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Transcriptional profiling of human skin fibroblast cell line Hs27 induced by herbal formula *Astragali Radix* and *Rehmanniae Radix*

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ABSTRACT

Ethnopharmacological relevance: The herbs *Astragali Radix* (AR) and *Rehmanniae Radix* (RR) have long been used in traditional Chinese Medicine and serve as the principal herbs in treating diabetic foot ulcer.
Aim of the study: Chinese herbal formulus comprising *Astragali Radix* (AR) and *Rehmanniae Radix* (RR) have been shown to improve the healing of diabetic foot ulcer through enhancing the viability of primary fibroblasts in diabetic patients suffering insulin resistance. Our previous study demonstrated that the herbal formula NF3 comprising of AR and RR in the ratio of 2:1 was effective in promoting wound healing in diabetic rats, and *in vitro* data indicated that the wound healing effects of NF3 might be due to the regulation and coordination of inflammation, angiogenesis and tissue regeneration. However, the underlying molecular mechanism has not been well investigated. In this study, we investigated the cellular and molecular effects of the herbal formula NF3 on human skin fibroblast cells.
Materials and methods: Human skin fibroblast cells Hs27 were treated with NF3 ranging from 0 to 8 mg/ml for 24 h, and the cells without NF3 treatment were used as control. Cell proliferation assay and cell cycle analysis were performed. Transcriptional profiles of Hs27 cells upon NF3 treatment were acquired by using a human cDNA microarray containing 10,000 genes, and the signaling pathways differentially regulated by NF3 were identified and analyzed.
Results: NF3 promoted Hs27 cell proliferation and cell cycle progression. Microarray analysis revealed that 116 genes were differentially expressed upon NF3 treatment. Functional analysis of the genes indicated that NF3 mainly activated Wnt and angiogenesis related pathways, which are directly related to cell proliferation, angiogenesis, extracellular matrix (ECM) formation and inflammation during the process of wound healing.
Conclusion: This study provides insight into the molecular mechanism of how the herbal formula *Astragali Radix* and *Rehmanniae Radix* may serve as potential therapeutics for wound healing.

1. Introduction

Chinese herbal formulus containing *Astragali Radix* (AR) and *Rehmanniae Radix* (RR) were found to promote the formation of granulation tissue at the diabetic foot ulcer bed (Wong et al., 2001; Leung et al., 2008), and to stimulate the viability of primary fibroblasts in patients with insulin resistance (Lau et al., 2007, 2008, 2009). In particular, the innovative formula used in these studies, NF3 (AR:RR = 2:1, w/w), has been demonstrate to exhibit wound healing effects on the diabetic foot ulcer animal model (Tam et al., 2011). The results implied that NF3 was effective in promoting wound healing. However, the molecular mechanisms involved are not very well understood.

Wound healing is a complex process involving several physiological events. Once a wound occurs, a number of different types of cells are recruited to participate in the healing process. Fibroblasts are predominant cells in the proliferation phase of wound healing,

Abbreviations: GLUT4, glucose transporter type 4; PDGFA, platelet-derived growth factor subunit A; BMP6, bone morphogenetic protein 6; COL1A1, collagen, type I, alpha 1; FZD1, frizzled-1; VEGF, vascular endothelial growth factor; Cyclin D1, G1/S-specific cyclin-D1; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor.

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which could proliferate and migrate to the wound through the stimulation of local growth factors and cytokines produced by platelets, macrophages and fibroblasts themselves. In this study, we used human skin fibroblasts Hs27 cell line as a cell model to investigate the effects and action mechanisms of NF3 on cell proliferation and progression.

The proliferation and cell cycle progression of fibroblast cells were measured by Bromodeoxyuridine (BrdU) assay and flow cytometry, respectively. The transcriptional profile of Hs27 cells treated with NF3 was measured using a high density cDNA microarray. The transcriptional profiles provided a comprehensive molecular signature on the effect of NF3 stimulation to fibroblast cells. Functional analysis of the differentially transcribed genes, with further quantitative reverse transcription real-time PCR (qRT-PCR) and western blot analysis, may revealed the important signaling pathways involved in the NF3 effects, which will provide insight understanding of the therapeutic potential of NF3 for wound healing.

2. Materials and methods

2.1. Herbal materials preparation for treatment

Herb formula NF3 composed of *Radix Astrgali* (AR) and *Radix Rehmanniae* (RR) in the ratio of 2:1 (w/w) were prepared as described in a previous publication, and chemical fingerprinting study on NF3 was also conducted using HPLC analysis (Tam et al., 2011). Before each experiment, NF3 was freshly prepared by dissolving in Dulbecco's Modified Eagle Medium (DMEM) and sterilized by syringe filter (0.22 μm).

2.2. Cell proliferation assay

Human skin fibroblast Hs27 was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were maintained in high-glucose Modified Eagle Medium (DMEM, 4500 mg/l) supplemented with 10% of fetal bovine serum and 1% of penicillin-streptomycin in a humidified 5% CO_2 incubator at 37 °C.

To investigate the effect of NF3 on Hs27 cell proliferation, 5000 cells were seeded onto 96-well tissue culture plate and allowed to grow overnight in complete DMEM. The culture medium was then removed and the cells were treated with different dosages of NF3, at 0, 0.25, 0.5, 1, 2, 4 and 8 mg/ml, respectively, for 24 h at 37 °C. Cell Proliferation ELISA, BrdU (colorimetric) Kit (Roche Diagnostics, USA) was used to measure the proliferating cells according to the manufacturer's instructions. The NF3 concentration with the highest proliferating effect was used for other experiments.

2.3. Cell cycle analysis

Cells were treated with NF3 at 4 mg/ml for 24 h, and cells without NF3 treatment were used as the control. Cell cycle was measured by flow cytometry after staining the cells with Propidium Iodide (PI). Briefly, the cells were first collected and washed twice using 1× D-PBS, and the cell pellets were resuspended and fixed with ice-cold 70% ethanol for 60 min. Before analysis, the cells were washed twice using 1× D-PBS and RNase A was added to digest the cell RNA in 37 °C for 15 min. Subsequently, PI buffer at concentration of 50 μg/ml was added to stain the DNA, and the stained cells were analyzed by a flow cytometer (EPICS Elite *ESP*, Coulter Electronic, USA) at excitation 488 nm/emission 600 nm. Data were analyzed using a cycle distribution software (ModFit LT version 2.0, Verity Software House, USA) to determine the proportion of cells in each cell cycle phase (G0/G1, S, G2/M).

Table 1
Primer sequences of genes PDGFA, COL1A1, BMP6, GLUT4, and 18S for quantitative real-time PCR analysis.

Gene symbol	Accession no.	Primer sequence	Product size
PDGFA	M19989.1, NM_033023	5′-ggtggtcacaggtgcttttt-3′ 5′-tccaagacattcctgcttcc-3′	110 bp
COL1A1	NM_000088.2	5′-gtgctaaaggtgccaatggt-3′ 5′-accaggttcaccgctgttac-3′	128 bp
BMP6	NM_001718	5′-acagcataacatggggcttc-3′ 5′-gaagggctgcttgtcgtaag-3′	112 bp
GLUT4	M20747.1	5′-atgactgtggctctgctcct-3′ 5′-aagatggccacaatggagac-3′	68 bp
18S	NM022551	5′-gaggatgaggtggaacgtgt-3′ 5′-agaagtgacgcagccctcta-3′	199 bp

2.4. cDNA microarray analysis

Cells were treated with NF3 at the concentration of 4 mg/ml in serum-free DMEM for 24 h, and un-treated cells were used as the control. Total RNA were extracted by TRIzol reagent (Ambion) and the gene expression was analyzed using a cDNA microarray containing 10,000 genes according to published protocols (Wang et al., 2009). Briefly, with the use of SuperScript III Reverse Transcriptase (Invitrogen), cDNA was synthesized with the incorporation of Cy5-dUTP and Cy3-dUTP in pairs. Labeled cDNA was then mixed and purified by Microcon 30 column (Millipore). Purified cDNA samples were added to the cDNA microarray at 65 °C in moist chambers for 20 h. Microarray images from the two-color fluorescence hybridization were obtained with a confocal laser scanner (ScanArray 4000, GSI Lumonics, MA). The microarray results were analyzed using an image analysis software (GenePix Pro 4.0, Axon, Union City, CA). The raw data were filtered according to the following criteria: spots with small diameters (<120 μm), low signal intensity (<300 fluorescence intensity units), and low signal to noise ratio (<1.5) were discarded. Fluorescence ratios (Cy5 over Cy3) were used to determine the relative level of gene expression. Genes showing a greater than 2-fold induction or repression (Cy5/Cy3 ratios above 2 or below 0.5) were selected for further analysis. The cutoff value of 2-fold is conventionally used by other investigators (Quackenbush, 2001).

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA extracted from Hs27 cells with or without NF3 treatment were purified first with the digestion of DNA with DNase I (Deoxyribonuclease I, Amplification Grade, Invitrogen), cDNA was then synthesized using a Transcription First Strand cDNA Synthesis Kit (Roche Diagnostics). Quantitative assessment of cDNA was carried out by real-time PCR with a SYBR Green Master Mix Kit (Applied Biosystems). Human 18S ribosomal gene was used as internal control to normalize the amount of sample cDNA. Specific primers for selected genes were designed using Primer 3 (Table 1). Briefly, gene amplification was carried out in 10 μl reaction with the following program: 45 s of an initial denaturation; 45 cycles of 10 s denaturation at 95 °C and 30 s annealing and extension of at 60 °C. After amplification, a melting curve analysis from 65 to 95 °C with heating rate of 0.1 °C/s with a continuous fluorescence acquisition was carried out. Quantification of cDNA was repeated four times. The resulting calibration graph [the fluorescent threshold cycle value (CT) vs. log unit of the standard template (UST), which is a relative value for the amount of cDNA] correlates Ct values with the relative amount of cDNA template in the PCR.