© 2015. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2015) 218, 2505-2509 doi:10.1242/jeb.120113

METHODS & TECHNIQUES

siRNA transfection in larvae of the barnacle *Amphibalanus amphitrite*

Gen Zhang[1,2], Li-Sheng He[2,3], Yue Him Wong[2], Li Yu[1,2] and Pei-Yuan Qian[1,2,*]

ABSTRACT

RNA interference (RNAi) provides an efficient and specific technique for functional genomic studies. Yet, no successful application of RNAi has been reported in barnacles. In this study, siRNA against p38 MAPK was synthesized and then transfected into *A. amphitrite* larvae at either the nauplius or cyprid stage, or at both stages. Effects of siRNA transfection on the p38 MAPK level were hardly detectable in the cyprids when they were transfected at the nauplius stage. In contrast, larvae that were transfected at the cyprid stage showed lower levels of p38 MAPK than the blank and reagent controls. However, significantly decreased levels of phosphorylated p38 MAPK (pp38 MAPK) and reduced settlement rates were observed only in 'double transfections', in which larvae were exposed to siRNA solution at both the nauplius and cyprid stages. A relatively longer transfection time and more larval cells directly exposed to siRNA might explain the higher efficiency of double transfection experiments.

KEY WORDS: Transfection, p38 MAPK, RNA interference, *Amphibalanus amphitrite*, Larval settlement

INTRODUCTION

Barnacles are typical marine fouling organisms and have been used as model organisms in biofouling and antifouling research for many years (Holm, 2012). So far, all the reported functional studie of genes, signal molecules and pathways in barnacle larval settlement have solely relied on chemical inhibitors, activators or analogues (Clare et al., 1995; He et al., 2012; Zhang et al., 2012, 2013). However, these chemicals, which are often claimed to be highly specific and active, might have unexpected or unnoticed effects and thus raise serious concerns about the reliability of previous results. Furthermore, not all genes and signal molecules have specific inhibitors, activators or analogues. These limitations in conventional functional analysis of genes and pathways hampered progress in molecular mechanism studies of barnacle settlement and development.

RNA interference (RNAi) is a powerful tool for studying gene function (Premsrirut et al., 2011) and is more specific to the target gene than chemical treatments. The manipulation of RNA in conventional model organisms is well developed. Lipofection (Zhang et al., 2014), viral infection (Ben-Shoshan et al., 2014) and electroporation (Xu et al., 2014) are widely applied to cultured cells.

[1]Environmental Science Programs, School of Science, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China. [2]KAUST Global Collaborative Research Program, Division of Life Science, School of Science, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China. [3]Sanya Institute of Deep-sea Science and Engineering, Chinese Academy of Science, No. 62, Fenghuang Road, Sanya City, Hainan Province 572000, China.

*Author for correspondence (boqianpy@ust.hk)

Received 2 February 2015; Accepted 15 June 2015

Microinjection of small interfering RNA (siRNA) into the animal tissue of interest has been successfully used in insects such as *Drosophila* (Zhang et al., 2006), *Acyrthosiphon pisum* (Jaubert-Possamai et al., 2007), *Aedes aegypti* (Isoe et al., 2007) and *Thermobia domestica* (Ohde et al., 2009), Crustacea such as *Daphnia magna* (Kato et al., 2011), *Artemia franciscana* (Copf et al., 2004) and *Parhyale hawaiensis* (Liubicich et al., 2009), the arachnids *Tetranychus urticae* (Khila and Grbić, 2007) and *Cupiennius salei* (Schoppmeier and Damen, 2001) and the oligochaete *Enchytraeus japonensis* (Takeo et al., 2010). Environmental exposure to siRNA solution (environmental RNAi) was also reported to be effective in the flatworms *Caenorhabditis elegans* (Timmons and Fire, 1998) and *Macrostomum lignano* (De Mulder et al., 2009), and the rotifer *Brachionus manjavacas* (Snell et al., 2011). Electroporation is successfully used to silence genes in the cnidarian *Hydra* (Lohmann et al., 1999). However, RNAi has not yet been successfully applied to barnacles.

In this study, we sought to develop a method to introduce siRNA into *Amphibalanus amphitrite* (Darwin 1854) larvae. The p38 mitogen-activated protein kinase (p38 MAPK), which regulates larval settlement in *A. amphitrite* through dual phosphorylation on the activated sites (He et al., 2012) was chosen as the target gene. The designed double-stranded siRNA against p38 MAPK was synthesized chemically and labelled with the red fluorescent dye ROX at both 5′ ends. By means of two commercial lipofection reagents, larvae were fed with siRNA at either nauplius VI or cyprid stage, or both. The efficiency of transfection was tested using western blots against p38 MAPK and phosphorylated p38 MAPK (pp38 MAPK) as well as real-time quantitative PCR and settlement bioassays.

RESULTS AND DISCUSSION

To transfect siRNA into the animals, larvae were fed with siRNA at nauplius VI stage (nauplius transfection), early cyprid stage (cyprid transfection), or both (double transfection). The concentration of lipofectin and RNAfectin was set as 4 μl ml^{-1} and 1 μl ml^{-1}, respectively, based on a previous concentration test (supplementary material Fig. S1). After 6 h, both lipofectin and RNAfectin effectively brought siRNA into the larvae (Fig. 1). For nauplius VI larvae, the red fluorescence was mainly concentrated in guts, but widely spread throughout the whole bodies (Fig. 1A,B). The red fluorescent signals were detected almost everywhere in the larval bodies in transfected cyprids (Fig. 1D,E). Double transfection allowed siRNA to diffuse into the larvae remarkably well, with strong fluorescent signals in guts, limps, antennules and near the ventral part of the carapace (Fig. 1G,H). There was no visual difference in signal distribution or intensity between lipofectin and RNAfectin treatments. In the blank controls, no obvious red fluorescent signal was detected (Fig. 1C,F,I).

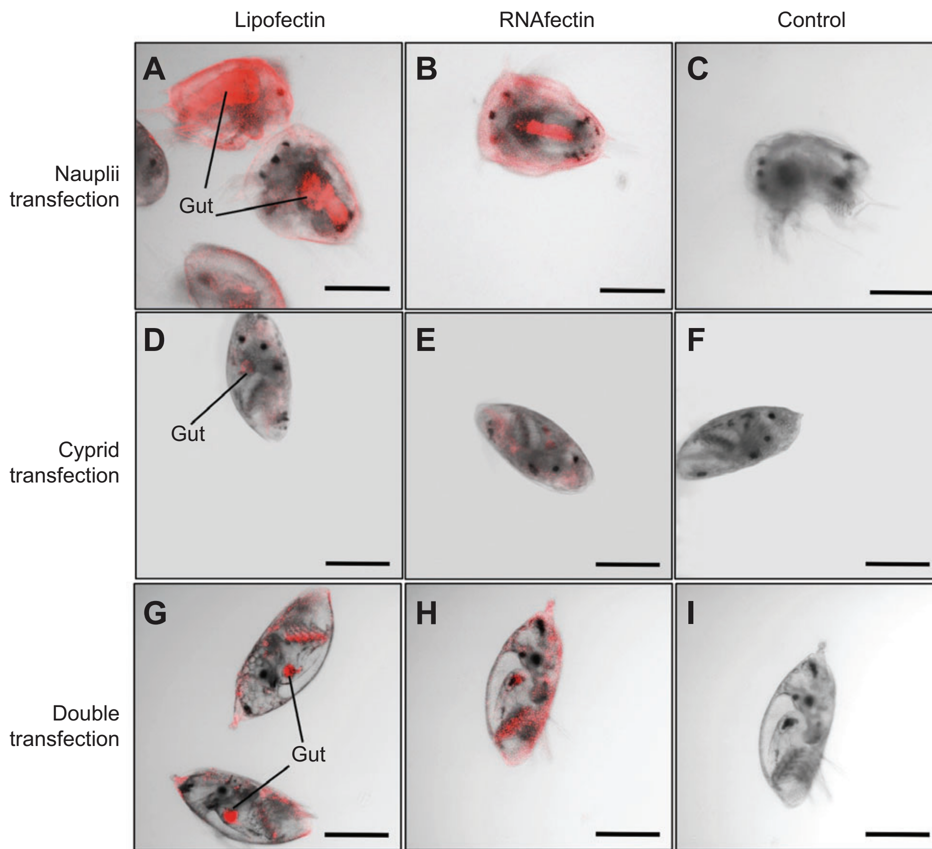

Fig. 1. Transfection reagents allow siRNA to diffuse into *Amphibalanus amphitrite* larvae. Nauplius or cyprid larvae were treated for 6 h with 0.8 μg ml^{-1} ROX-labelled siRNA against p38 MAPK and either 4 μl ml^{-1} lipofectin or 1 μl ml^{-1} RNAfectin. (A,B) Nauplius VI larvae transfected with siRNA using lipofectin or RNAfectin, respectively. Fluorescent signal (ROX) was detected throughout the whole body of larvae and highly concentrated in the gut. (D,E) Cyprids that were directly transfected using lipofectin or RNAfectin. ROX signal is widely detected in their bodies. (G,H) Cyprids dually transfected at both nauplius VI and cyprid stage with siRNA using lipofectin or RNAfectin. A high intensity of red fluorescence is observed in the guts, limps, antennules and the ventral part of the carapace. (C,F,I) Untransfected larvae. Scale bars: 200 μm.

Generally, siRNA diffuses first into outer cells in multicellular organisms by forming liposomes, followed by systemic RNAi, which means gene-specific silencing information is transmitted from a primary group of cells into a secondary group of cells (Winston et al., 2002). In the present study, both nauplii and cyprids have a thick carapace. However, the red fluorescent signals were found to attach to the surface of the larval body and concentrated in guts, revealing that siRNA might diffuse into the surface cells and also gut cells after uptake. Then, systemic RNAi may spread to other cells of barnacle larvae, because weaker ROX fluorescent signals were detected in other tissues.

Western blot analysis showed that both p38 and pp38 MAPK were slightly reduced when lipofectin was applied, and sharply dropped in RNAfectin treatments (Fig. 2) during double transfection. Transfection of control siRNA did not affect the levels of p38 and pp38 MAPK (Fig. 2). Nauplius transfection did not significantly affect the levels of p38 or pp38 (supplementary material Fig. S2A,B). For cyprid transfection, 0.4 and 0.8 μg ml^{-1} of siRNA decreased the level of p38 MAPK when either lipofectin or RNAfectin was used. However, the level of pp38 MAPK showed no apparent changes under the same conditions (supplementary material Figure S2C,D).

Further examination using real-time quantitative PCR revealed that double transfection using either lipofectin or RNAfectin significantly suppressed the mRNA level of p38 MAPK, compared with reagent controls or control siRNA treatments (Fig. 3). These results suggested that the downregulation of the protein level of p38 MAPK in these treatments resulted from the decreased mRNA level of p38 MAPK.

Similarly, settlement bioassay results revealed that double transfection of 0.4 and 0.8 μg ml^{-1} siRNA using RNAfectin significantly inhibited the larval settlement (Fig. 4B), compared with the reagent control. However, no effects were observed when lipofectin was used (Fig. 4A). Nauplius transfection and cyprid transfection had no effects on settlement and mortality, regardless of the transfection reagent (supplementary material Fig. S4).

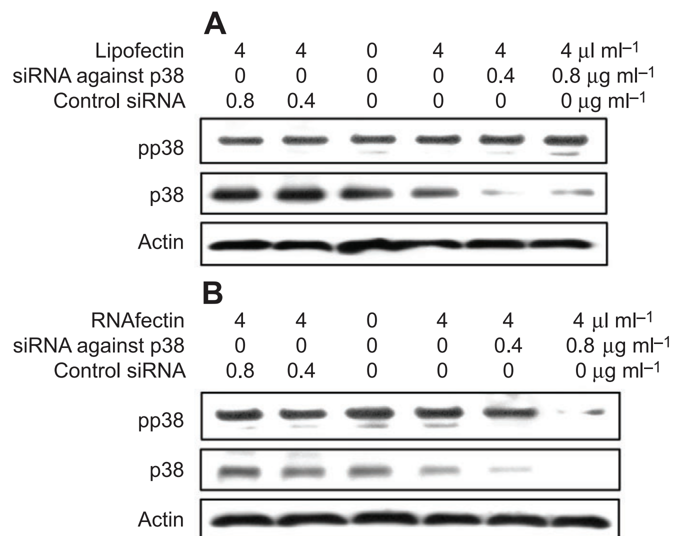

Fig. 2. Double transfection decreases the protein levels of p38 and pp38 MAPK in *A. amphitrite* larvae. Transfection with siRNA against p38 MAPK or control siRNA and either lipofectin (A) or RNAfectin (B) was carried out at both nauplius VI and cyprid stages. pp38 MAPK and p38 MAPK levels were detected on western blots. Representative blots of three replicates are shown. The statistical results of western blot analysis are shown in supplementary material Fig. S3.

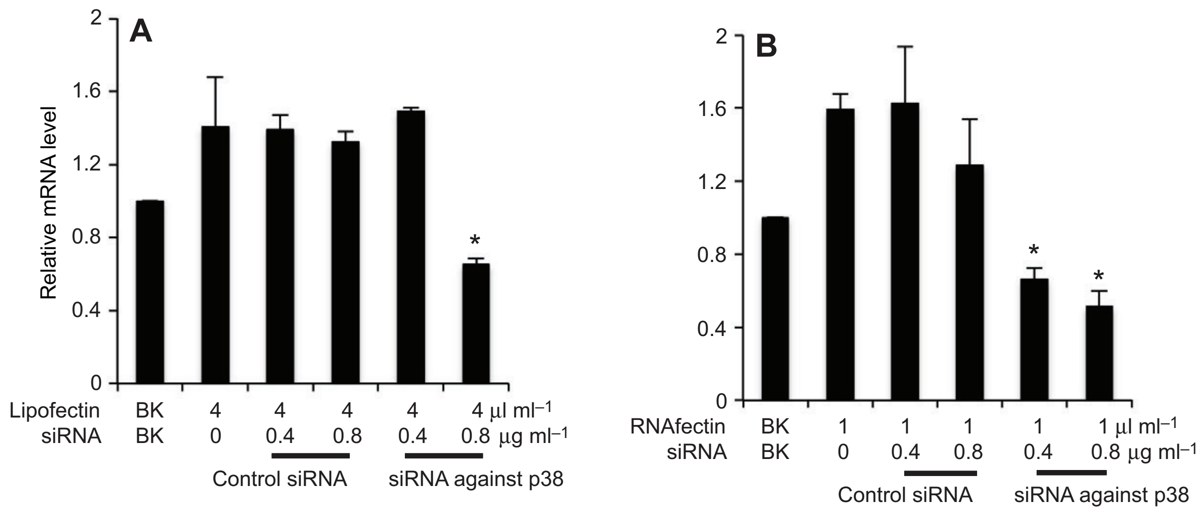

Fig. 3. Double transfection decreases mRNA levels of p38 and pp38 MAPK in *A. amphitrite* larvae. Transfection with siRNA against p38 MAPK or control siRNA and either lipofectin (A) or RNAfectin (B) was carried out at both nauplius VI and cyprid stages. Levels of mRNA relative to blank (BK) control with no siRNA are shown (means±s.e.m.; *N*=3). **P*<0.05.

The p38 MAPK executes biological functions through the dual phosphorylation of p38 protein molecules at Thr180 and Tyr182 (He et al., 2012). In barnacle larvae, p38 MAPK molecules are stored in the cells after being translated from the mRNA. In this state, p38 MAPK molecules do not have any functions. Once the larvae are stimulated, p38 MAPK will be phosphorylated by MAP kinase kinase 3 (MKK3), which then promotes larval settlement (Zhang et al., 2013). Using double transfection, the levels of p38 MAPK were decreased in lipofectin treatments and in treatments with 0.4 µl ml^{-1} siRNA and RNAfectin. However, the levels of pp38 MAPK were not changed, because the level of residual p38 MAPK molecules was still high enough to be phosphorylated, ensuring larval settlement rates in these treatments as high as that in the control. This viewpoint can be partially supported by the fact that only a part of p38 MAPK molecules needs to be phosphorylated to fully execute biological functions in cyprids under normal conditions, as the level of pp38 MAPK can be still elevated in response to crude extracts of adult barnacles, compared with that in the control (He et al., 2012).

Several possibilities might explain why double transfection was more efficient in gene knockdown. Firstly, double transfection enlarged the interaction area between liposome and larvae. Nauplius VI larvae moult once before transforming to cyprids. The body surfaces of both nauplii and the newly transformed cyprids were exposed to siRNA in double transfection, meaning that more surface cells interacted with siRNA directly. Moreover, nauplii ingested siRNA into their guts, but cyprid is a non-feeding stage, so less siRNA could be ingested into guts in cyprid transfection only. In double transfection, siRNA firstly got into the nauplius guts. After transforming to cyprids, larvae still retained part of these siRNA molecules in their guts, as a high intensity of ROX was detected there, providing another opportunity for cyprids in double transfections to obtain more siRNA than those transfected only at the cyprid stage. Secondly, double transfection extended the time for siRNA uptake. The singly transfected larvae were only exposed

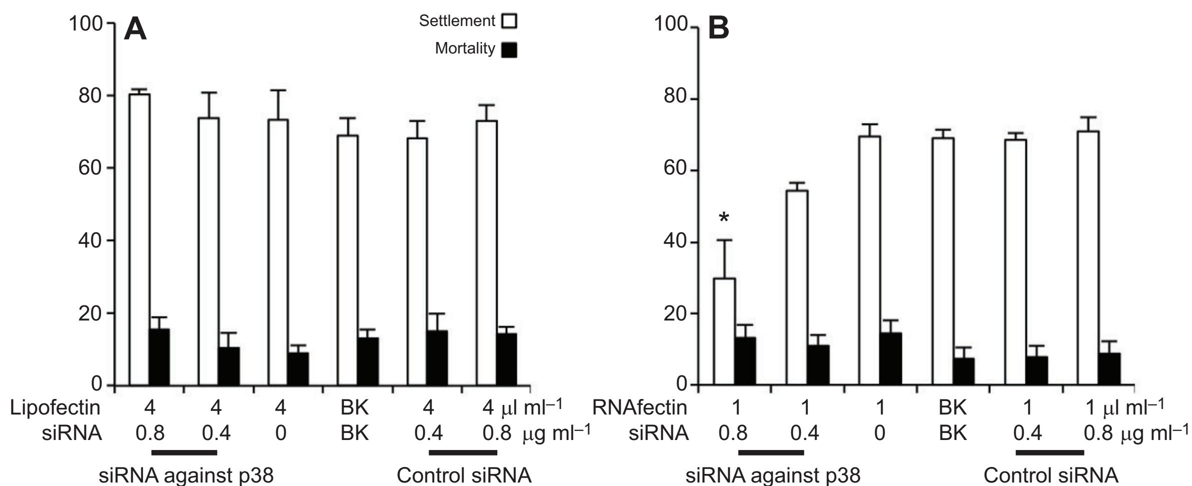

Fig. 4. RNAi against p38 MAPK affected larval settlement of *A. amphitrite*. (A) Transfection at both nauplius VI and cyprid stages with lipofectin showed no effects on larval settlement (means±s.e.m.; *N*=3). (B) Transfection of 0.8 µg ml^{-1} siRNA at both nauplius VI and cyprid stages using RNAfectin significantly inhibited larval settlement (means±s.e.m.; *N*=3). BK, blank control. **P*<0.05.

to siRNA solution for 12–24 h, but exposure was for 36 h in the double transfection. In the nauplius transfection, the level of p38 MAPK returned to a normal level 24 h after the initiation of transfection (supplementary material Fig. S2E). The second transfection in double transfections is essential to maintain a low level of p38 MAPK. Thirdly, doubly transfected larvae had a longer time for degradation of the residual p38 MAPK molecules. The p38 MAPK is synthesized and stored in cytoplasm. Knockdown of p38 MAPK minimized the production of mRNA and blocked the synthesis of new p38 MAPK protein, but did not affect the phosphorylation process on p38 MAPK. Longer treatment time in double transfections ensured a longer time to break down the residual p38 MAPK, resulting in reduced levels of pp38 MAPK, and hence, settlement rate. In conclusion, the present study developed a method for siRNA transfection of barnacle larvae, which would be helpful for future functional genomics and biofouling mechanism studies on barnacles.

MATERIALS AND METHODS

Larval collection and culture

The procedures for adult barnacle collection and larvae culture are described in Zhang et al. (2013). Nauplii developed to the VI stage after 3 days and transformed into cyprids after 4 days. To collect nauplius VI larvae and cyprids, the culture system was filtered through a series of different pore-sized meshes (355, 280, 180, 154 and 110 μm) 1–2 times. Larvae on the 355 and 280 μm pore meshes were mainly nauplius VI larvae, with cyprids mainly retained by 154 μm pore mesh.

Concentration test for transfection reagents

Two transfection reagents, lipofectin and RNAfectin, were purchased from Tiangen Biotechnology Company (Beijing, China). To test possible adverse effects of these two reagents, cyprids were treated with a series of dilutions of lipofectin or RNAfectin (1, 2, 4 and 8 μl ml^{-1} for lipofectin; 0.5, 1, 2 and 4 μl ml^{-1} for RNAfectin). For each treatment, 20 newly formed cyprids (<4 h) were introduced into 1 ml dilution of reagents in filtered seawater (FSW). The experiments were conducted in 24-well polystyrene plates (Becton Dickinson Labware). All the plates were kept at room temperature in the dark. After 24 h, the numbers of dead, settled and swimming larvae were counted.

siRNA synthesis

The siRNA for *A. amphitrite* p38 MAPK (KC287236) spans nucleotides 472–490 and the target sequence is 5′-GACTGCGAGCTCAAGATCC-3′ (Hu et al., 2007). A BLAST search of the NCBI database confirmed this segment to be specific for *A. amphitrite* p38 MAPK. The sense and antisense siRNA sequences were synthesized by Genscript Company (Nanjing, China), separately. To enable visual observation of the transfection process, the two siRNA strands were labelled with red fluorescent dye ROX at the 5′ ends and then purified using high-performance liquid chromatography (HPLC). Finally, these two strands were annealed to form double-strand siRNA. The non-sense siRNA (control siRNA; 5′-ROX-UUCUCCGAAGGUGUCACGUtt-3′) (Hu et al., 2007) was synthesized simultaneously. This sequence of control siRNA was compared with our transcriptome database of *A. amphitrite* and no hits were found.

siRNA transfection

Based on the results of preliminary tests, the concentration of lipofectin and RNAfectin was set at 4 μl ml^{-1} and 1 μl ml^{-1}, respectively, and two concentrations of siRNA (0.4 and 0.8 μg ml^{-1}) were tested. For a 1 ml culture volume, a suitable amount of siRNA and 4 μl lipofectin or 1 μl RNAfectin was diluted in 250 μl Milli-Q water, respectively, and then mixed well by vortexing. The mixture was incubated at room temperature for 20 min to form siRNA–liposome complexes. At the same time, reagent and blank controls were also prepared, using the same quantity of transfection reagent or FSW instead of transfection mixture, respectively.

Three groups of transfection: cyprid transfection, nauplius transfection and double transfection were conducted. For the cyprid transfection, 20 newly formed cyprids were placed into one polystyrene well containing 0.5 ml FSW and fed with siRNA–liposome complex. For the nauplius transfection, around 100 nauplius VI larvae were introduced into 2 ml FSW with 1×10^6 cells ml^{-1} of algae, and then incubated with siRNA–liposome complex. After 12 h, transformed cyprids were collected for real-time quantitative PCR, western blot and settlement bioassays. Cyprids transformed from the nauplius tranfection were moved to new wells and transfected with siRNA for a second time (as in the cyprid only transfection), for ‘double’ transfection. All experiments were repeated at least three times. During the transfection process, larvae were placed under a fluorescent microscope to observe the diffusion of siRNA into larval bodies.

Western blot

Unsettled cyprids in all the treatments were collected for western blot analysis. The protocols for total protein extraction, protein concentration determination and western blot are described in Zhang et al. (2013). Antibodies against pp38 MAPK (Cell Signaling Technology, USA) and p38 MAPK (raised by He et al., 2012) were used to determine the level of pp38 MAPK and p38 MAPK, respectively. Actin (Cell Signaling Technology, USA) was detected as a loading control. The intensity of each band was determined using ImageJ software.

RNA extraction, cDNA synthesis and real-time quantitative PCR

To confirm the positive results in the double transfection, total RNA from these samples was extracted for real-time qPCR according to the protocol described previously (Zhang et al., 2012). Primers used are shown in supplementary material Table S1. The gene cytochrome b (*cyt b*) was selected as the internal reference (Bacchetti De Gregoris et al., 2009). The final relative fold changes were calculated using the $2(-\Delta\Delta C_t)$ method (Livak and Schmittgen, 2001).

Settlement bioassay

To test the effects of siRNA transfection against p38 MAPK on the phenotypic changes of larvae, settlement bioassay were conducted for each transfection treatment. Twenty cyprids from each treatment were placed in a polystyrene well with (for cyprid and double transfection) or without (nauplius transfection) transfection mixture. After 24 h, the numbers of swimming, settled and dead cyprids were counted under a dissecting microscope, respectively. Mortality was calculated as the ratio of dead cyprids to all larvae in the well; and the settlement rate was represented as the percentage of settled cyprids to all larvae alive (including settled and swimming larvae).

Statistical analysis

Data were analysed using SPSS 11.5 software. Means of transfection treatments were compared with the reagent control using one-way analysis, followed by stepwise multiple comparisons using the Student–Newman–Keuls (SNK) method to identify sample means that are significantly different. $P<0.05$ was set as the significance criterion.

Competing interests

The authors declare no competing or financial interests.

Author contributions

G.Z., P.-Y.Q. and L.-S.H. designed the experiments. G.Z. and L.Y. collected samples and conducted the experiments. G.Z., Y.H.W. and P.-Y.Q. analysed the data and wrote the manuscript.

Funding

This work was supported by grants from China Ocean Mineral Resources Research and Development Association (DY125-15-T-02), the King Abdullah University of Science and Technology (SA-C0040/UK-C0016) and the Research Grants Council of the Hong Kong Special Administrative Region (GRF661611 and GRF662413) to P.-Y.Q. as well as the National Natural Science Foundation of China (31460092) to L.-S.H.

Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.120113/-/DC1

References

Bacchetti De Gregoris, T., Borra, M., Biffali, E., Bekel, T., Burgess, J. G., Kirby, R. R. and Clare, A. S. (2009). Construction of an adult barnacle (*Balanus amphitrite*) cDNA library and selection of reference genes for quantitative RT-PCR studies. *BMC Mol. Biol.* **10**, 62.

Ben-Shoshan, S. O., Simon, A. J., Jacob-Hirsch, J., Shaklai, S., Paz-Yaacov, N., Amariglio, N., Rechavi, G. and Trakhtenbrot, L. (2014). Induction of polyploidy by nuclear fusion mechanism upon decreased expression of the nuclear envelope protein LAP2beta in the human osteosarcoma cell line U2OS. *Mol. Cytogenet.* **7**, 9.

Clare, A., Thomas, R. and Rittschof, D. (1995). Evidence for the involvement of cyclic AMP in the pheromonal modulation of barnacle settlement. *J. Exp. Biol.* **198**, 655-664.

Copf, T., Schroder, R. and Averof, M. (2004). Ancestral role of caudal genes in axis elongation and segmentation. *Proc. Natl. Acad. Sci. USA* **101**, 17711-17715.

De Mulder, K., Pfister, D., Kuales, G., Egger, B., Salvenmoser, W., Willems, M., Steger, J., Fauster, K., Micura, R., Borgonie, G. et al. (2009). Stem cells are differentially regulated during development, regeneration and homeostasis in flatworms. *Dev. Biol.* **334**, 198-212.

He, L.-S., Xu, Y., Matsumura, K., Zhang, Y., Zhang, G., Qi, S.-H. and Qian, P.-Y. (2012). Evidence for the involvement of p38 MAPK activation in barnacle larval settlement. *PLoS ONE* **7**, e47195.

Holm, E. R. (2012). Barnacles and biofouling. *Integr. Comp. Biol.* **52**, 348-355.

Hu, J.-H., Chen, T., Zhuang, Z.-H., Kong, L., Yu, M.-C., Liu, Y., Zang, J.-W. and Ge, B.-X. (2007). Feedback control of MKP-1 expression by p38. *Cell Signal.* **19**, 393-400.

Isoe, J., Kunz, S., Manhart, C., Wells, M. A. and Miesfeld, R. (2007). Regulated expression of microinjected DNA in adult *Aedes aegypti* mosquitoes. *Insect Mol. Biol.* **16**, 83-92.

Jaubert-Possamai, S., Le Trionnaire, G., Bonhomme, J., Christophides, G. K., Rispe, C. and Tagu, D. (2007). Gene knockdown by RNAi in the pea aphid *Acyrthosiphon pisum*. *BMC Biotechnol.* **7**, 63.

Kato, Y., Shiga, Y., Kobayashi, K., Tokishita, S.-i., Yamagata, H., Iguchi, T. and Watanabe, H. (2011). Development of an RNA interference method in the cladoceran crustacean *Daphnia magna*. *Dev. Genes Evol.* **220**, 337-345.

Khila, A. and Grbić, M. (2007). Gene silencing in the spider mite *Tetranychus urticae*: dsRNA and siRNA parental silencing of the *Distal-less* gene. *Dev. Genes Evol.* **217**, 241-251.

Liubicich, D. M., Serano, J. M., Pavlopoulos, A., Kontarakis, Z., Protas, M. E., Kwan, E., Chatterjee, S., Tran, K. D., Averof, M. and Patel, N. H. (2009). Knockdown of *Parhyale Ultrabithorax* recapitulates evolutionary changes in crustacean appendage morphology. *Proc. Natl. Acad. Sci. USA* **106**, 13892-13896.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402-408.

Lohmann, J. U., Endl, I. and Bosch, T. C. G. (1999). Silencing of developmental genes in *Hydra*. *Dev. Biol.* **214**, 211-214.

Ohde, T., Masumoto, M., Yaginuma, T. and Niimi, T. (2009). Embryonic RNAi analysis in the firebrat, *Thermobia domestica*: *Distal-less* is required to form caudal filament. *J. Insect Biotechnol. Sericol.* **78**, 99-105.

Premsrirut, P. K., Dow, L. E., Kim, S. Y., Camiolo, M., Malone, C. D., Miething, C., Scuoppo, C., Zuber, J., Dickins, R. A., Kogan, S. C. et al. (2011). A rapid and scalable system for studying gene function in mice using conditional RNA interference. *Cell* **145**, 145-158.

Schoppmeier, M. and Damen, W. G. M. (2001). Double-stranded RNA interference in the spider *Cupiennius salei*: the role of *Distal-less* is evolutionarily conserved in arthropod appendage formation. *Dev. Genes Evol.* **211**, 76-82.

Snell, T. W., Shearer, T. L. and Smith, H. A. (2011). Exposure to dsRNA elicits RNA interference in *Brachionus manjavacas* (Rotifera). *Mar. Biotechnol.* **13**, 264-274.

Takeo, M., Yoshida-Noro, C. and Tochinai, S. (2010). Functional analysis of *grimp*, a novel gene required for mesodermal cell proliferation at an initial stage of regeneration in *Enchytraeus japonensis* (Enchytraeidae, Oligochaete). *Int. J. Dev. Biol.* **54**, 151-160.

Timmons, L. and Fire, A. (1998). Specific interference by ingested dsRNA. *Nature* **395**, 854.

Winston, W. M., Molodowitch, C. and Hunter, C. P. (2002). Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. *Science* **295**, 2456-2459.

Xu, Y., Lu, Y. and Xing, W. (2014). An individually addressable suspended-drop electroporation system for high-throughput cell transfection. *Lab. Chip* **14**, 686-690.

Zhang, X., Scott, M. P., Quate, C. F. and Solgaard, O. (2006). Microoptical characterization of piezoelectric vibratory microinjections in *Drosophila* embryos for genome-wide RNAi screen. *J. Microelectromech. Syst.* **15**, 277-286.

Zhang, Y., He, L.-S., Zhang, G., Xu, Y., Lee, O.-O., Matsumura, K. and Qian, P.-Y. (2012). The regulatory role of the NO/cGMP signal transduction cascade during larval attachment and metamorphosis of the barnacle *Balanus* (=*Amphibalanus*) *amphitrite*. *J. Exp. Biol.* **215**, 3813-3822.

Zhang, G., He, L.-S., Wong, Y. H. and Qian, P.-Y. (2013). MKK3 was involved in larval settlement of the barnacle *Amphibalanus amphitrite* through activating the kinase activity of p38MAPK. *PLoS ONE* **8**, e69510.

Zhang, W., Liu, J., Tabata, Y., Meng, J. and Xu, H. (2014). The effect of serum in culture on RNAi efficacy through modulation of polyplexes size. *Biomaterials* **35**, 567-577.