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The up-regulation of two identified wound healing specific proteins-HSP70 and lysozyme in regenerated *Eisenia fetida* through transcriptome analysis

Yuwei Yang, Yujie Sun, Na Zhang, Jianhao Li, Chenning Zhang, Xiaojie Duan, Yuting Ding, Renyun Zhao, Zhuhong Zheng, Di Geng^{*,*}, Yikun Sun^{*}

School of Chinese Material Medica, Beijing University of Chinese Medicine, Yangguang South Road, Fangshan District, Beijing, China

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ABSTRACT

Ethnopharmacological relevance: Disposed earthworm has been used to treat various common ailments including burns, arthritis, itching, and inflammation for thousands of years in China. As their remarkable ability to fully regenerate in a scar-free manner, regenerated tissue homogenate of amputated *Eisenia fetida* (*E. fetida*) have been considered as an excellent wound repair therapy in our previous study. We have demonstrated that regenerated earthworm (G-90') can perform higher wound repair ability to non-regeneration tissue (G-90) through significant promotion of cutaneous wound repair in mice after their administration into wound beds.

Objective: In the present study, we aimed to reveal the mechanism of G-90' and to explore a potential wound healing accelerated strategy.

Methods and results: Two functional proteins- HSP70 and lysozyme in G-90' were confirmed by cross-identification of LC-MS/MS and transcriptome analyses. Followed with semi-quantitative PCR and western blot, their expression were validated to up-regulate in 3-day regenerated tissues (G-90').

Conclusion: This study implies the therapeutic potency of G-90' for wound recovery and provides a new strategy to assess other natural materials targeting wound healing with the tail-amputated *E. fetida* as a model organism.

1. Introduction

Wound problems cause severe morbidity and mortality worldwide with the deficiency of structural integrity of skin and accompanying homeostasis disorder (Gurtner et al., 2008; Powers et al., 2016). However, current treatment therapies based on growth factors and cytokines hold multifarious disadvantages including complicated purification and expression process, enormous cost, burst release at wound area and deleterious side effects (Dreifke et al., 2015). Due to the increasing incidence and enormous financial burden of treating wounds, the exploration of effective remedies is urgent recently.

Development of potential therapeutic drugs from natural materials to accelerate wound healing will be useful for treating wounds (Jaric et al., 2018; Agyare et al., 2016). Natural resources provide cost-effective, accessible and reliable medical substances. For instance, in ancient China, disposed earthworms are relied on as home remedies to treat burns, arthritis, itching and inflammation (Zhenjun et al., 1997). Modern researches also revealed the glycolipoprotein extract from *E. fetida*, G-90, stimulates cell proliferation and migration promoting

wound recovery (Grdisa et al., 2004). Furthermore, accelerated wound healing process was observed in 3-day post-amputated regeneration section of *E. fetida* (G-90') compared to G-90 in our previous study (Yang et al., 2017). However, the mechanism contributes to augment therapeutic effect in G-90' to G-90 on wound repair is not clear. It was hypothesized as up-regulated expression of specific functional proteins in regeneration process of amputated *E. fetida*. Thus, in the present study, we aimed to elucidate up-regulated materials in regenerated earthworm tissue through several analytic methods. The experimental design is presented in Fig. 1.

To primarily identify wound healing therapeutic proteins, the most functional fraction in G-90' was separated as the previously determined method with prominent fibroblast proliferative property. In addition to functional protein identification, transcriptome analysis was performed to discover differentially expressed genes (DEGs) between 3-day regenerated (G-90') and 0-day regenerated (G-90) tissue homogenate from tail-amputated *E. fetida*. RNA-Sequencing (RNA-Seq) is a highly sensitive and accurate approach for analysing the whole-transcriptome by using the next generation sequencing technology (Dundar et al.,

* Corresponding author.

** Corresponding author.

E-mail addresses: piece124@sohu.com (D. Geng), sunyk@bucm.edu.cn (Y. Sun).

Fig. 1. Experimental design.

2014b, 2014a; Yamada et al., 2018). Due to its incomparable advantages in broader dynamic range, not limitation by prior knowledge and compatibility to all species, RNA-Seq is capable of identifying previously undetected genes even without the reference genome sequences (Thunders et al., 2017). In this study, we performed RNA-Seq analysis in the absence of a reference genome to discover DEGs in regenerated and non-regenerated *E. fetida*. Identified functional proteins combined with RNA-Seq analysis in G-90 and G-90', two differentially expressed functional proteins- HSP70 and lysozyme were considered up-regulation after regeneration process. Therefore, their up-regulated expression was validated through semi-quantitative Polymerase Chain Reaction (semi-q PCR) and western blot analysis.

In our own previous study, we successfully isolated bioactive components from G-90', and further elucidated their wound repair ability represented by superior proliferation potential to fibroblasts and keratinocytes as well as cutaneous incision wound model (Yang et al., 2017). The results indicate that bioactive compounds in G-90' might be a promising natural source for wound healing therapeutics. To elucidate the underlying mechanism for increased healing ability after regeneration and to further exploit effective and pleiotropic proteins in *E. fetida*, we performed LC-MS/MS and RNA-Seq analyses to specifically identify two differentially expressed proteins followed with validation through semi-q PCR and western blot approaches.

2. Materials & methods

2.1. Identification of functional proteins in 3-day regenerated tissue of *E. fetida*

2.1.1. Functional protein extract from G-90'

G-90' is the protein extract from regenerated tissue of earthworm (*Eisenia fetida*) after tail-amputation for 3 days, while G-90 is extracted from 0-day regenerated earthworm body. They were prepared as our previous study (Yang et al., 2017). Proteins in G-90' were initially fractionated by their solubility. Three fractionating proteins, named

ES1, ES2 and ES3, were precipitated by saturated ammonium sulfate solution $(\text{NH}_4)_2\text{SO}_4$, pH 7.2 in the concentration of 40%, 40%–60% and 60%–80% and de-salted in deionized water for another 2 days at 4 °C.

2.1.2. NIH 3T3 cell proliferation assay

NIH 3T3 cell line was obtained from Peking Union Medical College Cell Resource Centre and grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco (U.S.A.) with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin Biodee biotechnology Co., Ltd. (Beijing, R.P. China). Cells were seeded 100 μL at a density of 5000 cells/well in 96-well plates and cultured for 24 h in humidified 5% CO_2 incubator at 37 °C. Cultured cells were treated with 10 μL different groups for another 48 h: basic fibroblast growth factor (bFGF) PeproTech Inc. (0.1 $\mu\text{g}/\text{mL}$) as positive group, ES1, ES2, and ES3 in the concentration of 2, 4, 6, 8, 10, and 12 $\mu\text{g}/\text{mL}$, and negative group treated with DMEM in the same volume. Cell viability was determined using Cell Counting Kit-8 (CCK-8) Dojindo CO., Ltd (Japan). Optical density (OD) was determined at 450 nm by SPECTROstar^{Nano}, BMG LABTECH GmbH (Germany). Cell viability assay was conducted at three independent times.

2.1.3. Purification of functional proteins in ES2

As ES2 showed most vigorous proliferation ability to fibroblast (NIH 3T3) (Fig. 1), this protein fraction was further separated on anion exchange chromatography packed with DEAE Sepharose Fast Flow (GE Healthcare, USA) as previously illustrated (Yang et al., 2017). Most effective eluate in this process was determined by NIH 3T3 cell viability assay with the protein concentration of 0.5, 1, 1.5, 2, 2.5, and 3 $\mu\text{g}/\text{mL}$.

2.1.4. Identification of functional proteins through NANO LC-MS/MS analysis

Proteins (100 μg) were digested with 15 ng/ μL trypsin (diluted by 25 mM ammonium bicarbonate). The digestion was performed at 4 °C for 40 min and 37 °C overnight. Peptides were extracted in 50%

acetonitrile with 45% Milli-Q water and 5% trifluoroacetic acid at 37 °C for 1 h, centrifuged for 5 min and the supernatant were lyophilized. Dried peptides were suspended in 50 µL of 0.1% formic acid before nanoscale liquid chromatography-tandem MS (LC-MS/MS) analysis.

Tryptic peptides were analyzed on a 100 µm × 10 cm in-house made column packed with a reversed-phase ReproSil-Pur C18-AQ resin (3 µm, 120 Å, Dr. Maisch GmbH, Germany) using Easy-nLC1000 (ThermoFisher Scientific, U.S.A.) and a flow rate of 300 nL/min with loaded sample volume of 5 µL. The solvent system was 0.1% formic acid in Milli-Q water (solvent A) and 0.1% formic acid in 80% acetonitrile (solvent B). The elution was conducted in a gradient: 5% B for 5 min, 5%–25% B for 20 min, 50%–90% B for 5 min, 90% B for 5 min and 5% B for 10 min.

Q-Exactive mass spectrometry (ThermoFisher Scientific, U.S.A.) was used to analyze peptide fractions from the UPLC system with spray voltage of 2.2 kV and capillary temperature of 270 °C. MS spectra were recorded with resolution of 70,000 and scan range of m/z 350.0–1800.0 and MS/MS spectra were recorded with resolution of 17,500.

MS/MS spectra were interpreted by UniProt *E. fetida* database (<http://www.uniprot.org/>) to identify proteins. The searching parameters were set as follows: the fixed modification of carbamidomethyl (C) and variable modification of oxidation (M), two missing cleavage sites, a mass tolerance of 20 p.p.m. for peptide tolerance, 0.6 Da for MS/MS tolerance. Only high confident identified peptides were chosen for downstream protein identification analysis.

2.2. Identification of DEGs between G-90 and G-90' through transcriptome analysis

2.2.1. RNA isolation

Total RNA was isolated from previously flash-frozen 3-day regenerated tissue (G-90') and 0-day regenerated tissue (G-90) of amputated *E. fetida* respectively through RNAeasy™ Plus Animal RNA Isolation Kit with Spin Column (Beyotime Institute of Biotechnology, China). RNA quality was evaluated by RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, U.S.A.) and quantified by Qubit® RNA Assay Kit in Qubit®2.0 Fluorometer (Life Technologies, CA, U.S.A.).

2.2.2. RNA-Seq

Sequencing libraries were generated using NEBNext®Ultra™ RNA Library Prep Kit for Illumina® (NEB, U.S.A.) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Library quality was assessed on the Agilent Bioanalyzer 2100 system and quantified by quantitative PCR (qPCR). Cluster generation was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina). Libraries were sequenced on an Illumina HiSeq 2000 platform and paired-end reads were generated.

2.2.3. RNA-Seq data processing

Raw data in FASTQ files were trimmed to remove reads containing adapter, ploy-N and low quality reads. Obtained clean data were calculated with quality score (Q20, Q30), GC-content and their sequence duplication level. Transcriptome assembly was accomplished to mapped reads based on clean data with high quality using Trinity version 2.6.5 by the default parameters (Grabherr et al., 2011).

2.2.4. Bioinformatics analysis

Assembled unigenes were annotated in several databases including NR (NCBI non-redundant protein sequences), Pfam (Protein family), KOG/COG/eggNOG (Clusters of Orthologous Groups of proteins), Swiss-Prot (A manually annotated and reviewed protein sequence database), KEGG (Kyoto Encyclopedia of Genes and Genomes), and GO (Gene Ontology). Differential gene expression analysis was analyzed by edgeR to discover DEGs in G-90 and G-90' RNA-Seq dataset (Robinson

et al., 2010). Read counts were adjusted by edgeR program package through one scaling normalized factor. Pearson's correlation's test between G-90 and G-90' was performed and ranked. Differential expression analysis of two samples was performed using the DESeq R package (false discovery rate < 0.01 and fold change ≥ 2) (Leng et al., 2013). GO enrichment analysis of the DEGs was implemented by the topGO R packages version 2.18.0 based Kolmogorov-Smirnov test. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (<http://www.genome.jp/kegg/>) (Alexa and Rahnenfuhrer, 2006; Kanehisa et al., 2004). The statistical enrichment of DEGs in KEGG pathways was tested by KOBAS software (Xie et al., 2011). To obtain the predicted protein-protein interaction (PPI) of these DEGs, their sequences were blasted to the genome of a related species in STRING database (<http://string-db.org/>). Furthermore, the PPI of these DEGs were visualized in Cytoscape (<http://www.cytoscape.org/>).

2.3. Validation through Western blot and semi-quantitative PCR analyses

2.3.1. cDNA synthesis, PCR amplification and quantification analysis

To validate the up-regulated expression of HSP70 and lysozyme in 3-day regenerated tissue, 300 tail-amputated *E. fetida* were equally divided to 6 groups for different regeneration days (0-day, 12-h, 1-day, 2-day, 3-day and 4-day). RNA isolation was performed in each group with the same instructions in RNA-Seq analysis. cDNA was synthesised using BeyoRT™ II cDNA first strand synthesis kit (Beyotime Institute of Biotechnology, China) according to manufacturer's instructions. A final 20 µL cDNA was generated from 4 µg of RNA per sample. PCR amplification was performed with 0.1–1 µg final concentration of cDNA template, 0.8 µM Primer Mix (Table 6) and 1× PCR Master Mix (Beyotime, China) on Mastercycler Pro PCR (Eppendorf AG., Germany) using the following cycling condition: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 10 min. The PCR products were analyzed on 1% agarose gel (Beyotime, China) with Sub-Cell GT cell (Bio-Rad Laboratories, Inc., U.S.A) under 100 V for 40 min. The results were analyzed by ImageJ version k 1.45 with the reference gene β -actin and the ratio of target genes/ β -actin was calculated. A paired Student's t-test by using Prism 6 software was performed to determine the significance between different groups ($P < 0.05$) from three individual experiments.

2.3.2. Western blot

Proteins were extracted from 6 independent groups (0-day, 12-h, 1-day, 2-day, 3-day and 4-day). Tissue homogenate was prepared in 10 mL radio immune precipitation assay (RIPA) lysis buffer (Beijing Solarbio Science & Technology Co., Ltd, China) supplemented with 100 µL protein inhibitor PMSF (Phenylmethanesulfonyl fluoride) (Solarbio, China) for each 1.33 g liquid nitrogen-preserved tissue for 10 min using T 10 basic ULTRA-TURRAX homogeniser (IKA Experimental Equipment Co., Ltd, Germany). Proteins were extracted from homogenate followed by centrifugation at 4 °C, 14,000× g for 2 min and the supernatant was filtrated on 0.45 µm filter (Millipore, Merck KGaA, Germany). Protein concentration was measured by bicinchoninic acid (BCA) protein quantitative kit (Biomiga, Inc, U.S.A). Protein samples were tested for the expression of HSP70 and lysozyme with β -actin as loading control. The primary antibodies including HSP70 Rabbit Polyclonal antibody (in the dilution of 1:3000), Lysozyme Rabbit Polyclonal antibody (in the dilution of 1:200) and beta Actin Rabbit Polyclonal antibody (in the dilution of 1:2500) (Proteintech Group, Inc., U.S.A) were used for incubating with transferred 0.45 µm nitrocellulose membrane (Millipore, Merck KGaA, Germany) for 22 h. IgG HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H + L) (Proteintech Group, Inc., U.S.A) was used at 1 in 10,000 dilution. The band volume for each identified protein was analyzed

Fig. 2. Identification of wound healing specific proteins in G-90', the homogenate of 3-day regenerated tissue from tail-amputated earthworm *E. fetida*. (A) Effects of protein fractions (ES1, ES2, and ES3) from G-90' on the proliferation activity of fibroblast (NIH 3T3 cells). The proliferative effects in response to various protein concentrations to NIH 3T3 cells were measured by cell counting Kit-8 under the absorbance of 450 nm. (B) Elution profile of protein fraction ES2 on anion exchange chromatography packed with DEAE Sepharose Fast Flow column. The gradient elution was conducted by series concentrations of NaCl solution: 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M. (C) Effects of eluates (peak 1–5) from most functional proteins ES2 on the proliferation activity of fibroblast cells. (D) Nanoscale liquid chromatography-tandem MS analysis of functional proteins from peak 3 eluate. Bars represent mean $\pm$ S.E.M from three individual experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ ($n = 3$).

using ImageJ version k 1.45 with the reference protein β -actin and the ratio of target proteins/ β -actin was calculated. A paired Student's t-test by using Prism 6 software was performed to determine the significance between different groups ($P < 0.05$) from three individual experiments.

3. Results

3.1. Identification of functional proteins from 3-day regenerated tissue of *E. fetida*

To identify wound healing specific proteins in G-90', a protein homogenate extracted from 3-day regenerated tissue of amputated earthworm *E. fetida*, proteins were initially fractionated by their solubility to three fractions namely ES1, ES2 and ES3. The functional fraction was evaluated by fibroblast (NIH 3T3) viability assay. The cell proliferation rate treated with ES2 was the highest than ES1 or ES3 under the optimum concentration of 6 $\mu\text{g/mL}$ (Fig. 2A). Therefore, we considered ES2 fraction possessed most wound healing specific proteins and separated it in next step through anion exchange chromatography (Fig. 2B). As shown in Fig. 2C, eluate in peak 3 displayed the highest proliferation rate on NIH 3T3 cells under the optimum treated concentration of 1.5 $\mu\text{g/mL}$. Proteins in eluate (peak 3) were further identified through nanoscale LC-MS/MS analysis (Fig. 2D). After the interpretation of MS/MS spectra with Uniprot protein database, twenty-three proteins were identified, including six proteins from *E. fetida* species and eighteen from others which may novel proteins have not been discovered in *E. fetida* before (Table 1).

3.2. Identification of differentially expressed genes (DEGs) in G-90 and G-90' homogenate

In last step, we confirmed several functional proteins in G-90' through LC-MS/MS analysis. To identify DEGs and reveal regenerative mechanism from molecular level, transcriptome analysis was performed in G-90' and G-90. Total RNAs were successfully isolated from them and analyzed by RNA-Seq on an Illumina HiSeq 2000. The correlation between two samples was evaluated by Pearson's Correlation's test and ranked with coefficient r . When r^2 is close to 1, two samples were considered high correlated. As shown in Fig. 3A, a positive correlation was observed between 0-day (G-90) and 3-day (G-90') regenerated tissue homogenate of amputated *E. fetida* with r^2 equaling to 0.9529. Significant DEGs between G-90 and G-90' were indicated in volcano plot with fold change ≥ 2.0 and an adjusted p -value ≤ 0.01 (Fig. 3B). Of the 557 DEGs identified, 469 were up-regulated and 88 were down-regulated in 3-day regenerated group (G-90') compared to G-90 (Table 2). A heatmap generated from sequenced dataset showed distinct expression patterns of these DEGs between G-90' and G-90 (Fig. 3C). These analyses indicate that numerous genes are interfered in amputation process of earthworm *E. fetida* and the regeneration specific genes are worth to be identified for wound healing study.

3.3. Functional annotation and enrichment analysis of DEGs

To further explore biological function of these DEGs, a series protein databases were implemented including COG, GO, KEGG, KOG, Pfam, Swiss-Prot, EggNOG and BLASTnr (Table 3). Sequenced DEGs were translated into proteins through BLAST and annotated by searching in those protein databases. Of the 557 DEGs identified, 337 were annotated between G-90 and G-90'. Proteins annotated in GO, COG and

Table 1

Identification results of LC-MS/MS analysis for functional proteins in 3-day regenerated tissue homogenate (G-90') of tail-amputated earthworm *E. fetida*. '#' represent proteins recognised in UniProt database of *E. fetida* species.

Description	Species	MW (kDa)	UniProt ID	GeneBank ID
Lombricine kinase [#]	<i>Eisenia fetida</i>	41.8	O15991	
Heat shock protein 70 [#]	<i>Eisenia fetida</i>	72.9	E9NPR6	DQ286711
Cytochrome c oxidase subunit 1 [#]	<i>Eisenia fetida</i>	23.6	H6VSN8	
Fibrinolytic protease P-III-1 [#]	<i>Eisenia fetida</i>	25.6	Q308Q8	
Lysozyme [#]	<i>Eisenia fetida</i>	17.9	M9VY46	KC493575.1
1,3-beta-glucanase [#]	<i>Eisenia fetida</i>	44.2	A0A0K2RV70	
Keratin, type II cytoskeletal 1	<i>Homo sapiens</i>	66.0	P04264	
Keratin, type I cytoskeletal 9	<i>Homo sapiens</i>	62.0	P35527	
Keratin, type II cytoskeletal 2 epidermal	<i>Homo sapiens</i>	16.8	P35908	
Tropomyosin	<i>Turbo cornutus</i>	28.6	Q7M3Y8	
14-3-3-like protein	<i>Nicotiana tabacum</i>	65.4	Q41246	
Heat shock protein HSP 90-alpha	<i>Oryctolagus cuniculus</i>	79.7	P30946	
Extracellular globin-2	<i>Lumbricus terrestris</i>	16.2	P02218	
Actin	<i>Chionoecetes opilio</i>	20.8	P86700	
GTP-binding protein ypt2	<i>Schizosaccharomyces pombe</i>	22.7	P17609	
Keratin, type I cytoskeletal 10	<i>Canis lupus familiaris</i>	57.7	Q6EI20	
Fructose-bisphosphate aldolase, cytoplasmic isozyme	<i>Zea mays</i>	38.6	P08440	
Ezrin	<i>Mesocricetus auratus</i>	11.1	P86232	
14-3-3 protein homolog 2	<i>Schistosoma mansoni</i>	24.6	Q26537	
Voltage-dependent L-type calcium channel subunit alpha-1D	<i>Gallus gallus</i>	249.2	O73700	
Replicase polypeptide 1a	<i>Bat coronavirus</i>	491.5	P0C6T4	
Putative uncharacterized protein YGL217C	<i>Saccharomyces cerevisiae</i>	12.1	P53085	
UDP-glucose 6-dehydrogenase 4	<i>Oryza sativa subsp</i>	52.8	Q2Q814	

KEGG databases were performed GO enrichment, eggNOG classification and KEGG pathway analyses to investigate biological function of DEGs. As a major bioinformatics, Gene Ontology (GO) aims to unify the representation of gene and gene product attributes across all species through three categories-cellular component (CC), molecular function (MF) and biological process (BP). The terms in each of the GO categories were presented in Fig. 4A. This analysis indicates that DEGs are more enriched with terms related to metabolic process, locomotion and biological adhesion in BP, extracellular region, matrix and synapse in CC as well as catalytic activity, transport and antioxidant activity in MF, respectively (Fig. 4A). Top GO terms of identified DEGs in three categories were presented in Table 4 (p -value < 0.01). Moreover, annotated DEGs in Kyoto Encyclopedia of Genes and Genomes (KEGG) database were conducted KEGG pathway analysis to better understand the molecular interaction, reaction and relation networks of DEGs in studied organism. As shown in Fig. 4B, there are 50 KEGG pathways included more than 46 annotated DEGs. Of these, 9 KEGG pathways contained genes which tended to be down-regulated, 37 contained genes which tended to be up-regulated and the remaining 4 KEGG pathways contained genes which tended to be differently expressed without regard of direction. Identified DEGs were enormously concentrated in the metabolism, cellular processes and environmental information processing pathways. Among those annotated DEGs, the typically up-regulated gene expression for cathepsins in lysosome pathway and Heat Shock 70 kDa Proteins, the down-regulated expression for collagen and protein kinase A, as well as mix-regulated carbonic anhydrase in nitrogen metabolism were presented in Table 5. By the combination of LC-MS/MS and RNA-Seq analyses between 3-day (G-90') and 0-day (G-90) regenerated tissue homogenate of amputated earthworm *E. fetida*, identified specific proteins HSP70 and lysozyme were speculated to up-regulation in G-90'. Their expression levels were further validated through semi-quantitative PCR analysis and Western Blot.

3.4. Validation through Western blot and semi-quantitative PCR analyses

Several functional proteins selected from NIH 3T3 cell proliferation assay and identified by LC-MS/MS analysis in G-90', were hypothesized to up-regulated expression in 3-day regenerated tissue of tail-amputated *E. fetida*. To gain further insight into gene alteration of these proteins,

RNA-Seq analysis was performed between 3-day (G-90') and 0-day (G-90) regenerated tissue homogenate. The validation of up-regulated expression of HSP70 and lysozyme in G-90' were conducted semi-q PCR and Western blot analyses. The DEGs of HSP70 and lysozyme were involved in a range of functions including endocytosis, MAPK signaling pathway, protein processing in endoplasmic reticulum, and lysosome process (Table 5). Of the genes tested by semi-q PCR, HSP70 and lysozyme were significantly up-regulated in 3-day (G-90') and 4-day regenerated tissue of tail-amputated *E. fetida* respectively compared to 0-day regeneration (G-90) (Fig. 5B and C). Their down-regulation on 12-h to 1-day post-amputation was speculated by the impaired metabolism. Two expressed proteins in HSP70 family were also found significantly increased after 3-day (G-90') and 4-day post-amputation (Fig. 5E and F). The non-detected expression of lysozyme through western blot analysis was considered as the absence of its specific primary antibody to this species. The validation of up-regulated expression of HSP70 and lysozyme in 3-day (G-90') and 4-day compared to 0-day (G-90) regenerated tissue illustrated that some functional proteins identified in G-90' was triggered expression after amputation process and these proteins may also explain regeneration mechanism of amputated earthworms.

4. Discussion

Earthworms appear to have a remarkable ability to fully regenerate tail-amputated tissues in a scar-free manner (Xiao et al., 2011). Previously, we have demonstrated that regenerated earthworm can perform higher wound repair ability to non-regeneration tissue through significant promotion of cutaneous wound repair in mice after the administration of G-90' into wound beds compared to G-90 (Yang et al., 2017). In the present study, a series of systematic and comprehensive strategies were performed to reveal the accelerated mechanism of G-90' to G-90. Two functional proteins- HSP70 and lysozyme were identified and validated to up-regulated expression in regeneration process of tail-amputated *E. fetida*.

Twenty-three functional proteins were firstly identified in isolated fraction of G-90' through LC/MS-MS analysis. Coupled with RNA-Sequencing approach between G-90 and G-90', 557 DEGs were detected with 469 up-regulated and 88 down-regulated genes. After functional annotation and KEGG pathway analysis of DEGs, several extraordinary

Fig. 3. RNA-Seq analysis for 3-day (G-90') and 0-day (G-90) regenerated tissue homogenate of amputated *E. fetida*. (A) Pearson's Correlation's test revealed the high correlation between G-90 and G-90'. FPKM means Fragments Per Kilobase of transcript per Million mapped reads. (B) The volcano plot displayed all detected genes with 469 up-regulated genes indicated in red dots and 88 down-regulated in green (fold change ≥ 2.0 and an adjusted p -value ≤ 0.01). (C) The heatmap illustrated different expression level of DEGs in two groups. Column represents each sample and row represents each gene. Yellow color represents higher expression of genes than the overall mean. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
DEGs between G-90 and G-90'. The up-regulation and down-regulation of gene refer to expression level in G-90' compared to G-90.

DEG set	All DEGs	Up-regulated gene	Down-regulated gene
G-90'/G-90	557	469	88

annotated proteins were selected including HSP70s, cathepsins and carbonic anhydrase correlated with pathway of endocytosis, protein processing in endoplasmic reticulum as well as lysosome (Table 5). By the combination of LC-MS/MS and transcriptome analytic results, proteins-HSP70 and lysozyme were successfully determined as up-regulation in regeneration process.

Further validation of their expression patterns after tail-amputation was conducted with semi-q RT-PCR and western blot analyses. Referring to the protocol by Maria Marone et al., semi-q RT-PCR was performed to assess mRNA expression levels of HSP70 and lysozyme on

different regeneration time of amputated *E. fetida* (Marone et al., 2001). A decreased mRNA levels in both HSP70 and lysozyme was detected after amputation for 12 h to 1 day, while their expression progressed incrementally in regenerated wound tissues and reached a high level after 3–4 days regeneration. The explanation for the sharply reduced mRNA levels may be the impaired metabolism after tail-amputation. Therefore, enzymatic reactions including DNA transcription, translation and RNA expression processes were delayed for the absence of timely energetic supply. The cross-reaction of HSC73 and HSP72 in HSP70 family may due to the highly homogenous recognition site for purchased HSP70 Rabbit Polyclonal primary antibody. This phenomenon also demonstrated by Barbara S. Polla et al. (Polla et al., 2007). In this study, up-regulated expression of HSP70s occurred after 3–4 days of regeneration in amputated *E. fetida*. However, lysozyme was not successfully detected presumably due to the low specific binding between protein and its antibody.

Although this is the first research to discuss HSP70 and lysozyme in

Table 3
Annotated DEGs between G-90 and G-90' in different protein databases.

DEG Set	annotated	COG	GO	KEGG	KOG	Pfam	Swiss-Prot	eggNOG	nr
G-90'/G-90	337	92	102	117	196	271	190	268	319

Fig. 4. Functional annotation and enrichment analyses of DEGs detected between 3-day (G-90') and 0-day (G-90) regenerated tissue homogenate of amputated earthworm *E. fetida*. (A) Gene Ontology enrichment analysis. '*' represents significantly differential terms with the enrichment of annotated DEGs higher than all annotated genes. (B) KEGG pathway analysis. '#' represents the up-regulated genes and '▲' represents the down-regulated genes.

regeneration process of tail-amputated *E. fetida*, their functional roles have been ubiquitous elucidated in other species. Lysozyme is widely distributed in nature from eukaryotes to prokaryotes (Jollès, 1996; Prager and Jolles, 1996; Van Herreweghe and Michiels, 2012; Goto et al., 2007; Beintema and Terwisscha van Scheltinga, 1996; Callewaert and Michiels, 2010). Because of their original source, catalytic character and structure, lysozymes are divided into six groups, in which earthworm derived lysozyme belongs to invertebrate-type lysozyme (i-type lysozyme) (Joskova et al., 2009; Zavalova et al., 2000). Radka Joskova et al. successfully expressed recombinant *Eisenia andrei*

lysozyme by using *Escherichia coli* BL21(DE3) cells and characterised it in innate defence system with lysozyme, isopeptidase, as well as anti-bacterial activities (Bachali et al., 2002). As to heat shock proteins (HSPs), they are generally grouped into six major families based on molecular weight, including HSP100, HSP90, HSP70, HSP60, HSP40 and the small Heat Shock Protein family (Vos et al., 2008). Among them, HSP70 are the most studied HSPs due to its ubiquitous and highly conserved in all organisms (Feder and Hofmann, 1999; Morimoto, 1998). Dugaard et al. demonstrated that in eight members of HSP70 family, HSC73 (heat shock cognate) participates in cellular homeostasis

Table 4

Top GO terms identified from DEGs between G-90 and G-90'.

Category	GO.ID	Term	DEGs/Annotated all genes	p-value
BP	0007015	actin filament organization	0.012346	1.70E-05
	0016310	Phosphorylation	0.010471	0.00023
	0044087	regulation of cellular component biogenesis	0.009615	0.00068
	0007596	blood coagulation	0.714286	0.00089
	0045010	actin nucleation	0.055556	0.00121
	0071310	cellular response to organic substance	0.011364	0.002
	0006520	cellular amino acid metabolic process	0.005435	0.00245
	0055114	oxidation-reduction process	0.005141	0.00311
	0050830	defense response to Gram-positive bacterium	0.666667	0.00583
	0048522	positive regulation of cellular process	0.003571	0.0099
	0005634	Nucleus	0.002165	4.50E-10
	0005622	Intracellular	0.001687	3.30E-05
	0043231	intracellular membrane-bounded organelle	0.002135	0.00047
	0005783	endoplasmic reticulum	0.010101	0.00057
CC	0044424	intracellular part	0.001782	0.00455
	0003824	catalytic activity	0.011712	1.50E-08
	0004674	protein serine/threonine kinase activity	0.002597	4.00E-08
	0046872	metal ion binding	0.004886	0.00067
MF	0016706	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors	0.166667	0.00173
	0003779	actin binding	0.012987	0.00182
	0004252	serine-type endopeptidase activity	0.290323	0.00184
	0016301	kinase activity	0.009001	0.00193
	0043565	sequence-specific DNA binding	0.003774	0.00585
	0016491	oxidoreductase activity	0.004418	0.0087

and HSP72 is induced by stress conditions (Daugaard et al., 2007). Consistent with these analyses, we discovered their up-regulated expression in 3–4 days of regeneration of tail-amputated *E. fetida* by the cross-validation of semi-q PCR and western blot methods. Up-regulation of these proteins in earthworm regeneration period suggests their wound healing ability and reveals accelerated wound recovery mechanism for G-90' compared to G-90 in previous study.

Earthworms are widely used as indicator organism in ecotoxicological studies to investigate the soil environmental quality for it hypersensitive to toxic chemicals (Brulle et al., 2006; OECD, 1984; Ji et al., 2014). For instance, RNA-Seq analysis was performed in juvenile and adult *E. fetida* to analyze the impact of toxin exposure on genome wide gene expression (Thunders et al., 2017). In this study, the detection of two up-regulated proteins in regeneration process of tail-amputated *E. fetida* can provide a new strategy to assess potential natural

Table 6

Primer sequences in PCR amplification analysis. These sequences were designed in NCBI Primer-BLAST website (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

Name/Gene ID	Specific Primer	Sequence	Product Length (bp)
HSP70/DQ286711	forward	TTTACCACCTACTCGGACAAC	395
	reverse	TTGAGCTTCTCATCCTCGAC	
Lysozyme/KC493575.1	forward	ACAACCTCAGATGGCCTGCTC	249
	reverse	CTCTTCGATGTCTACCGCCC	
β-actin/DQ286722	forward	TCTCCACCTTCCAGCAGATG	209
	reverse	CGAAAAATGTCTCCGCAAG	

Table 5

Several selected DEGs in KEGG pathway analysis.

Tendency	Annotated proteins	Gene ID	KEGG Orthology	KEGG pathways
Up-regulated	HSP70s	c124124.graph_c2	K03283	Endocytosis; MAPK signaling pathway; protein processing in endoplasmic reticulum
	Cathepsin A	c111943.graph_c0	K13289	Lysosome
	Cathepsin L	c119433.graph_c0	K01365	
	hexosaminidase	c116820.graph_c0	K12373	
	sphingomyelin phosphodiesterase	c108967.graph_c0	K12350	
		c115663.graph_c0, c1; c116134.graph_c0, c1		
Down-regulated	collagen	c119561.graph_c1	K06238	ECM-receptor interaction
	protein kinase A	c131966.graph_c0	K04345	Gap junction;
				Progesterone-mediated oocyte maturation;
Mix regulated				Wnt signaling pathway;
				Melanogenesis;
				Calcium signaling pathway;
				Insulin signaling pathway;
				MAPK signaling pathway;
				Apoptosis;
				Hedgehog signaling pathway;
				GnRH signaling pathway;
				Oocyte meiosis
	carbonic anhydrase	c105513.graph_c0 c127724.graph_c0	K01672	Nitrogen metabolism

Fig. 5. The validation of up-regulated expression of HSP70 and lysozyme through Semi-q PCR and Western blot analyses. (A, B, and C) Cropped images of semi-q PCR products analyzed by agarose gel electrophoresis. Relative quantification of PCR products using β -actin as a reference gene displayed a significant up-regulation of HSP70 and lysozyme in 3-day and 4-day regenerated tissue of tail-amputated *E. fetida* compared to 0-day regeneration. (D, E, and F) Cropped images of Western blot analysis showing the detected bands of targeted proteins. Relative quantification of western blot using β -actin as a reference protein showed a significant up-regulation of HSP70s in 3-day and 4-day regenerated tissue of tail-amputated *E. fetida* compared to 0-day regeneration. Bars represent mean $\pm$ S.E.M from three individual experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

materials targeting wound healing. With the tail-amputated *E. fetida* as wound healing model organism, HSP70 and lysozyme as biomarkers and established analytic method, the unique value of this worldwide distributed species should be deeply explored.

In summary, we identified two wound healing specific proteins-HSP70 and lysozyme in 3-day regenerated earthworm tissue (G-90') by the cross-identification with LC-MS/MS and RNA-Seq analyses. Moreover, their up-regulated expression patterns in G-90' to G-90 were validated using semi-q PCR and western blot approaches. This reveals the mechanism for accelerated wound repair in mice occurred after the administration of G-90' into wound beds compared to G-90 because of HSP70 and lysozyme up-regulation (Yang et al., 2017). This study indicated the therapeutic potential of G-90' on wound recovery and provide a new strategy to assess other natural materials targeting wound healing with the tail-amputated *E. fetida* as wound healing model organism.

Conflicts of interest

The authors declare no conflict of interest.

Biological significance

Understanding the up-regulation of wound healing specific proteins in tail-amputated *E. fetida* compared to normal *E. fetida* is essential to explore therapeutic agents of G-90' on wound recovery process. Furthermore, the experimental methods of HSP70 and lysozyme through semi-q PCR and western blot, provide a new strategy to assess other natural materials targeting wound healing with the tail-amputated *E. fetida* as wound healing model organism.

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