

# Characterization of an amphioxus heat-shock protein gene promoter and its application *in vivo*

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**ABSTRACT** Amphioxus is a good proxy for studying the evolution and development of mechanisms during the invertebrate to vertebrate transition. However, one of the major limitations of amphioxus as a model organism is the lack of well-developed technical approaches. Promoters of genes encoding heat shock proteins are useful tools for gene function studies. In the present study, we tested heat shock temperatures on 4-cell, 128-cell, late blastula, mid-gastrula and late gastrula amphioxus embryos and optimized the heat shock conditions. We then examined the expression profile of the endogenous *Hsp70* gene in embryos heat-shocked under optimum conditions. RT-qPCR revealed that the expression of *Hsp70* could be induced in embryos by heat shock after the 128-cell stage, and *in situ* hybridization showed that the induced *Hsp70* expression was predominantly detected in epidermal ectoderm. Further, we constructed two reporter gene expression plasmids carrying the *Hsp70* promoter to evaluate its efficiency *in vivo*. Microinjection experiments showed strong red fluorescence or *LacZ* staining signals in injected embryos after heat shock treatment. In contrast to endogenous *Hsp70*, reporter gene expression was found in all three germ layers at equivalent levels. Finally, using the *Hsp70* promoter, we conducted a preliminary functional analysis of two amphioxus genes, *Vg1* and *Cer*, which play essential roles in left-right patterning in vertebrates. After heat shock at late blastula, mid-gastrula or early neurula stages, ectopic expression of *Vg1* was detected in each group of treated embryos. Phenotype observation at 3-gill-slit larvae stage showed that ectopic *Vg1* expression led to two-left-side or left-right reversal phenotypes, and that of *Cer* caused a two-right-side phenotype. Our study establishes a thermo-inducible gene expression system which will be a useful tool for gene function investigation in amphioxus embryos.

**KEY WORDS:** *amphioxus*, *Hsp70* promoter, inducible gene expression, embryo development, heat shock

## Introduction

Amphioxus, being a sister group of urochordates and vertebrates, represents the most basally-diverged lineage of chordates (Bourlat *et al.*, 2006, Delsuc *et al.*, 2006). Like vertebrates, amphioxus possesses a dorsal hollow neural tube, a notochord, a perforated pharynx with gill slits and segmented axial muscles (Koop and Holland, 2008). Moreover, the amphioxus genome has not undergone the two-rounds of genome duplications that occurred during the early evolution of vertebrates, and has maintained most features of the ancestral chordate (Putnam *et al.*, 2008). Because of this morphological and genetic simplicity, amphioxus has been regarded as a valuable model for studies in genetics, developmental biology and evolutionary biology (Bertrand and Escriva, 2011, Holland *et*

*al.*, 2004, Koop and Holland, 2008). Techniques such as *in situ* hybridization and immunohistochemical staining have been applied in amphioxus for many years, followed by whole genome sequencing of two amphioxus species (Huang *et al.*, 2014, Putnam *et al.*, 2008), and methods for acquirement of the second filial generation of amphioxus (Wang *et al.*, 2006, Zhang *et al.*, 2007), year-round spawning (Benito-Gutierrez *et al.*, 2013, Li *et al.*, 2012) as well as induced breeding (Li *et al.*, 2015) and genome editing in *B. belcheri* and *B. floridae* (Li *et al.*, 2014, Wang *et al.*, 2015). All those achievements made amphioxus more applicable as a laboratory model organism. However, molecular tools for amphioxus, particularly effectively working in amphioxus, are obviously lacking

*Abbreviations used in this paper:* HSP, heat shock protein.

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and their development is required for subsequent experimental studies using this new model animal (Bertrand and Escriva, 2011, Garcia-Fernandez and Benito-Gutierrez, 2009).

Activation of target genes of interest in a temporal and spatial manner is frequently required in embryonic development studies, and can be achieved using heat-shock inducible promoters. Those promoters have been used in most well-developed model animals, such as *Drosophila* (Pelham and Bienz, 1982), zebrafish (Halloran *et al.*, 2000), mouse (Hunt and Calderwood, 1990), medaka (Oda *et al.*, 2010) and *Xenopus* (Sauert *et al.*, 2012). Nevertheless, in our preliminary experiments, current commercial heat-shock promoters are not efficacious enough for amphioxus studies. For this reason, Li and colleagues have cloned a heat-shock promoter from amphioxus *B. belcheri* and evaluated its activity in Epithelioma Papulosum Cyprini (EPC) cell line and zebrafish embryos, but not in amphioxus embryos due to technical limitations in unfertilized egg acquirement and microinjection for amphioxus at that time (Li *et al.*, 2012). Thus, the activity and characters of this promoter remain to be verified in amphioxus embryos. Besides, the heat-inducible features of amphioxus endogenous *Hsp70* gene in amphioxus also need to be characterized, which is valuable for the application of this promoter in ectopic gene expression studies. In the present study, we tested different shock conditions on five developmental stages of *B. floridae* embryos and identified the optimum ones for each stage according to malformed embryo ratios. Further, we examined the expression profile of endogenous *Hsp70* gene after heat shock at different embryonic development stages under a certain heat shock condition, and evaluated the activity of the promoter in amphioxus embryos. The result showed that both endogenous *Hsp70* gene and exogenous genes under the control of *Hsp70* promoter could be induced to express throughout the embryo after heat shock. Additionally, using the promoter we conducted a preliminary functional analysis on amphioxus *Cer* and *Vg1* genes and demonstrated that ectopic activation of either of the two genes caused left-right patterning defects in amphioxus embryos.

## Results

### Optimization of heat-shock conditions for developing amphioxus embryos

To determine the optimum heat shock condition, we heat-shocked amphioxus embryos (which were originally reared at 25°C) at 31°C or 35°C for a period of time. The effect of heat shock was evaluated by counting the deformity ratio of the embryos at early larval stage. As shown in Fig. 1, the deformity ratios of those embryos heat-shocked at 31°C are low and approximate to that of negative control, no matter at what embryonic stages or for how long of heat shock duration. However, most embryos died soon and the remainder deformed seriously if 4-cell or 128-cell stage embryos were heat-shocked at 35°C. By contrast, the deformity ratios significantly decreased to 10% to 25% when the treatment (35°C) was performed on the embryos at LB, MG or LG stages. The result indicates that the early embryos are more sensitive to high temperature than LB or later embryos. Thus for the overall consideration of heat shock effect and embryonic deformity, we adopted respectively 31°C-30 min and 35°C-30 min combinations as optimum heat shock conditions respectively for early cleavage stage embryos (4-cell and 128-cell stages) and late stage embryos (LB, MG, LG or subsequent stages).

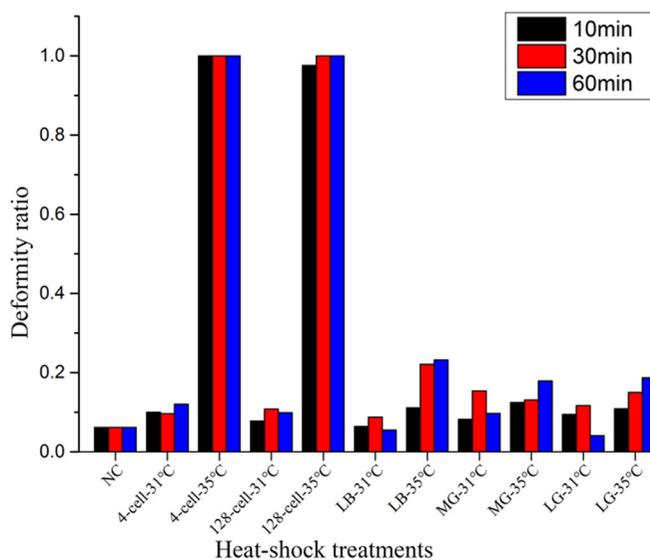
### Endogenous Hsp70 expression profile after heat shock

The expression of *Hsp70* gene can be efficiently induced by heat shock in adult amphioxus (Li *et al.*, 2012) and in many other species (Oda *et al.*, 2010, Stringham and Candido, 1993, Wysocka and Krawczyk, 2000). To evaluate the response of amphioxus embryos to heat shock under the above optimized conditions, we analyzed endogenous *Hsp70* expression profiles using RT-qPCR. The results showed that almost no expression of the endogenous *Hsp70* was detectable in all examined embryonic stages without heat shock in the negative control. By contrast, gene expression was strongly up-regulated in embryos after heat shock at LB, MG and 3-somite stages. In all experiments, induced expression reached the highest peak within one hour after heat shock, and then gradually decreased to a normal level in the subsequent five hours (Fig. 2A). The results also indicated no significant up-regulation of *Hsp70* expression was detected in the embryos heat-shocked at 4-cell stage (Fig. 2A).

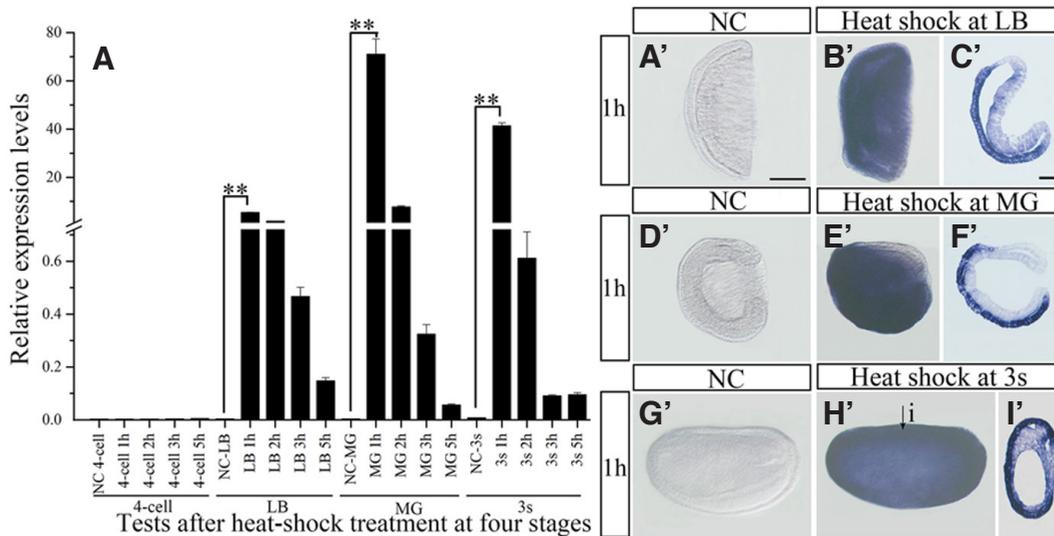
We further determined the spatial distribution of *Hsp70* mRNA in normal and heat-shocked embryos using whole-mount *in situ* hybridization plus histological sections. The results revealed no *Hsp70* expression in the un-heat-shocked control embryos (Fig. 2 A', D', G') but strong expression in the embryos heat-shocked at 35°C (Fig. 2 B', E', H'). The induced expression level was much higher in epithelial ectoderm than in neural ectoderm, mesoderm and endoderm (Fig. 2 C', F', I').

### No endogenous Hsp70 expression in early embryos

The gene expression profile indicated that endogenous *Hsp70* expression could not be induced by heat shock at 31°C in the 4-cell stage embryos (Fig 2A). To determine when the gene began to respond to the heat stimulation, we set the heat shock condition to 33°C for 30 min and used it to treat early stage embryos (4-cell, 16-cell, 32-cell, 64-cell, 128-cell, 256-cell and LB embryos). One hour after heat shock, we collected embryos and extracted total



**Fig. 1. Embryo deformity ratio at the larval stage after heat shock.** The color columns show the deformity ratio with different heat-shock durations, as indicated in the corner. NC is a negative control without heat shock treatment, 4-cell, 128-cell, LB, MG and LG indicate different embryonic stages, and 31°C or 35°C denotes the heat shock temperature.



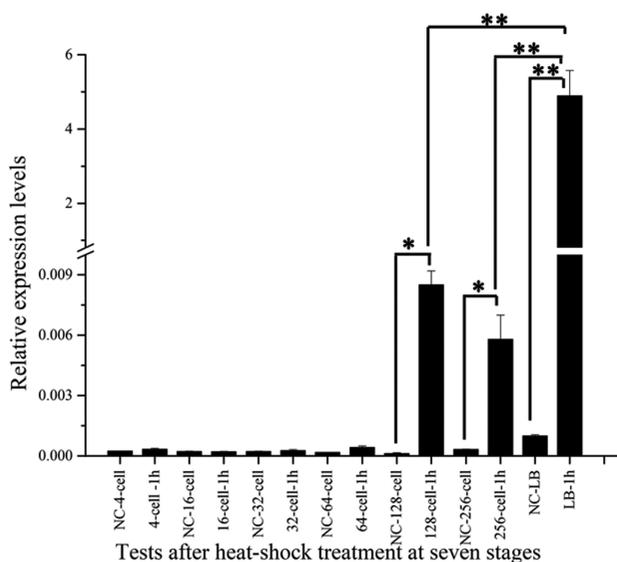
**Fig. 2. Temporal and spatial expression pattern of endogenous *Hsp70* in heat-shocked embryos.** (A) RT-qPCR analysis of *Hsp70* gene expression in the embryos during the five hours (1 h, 2 h, 3 h, 5 h) after heat shock at four developmental stages. \*\* indicates  $P < 0.01$  compared with the negative control (NC). (B', E', H') Distribution of *Hsp70* mRNA in the embryos one hour after heat shock at LB, MG, and 3-somite (3s) stages. (A', D', G') Negative controls without heat shock. (C', F') Coronal sections of the embryo in image C and F. (I') Cross section through level *i* in image H'. All panels are from the left lateral view. Scale bars in A', B', D', E', G', H' are 50  $\mu\text{m}$ , and that in C', F', I' are 20  $\mu\text{m}$ .

through level *i* in image H'. All panels are from the left lateral view. Scale bars in A', B', D', E', G', H' are 50  $\mu\text{m}$ , and that in C', F', I' are 20  $\mu\text{m}$ .

RNA for RT-qPCR analysis. The result showed that the *Hsp70* expression significantly increased in those embryos heat-shocked at 128-cell or 256-cell stages although the increments at these two stages were much lower than that in the embryos treated at LB stage (Fig. 3). However, compared with the control, no gene expression change appeared in the embryos heat-shocked at 4-cell, 16-cell, 32-cell or 64-cell stages (Fig. 3). Thus, amphioxus embryos began to respond to the thermal stimulation from the 128-cell stage.

#### Use of the *Hsp70* promoter for exogenous gene expression in *B. floridae* embryos

The *B. belcheri* *Hsp70* promoter is inducible by heat stimuli in zebrafish embryos or carp EPC cell line (Li *et al.*, 2012). The core



**Fig. 3. RT-qPCR analysis of endogenous *Hsp70* expression in embryos after heat shock at seven stages.** NC, negative control. "\*" represents the  $P$  value is less than 0.05. "\*\*" indicates the  $P$  value is less than 0.01.

elements of the *Hsp70* promoter in the corresponding region are also highly conserved between *B. belcheri* and *B. floridae*. We thus presumed that the *B. belcheri* *Hsp70* promoter should also work in *B. floridae* embryos. To detect the activity of the *Hsp70* promoter in amphioxus embryos, we integrated the *B. belcheri* *Hsp70* promoter upstream of the reporter genes *mCherry* or *LacZ*, and microinjected the constructs into *B. floridae* unfertilized eggs and then dropped a few sperm for fertilization. Based on the observation in our pilot experiments, we heat-shocked all embryos at required stages under 33°C for 30 min, and then collected samples for detection of reporter gene expression. In the embryos microinjected with the construct *Hsp70::mCherry*, red fluorescent signal initially appeared in several embryos 1.5 h after heat shock at the LB stage. By 2 h after the treatment, 87% (87/100) of embryos emitted strong red fluorescence in a dotted manner throughout the body (Fig. 4 J). Similarly, 85.7% (78/91) of embryos heat-shocked at the 3-somite stage emitted bright red fluorescence 2 hours after the treatment (Fig. 4 V). The red fluorescence was slightly increased 3 hours after heat shock (Fig. 4 K, W) and still very bright till the early larva stage (Fig. 4 L, X). In addition, we also observed a very weak red fluorescence signal in few embryos injected *Hsp70::mCherry* construct without heat shock (data not shown), indicating a leakiness of the promoter.

We also injected embryos with the construct *Hsp70::LacZ*, heat-shocked them at LB, MG, or 3-somite stages, and examined them for *LacZ* gene expression. Similar to *mCherry* expression shown above, mosaic or regional *LacZ* staining signal was initially detected 2 hours after heat shock (Fig. 4 I', Q', K', S', N', V'), and sustained till 12 hours later (Fig. 4 M', U', P', X').

#### Application of the *Hsp70* promoter for gene function examination in *B. floridae* embryos

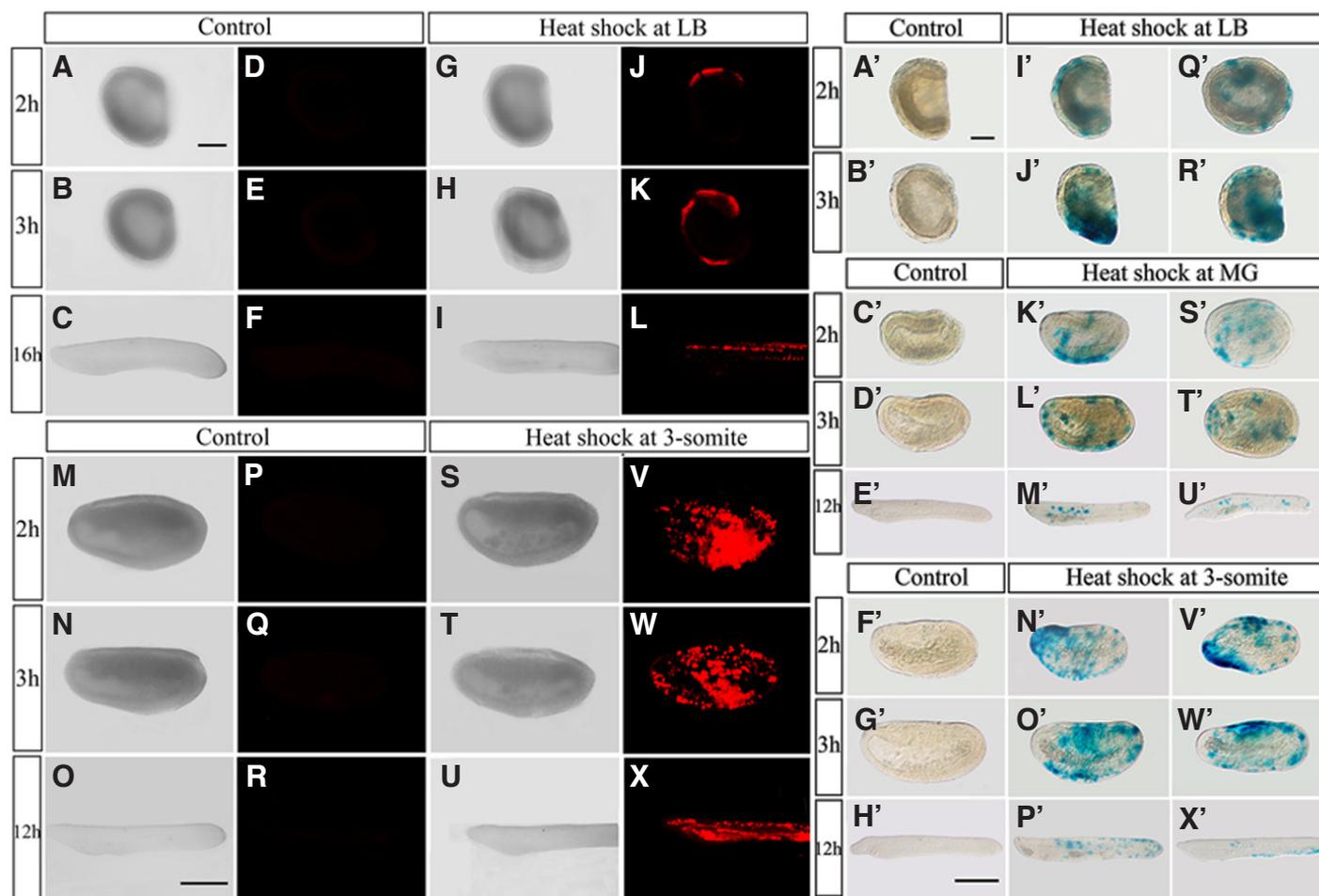
Encouraged by the high activity of the *Hsp70* promoter *in vivo*, we used it to examine gene function during amphioxus embryo development, and selected *Vg1* and *Cer* for this primary analysis. *Cer* was recently shown to be required for the formation of left-right asymmetry in amphioxus (Li *et al.*, 2017). Similar to *Cer*, *Vg1* is also expressed asymmetrically during amphioxus embryonic de-

velopment (Onai *et al.*, 2010, Soukup *et al.*, 2015), but whether it is involved in amphioxus left-right determination remains unknown. We injected *Hsp70::Vg1* or *Hsp70::Cer* plasmids into unfertilized eggs and heat-shocked them after fertilization at LB, MG and 3-somite stages respectively. *In situ* hybridization results showed that the induced expression of *Vg1* appeared in all those three treatments within the subsequent five hours (Fig. 5). Further, we evaluated the effects of the expression of these two exogenous genes via morphological observation of the developing larvae. In most control larvae (injected but not heat-shocked), the mouth formed on the left side (Fig. 6 A). However, among the heat-shocked larvae injected with *Hsp70::Vg1*, around 21.7% to 45.8% of them developed a mouth on both sides, or on reverse (right) side (Fig. 6 B-D), whereas among the heat-shocked larvae injected with *Hsp70::Cer*, about 52.9% to 64.3% showed no mouth forming on either side (Fig. 6 F-H). Similar phenotypes were also found in few injected but not heat-shocked control larvae (1/44 in the *Hsp70::Vg1*-injected control larvae and 3/20 in the *Hsp70::Cer*-injected control larvae), which might be caused by the leakiness of the promoter as evidenced by ectopic expression of *Vg1* in a few cells of the injected embryos.

The results indicate that *Vg1* and *Cer* genes were important for the left-side identity development in amphioxus embryos. These results demonstrate that the *Hsp70* promoter is a useful molecular tool for gene function studies in amphioxus.

## Discussion

Given its key phylogenetic position and morphological characteristics, amphioxus has been used as a model for studying the development and evolution of vertebrates for many years (Acmel *et al.*, 2016, Koop and Holland, 2008). In the past few years, several important steps have been made in amphioxus research, including genome sequencing of two amphioxus species (Huang *et al.*, 2014, Putnam *et al.*, 2008), establishing methods for inducing amphioxus spawning all year around (Li *et al.*, 2012, Li *et al.*, 2013, Li *et al.*, 2015,) and generating amphioxus mutant at desired gene loci (Li *et al.*, 2014, Wang *et al.*, 2015). Nevertheless, many molecular tools like tissue-specific promoters and inducible gene expression systems are still lacking. We here report an efficient heat-shock inducible gene expression system using the amphioxus

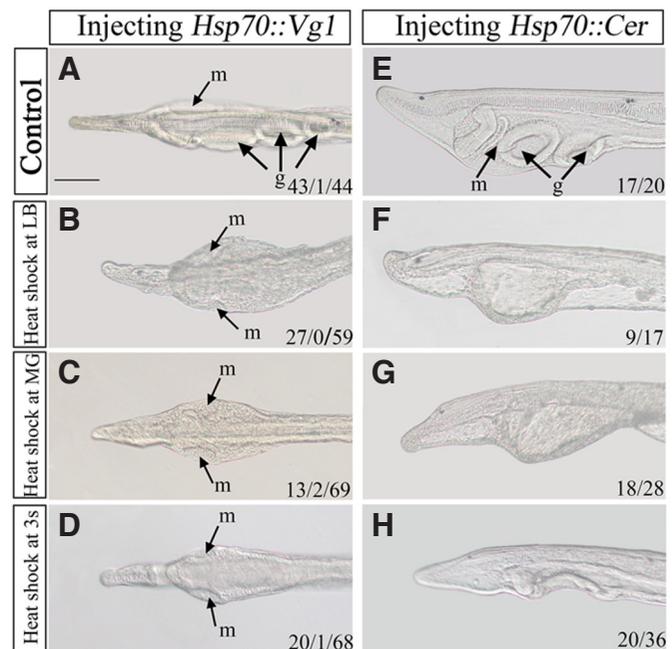
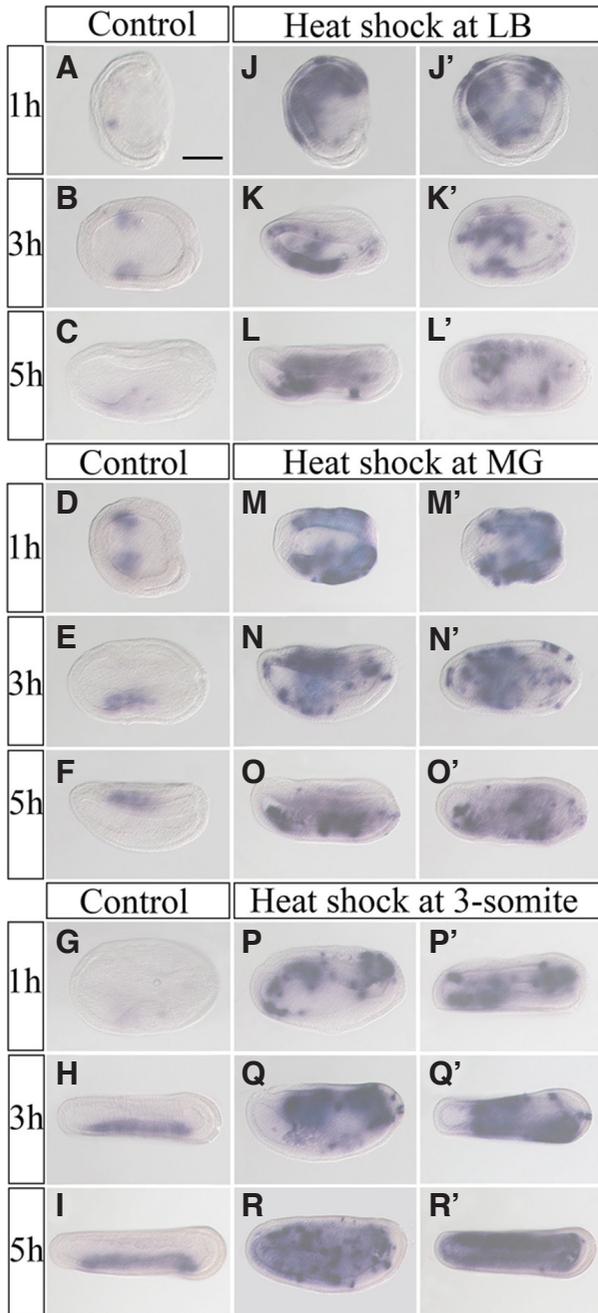


**Fig. 4. Reporter gene expression in embryos injected with construct *Hsp70::mCherry* or *Hsp70::LacZ*.** (A-F, M-R) show the control embryos injected *Hsp70::mCherry* without heat shock. Red fluorescence brightly emits 2 h (J, V), 3 h (K, W), 12 h (X) or 16 h (L) after the heat shock respectively at LB stage or 3-somite stage. (G-I, S-U) are corresponding bright field images of (J-L, V-X). (A'-H') show the control embryos injected *Hsp70::LacZ* without heat shock. The injected embryos display mosaic or regional staining signal 2 h (I', Q', K', S', N', V'), 3 h (J', R', L', T', O', W'), and 12 h (M', U', P', X') after heat shock respectively at LB, MG, 3-somite stages. Scale bars are 100  $\mu$ m in images 12 h or 16 h after heat shock and 50  $\mu$ m in others.

*Hsp70* promoter. We evaluated the system to control exogenous *Cer* and *Vg1* expression during amphioxus embryo development, and found that ectopic activation of either *Cer* or *Vg1* could disrupt normal LR asymmetric development. This observation is consistent

with our recent finding that *Cer* is required for correct right-side development in amphioxus embryogenesis (Li *et al.*, 2017).

The embryonic expression pattern of *Hsp70* gene has been widely studied in many different animals. Comparison of these data with the result presented in this study reveals several conserved expression features of *Hsp70* between amphioxus and other animals. Firstly, as it in fish or *Xenopus* (Michiue and Asashima, 2005, Oda *et al.*, 2010, Santacruz *et al.*, 1997), *Hsp70* in amphioxus is barely expressed during normal development but can be significantly induced by an approximately 10°C temperature increment. Secondly, like in vertebrates (Bienz, 1984a, Lele *et al.*, 1997, Lang *et al.*, 2000), induced *Hsp70* expression in amphioxus also reaches its maximum level within one hour after heat shock, and then gradually decreases in subsequent development. Thirdly, in both amphioxus and *Xenopus* (Bienz, 1984a, Heikkila *et al.*, 1985), as well as in sea urchin (Giudice, 1989) and *Drosophila* (Wang and Lindquist, 1998), *Hsp70* expression cannot be activated by heat shock at very early developmental stages. In *Xenopus*, the earliest stage when *Hsp70* can be induced by heat shock corresponds to the stage when zygotic gene expression is activated (Lang *et al.*, 2000). This is probably the same case in amphioxus, since heat-shock-induced *Hsp70* mRNA was detected only from the 128-cell stage on, when the zygotic genome is thought to be activated (Yang *et al.*, 2016). Lastly, as it is in zebrafish (Lele *et al.*, 1997) and *Xenopus* embryos (Lang *et al.*, 2000), the induced *Hsp70* expression level by heat shock in amphioxus embryos is not identical among different tissues.



**Fig. 5 (left).** Exogenous gene expression in embryos injected with *Hsp70::Vg1* plasmids after heat shock. (A-I) show the control embryos injected with *Hsp70::Vg1* plasmids but not heat-shocked, showing a normal expression pattern of *Vg1* gene in developing amphioxus embryos. (J-R) and (J'-R') are lateral and dorsal view images, showing exogenous *Vg1* expression in the embryos injected with *Hsp70::Vg1* and heat-shocked at LB, MG and 3-somite stages. "1h," "3h" and "5h" indicate samples collected at different times after heat shock. Scale bar in (A) is 50  $\mu$ m and applicable all images.

**Fig. 6 (right).** Morphological observation on 3-gill slit larvae injected with *Hsp70::Vg1* or *Hsp70::Cer* plasmids and heat-shocked at LB, MG or 3-somite stages. (A, E) are control larvae injected but not heat-shocked. (B-D) are ventral views of the larvae injected with *Hsp70::Vg1* and heat-shocked at different developing stage, showing two-mouth phenotypes with a on both sides. (F-H) are lateral views of the larvae injected with *Hsp70::Cer* and heat-shocked, showing no mouth on either side. The first numbers indicate individuals as shown in this image, the middle ones represent deformed individuals with mouth on the right, and the last ones denote observed total larvae. m, mouth; g, gill slit; Scale bar is 50  $\mu$ m and applicable to all images.

The highly inducible activity of the endogenous *Hsp70* gene by heat shock has made its promoter a useful tool for temporal manipulation of exogenous gene expression in many different species (Michiue and Asashima, 2005, Oda et al., 2010, Santacruz et al., 1997). To develop such a tool for amphioxus studies, we previously isolated a *Hsp70* promoter from *B. belcheri* and showed its heat-inducible activity in zebrafish embryos and carp EPC cell line (Li et al., 2012). In the present study, we further demonstrated that this promoter could be used to activate exogenous gene expression very efficiently in *B. floridae* embryos. Moreover, compared with endogenous *Hsp70* expression, exogenous gene expression driven by the promoter showed no obvious propensities among different tissues in the *B. floridae* embryos. This difference might be caused by cross-species utility of the *B. belcheri* *Hsp70* promoter in the *B. floridae* embryos. It is also possible that the promoter used does not include the regulative sequence responsible for tissue-specific expression. A similar ubiquitous induction of exogenous gene expression by the *Hsp70* promoter is also observed in *Xenopus* and fishes (Bajoghli et al., 2004, Michiue and Asashima, 2005, Oda et al., 2010), although their endogenous *Hsp70* genes are not uniformly expressed in different tissues (Lele et al., 1997, Lang et al., 2000).

Taking all properties discussed above into consideration, we suggest three matters should be noted for a better application in amphioxus embryos. First, since the embryos are more susceptible to heat shock at early stage than those at late stage, a moderate heat shock condition should be taken for embryos between 128-cell and late blastula stages. Among the four tested heat shock conditions (31°C for 30 min, 31°C for 60 min, 33°C for 30 min and 35°C for 15 min), we found that 33°C for 30 min seems to be the best since it caused only mild levels of embryonic deformation, but still induced robust *mCherry* expression. As for embryos after the gastrula stage, 35°C for 30 min is recommended as most embryos are resistant to this shock condition and express the exogenous gene efficiently. Secondly, as observed in other animals, the *Hsp70* promoter in amphioxus embryos also led to a very low level of leakiness of exogenous gene expression even without heat shock. Such leakiness will not be a problem if a high expression level of a gene is required to produce phenotypes since low leaking expression can not produce evident side-effects. However, if a gene functions at a very low expression level, the side-effects by leakiness should be considered during the experiment. Researchers could set delicate controls, such as *Hsp70* construct-injected but non-shocked and/or un-injected but shocked controls, to exclude potential side-effects from the leakiness. Thirdly, the promoter is not suitable for genes that function in the early embryos before the 128-cell stage, because it cannot be activated by heat shock at such early stages. Fortunately, such inability would not matter in this promoter application for functional studies of many amphioxus genes because most developmentally essential genes execute their roles after the 128-cell stage. Moreover, overexpression of a certain gene at the early cleavage stages can be achieved by direct mRNA injection into unfertilized eggs.

## Materials and Methods

### Animals

Amphioxus *Branchiostoma floridae* was originally introduced from Dr. Jir-Kai Yu's lab and now is bred in our aquarium room. Animal cultivation

and spawning induction were performed according to the previous reports (Li et al., 2013, Li et al., 2012, Zhang et al., 2007). Fertilization and embryo rearing were performed essentially according to previous description (Liu et al., 2013) with culture temperature adjusted to 25°C before or after heat shock.

### Heat shock experiments

Generally, we transferred embryos at the required developmental stage into a beaker containing 100 ml preheated seawater for heat shock. After that, we put the beaker together with those embryos into a 25°C incubator and cultured them till morphological observation. To find an optimum condition, we set seawater temperature at 31°C or 35°C and heat-shock duration for 10 min, 30 min or 60 min respectively. We treated about 100 embryos for each temperature-duration combination, and also included a similar number of non-shocked embryos from the same clutch as a negative control. When the embryos developed into early larval stage, we evaluated the ratio of malformed larva number to the total number of treated larvae according to the morphological count of each treatment.

### Real-time quantitative PCR (RT-qPCR)

To quantify the dynamic of the endogenous *Hsp70* gene, we respectively treated embryos at the 4-cell stage (31°C for 30 min) or at late blastula (LB), middle gastrula (MG) and 3-somite (3s) stages (35°C for 30 min). From each treatment, about 200 embryos at 1, 2, 3 or 5 hours after heat-shock were collected for total RNA extraction using Trizol reagent (Invitrogen Co., USA). The mRNA was reverse-transcribed using PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Co., Japan), and RT-qPCR was carried out on a Rotor-Gene 6000 (Corbett Robotics Co., USA) using SYBR Green (Promega Co., USA) with primers BfHsp70-RT-F1: 5'-AG-CAAGTCGCTAGGAACCCCAA-3' and BfHsp70-RT-R1: 5'-CCTCCGC-CGTCTCCTTCATCT-3'. The expression level of *Gapdh* was adopted as an internal control with primers Gapdh-RT-F1: 5'-GGTGGAAAGGTCCT-GCTCTC-3' and Gapdh-RT-R1: 5'-CTGGATGAAAGGGTCGTTAATGG-3' (Liu et al., 2015). Three replications were performed for each sample. The reaction tubes were denatured at 95°C for 2 min followed by 40 cycles of 15 s at 95°C, 15 s at 60°C, and 15 s at 72°C. The data were quantified using the comparative *Ct* method to calculate the fold increase (Livak and Schmittgen, 2001).

### In situ hybridization

*Hsp70* coding sequence was amplified from cDNA templates with primer BfHsp70-F1: 5'-TCATCACCGTCCCGGCTACT-3' and BfHsp70-R1: 5'-GCGTCCTGCTCTTTGTGCTTC-3', and then subcloned into a pGEM-T easy vector. The resulting plasmid was then used to synthesize a digoxigenin-labeled antisense RNA probe using Sp6 RNA polymerase (Promega Co., USA). To examine the expression patterns of exogenous *Hsp70* promoter-driven genes, we also subcloned a segment of the coding sequence of *Vg1* into the pGEM-T easy vector for RNA probe synthesis. Whole mount *in situ* hybridization was performed following the description by Yu and Holland (Yu and Holland, 2009).

### Construct preparation and microinjection

A 791 bp promoter region of *Hsp70* gene was amplified from the reporter construct P1 using primer pairs BbHsp70-promoter-F1: 5'-AAGCTTCAATG-GCTAACACGACATCAC-3' and BbHsp70-promoter-R1: 5'-CGGCCGC-CAATCGCTCTACGTTCTACT-3' (Li et al., 2012). The amplicon was digested with restriction enzymes *Hind* III and *Not* I (NEB, Co., USA) and then ligated into a *lacZ* reporter vector derived from plasmid  $\beta$ -actin::LacZ (Feng et al., 2014) to generate *Hsp70*::LacZ construct. Meanwhile, the segment was also cloned to pmCherry-1 vector (Clontech Co., Japan) at *Xho*I and *Eco*RI restriction sites using a similar strategy. To induce expression of a target gene, the *Hsp70*::mCherry construct was further used to make *Hsp70*::*Cer* and *Hsp70*::*Vg1* by replacing the mCherry sequence with the coding sequences of *Cer* or *Vg1* genes from *B. floridae*.

The injection cocktail containing 20% glycerol (Sangon Co., China), 2 mg/ml Texas red dextran (Invitrogen Co., USA) and 200-300 ng/ $\mu$ l recombinant plasmid DNA was microinjected into unfertilized eggs according to our previous report (Liu *et al.*, 2013). After injection, the eggs were fertilized immediately and then cultured in an incubator at 25°C with 95% humidity. Fluorescent signals were observed under a stereoscope (SZX10, Olympus Co., Japan) and photographed under a high sensitivity confocal microscope (LSM 780-1, Zeiss Co., Germany).

#### Detection of $\beta$ -galactosidase activity

The detection of  $\beta$ -galactosidase ( $\beta$ -gal) activity was after the previous description (Yu *et al.*, 2004) with a slight modification. In brief, embryos injected with *Hsp70::LacZ* plasmid were fixed in 1% glutaraldehyde for 30 min at room temperature, and then washed with PBST (0.1% Tween-20 in 1× PBS, pH 7.4) four times (10 min each). After that, the samples were stained with freshly prepared staining solution (4 mM  $K_4Fe(CN)_6$ , 4 mM  $K_3Fe(CN)_6$ , 40 mg/ml X-gal and 0.1% Tween-20 in 1× PBS) at 37°C in the dark for 2 hours or longer, and subsequently were washed at least three times (10 min each) with 1× PBST (pH 7.4) and post-fixed in 4% paraformaldehyde in 1× PBST for 1 hour at room temperature. After removing the paraformaldehyde, the embryos were quickly rinsed in 1× PBST and then mounted on slides in 80% glycerol. Finally, observation and photography were performed under an inverted microscope (IX71, Olympus Co., Japan).

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#### Author Contributions

C.X., G.L. and Y.W. designed research; C.X., G.L., X.L. and X.D. performed experiments; C.X., G.L. and Y.W. analyzed data; and C.X., G.L. and Y.W. wrote the paper.

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