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Djhsp90s are crucial regulators during planarian regeneration and tissue homeostasis

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ABSTRACT

Heat shock protein 90 family members (HSP90s), as molecular chaperones, have conserved roles in the physiological processes of eukaryotes regulating cytoprotection, increasing host resistance and so on. However, whether HSP90s affect regeneration in animals is unclear. Planarians are emerging models for studying regeneration in vivo. Here, the roles of three *hsp90* genes from planarian *Dugesia japonica* are investigated by WISH and RNAi. The results show that: (1) *Djhsp90s* expressions are induced by heat and cold shock, tissue damage and ionic liquid; (2) *Djhsp90s* mRNA are mainly distributed each side of the body in intact worms as well as blastemas in regenerative worms; (3) the worms show head regression, lysis, the body curling and the regeneration arrest or even failure after *Djhsp90s* RNAi; (4) *Djhsp90s* are involved in autophagy and locomotion of the body. The research results suggest that *Djhsp90s* are not only conserved in cytoprotection, but also involved in homeostasis maintenance and regeneration process by regulating different pathways in planarians.

1. Introduction

Heat-shock proteins (HSPs) are a highly conserved family of proteins best characterized as molecular chaperones that prevent the formation of misfolded protein structures [1], which are critical in dealing with stress response, including heat and cold shock, heavy metals, tissue trauma, bacterial infection [2,3]. HSP90s are key members of the heat-shock protein family. As molecular chaperones, HSP90s play crucial roles in cytoprotection by preventing protein aggregation, promoting the folding, stabilizing and refolding of proteins in newly synthesized proteins, and targeting the misfolded proteins to specific degradation pathways. HSP90s are ATP-dependent partners that interact with the client protein through formed a hydrophobic pocket homodimer. When HSP90s are activated, ATP binds to the nucleic acid binding domain and increases the affinity of the corresponding binding domain to different substrates by the hydrolysis of ATP [4]. HSP90s can form a stable complex with multiple protein kinases involved in regulation of a variety of life activity, such as cell cycle regulation, cell proliferation, autophagy, apoptosis, tumorigenesis [5–8].

Planarians are ideal model organisms for regeneration research due to their amazing regenerative capacity. Here, we have screened *hsp90* family members from *Dugesia japonica* transcriptome data, in which *Djhsp90-1*, *Djhsp90-2* and *Djhsp90-3*, exhibit dynamic changes of expression levels during regeneration. Therefore, their expression patterns and functions are investigated in intact and regenerating worms. The research results demonstrate that *Djhsp90s* are essential for regeneration, tissue homeostasis, movement and cytoprotection in the *D. japonica*.

2. Materials and methods

2.1. Animals and treatments

Planarians used in this study belong to the species *D. japonica* and are collected from Shilaogong, Hebi City, Henan Province, China. Animals are kept in autoclaved tap water in dark at 20 °C and starved for at least 1 week before being used in all experiments. Regenerating fragments are obtained by transverse amputation at the pre-pharyngeal and post-auricle level from intact worms.

For stress response experiments, 200 worms are divided into 10 groups (20 worms per group) and exposed to different stressors according to the pre-experiments. For thermal stress, the worms are cultured in dark at 4 °C, 10 °C, 20 °C (the control) and 30 °C for 2 days. For tissue damage stress, the regenerative fragments are pooled at 12 h, 24 h and 36 h after amputation respectively. For

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chemicals, the worms are exposed to 1-octyl-3-methylimidazolium bromide ([C8mim]Br) at 0 mg/L (the control), 15.6 mg/L, 31.2 mg/L and 46.8 mg/L in the dark at 20 °C for 2 days, respectively.

2.2. Homology analysis and phylogenetic tree reconstruction

Amino acid sequences of *Djhsp90s* are deduced from their cDNA sequences. Multiple HSP90 protein sequences are aligned using the MegAlign program of DNAStar and DNAMAN software. Phylogenetic tree analysis is carried out from the amino acid sequence alignments by the neighbor-joining (NJ) method using Mega 5.0 program (http://www.Megasoftware.net/), statistical support is provided by 1000 bootstrap replications. *Djhsp90-1*, *Djhsp90-2* and *Djhsp90-3* sequences have been deposited in GenBank, corresponding accession numbers are MG334593, MG334594 and MG334595, separately. The HATPase c domains are predicted by the Motif Scan program (https://myhits.isb-sib.ch/cgi-bin/motif_scan).

2.3. Quantitative real-time PCR (qPCR)

Quantitative real-time PCR is performed as previously described [9]. The real-time PCR primers of *Djhsp901-3* and some marker genes are specially designed using Primer Premier 5.0 software. *Djef2* is used as the reference gene in all experiments. All primer sequences used in this study can be found in Supplementary Table 1. The levels of relative expression are calculated and quantified with the $2^{-\Delta\Delta Ct}$ method.

2.4. Whole-mount in situ hybridization

Whole-mount in situ hybridization (WISH) is carried out as previously described [10,11]. The digoxigenin-labelled probes are designed according to *Djhsp90-1* (from position 289 to 1004), *Djhsp90-2* (from position 844 to 1777) and *Djhsp90-3* (from position 880 to 1675) sequences, respectively. The probes are synthesized using the RNA in vitro labeling kit (Roche). Intact, regenerative fragments (1, 3, 5, and 7 days after amputation) and RNAi worms are performed by WISH. Control experiments are performed using the sense probes.

2.5. RNA interference

Planarians are fed bacteria induced to express double stranded RNA (dsRNA) against the *Djhsp90-1*, *Djhsp90-2* and *Djhsp90-3* genes as previously described [12]. The conserved domains of *Djhsp90-1*, *Djhsp90-2* and *Djhsp90-3* are amplified from cDNA clone using primers (*Djhsp90-1* RNAi; *Djhsp90-2* RNAi; *Djhsp90-3* RNAi) and then are cloned into the L4440 vector using SacII and KpnI, SacII and KpnI, XbaI and KpnI, respectively. For the RNA interference (RNAi) experiment, the worms are fed 3 times over 5 days (1st, 3rd and 5th) and are amputated into 2 fragments pre-pharyngeally at 24 h after the last feeding (Fig. s1). The regeneration rate is detected by measuring the length of the blastema during the regeneration. The effectiveness of RNAi is confirmed by WISH and qRT-PCR.

2.6. Statistical analysis

The SPSS13.0 software is used as statistical analysis. Data are analyzed by one-way ANOVA followed by post-hoc multiple comparisons using the LSD test. The differences are regarded as statistically significant at $P < 0.05$ and highly significant at $P < 0.01$.

3. Results

3.1. Gene structure and homology analysis of Djhsp90s

Four *hsp90* sequences, including the *Djhsp90-1*, *Djhsp90-2, Djhsp90-3* and *Djhsp90* (GenBank accession number: ACM91724.1), are found in the *D. japonica.* Multiple alignment of putative proteins show that the four genes differ significantly from each other (Fig. s2). The amino acid sequences deduced from *Djhsp90-1*, *Djhsp90-2* and *Djhsp90-3* contain five well-conserved signature motifs of HSP90 family and HATPase_c domain, which is typical histidine kinase-like ATPase domain and ubiquitous in all HSP90 family members (Fig.s3). Multialignments of both nucleotide and predicted protein sequences reveal that the three genes are significantly different from each other, as indicated by the percentages of identity and similarity shown in Fig.s2. The amino acid sequence alignment analysis by DNAMAN show that the five sequence tags are not only highly conserved in different species, but also the sequence tag sites are the same or similar (Fig.s4). Based on above data, the *Djhsp90-1*, *Djhsp90-2* and *Djhsp90-3* are affirmed the different members of the HSP90 family.

Sequence homology search using BLASTn and BLASTp reveal that the *Djhsp90s* sequence share high similarity with other eukaryotic organisms' *hsp90* sequences. The deduced amino acid sequence of *Djhsp90-1* gene show identity of 67.7% with *Rhizopus delemar*, 65.3% with *Djhsp90* and 67% with *Mucor circinelloides*, respectively. The amino acid sequence of *Djhsp90-2* display identity with *Djhsp90-3* (62.6%), *Gavialis gangeticus* and *Oreochromis niloticus* are the same (57.8%), *Capitella teleta* (61.8%) and *Helobdella robusta* (58%). The amino acid sequence of *Djhsp90-3* display identity with those from *Capitella teleta* (64.2%), *Helobdella robusta* (64.3%), *Bos taurus* (64.2%), and *Lottia gigantea* (63.9%).

The phylogenetic trees are constructed using the amino acid sequences of *hsp90s* (Fig. s5). The results show that the deduced amino acid sequences of *Djhsp90-1*, *Djhsp90-2* and *Djhsp90-3* cluster together with other HSP90 family members, the members of the HSP70 and HSP110 family cluster together, and the members of the HSP40 and HSP60 family cluster together respectively. The above results demonstrate that *Djhsp90s* belong to *hsp90* family members and are conserved.

3.2. External stressors induce Djhsp90s expression

In order to determine whether external stressors induce *Djhsp90s* expression in planarians, expression levels of *Djhsp90-1, 2, 3* in response to diverse external stressors are examined by quantitative real-time PCR. For the cold and thermal stress, the transcriptional levels of *Djhsp90-1*, *2*, *3* increase significantly when worms are cultured at 4 °C and 30 °C compared to the control (20 °C), and reach the peak at 30 °C (Fig. 1a). For tissue damage stress, the expression values of *Djhsp90-1*, *2*, *3* exhibit a gradual upregulation at 12 h, 24 h and 36 h after amputation (Fig. 1b). For ionic liquid-induced stress, the expression patterns of *Djhsp90-1, 2, 3* exhibit a significant up-regulation in a dose-dependent manner (Fig. 1c). These results show that *Djhsp90-1*, *2*, *3* exert cytoprotection in response to external stress in planarians.

3.3. Expression patterns of Djhsp90-1, 2, 3 in intact and regenerative worms

The WISH is performed to analyze transcripts distribution of *Djhsp90s* in intact and regenerative animals. The spatial and temporal expression patterns of *Djhsp90-1*, *2*, *3* in intact and regenerative worms are highly similar (Fig. 2a, b, c). In intact worms, *Djhsp90-1, 2, 3* are ubiquitous and mainly distribute each side of the