component is a synthetic peptide of CAMA-syn. Therefore, it might be possible to use the lung epithelium special promoter and INF gamma signal sequence to produce and secrete proteins at the site of detection of foreign antigens. The

results indicate that the lung epithelium specific expression system will enable creation and secretion of AMPs to be as therapeutic agents.

Cell-specific expression of therapeutic genes in targeted cells is an excellent strategy for the absence of expression genes in non-targeted cells could reduce side effects on normal cells and even the whole body. The lung epithelium specific promoter can drive objective gene expression in lung epithelial cells exclusively, making it a convenient method for targeted expression. Indeed, it has already been applied to express PDGF (Platelet-Derived Growth Factor) in lung to study the effect on lung development and in the pathogenesis of fibrotic lung disease (Hoyle *et al*., 1999; Zhuo *et al*., 2006). SP-C Promoter was able to mediate nuclear localization of plasmid DNA specifically in ATII cells but not in other cell types (Degiulio *et al*., 2010). Thus, overexpression of CAMA-syn in lung epitheliumis a reasonable way to improve their respiratory epithelial cells bactericidal ability and a desirable approach that can be applied as a promising therapeutic strategy for refractory blood infections in the future.

Recombinant expressed AMPs have displayed a broad spectrum of antibacterial activity. Human keratinocyte cell line were transduced with adenovirus encoding for Human β-Defensin 3 (HBD-3) or LL-37 and various bacteria were allowed to adsorb onto the surface (Carretero *et al*., 2004). When LL-37 or HBD-3 was overexpressed by the cells, *Escherichia coli* viability was reduced by 28-68 and 34-72%, respectively. For *Staphylococcus aureus*, the cell expressing LL-37 and HBD-3 showed 20-45 and 20-85% inhibitory activity, respectively. *Pseudomonas aeruginosa* viability was inhibited by 43-53 and 28-35% when over-expressed LL-37 or HBD-3, respectively (Carretero *et al*., 2004). Antimicrobial assays demonstrated that recombinant cecropin B has antimicrobial activity against both gram positive and negative bacteria (Wang *et al*., 2011). CAMA hybrid peptide for fusion expression in *E. coli* showed a certain inhibitory effect on *Staphylococcus* (Ping *et al*., 2008). In the study, CAMA-syn produced served increased antibacterial activity against both gram-positive and gram-negative bacterial strains.

CONCLUSION

In this study, researchers expressed antimicrobial peptide CAMA-syn in A549 and 293cells with two different promoters which implied the system working well. The antibacterial assay of CAMA-syn peptide in both cell lysates and supernatants gave evidences of the biology function of the peptide. The results offer an option that transgenic animal with *in vivo* expressed CAMA-syn peptide may obtain the antimicrobial capability and prevent the bacterial infection.

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REFERENCES

Carretero, M., M. Del Rio, M. Garcia, M.J. Escamez and I. Mirones *et al*., 2004. A cutaneous gene therapy approach to treat infection through keratinocyte-targeted overexpression of antimicrobial peptides. FASEB. J., 18: 1931-1933.

Corsi, B., F. Perrone, M. Bourgeois, C. Beaumont and M.C. Panzeri *et al*., 1998. Transient overexpression of human H- and L-ferritin chains in COS cells. Biochem. J., 330: 315-320.

Degiulio, J.V., C.D. Kaufman and D.A. Dean, 2010. The SP-C promoter facilitates alveolar type II epithelial cell-specific plasmid nuclear import and gene expression. Gene Ther., 17: 541-549.

Hoyle, G.W., J. Li, J.B. Finkelstein, T. Eisenberg and J.Y. Liu *et al*., 1999. Emphysematous lesions, inflammation and fibrosis in the lungs of transgenic mice overexpressing platelet-derived growth factor. Am. J. Pathol., 154: 1763-1775.

Huang, G.T., H.B. Zhang, D. Kim, L. Liu and T. Ganz, 2002. A model for antimicrobial gene therapy: Demonstration of human β-defensin 2 antimicrobial activities *In vivo*. Hum. Gene Ther., 13: 2017-2025.

Jacobsen, F., D. Mittler, T. Hirsch, A. Gerhards and M. Lehnhardt *et al*., 2005. Transient cutaneous adenoviral gene therapy with human host defense peptide hCAP-18/LL-37 is effective for the treatment of burn wound infections. Gene Ther., 12: 1494-1502.

Jeong, K.W., S. Shin, J.K. Kim and Y. Kim, 2009. Antibacterial activity and synergism of the hybrid antimicrobial peptide, CAMA-syn. Bull. Korean Chem. Soc., 30: 1839-1844.

Lehrer, R.I. and T. Ganz, 1999. Antimicrobial peptides in mammalian and insect host defence. Curr. Opin. Immunol., 11: 23-27.

Liu, L., H.M. Xu, H. Jiang, J. Wang, N. Song and J.X. Xie, 2010. Recombinant adenovirus-mediated expression of GHS-R1a in HEK 293 cells. Neurosci. Bull., 26: 225-231.

Miyasaki, K.T. and R.I. Lehrer, 1998. Beta-sheet antibiotic peptides as potential dental therapeutics. Int. J. Antimicrob. Agents, 9: 269-280.

Oh, D., S.Y. Shin, J.H. Kang, K.S. Hahm, K.L. Kim and Y. Kim, 1999. NMR structural characterization of cecropin A(1-8)-magainin 2(1-12) and cecropin A (1-8)-melittin (1-12) hybrid peptides. J. Pept. Res., 53: 578-589.

Oh, D., S.Y. Shin, S. Lee, J.H. Kang amd S.D. Kim, P.D. Ryu, K.S. Hahm and Y. Kim *et al*., 2000. Role of the hinge region and the tryptophan residue in the synthetic antimicrobial peptides, cecropin A(1-8)-magainin 2(1-12) and its analogues, on their antibiotic activities and structures. Biochemistry, 39: 11855-11864.

Ping, F.W., H.Y. Wu and R. Hui-Zi, 2008. Construction and expression of cecropin A (1-8)-magainin (1-12) hybrid peptide mutant and antibacterial activity of expressed product. Chinese J. Biol., 21: 4-4.

Reed, W.A., P.H. Elzer, F.M. Enright, J.M. Jaynes, J.D. Morrey and K.L. White, 1997. Interleukin 2 promoter/enhancer controlled expression of a synthetic cecropin-class lytic peptide in transgenic mice and subsequent resistance to *Brucella abortus*. Transgenic. Res., 6: 337-347.

Schoenborn, J.R. and C.B. Wilson, 2007. Regulation of interferon-gamma during innate and adaptive immune responses. Adv. Immunol., 96: 41-101.

Soballe, P.W., W.L. Maloy, M.L. Myrga, L.S. Jacob and M. Herlyn, 1995. Experimental local therapy of human melanoma with lytic magainin peptides. Int. J. Cancer, 60: 280-284.

Verma, R., E. Boleti and A.J. George, 1998. Antibody engineering: comparison of bacterial, yeast, insect and mammalian expression systems. J. Immunol. Methods, 216: 165-181.

Wang, X., M. Zhu, G. Yang, C. Su, A. Zhang, R. Cao and P. Chen, 2011. Expression of cecropin B in Pichia pastoris and its bioactivity *In vitro*. Exp. Ther. Med., 2: 655-660.

Wen, Y.A., X.L. Yu, Q.F. Xia, D. Cen, J.B. Liu and Z.G. Tu, 2009. Macrophage-specific overexpression of antimicrobial peptide PR-39 inhibits intracellular growth of *Salmonella enterica* serovar *Typhimurium* in macrophage cells. Int. J. Antimicrob. Agents, 34: 315-321.

Wurm, F. and A. Bernard, 1999. Large-scale transient expression in mammalian cells for recombinant protein production. Curr. Opin. Biotechnol., 10: 156-159.

Yarus, S., D. Hadsell and J.M. Rosen, 1996. Engineering transgenes for use in the mammary gland. Genet. Eng. NY., 18: 57-81.

Yi, N. and N. Li, 2010. Transient expression of chicken antimicrobial peptides by mouse mammary carcinoma cells C127. Protein Pept. Lett., 17: 1517-1523.

Zasloff, M., 1987. Magainins, a class of antimicrobial peptides from Xenopus skin: Isolation, characterization of two active forms and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. USA., 84: 5449-5453.

Zhuo, Y., G.W. Hoyle, B. Shan, D.R. Levy and J.A. Lasky, 2006. Over-expression of PDGF-C using a lung specific promoter results in abnormal lung development. Transgenic. Res., 15: 543-555.