Identification of Target Genes Involved in Wound Healing Angiogenesis of Endothelial Cells with the Treatment of a Chinese 2-Herb Formula

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Abstract

Angiogenesis is vitally important in diabetic wound healing. We had previously demonstrated that a Chinese 2-herb formula (NF3) significantly stimulated angiogenesis of HUVEC in wound healing. However, the molecular mechanism has not yet been elucidated. In line with this, global expression profiling of NF3-treated HUVEC was performed so as to assess the regulatory role of NF3 involved in the underlying signaling pathways in wound healing angiogenesis. The microarray results illustrated that different panels of differentially expressed genes were strictly governed in NF3-treated HUVEC in a time-regulated manner. The microarray analysis followed by qRT-PCR and western blotting verification of NF3-treated HUVEC at 6 h revealed the involvement of various genes in diverse biological processes, e.g., MAP3K14 in anti-inflammation; SLC5A8 in anti-tumorogenesis; DNAJB7 in protein translation; BIRC5, EPCAM, INSL4, MMP8 and NPR3 in cell proliferation; CXCR7, EPCAM, HAND1 and MMP8 in migration; CXCR7, EPCAM and MMP8 in tubular formation; and BIRC5, CXCR7, EPCAM, HAND1, MMP8 and UBD in angiogenesis. After 16 h incubation of NF3, other sets of genes were shown with differential expression in HUVEC, e.g., IL1RAPL2 and NR1H4 in anti-inflammation; miR28 in anti-tumorogenesis; GRIN1 and LCN1 in anti-oxidation; EPB41 in intracellular signal transduction; PRL and TFAP2A in cell proliferation; miR28, PRL and SCG2 in cell migration; PRL in tubular formation; and miR28, NR1H4 and PRL in angiogenesis. This study provided concrete scientific evidence in support of the regulatory role of NF3 on endothelial cells involved in wound healing angiogenesis.
Introduction

Angiogenesis is vitally important in wound healing, which describes the formation of new blood vessels from the existing vasculature for restoring blood flow to wound tissues after injury for diffusion exchange of nutrients and metabolites. The luminal surface of circulation system in closely contact with blood is a single layer of endothelial cells (ECs) which are actively involved in the overall process of angiogenesis. It is tightly governed by pro- and anti-angiogenic factors and involves a cascade of signaling events [1]. Angiogenesis begins at the moment of injury. Resident ECs are responsive to a number of angiogenic growth factors for ECs activation after receptor binding. Four fundamental and essential steps of angiogenesis take place: (i) protease synthesis by ECs for degradation of basal lamina in the parent vessel associated with dissolving the tissue extracellular matrix (ECM); (ii) migration of ECs into the wound bed; (iii) proliferation and sprouting of ECs for restoring the tubular channels and forming the vascular loops; and finally (iv) remodeling and differentiation [2]. Normal wound healing is a highly orchestrated and dynamic mechanism which is comprised of the coordination of various cells including platelets, inflammatory cell, ECs, epidermal cells and parenchyma cells. The process involves hemostasis, coagulation, inflammation, regeneration, migration and proliferation of connective tissue associated with extracellular matrix proteins synthesis and deposition for enhancement of wound tensile strength. However, numerous studies reported that angiogenesis was seriously hampered in diabetes with diminished level of growth factors, persistent inflammatory response and ECs dysfunction [3]. Defects in the angiogenesis adversely affected the subsequent tissue granulation and healing process, leading to wound healing failure.

Astragali Radix (AR) and Rehmanniae Radix (RR) have long been used in Traditional Chinese Medicines (TCM) formulae and serve as the principal herbs in treating diabetic foot ulcer. Both clinical and scientific studies provided strong and equivocal evidence of AR and RR as alternatives to conventional medicines in the treatment of diabetic ulcer with wound modulating and pro-angiogenic effects. In the clinic, Wong et al. (2001) applied two herbal formulae F1 and F2 in promoting wound closure and preventing limb amputation in diabetic foot ulcer patients [4]. In the laboratory, Tam et al. (2011) demonstrated a modified herbal formula NF3 (AR and RR in the ratio of 2 to 1) that imposed promising wound healing effect in streptozotocin-induced diabetic foot ulcer rats [5]. Subsequent evidence further supported the pro-angiogenic capacity of NF3 in diabetic wound healing [6–9].

Previously, we had demonstrated that NF3 could significantly enhance the in vitro cell migration and tube formation ability of human umbilical vein endothelial cells (HUVEC) [5]. This revealed the pro-angiogenic capacity of HUVEC after NF3 treatment, promoting blood vessel formation in wound healing mechanism. However, the underlying mechanisms of the functional changes and modulation of the pathways had not yet been fully addressed. The advent of powerful technology of functional genomics has accelerated the investigations of gene identification and cellular function in a high-throughput and global scale. Making use of the advantages of DNA microarray technology, our ultimate aim was to identify the target genes involved in pathway resulting from the therapeutic effects of NF3.

In line with this, we focused on understanding the regulatory mechanisms of NF3 on HUVEC at transcriptional level. The 6 h and 16 h incubation of NF3-HUVEC chosen for microarray study were based on our previous study of pro-angiogenic activity of NF3-treated HUVEC on tube formation and migration scratch assay at 6 h and 16 h, respectively [5]. This study aimed to identify genes which were responsible for wound healing, as well as angiogenesis-associated pathways in different time frame. This would allow the identification of a large number of molecular targets closely related to the biological effects of NF3. Associated with the
bioinformatic data mining tools, the identified target genes from microarray analysis of HUVEC would be further validated by qRT-PCR and western blotting so as to elucidate the role of NF3 in governing the angiogenic responses of the mature endothelial cells at the transcriptional level in wound healing angiogenesis.

**Materials and Methods**

**Authentication and extracts preparation of NF3**

Herbal formula NF3 was composed of Astragali Radix (AR) and Rehmanniae Radix (RR) in the ratio of 2:1 (w/w) and was prepared as described previously [5–7]. AR was derived from the dried root of *Astragalus membranaceus* (Fisch.) Bge., while RR was derived from the dried root of *Rehmannia glutinosa* Libosch. The raw herbs of AR and RR were purchased from mainland China. They were authenticated by morphological characterizations and thin layer chromatography in accordance with the Chinese Pharmacopoeia [10]. Voucher specimens of AR and RR were deposited in the museum of Institute of Chinese Medicine, The Chinese University of Hong Kong, with voucher specimen numbers: 2008–3201 for AR and 2008–3200 for RR.

For NF3 preparation, raw herbs of AR and RR were cut into small pieces and mixed in the ratio of 2:1 (w/w). After being soaked in 10 volumes of distilled water for 30 min, they were boiled under reflux for 1 h twice. The extracts were pooled, filtered and lyophilized into dry powder. The extraction yield was around 34% (w/w). The NF3 preparation was under strict quality control to ensure the identity throughout all the experiments. The chemical fingerprinting of NF3 was conducted by high performance liquid chromatography-mass spectrometry analysis and the details were described previously [11].

**Cell culture**

The culture conditions of human umbilical vein endothelial cells (HUVEC) were followed by our previous method [5, 8]. In brief, HUVEC were subcultured to confluence in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Life technologies, USA) supplemented with 0.1 mg/ml heparin (Sigma-Aldrich, USA), endothelial cell growth supplement (Sigma-Aldrich, USA) and 10% fetal bovine serum (GIBCO, USA) and 1% penicillin-streptomycin (GIBCO, USA). Cells were maintained at 37°C, 5% CO2 humidified incubator.

**Sample preparation for microarray study**

HUVEC (6 x 10^5 cells) were seeded in 75 cm^2 culture flask with complete medium. After 24 h of incubation, the cells were starved in medium with 0.5% of FBS for 24 h. The medium was replaced with fresh medium in the absence (control HUVEC) or presence of 75 μg/ml NF3 (NF3-treated HUVEC). After 6 h and 16 h incubation, cells were collected with addition of Trizol reagent and stored at -80°C for further RNA extraction.

**RNA isolation**

Total cellular RNA was extracted by TRIzol® reagent (Invitrogen, USA) according to the manufacturer’s instructions. Freshly extracted RNA was measured by using a NanoDrop™ 2000 UV-visible spectrophotometer (Thermo Scientific, USA). RNA integrity was additionally assessed by using a Eukaryote total RNA nano chip and the Agilent 2100 Bioanalyser (Agilent Technologies, Germany).
Microarray hybridization

cRNA was transcribed in vitro by incorporating a biotinylated pseudouridine molecule by using the Ambion WT expression kit (Applied Biosystem, USA) and Affymetrix reagent kits (Affymetrix, USA). Hybridization was performed by using GeneChip Human Gene 1.0 ST Array (Affymetrix, USA) containing 764,885 distinct probes corresponding to 28,869 well-annotated genes. After washing, the chips were stained with streptavidin–phycoerythrin in accordance with the Affymetrix EukGE-WS2v5 protocol by using a Fluidic FS450 station. The microarrays were read with the GeneChip Scanner 3000 (Affymetrix, USA). The Affymetrix GeneChip Operating Software version 1.2 was used to manage the Affymetrix GeneChip array data and to automate the control of the GeneChip fluidics stations and scanners.

Analysis for genomic study

The acquisition and initial quantification of array images were conducted using the AGCC software (Affymetrix, USA). Subsequent data analyses were performed using Partek Genomics Suite Version 6.4 (Partek, USA). We firstly performed a one-way ANOVA to identify the genes between the groups at a P value of less than 0.05, and then calculated the relative difference in fold change between the groups. Although these criteria were used with low stringency, this was expected to detect more genes truly associated with NF3 treatment at the expense of increasing the number of false-positives to be validated by bio-informatics and qRT-PCR experimental means. Cluster analyses and principal component analysis (PCA) were conducted with Partek default settings.

Bioinformatic analysis

The gene lists were further analyzed by Genomatix Pathway System (GePS; http://www.genomatix.de/index.html) which gave all canonical pathways, interactions and network construction with significant enrichment of the input proteins based on data from the NCI-Nature Pathway Interaction Database, Biocarta, etc. The selection parameter of the differentially expressed genes of molecular functions classification and connectivity network identification was based on the gene enrichment analysis with p < 0.05. The Universal Protein Resource (UniProt; http://www.uniprot.org/) was a comprehensive resource for annotating and identifying genes with respective to the gene ontology (GO), pathway and protein families from the input of gene list, providing a platform for the understanding of molecular functions and signaling pathways involved with the regulatory genes.

Detection of mRNA expression level by quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR)

The preparation and isolation of RNA were previously described. HUVEC were used in the quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR) for confirmation of the selected genes. qRT-PCR was performed by using iScript™ one-step RT-PCR kit with SYBR® Green (Bio-Rad, USA) on CFX96 Real-Time PCR Detection System (Bio-Rad, USA) in accordance to the manufacturer’s instructions. Specific primers for target and reference genes were designed using OligoPerfectTM Designer (Life technologies, USA) or PrimerQuest (Integrated DNA technologies, USA). The primer sets for 6 h and 16 h time points were listed in Tables 1 and 2, respectively. Gene amplification was carried out in 10 μl reaction with the following program: 10 min of cDNA synthesis at 50°C; 5 min of initial denaturation at 95°C; 49 cycles of 10 s of denaturation at 95°C and 30 s of annealing and extension at 60°C. After amplification, a melting curve analysis from 65 to 95°C with heating rate of 0.5°C per 5 s
with a continuous fluorescence acquisition was carried out. The experiments were repeated three times each. Relative expression of the qRT-PCR product was calculated by using the comparative $2^{-\Delta\Delta C_T}$ method. The gene expression level was determined by normalizing to the reference gene GAPDH mRNA level. The fold difference of NF3 treatment group was then determined by normalizing all values to the mean of the relative expression in control group.

### Table 1. Primer list of NF3-treated HUVEC versus control at 6 h.

| Gene     | Primer sequence (5’→3’)                                      | Forward       | Reverse       |
|----------|---------------------------------------------------------------|---------------|---------------|
| BIRC5    | TCATAGAGCTGCAGGATCTTGTGATTTGAGA                              | AGTAGGCTGACAGAGGTGTTTGAGA |
| CXR7     | ACCTGCTGTCACCTTCTTGTGAGA                                      | AGCAGGCTGACAGAGGTGTTTGAGA |
| DNAJB7   | TCGAATCTTCACAGAGGTGTTTGAGA                                    | AGCAGGCTGACAGAGGTGTTTGAGA |
| EPCAM    | AGACTGGGCTTACAGCTTTGTGAGA                                     | AGCAGGCTGACAGAGGTGTTTGAGA |
| GAPDH    | AGCTACGACAGCTTCTTGTGAGA                                       | AGCAGGCTGACAGAGGTGTTTGAGA |
| GUCY2C   | TGCTGCTGTCACCTTCTTGTGAGA                                      | AGCAGGCTGACAGAGGTGTTTGAGA |
| HAND1    | ATGAAACATGGCTACAGCTTTGTGAGA                                   | ATGAAACATGGCTACAGCTTTGTGAGA |
| HLA-DQB1 | ATGACCTGTCATGGCTACAGCTTTGTGAGA                               | ATGAAACATGGCTACAGCTTTGTGAGA |
| INSL4    | AGCTGCTGTCACCTTCTTGTGAGA                                      | ATGAAACATGGCTACAGCTTTGTGAGA |
| MAP3K14  | TGCAGCTGTCGATCTTCTTGTGAGA                                     | ATGAAACATGGCTACAGCTTTGTGAGA |
| MPP8     | AAATCTGCTGTCACCTTCTTGTGAGA                                    | ATGAAACATGGCTACAGCTTTGTGAGA |
| NPR3     | TGCTGCTGTCACCTTCTTGTGAGA                                      | ATGAAACATGGCTACAGCTTTGTGAGA |
| PGC      | ATGACCTGTCATGGCTACAGCTTTGTGAGA                               | ATGAAACATGGCTACAGCTTTGTGAGA |
| SLC5A8   | GGTGCTGTCGATCTTCTTGTGAGA                                      | ATGAAACATGGCTACAGCTTTGTGAGA |
| UBD      | TGCTGCTGTCACCTTCTTGTGAGA                                      | ATGAAACATGGCTACAGCTTTGTGAGA |

### Table 2. Primer list of NF3-treated HUVEC versus control at 16 h.

| Gene     | Primer sequence (5’→3’)                                      | Forward       | Reverse       |
|----------|---------------------------------------------------------------|---------------|---------------|
| ABCG8    | TGCTGCTGTCACCTTCTTGTGAGA                                      | ATGAAACATGGCTACAGCTTTGTGAGA |
| EPB41    | TTCCTGAGCTGCTTCTTGTGAGA                                       | ATGAAACATGGCTACAGCTTTGTGAGA |
| GAPDH    | ACCTGCTGTCGATCTTCTTGTGAGA                                     | ATGAAACATGGCTACAGCTTTGTGAGA |
| GRIN1    | GTCTGCTGTCGATCTTCTTGTGAGA                                     | ATGAAACATGGCTACAGCTTTGTGAGA |
| GYP4A    | GCATTGCTGTCGATCTTCTTGTGAGA                                    | ATGAAACATGGCTACAGCTTTGTGAGA |
| IL1RAP1  | AACCTGCTGTCGATCTTCTTGTGAGA                                    | ATGAAACATGGCTACAGCTTTGTGAGA |
| LCN1     | CAGCTGCTGTCGATCTTCTTGTGAGA                                    | ATGAAACATGGCTACAGCTTTGTGAGA |
| LRFN2    | TGTGCTGCTGTCGATCTTCTTGTGAGA                                   | ATGAAACATGGCTACAGCTTTGTGAGA |
| MIR28    | CAGCTGCTGTCGATCTTCTTGTGAGA                                    | ATGAAACATGGCTACAGCTTTGTGAGA |
| NR1H4    | AACCTGCTGTCGATCTTCTTGTGAGA                                    | ATGAAACATGGCTACAGCTTTGTGAGA |
| PRL      | TGTGCTGCTGTCGATCTTCTTGTGAGA                                   | ATGAAACATGGCTACAGCTTTGTGAGA |
| SCG2     | GGCTGCTGTCGATCTTCTTGTGAGA                                     | ATGAAACATGGCTACAGCTTTGTGAGA |
| TFAP2A   | GGCTGCTGTCGATCTTCTTGTGAGA                                     | ATGAAACATGGCTACAGCTTTGTGAGA |
| TTC39B   | ATGAAACATGGCTGTCGATCTTCTTGTGAGA                               | ATGAAACATGGCTACAGCTTTGTGAGA |
| ZNF711   | TGCTGCTGTCGATCTTCTTGTGAGA                                     | ATGAAACATGGCTACAGCTTTGTGAGA |

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Protein extraction and western blotting
The NF3-treated cells at 6 and 16 h were collected and lysed in a RIPA buffer for 30 min on ice, and then centrifuged at 14000 rpm for 15 min at 4°C. Protein concentration was measured using Bio-Rad Dc Protein Assay (Bio-Rad, Hercules, CA USA). Equal amount (40 μg) of protein samples were separated on a 8% SDS-polyacrylamide gel and electrophoretically transferred (100 V, 2 h) onto a nitrocellulose membrane (Pall Gelman Laboratory, Ann Arbor, MI USA). Afterwards the membranes were blocked for 1 h using 5% non-fat dry milk, and then incubated overnight at 4°C with primary antibodies, including CXCR7 (GTX100027; GeneTex, USA), MMP8 (ab81286; Abcam, USA), NR1H4 (ab28676; Abcam, USA) and SCG2 (26101; QED Bioscience, USA). After washing the membranes, membranes were incubated with the secondary horseradish peroxidase-conjugated antibodies (Invitrogen, Carlsbad, CA, USA) for 1 h, detection was performed using enhanced chemiluminescence assay kit (GE Healthcare, UK).

Statistical analysis
The differences in the gene expression level between the NF3-treated and the control group were compared by a Student’s t test with p < 0.05 as considered statistically significant. All data were expressed as mean ± standard deviation (SD). Statistical analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad software, USA).

Results
Comparison of gene expression profile by principal component analysis (PCA)
Principal component analysis (PCA) is a statistical technique for determining the key variables in a multidimensional data set. It clearly identifies the inter-experimental variations of the differential expression pattern of two groups associated with the validation of intra-experimental variations of the microarray experiment. The two distinct clusters of three red and blue balls represented the intra-experimental variation of control cells and NF3-treated cells, respectively. The PCA results illustrated that there was no overlap in the clusters of three repeated hybridizations (balls in the same color) of the control HUVEC (red) and NF3-treated HUVEC (blue) (Fig 1). The gene expression profile of NF3-treated HUVEC group was well separated from that of control HUVEC group.

Identification of global gene expression changes in 6 h and 16 h of NF3-treated HUVEC
The pro-angiogenic activities of HUVEC with different concentrations of NF3 were previously demonstrated [5]. A significant cell migration and tube formation effect were found in HUVEC treated with 75 μg/ml NF3. For comprehensive microarray study, 75 μg/ml concentration of NF3 was used for global genomic expression analysis in HUVEC for 6 h and 16 h incubation. The raw data generated by AGCC software were further processed by Partek Genomics Suite, which identified the genes between NF3-treated HUVEC and control HUVEC with criteria settings of fold change above 1.2 and p value lower than 0.05. Although these criteria were used with low stringency, more genes associated with NF3 treatment could be detected. The number of false-positive results would be validated further by bio-informatics analysis and qRT-PCR. The relative gene expression difference was presented as the fold change of NF3-treated HUVEC versus control HUVEC. From the Partek software analysis, 103 genes were identified with 61 up-regulated and 42 down-regulated in NF3-treated HUVEC versus
control HUVEC at 6 h. The panels of differentially expressed genes with accession number, gene symbol, gene name and p value were shown in S1 Table. At 16 h, we found a total of 96 genes with 32 up-regulated and 64 down-regulated in NF3-treated HUVEC versus control HUVEC, which were listed in S2 Table.

Functional categorization of genes
Genomatix Pathway System (GePS) was applied to classify the differentially expressed genes into molecular functions and biological processes based on an AmiGo gene ontology (GO) database with significance of $p < 0.05$. As shown in Fig 2A, the most significant molecular function of NF3-treated HUVEC versus control HUVEC at 6 h was toxin binding consisting of GUCY2C and HLA-DQB1 ($p = 8.3 \times 10^{-4}$). Delayed rectifier channel activity included KCNE1 and KCNH1 ($p = 1.21 \times 10^{-4}$). However, only a few genes were grouped into molecular functions with significances from the gene list of NF3-treated HUVEC versus control HUVEC at 6 h. Not much molecular information could be obtained. Surprisingly, more genes in NF3-treated HUVEC versus control HUVEC at 16 h were grouped into different significant molecular functions (Fig 2B). 18 genes were classified into signaling receptor activity ($p = 1 \times 10^{-5}$), while 17 genes belonged to transmembrane signaling receptor activity ($p = 1.55 \times 10^{-5}$). Besides, a number of genes had G-protein coupled receptor activity, signal transducer activity and receptor activity. The molecular function categorization provided molecular details among the genes, postulating the specific regulation of different panel of differentially expressed genes in NF3-treated HUVEC versus control HUVEC at different time frame.

Network connectivity identification
To elucidate the regulatory mechanisms of NF3 on the differentially expressed genes of HUVEC involved in wound healing angiogenesis, the genes were annotated by GePS according
to their relative interactions and frequent co-citation from data and literature mining so as to predict their biological processes and underlying signaling pathways. The connectivity map organized genes based on their interactions, which could further extend the input genes by transcription factors or transcriptional downstream targets. In Fig 3, genes with differential expression in HUVEC after 6 h and 16 h of NF3 treatment were linked in the connectivity network. The advantage of connectivity map by GePS was to sort out genes with interactions for subsequent pathway prediction. All the target genes were further analyzed using the Universal Protein Resource (UniProt) in order to categorize them according to gene ontology (GO) and respective protein families. As illustrated in Table 3 of HUVEC after 6 h of NF3 treatment, BIRC5 was annotated to regulate cell cycle and migration behavior (microtubule organizing center). CXCR7 and HAND1 were responsible for neovascularization of endothelial cells. Some genes (DNAJB7 and HAND1) possessed DNA binding transcription factor activity. EPCAM, GUCY2C and INSL4 were directly involved in cell proliferation. Genes with protease activity participating in different cellular reaction included MAP3K14, MMP8 and PGC. On the other hand, after 16 h of NF3 treatment (Table 4), different genes were triggered and expressed in HUVEC. EPB41 was identified to participate in actin cytoskeleton organization, governing cell motility. IL1RAPL2 contained interleukin-1 receptor activity, transmitting inflammatory signal of interleukin-1. SCG2 was the candidate gene responsible for angiogenesis as well as regulation of proliferation and migration of endothelial cells. NR1H4, TFAP2A and ZNF711 belonged to DNA binding transcription factor activity or regulation of transcription. From the GO analysis, the differentially expressed genes in HUVEC would provide insight of the regulatory mechanisms as well as the prediction of the signaling pathways elicited by NF3 on mature endothelial cells in modulation of wound healing angiogenesis.

Microarray analysis and data verification by qRT-PCR

qRT-PCR was employed to validate the expression level of the selected genes from microarray analysis. We determined the mean value of expression of the selected genes in 3 independent NF3-treated HUVEC samples at 6 h and 16 h. The qualitative changes in gene expression levels
Fig 3. Network connectivity of identified genes in NF3-treated HUVEC by GePS. (A) Differentially expressed genes with frequent co-citations and interactions in NF3 treated HUVEC at 6 h. (B) Differentially expressed genes with frequent co-citations and interactions in NF3 treated HUVEC at 16 h. Genes were identified by their gene symbols (S1 and S2 Tables). Dashed lines indicated association by co-citation. Up arrow and down arrow corresponded to up- or down-regulation, respectively.

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Table 3. Gene ontology classification of the identified genes in NF3-treated HUVEC at 6 h by UniProt.

| Gene       | Protein names                  | Gene ontology (GO)                                                                 | Protein families                      |
|------------|--------------------------------|-----------------------------------------------------------------------------------|---------------------------------------|
| BIRC5      | Baculoviral IAP repeat-containing protein 5 | G2/M transition of mitotic cell cycle; microtubule organizing center               | IAP family                           |
| CXCR7      | C-X-C chemokine receptor type 7  | Vasculogenesis; angiogenesis; cell adhesion                                       | G-protein coupled receptor 1 family   |
| DNAJB7     | DnaJ homolog subfamily B member 7 | DNA binding transcription factor activity                                           | —                                     |
| EPCAM      | Epithelial cell adhesion molecule | Positive regulation of cell proliferation                                        | EPCAM family                         |
| GUCY2C     | Heat-stable enterotoxin receptor  | Intracellular signal transduction; regulation of cell proliferation                 | Adenylyl cyclase class-4/guanylyl cyclase family |
| HAND1      | Heart- and neural crest derivatives-expressed protein 1 | Angiogenesis; embryonic heart tube formation; mesoderm formation; DNA binding transcription factor activity | —                                     |
| HLA-DQB1   | major histocompatibility complex, class II, DQ beta 1; similar to major histocompatibility complex, class II, DQ beta 1 | —                                                                                   | —                                     |
| INSL4      | Early placenta insulin-like peptide | Cell proliferation; cell-cell signaling; insulin-like growth factor receptor binding | Insulin family                       |
| MAP3K14    | Mitogen-activated protein kinase kinase kinase 14 | MAPK cascade; NF-kappab-inducing kinase activity; T cell costimulation; activation of MAPKK activity | Protein kinase superfamily          |
| MMP8       | Matrix metalloproteinase-8        | Collagen catabolic process; extracellular matrix disassembly                      | Peptidase M10A family                |
| NPR3       | Natriuretic peptide receptor C    | Natriuretic peptide receptor activity                                             | —                                     |
| PGC        | Pepsinogen C                      | Aspartic-type endopeptidase activity; proteolysis                                  | Peptidase A1 family                  |
| SLC5A8     | Sodium-coupled monocarboxylate transporter 1 | Sodium ion transport; symporter activity; transmembrane transport                  | Sodium:solute symporter (SSF) (TC 2. A.21) family |
| UBD        | Ubiquitin D                       | Positive regulation of I-kappab kinase/NF-kappab cascade; positive regulation of apoptotic process; proteasome binding; protein ubiquitination | —                                     |

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were consistent between the analyses. Quantitative difference was greater in qRT-PCR than in microarray analysis. The expression levels of the target genes in NF3-treated HUVEC versus control HUVEC at 6 and 16 h were listed in Tables 5 and 6, respectively.

### Protein expression of the target genes by western blotting

Western blotting of the protein expression was conducted so as to confirm the differentially expressed gene targets revealed from microarray data. Since a panel of different sets of genes was believed to be involved in HUVEC after NF3 treatment for 6 and 16 h, two representative proteins at each time point were selected for further confirmation. The selection criteria of the proteins from these sets of genes were based on the role of the genes and the availability of antibody for western blotting. CXCR7 and MMP8 were used at 6 h while NR1H4 and SCG2 were examined at 16 h. As shown in Fig 4, NF3 caused a strong induction of the protein expression of CXCR7 in HUVEC associated with an increase in the protein expression of MMP8 and NR1H4 in HUVEC. A mild increase in the protein expression of SCG2 was observed in NF3-treated HUVEC. The results demonstrated that both genes and proteins were tightly involved and regulated in HUVEC after NF3 treatment, promoting the overall wound healing angiogenesis.

### Discussion

The individual herb AR and RR had been historically used in diabetic foot ulcer healing. Increasing studies provided concrete scientific proof for the diabetic wound healing efficacy and pro-angiogenic capacity of formula NF3 [5–9].
The merit of Traditional Chinese Medicine (TCM) was its holistic pharmaceutical characteristic with multi-target approach, improving the whole biological system and making TCM as promising alternative therapies in different diseases. Substantial \textit{in vitro} and clinical studies demonstrated the involvement of diverse signaling pathways of NF3 involved in diabetic

### Table 5. \textit{qRT-PCR} analysis of the relative gene expression level of NF3-treated HUVEC versus control HUVEC at 6 h. RNA from new preparation of HUVEC samples were used for confirmation of microarray analysis.

|       | NF3  | Control | P-value |
|-------|------|---------|---------|
| ABCG8 | 1.61 | 1.42    | 0.003   |
| CXCR7 | 1.93 | 0.86    | 0.039   |
| DNAJB7| 0.82 | 0.66    | 0.044   |
| EPCAM | 1.43 | 1.18    | 0.027   |
| GUCY2C| 2.62 | 2.16    | 0.216   |
| HAND1 | 0.74 | 0.48    | 0.043   |
| HLA-DQB1| 0.83| 0.70    | 0.235   |
| INSL4 | 0.44 | 0.64    | 0.013   |
| MAP3K14| 0.56| 0.67    | 0.014   |
| MMPB8 | 0.76 | 0.45    | 0.028   |
| NPR3  | 1.21 | 0.80    | 0.033   |
| PGC   | 0.79 | 0.87    | 0.191   |
| SLC5A8| 0.46 | 0.30    | 0.005   |
| UBD   | 0.35 | 0.52    | 0.016   |

Values are means ± SD from three individual experiments.

### Table 6. \textit{qRT-PCR} analysis of the relative gene expression level of NF3-treated HUVEC versus control HUVEC at 16 h. RNA from new preparation of HUVEC samples were used for confirmation of microarray analysis.

|       | NF3  | Control | P-value |
|-------|------|---------|---------|
| ABCG8 | 1.61 | 1.42    | 0.003   |
| EPB41 | 1.35 | 0.74    | 0.001   |
| GRIN1 | 1.20 | 2.65    | 0.036   |
| GYPB  | 1.70 | 0.98    | 0.078   |
| IL1RAPL2| 0.80| 1.44    | <0.001  |
| LCN1  | 2.46 | 1.32    | 0.027   |
| LRFN2 | 2.48 | 1.87    | 0.107   |
| MIR28 | 1.08 | 1.86    | 0.019   |
| NR1H4 | 0.79 | 0.41    | 0.032   |
| PRL   | 2.52 | 1.18    | 0.004   |
| SCG2  | 0.74 | 0.43    | 0.002   |
| TFAP2A| 1.02 | 0.47    | 0.006   |
| TTC39B| 1.48 | 1.32    | 0.296   |
| ZNF711| 1.45 | 1.62    | 0.387   |

Values are means ± SD from three individual experiments.

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wound healing. Zhang et al. (2012) showed that NF3 triggered a panel of genes in human skin fibroblast involved in various signaling pathways in wound healing [12]. Ko et al. (2014) further showed that NF3 was effective in healing of diabetic foot ulcer patients associated with the global changes in gene expression [13]. We therefore applied microarray technology to screen out the regulatory genes in endothelial cells with NF3 intervention in relation to angiogenesis and diabetic wound healing.

Endothelial cells activation followed by diverse intracellular signaling pathways of proliferation, adhesion, migration, tubular formation, release of growth factors and matrix metalloproteinases enabled proper angiogenesis in wound healing process. However, in diabetic ulcers, the angiogenesis was hampered by persistent hyperglycemic condition, causing destructive effects on cellular and molecular levels in endothelial cells.

In the present study, we investigated the effect of NF3 treatment on global gene expression of HUVEC governing angiogenesis in wound healing after 6 h and 16 h. From the microarray analysis associated with qRT-PCR and western blotting verification, target genes (BIRC5, CXCR7, DNAJB7, EPCAM, HAND1, MMP8, NPR3 and SLC5A8) were significantly up-regulated, while target genes (INSL4, MAP3K14 and UBD) were significantly down-regulated in NF3-treated HUVEC when compared to that of control HUVEC at 6 h. BIRC5 gene encoded the protein survivin, which regulated the apoptosis pathway. BIRC5 was crucial for optimal angiogenesis after brain injury in VEGF-treated HUVEC [14]. CXCR7, a member of CXC chemokine receptor family, possessed high binding affinity to chemokine stromal derived factor-1α (SDF-1α). Up-regulation of CXCR7 was vitally important in angiogenesis, stimulating the production of interleukin-8 (IL-8) and VEGF [15–17]. The protein of DNAJB7 gene belonged to the DNAJ/HSP40 family, which regulated molecular chaperone activity [18]. EPCAM mediated cell-cell adhesion, migration, proliferation and differentiation. EPCAM was asserted to possess oncogenic potential and up-regulation of EPCAM induced angiogenesis [19–21]. The protein encoded by HAND1 was classified into the basic helix-loop-helix family of transcription factors. Recent finding identified that thymosin β4 was an in vivo downstream target of HAND1, which was crucial for cell migration and angiogenesis as well as yolk sac vasculogenesis [22, 23]. The functional involvement of MMP8 (matrix metalloproteinase 8) in angiogenesis and tissue remodeling was extensively investigated [24, 25]. Up-regulation of NPR3 (natriuretic peptide receptor 3) activated ECs proliferation and meanwhile inhibited vascular smooth muscle cells (VSMCs) growth [26, 27]. SLC5A8 was identified as one of the tumor suppressor genes [28, 29]. For those down-regulated genes, INSL4 (insulin-like 4 protein) was the member of insulin superfamily. It was suggested that down-regulation of INSL4 might contribute to the cell survival and anti-apoptotic ability [30, 31]. MAP3K14 (mitogen-activated...
protein kinase kinase kinase 14) was a serine/threonine protein-kinase. Up-regulation of MAP3K14 had been linked to the persistent inflammatory response in diabetes \[32, 33\]. UBD was one of the components in the ubiquitin-proteasome system (UPS). It had been found that inhibition of UPS up-regulated endothelial nitric oxide synthase (eNOS) expression and activity, thus improving the endothelial function \[34, 35\]. By summarizing the biological function among the differentially expressed genes in NF3-treated HUVEC at 6 h, their respective roles were postulated, suggesting their beneficial effects on diverse mechanisms related to migration, tube formation, proliferation, angiogenesis as well as anti-inflammation in HUVEC (Fig 5).

As demonstrated by the gene expression results of NF3-treated HUVEC versus control HUVEC at 16 h, genes (EPB41, LCN1, NR1H4, PRL, SCG2 and TFAP2A) were significantly up-regulated while genes (GRIN1, IL1RAPL2 and miR28) were significantly down-regulated. Among those up-regulated genes of NF3-treated HUVEC at 16 h, protein 4.1 encoded by EPB41 gene was required for the regulation of channel activation and ion permeation for signaling transduction in ECs \[36, 37\]. LCN1 gene encoded a member of the lipocalin family of small secretory proteins. LCN1 was devoted to the cellular defense against oxidative stress \[38\]. NR1H4 (protein farnesoid X receptor (FXR)) was a member of the nuclear receptor superfamily. FXR was confirmed to induce eNOS expression at transcriptional level since up-regulation and subsequent NO production \[39\]. FXR activation in turn down-regulated inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which were the pro-inflammatory mediators. FXR signaling pathway played a pivotal role in vascular anti-inflammation and remodeling \[40\]. Substantial reports demonstrated the advantageous role of PRL (prolactin) in angiogenesis \[41–43\]. SCG2 gene encoded the protein secretogranin, which was converted to chemotactic active secretoneurin. Secretoneurin was involved in inflammation and wound healing \[44\]. Secretoneurin significantly induced the chemotaxis ability of ECs, confirming the regulatory role of secretoneurin in vascular cells migration \[45\]. The protein named transcription factor activator protein-2A (AP-2A) was encoded by TFAP2A gene, which was involved in transcriptional regulation and cell proliferation \[46, 47\]. For those down-regulated genes, study revealed that GRIN1 (N-methyl-D-aspartate receptor (NMDAR)) activation in human cerebral ECs promoted intracellular oxidative stress \[48\]. IL1RAPL2 (interleukin-1 receptor accessory protein-like 2) was the member of interleukin-1 receptor (IL-1R) family for signaling transduction of pro-inflammatory cytokines within cells \[49\]. miR28 was a member of the small non-coding RNAs called microRNAs (miRNAs). Elevation of endothelial miR28 impaired angiogenesis in diabetic patients \[50\]. Nrf2 regulated the expression of detoxifying enzymes, protecting cells towards tumorogenesis. Up-regulation of miR28 inhibited Nrf2 mRNA and protein expression, leading to breast cancer progression \[51\]. Besides, miR28 inhibited the translation of thrombopoietin receptor (TPOR). Activation of TPOR in HUVEC demonstrated enhanced cell motility through synthesis of 1-alkyl-/1-acyl-platelet-activating factor (PAF), IL-8, and phosphorylation of STAT1 and STAT5B \[52, 53\]. From numerous literature support, those differentially expressed genes at 16 h were proposed to devote pivotal role in wound healing angiogenesis, governing the diverse signaling pathways of transcriptional and translational regulation, anti-tumor activity, anti-oxidant activity, angiogenesis and anti-inflammation in NF3-treated HUVEC (Fig 6).

In summary, the molecular gene targets were identified in NF3-treated HUVEC and their expression was further confirmed by qRT-PCR. Different panels of genes were differentially expressed and strictly governed in NF3-treated HUVEC in a time-regulated manner. In the investigation of two time points, we showed that NF3 regulated different set of genes in HUVEC to achieve various biological processes such as ECs proliferation, migration, tube formation, transcriptional and translational regulation, anti-inflammation and anti-oxidation. This might explain the underlying mechanism of NF3-induced angiogenesis of endothelial...
cells involved in wound healing. Interestingly, the results revealed that different sets of genes were differentially expressed and regulated in HUVEC by NF3 in two time points. In general, there was a strong correlation between time series and gene expression in every cell. We had performed time series analysis of NF3-treated HUVEC in tube formation assay from 4 to 8 h and migration assay from 16 to 20 h (data not shown). NF3 significantly stimulated the tube formation.
formation and migration ability of HUVEC in the tested time points. In line with this, it was extremely important to choose the most appropriate time points for evaluating the global gene expression pattern changes in HUVEC after NF3 treatment. The tubular formation at 6 h and migration at 16 h of NF3 on HUVEC were shown to have profound significant pro-angiogenic effects [5]. Therefore 6 h and 16 h incubation of NF3 in HUVEC were selected for microarray analysis.

With reference to the assessment of molecular targets of HUVEC after NF3 treatment by microarray technology, we constructed novel hypothetical diagrams to illustrate the regulatory mechanism and signaling pathways of NF3 on endothelial cells involved in wound healing angiogenesis at transcriptional levels. The identified gene and targets enabled us to thoroughly understand the therapeutic efficacies of NF3 in diabetic wound healing angiogenesis. This study provided valuable genetic database with support of scientific evidences for microarray research using TCM on endothelial cells in different diseases.

Supporting Information
S1 Table. Differentially expressed genes in NF3-treated HUVEC versus control at 6 h from microarray analysis.
(DOCX)

S2 Table. Differentially expressed genes in NF3-treated HUVEC versus control at 16 h from microarray analysis.
(DOCX)

S1 Raw Data.
(XLSX)

S2 Raw Data.
(XLSX)

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Author Contributions
Conceived and designed the experiments: JCWT CHK CMK CPL CBSL. Performed the experiments: JCWT CHK CMK CPL ZC WHL. Analyzed the data: JCWT CHK. Contributed reagents/materials/analysis tools: JCWT PCL KPF WYC. Wrote the paper: JCWT WYC CBSL.

References
1. Madeddu P. (2005) Therapeutic angiogenesis and vasculogenesis for tissue regeneration. Exp Physiol 90: 315–326. PMID: 15778410
2. Adams RH, Alitalo K. (2007) Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol 8: 464–478. PMID: 17522591
3. Martin A, Komada MK, Sane DC. (2003) Abnormal angiogenesis in diabetes mellitus. Med Res Rev 23: 117–145. PMID: 12500286
4. Wong MW, Leung PC, Wong WC. (2001) Limb salvage in extensive diabetic foot ulceration—a preliminary clinical study using simple debridement and herbal drinks. Hong Kong Med J 7: 403–407. PMID: 11773675

5. Tam JC, Lau KM, Liu CL, To MH, Kwok HF, Lai KK, et al. (2011) The in vivo and in vitro diabetic wound healing effects of a 2-herb formula and its mechanisms of action. J Ethnopharmacol 134: 831–838. doi: 10.1016/j.jep.2011.01.032 PMID: 21291991

6. Liu CL, Tam JC, Sanders AJ, Ko CH, Fung KP, Leung PC, et al. (2013) Molecular angiogenic events of a two-herb wound healing formula involving MAPK and akt signaling pathways in human vascular endothelial cells. Wound Repair Regen 21: 579–587. doi: 10.1111/wrr.12055 PMID: 23755905

7. Tam JC, Ko CH, Lau KM, To MH, Kwok HF, Chan WY, et al. (2014) A chinese 2-herb formula (NF3) promotes hindlimb ischemia-induced neovascularization and wound healing of diabetic rats. J Diabetes Complications 28: 436–447. doi:10.1016/j.jdiacomp.2014.03.004 PMID: 24731763

8. Tam JC, Ko CH, Zhang C, Wang H, Lau CP, Chan WY, et al. (2014) Comprehensive proteomic analysis of a chinese 2-herb formula (astragali radix and rehmanniae radix) on mature endothelial cells. Proteomics 14: 2089–2103. doi:10.1002/pmic.201300547 PMID: 25044676

9. Tam JC, Ko CH, Lau KM, To MH, Kwok HF, Siu WS, et al. (2014) Enumeration and functional investigation of endothelial progenitor cells in neovascularization of diabetic foot ulcer rats with a chinese 2-herb formula. J Diabetes.

10. State Pharmacopoeia Commission of P.R. China. China Medico-Pharmaceutical Science & Technology Publishing House: Beijing 2010.

11. Or PM, Lam FF, Kwan YW, Cho CH, Lau CP, Yu H, et al. (2012) Effects of radix astragali and radix rehmanniae, the components of an anti-diabetic foot ulcer herbal formula, on metabolism of model CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 probe substrates in pooled human liver microsomes and specific CYP isoforms. Phytomedicine 19: 535–544. doi:10.1016/j.phymed.2011.12.005 PMID: 22261394

12. Zhang Q, Fong CC, Yu WK, Chen Y, Wei F, Koon CM, et al. (2012) Herbal formula astragali radix and rehmanniae radix exerted wound healing effect on human skin fibroblast cell line Hs27 via the activation of transformation growth factor (TG5-beta) pathway and promoting extracellular matrix (ECM) deposition. Phytomedicine 20: 9–16. doi: 10.1016/j.phymed.2012.09.006 PMID: 23083814

13. Ko CH, Yi S, Ozaki R, Cochrane H, Chung H, Lau W, et al. (2014) Healing effect of a two-herb recipe (NF3) on foot ulcers in chinese patients with diabetes: A randomized double-blind placebo-controlled study. J Diabetes 6: 323–334. doi: 10.1111/1753-0407.12117 PMID: 24330156

14. Conway EM, Zwerts F, Van Eygen V, DeVriese A, Nagai N, Luo W, et al. (2003) Survivin-dependent angiogenesis in ischemic brain: Molecular mechanisms of hypoxia-induced up-regulation. Am J Pathol 163: 935–946. PMID: 12937134

15. Wang J, Shiozawa Y, Wang J, Wang Y, Jung Y, Pienta KJ, et al. (2008) The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer. J Biol Chem 283: 4283–4294. PMID: 18057003

16. Zheng K, Li HY, Su XL, Wang XY, Tian T, Ren GS. (2010) Chemokine receptor CXCR7 regulates the invasion, angiogenesis and tumor growth of human hepatocellular carcinoma cells. J Exp Clin Cancer Res 29: 31. doi: 10.1186/1756-9966-29-31 PMID: 20980740

17. Yan X, Cai S, Xiong X, Sun W, Dai X, Chen S, et al. (2012) Chemokine receptor CXCR7 mediates human endothelial progenitor cells survival, angiogenesis, but not proliferation. J Cell Biochem 113: 1437–1446. doi: 10.1002/jcb.24015 PMID: 22173725

18. Qiu XB, Shao YM, Miao S, Wang L. (2006) The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. Cell Mol Life Sci 63: 2560–2570. PMID: 16952052

19. Osta WA, Chen Y, Mikhitarian K, Mitas M, Salem M, Hannun YA, et al. (2004) EpCAM is overexpressed in breast cancer and is a potential target for breast cancer gene therapy. Cancer Res 64: 5818–5824. PMID: 15313925

20. Shao YM, Huang YL, Xie YK, Tan YH, Chen BC, Zhou MT, et al. (2011) Angiogenesis and clinicopathologic characteristics in different hepatocellular carcinoma subtypes defined by EpCAM and alpha-feto-protein expression status. Med Oncol 28: 1012–1016. doi: 10.1007/s12032-010-9600-6 PMID: 20571836

21. Sankpal NV, Fleming TP, Gillanders WE. (2013) EpCAM modulates NF-kappaB signaling and interleukin-8 expression in breast cancer. Mol Cancer Res 11: 418–426. doi: 10.1158/1541-7786.MCR-12-0518 PMID: 23378678

22. Morikawa Y, Cserjesi P. (2004) Extra-embryonic vasculature development is regulated by the transcription factor HAND1. Development 131: 2195–2204. PMID: 15073150
23. Smart N, Dube KN, Riley PR. (2010) Identification of thymosin beta4 as an effector of Hand1-mediated vascular development. Nat Commun 1: 46. doi: 10.1038/ncomms1041 PMID: 20975697

24. Fang C, Wen G, Zhang L, Lin L, Moore A, Wu S, et al. (2013) An important role of matrix metalloproteinase-8 in angiogenesis in vitro and in vivo. Cardiovasc Res 99: 146–155. doi: 10.1093/cvr/cvt060 PMID: 23512982

25. Xiao Q, Zhang F, Lin L, Fang C, Wen G, Tsai TN, et al. (2013) Functional role of matrix metalloproteinase-8 in stem/progenitor cell migration and their recruitment into atherosclerotic lesions. Circ Res 112: 35–47. doi: 10.1161/CIRCRESAHA.112.274019 PMID: 23071158

26. Matsukawa N, Grzesik WJ, Takahashi N, Pandey KN, Pang S, Yamauchi M, et al. (1999) The natriuretic peptide clearance receptor locally modulates the physiological effects of the natriuretic peptide. Proc Natl Acad Sci U S A 96: 7403–7408. PMID: 10377427

27. Khandbhar RS, Panayiotou CM, Hobbs AJ. (2011) Natriuretic peptide receptor-3 underpins the dispatrate regulation of endothelial and vascular smooth muscle cell proliferation by C-type natriuretic peptide. Br J Pharmacol 164: 584–597. doi: 10.1111/j.1476-5381.2011.01400.x PMID: 21457229

28. Miyamura S, Gopal E, Fei YJ, Ganapathy V. (2004) Functional identification of SLC5A8, a tumor suppressor down-regulated in colon cancer, as a Na(+)-coupled transporter for short-chain fatty acids. J Biol Chem 279: 13293–13296. PMID: 14966140

29. Drummond DC, Noble KO, Kirpotin DB, Guo Z, Scott GK, Benz CC. (2005) Clinical development of histone deacetylase inhibitors as anticancer agents. Annu Rev Pharmacol Toxicol 45: 495–528. PMID: 15822269

30. Laurent A, Rouillac C, Delezoide AL, Giovangrandi Y, Vekemans M, Bellet D, et al. (1998) Insulin-like 4 (INSL4) gene expression in human embryonic and trophoblastic tissues. Mol Reprod Dev 51: 123–129. PMID: 9740319

31. Millar L, Streiner N, Webster L, Yamamoto S, Okabe R, Kawamata T, et al. (2005) Early placental insulin-like protein (INSL4 or EPIL) in placental and fetal membrane growth. Biol Reprod 73: 695–702. PMID: 15958731

32. Liu J, Sudom A, Min X, Cao Z, Gao X, Ayres M, et al. (2012) Structure of the nuclear factor kappaB-inducing kinase (NIK) kinase domain reveals a constitutively active conformation. J Biol Chem 287: 27326–27334. doi: 10.1074/jbc.M112.366658 PMID: 22718757

33. Donath MY, Shoelson SE. (2011) Type 2 diabetes as an inflammatory disease. Nat Rev Immunol 11: 98–107. doi: 10.1038/nri2925 PMID: 21233852

34. Stangl V, Lorenz M, Meiners S, Ludwig A, Bartsch C, Moobed M, et al. (2004) Long-term up-regulation of eNOS and improvement of endothelial function by inhibition of the ubiquitin-proteasome pathway. FASEB J 18: 272–279. PMID: 14769821

35. Stangl K, Stangl V. (2010) The ubiquitin-proteasome pathway and endothelial (dys)function. Cardiovasc Res 85: 281–290. doi: 10.1093/cvr/cvp315 PMID: 19762793

36. Wu S, Sangerman J, Li M, Brough GH, Goodman SR, Stevens T. (2001) Essential control of an endothelial cell ISOC by the spectrin membrane skeleton. J Cell Biol 154: 1225–1233. doi: 10.1083/jcb.154.5.1225 PMID: 11564759

37. Cioffi DL, Wu S, Alexeyev M, Goodman SR, Zhu MX, Stevens T. (2005) Activation of the endothelial store-operated ISOC Ca2+ channel requires interaction of protein 4.1 with TRPC4. Circ Res 97: 1164–1172. PMID: 16254212

38. Lechner M, Wojnar P, Redl B. (2001) Human tear lipocalin acts as an oxidative-stress-induced scavenger of potentially harmful lipid peroxidation products in a cell culture system. Biochem J 356: 129–135. PMID: 11336644

39. Li J, Wilson A, Kuruba R, Zhang Q, Gao X, He F, et al. (2008) FXR-mediated regulation of eNOS expression in vascular endothelial cells. Cardiovasc Res 77: 169–177. PMID: 18006476

40. Li YT, Swales KE, Thomas GJ, Warmer TD, Bishop-Bailey D. (2007) Farnesoid x receptor ligands inhibit vascular smooth muscle cell inflammation and migration. Arterioscler Thromb Vas Biol 27: 2606–2611. PMID: 18029909

41. Castilla A, Garcia C, Cruz-Soto M, Martinez de la Escalera G, Thebault S, Clapp C. (2010) Prolactin in ovarian follicular fluid stimulates endothelial cell proliferation. J Vasc Res 47: 45–53. doi: 10.1159/000231720 PMID: 19672107

42. Reuwer AQ, Nowak-Sliwinska P, Mans LA, van der Loos CM, von der Thuesen JH, Twickler MT, et al. (2012) Functional consequences of prolactin signalling in endothelial cells: A potential link with angiogenesis in pathophysiology? J Cell Mol Med 16: 2035–2048. doi: 10.1111/j.1582-4934.2011.01499.x PMID: 22128761

43. Yang X, Meyer K, Friedl A. (2013) STAT5 and prolactin participate in a positive autocrine feedback loop that promotes angiogenesis. J Biol Chem 288: 21184–21196. doi: 10.1074/jbc.M113.481119 PMID: 23729680
44. Kahler CM, Bellmann R, Reinisch N, Schratzberger P, Gruber B, et al. (1996) Stimulation of human skin fibroblast migration by the neuropeptide secretoneurin. Eur J Pharmacol 304: 135–139. PMID: 8813595

45. Kahler CM, Kirchmair R, Kaufmann G, Kahler ST, Reinisch N, Fischer-Colbrie R, et al. (1997) Inhibition of proliferation and stimulation of migration of endothelial cells by secretoneurin in vitro. Arterioscler Thromb Vasc Biol 17: 932–939. PMID: 9157958

46. Hilger-Eversheim K, Moser M, Schorle H, Buettner R. (2000) Regulatory roles of AP-2 transcription factors in vertebrate development, apoptosis and cell-cycle control. Gene 260: 1–12. PMID: 11137286

47. Ding X, Yang Z, Zhou F, Wang F, Li X, Hu X, et al. (2013) Transcription factor AP-2alpha regulates acute myeloid leukemia cell proliferation by influencing hoxa gene expression. Int J Biochem Cell Biol 45: 1647–1656. doi:10.1016/j.biocel.2013.04.024 PMID: 23660297

48. Sharp CD, Houghton J, Elrod JW, Warren A, Jackson TH, Jawahar A, et al. (2005) N-methyl-D-aspartate receptor activation in human cerebral endothelium promotes intracellular oxidant stress. Am J Physiol Heart Circ Physiol 288: H1893–H1899. PMID: 15576430

49. Born TL, Smith DE, Garka KE, Renshaw BR, Bertles JS, Sims JE. (2000) Identification and characterization of two members of a novel class of the interleukin-1 receptor (IL-1R) family. Delineation of a new class of IL-1R-related proteins based on signaling. J Biol Chem 275: 29946–29954. PMID: 10882729

50. Zampetaki A, Kiechl S, Drozdzov I, Willeit P, Mayr U, Prokopi M, et al. (2010) Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res 107: 810–817. doi: 10.1161/CIRCRESAHA.110.226357 PMID: 20651284

51. Yang M, Yao Y, Eades G, Zhang Y, Zhou Q. (2011) MiR-28 regulates Nrf2 expression through a Keap1-independent mechanism. Breast Cancer Res Treat 129: 983–991. doi: 10.1007/s10549-011-1604-1 PMID: 21638050

52. Girardot M, Pecquet C, Boukour S, Knoops L, Ferrant A, Vainchenker W, et al. (2010) miR-28 is a thrombopoietin receptor targeting microRNA detected in a fraction of myeloproliferative neoplasm patient platelets. Blood 116: 437–445. doi: 10.1182/blood-2008-06-165985 PMID: 20445018

53. Brizzi MF, Battaglia E, Montrucchio G, Dentelli P, Del Sorbo L, Garbarino G, et al. (1999) Thrombopoietin stimulates endothelial cell motility and neoangiogenesis by a platelet-activating factor-dependent mechanism. Circ Res 84: 785–796. PMID: 10205146