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# A potential wound healing-promoting peptide from frog skin

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ABSTRACT

Cutaneous wound healing is a dynamic, complex, and well-organized process that requires the orchestration of many different cell types and cellular processes. Transforming growth factor β1 is an important factor that plays a key role during wound healing. Amphibian skin has been proven to possess excellent wound healing ability, whilst no bioactive substrate related to it has ever been identified. Here, a potential wound healing-promoting peptide (AH90, ATAWDFGPHGLLPIRPIRIRPLCG) was identified from the frog skin of *Odorrana grahami*. It showed potential wound healing-promoting activity in a murine model with full thickness dermal wound. AH90 promoted release of transforming growth factor β1 through activation of nuclear factor-κB and c-Jun NH2-terminal kinase mitogen-activated protein kinases signaling pathways, while inhibitors of nuclear factor-κB and c-Jun NH2-terminal kinase inhibited the process. In addition, the effects of AH90 on Smads family proteins, key regulators in transforming growth factor β1 signaling pathways, could also be inhibited by transforming growth factor β1 antibody. Altogether, this indicated that AH90 promoted wound healing by inducing the release of transforming growth factor β1. This current study may facilitate the understanding of effective factors involved in the wound repair of amphibians and the underlying mechanisms as well. Considering its favorable traits as a small peptide that greatly promoting generation of endogenous wound healing agents (transforming growth factor β1) without mitogenic effects, AH90 might be an excellent template for the future development of novel wound-healing agents.



## 1. Introduction

Wound healing is essential for all organisms to survive and skin is the most frequently injured one among all tissues (Martin, 1997). Amphibians display ability for high efficiency wound repair, (Campbell and Crews, 2008; Matsuda et al., 2001; Radice, 1980; Satoh et al., 2008; Yannas et al., 1996) up to the point of regeneration of an entire limb or tail in juvenile animals (Alibardi, 2010; Fox, 1986; Yoshizato, 2007). Typically, wound healing proceeds incredibly quickly in urodele amphibians (Yokoyama et al., 2011), while in anuran amphibians (frogs and toads) healing is not as quick. However, skin-wound healing in the froglet forelimb appears to be histologically equivalent to that of urodele (Suzuki et al., 2005; Yokoyama et al., 2011). Recently, the healing process in young adult *Xenopus* "froglets" via experimental skin excision was studied by Hitoshi et al. (Yokoyama et al., 2011). It has been found that skin-wound healing in the froglet may require activation of *prx1* (a homeobox gene) limb enhancer. Nerve-dependent and -independent events in blastema formation during *Xenopus* froglet limb regeneration were also well investigated (Suzuki et al., 2005). Many genes including *prx1*, *Tbx5*, *Fgf8*, *Fgf10* and *Msx1* have been found to take part in amphibian skin wound healing and limb regeneration, but so far no bioactive peptide exerting skin wound healing function has ever been identified from amphibians.

It has been proved that fresh frog skin is efficient in the wound healing process. Recently, a formulation of frog skin powder was found to contain considerable healing and antibacterial effects on

*Abbreviations:* EGF, epidermal growth factor; DPBS, dulbecco's phosphate-buffered saline; DMEM, dulbecco's modified eagle medium; TBST, Tween 20/Tris-buffered saline; TGF-β1, transforming growth factor beta1; MAPK, mitogen-activated protein kinases; ERK, extracellular regulated protein kinases; JNK, c-Jun NH2-terminal kinase; NF-κB, nuclear factor Kappa B; α-SMA, α smooth muscle actin; TNF-α, tumor necrosis factor-α; PDTC, pyrrolidine dithiocarbamate; AP-1, activator protein-1; TLR4, toll-like receptor4; TRH, thyrotropin-releasing hormone; DAB, 3,3-diaminobenzidine; MTT, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide.

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wounds (Rezazade Bazaz et al., 2013). Frog skin secretions of *Rana ridibunda* were also demonstrated to be significantly effective in promoting wound healing process (Mashreghi et al., 2013). Lipid components from frog skin have been demonstrated to have pharmaceutical and therapeutic potential for wound repair (Raghavan et al., 2010). Although many literatures have proved that frog skins contain certain factors associated with wound healing-promoting ability, on molecular level, almost no bio-molecules were ever identified except thyrotropin-releasing hormone (TRH), which was recently reported in promoting wound re-epithelialization in frog and human skin (Meier et al., 2013).

In current study, a peptide named AH90 with wound healing-promoting ability was identified from the frog skin of *Odorrana grahami*. Besides its excellent wound healing-promoting ability in an *in vitro* wound scratch assay, it also showed potential wound healing-promoting activity in a murine model with full thickness dermal wound *in vivo*. It is the first wound healing-promoting peptide associated with TGF-β releasing ability ever identified from frog skin. Our results suggest that AH90 might be an excellent template for the development of novel wound-healing agent.

## 2. Materials and methods

### 2.1. Peptides and reagents

Many bioactive peptides have been identified from the skin of *O. grahami* in our previous work (Li et al., 2007). They were synthesized by solid phase synthesis on an Applied Biosystems model 433A peptide synthesizer and their ability to induce cell migration was screened as described below. The purity of the wound healing-promoting peptide AH90 was determined by MALDI-TOF MS analysis (Fig. S1). In addition, a scrambled version of AH90 called sAH90 (LLPLGRAIRCHWPGDTIFRPPGIA) was synthesized and used for testing the sequence specificity of AH90 in the murine wound healing model. The information of other reagents and antibodies was provided in the supplementary methods.

### 2.2. Cell culture

The murine macrophage cell line, Raw264.7 cells were cultured in high glucose dulbecco's modified eagle medium (DMEM, Invitrogen) containing 10% (v/v) fetal bovine serum (FBS, Hyclone) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in an atmosphere of 5% $CO_2$. Human epidermal keratinocyte cell line, HaCaT cells were cultured in high glucose DMEM supplemented with 10% (v/v) FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in an atmosphere of 5% $CO_2$.

### 2.3. Cell migration assay

The effects of AH90 and other synthesized bioactive peptides from the skin of *O. grahami* on HaCaT cell migration were examined by performing the wound scratch assay. The detailed information was listed in supplementary methods.

### 2.4. Wound healing model

The male Balb/c mice were exploited to make wound healing model. The experimental protocols (approval ID: SYDW-2011015) were approved by the Animal Care and Use Committee at Kunming Institute of Zoology, Chinese Academy of Sciences. The details were described in supplementary methods.

### 2.5. Tissue preparation and histological analysis

After the fixation in 10% paraformaldehyde, tissue samples were embedded in paraffin and sectioned into 5 μm thickness slices. For histological evaluation, all sections were deparaffinized, rehydrated and stained by hematoxylin and eosin (H&E). All slices were used to evaluate granulation and epidermal regeneration, by using a semi-quantitative score system (Galeano et al., 2003). In this system, four-point scales were used to evaluate granulation tissue formation (1, thin granulation layer; 2, moderate granulation layer; 3, thick granulation layer; and 4, very thick granulation layer). A three-point scale was used to evaluate dermal and epidermal regeneration (1, little regeneration; 2, moderate regeneration; and 3, complete regeneration). At day 7, mice wounds sections were incubated with antibodies against α-SMA and phosphorylated Smad3 (p-Smad3). The immunoreactivity was visualized with a horseradish peroxidase-conjugated secondary antibody and 3,3′-diaminobenzidine tetrachloride (DAB).

### 2.6. Cell proliferation assay

The effects of AH90 on Raw264.7 cell proliferation were assayed using 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-diphenytetrazoliumromide (MTT) method. The detail method was described in supplementary methods.

### 2.7. ELISA and Western blotting

The effects of AH90 on TGF-β secretion in Raw264.7 cells, and MAPK and TGF-β/SMAD signal pathways were assayed by ELISA and Western blotting. The detail method could be referred to supplementary methods.

### 2.8. Cell adhesion assay

The detailed description of the method was in supplementary material.

### 2.9. Flow cytometry assay

The surface expression of integrin subunits on keratinocyte cells was assessed by flow cytometry. Cells ($5 \times 10^5$ cells/well) were preincubated for 30 min with Dulbecco's phosphate buffered Saline (DPBS) containing 2% BSA, followed by washing three times with DPBS. The cells were then incubated with PE-conjugated antibodies on ice for 20 min in the dark. After a further three washes, cells were resuspended and analyzed with a FACScan® flow cytometer (Becton Dickinson, Frankin Lakes, NJ, USA).

### 2.10. Statistic analysis

All values were presented as mean ± SD. Differences between groups were determined using ONE-WAY ANOVA and Student's *t* test. A $P$ value < 0.05 was considered statistically significant.

## 3. Results

### 3.1. AH90 in vitro induced migration of HaCaT cells in wound model

Keratinocyte cell migration is the most critical factor in wound healing. It promotes re-epithelialization process and accelerates the wound closure. In order to identify the potential wound healing-promoting peptide, frog skin peptides reported in our previous work (Li et al., 2007) were synthesized and examined for