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Knock down of *gfp* and *no tail* expression in zebrafish embryo by *in vivo*-transcribed short hairpin RNA with T7 plasmid system

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Summary

A short-hairpin RNA (shRNA) expression system, based on T7 RNA polymerase (T7RP) directed transcription machinery, has been developed and used to generate a knock down effect in zebrafish embryos by targeting *green fluorescent protein* (*gfp*) and *no tail* (*ntl*) mRNA. The vector pCMVT7R harboring T7RP driven by CMV promoter was introduced into zebrafish embryos and the germline transmitted transgenic individuals were screened out for subsequent RNAi application. The shRNA transcription vectors pT7shRNA were constructed and validated by *in vivo* transcription assay. When pT7shGFP vector was injected into the transgenic embryos stably expressing T7RP, *gfp* relative expression level showed a decrease of 68% by analysis of fluorescence real time RT-PCR. As a control, injection of chemical synthesized siRNA resulted in expression level of 40% lower than the control when the injection dose was as high as 2 μg/μl. More importantly, injection of pT7shNTL vector in zebrafish embryos expressing T7RP led to partial absence of endogenous *ntl* transcripts in 30% of the injected embryos when detected by whole mount *in situ* hybridization. Herein, the T7 transcription system could be used to drive the expression of shRNA in zebrafish embryos and result in gene knock down effect, suggesting a potential role for its application in RNAi studies in zebrafish embryos.

Introduction

RNA interference (RNAi) is the phenomena of gene silencing in which double-stranded RNA triggers the degradation of a homologous mRNA. This post-transcriptional gene silencing phenomena was first met with Guo and Kemphues in 1995, but they did not give a reasonable explanation [1]. Until 1998, Fire and his colleagues validated that the phenomena of gene expression inhibition also by sense RNA was induced by contaminative double-stranded RNA generated during *in vitro* transcription [2]. This finding led to a series of RNAi-mediated gene silencing studies in many organisms such as worm [3, 4], fly [5, 6], mammalian [7–9] and plant [10] as well. Now RNAi has become a powerful tool to study gene function and explore the gene expression regulation mechanism, as well as provide a new approach for gene therapy.

The phage T7 transcription system is specific in that T7 promoter is recognized only by the T7 RNA polymerase existing in phage [11]. Davanloo et al. isolated the T7 RNA polymerase gene from T7 phage and analyzed its transcription activity [12]. T7 RNA polymerase has the identification stringency for its own promoter and specifically

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transcribes DNA linked to such a promoter. Based on this specificity, the T7 RNA polymerase/T7 promoter system has been successfully applied in the study for the expression and regulation of the foreign genes [13–15].

In recent years, many studies have shown that siRNAs transcribed *in vitro* by T7 system have a preferable effect in cells or organisms [16–19] and thus become an economic and effective method for production of siRNA instead of chemically synthesis. Nevertheless, *in vitro* synthesized siRNA could only give a temporary effect, and the T7 based siRNA transcription cassettes need to be introduced into the cell and organism in order to achieve a long-lasting effect. Recently, various investigators made attempts in this aspect and succeeded in *in vitro* cultured cells [20–22].

In one of the important animal models, zebrafish, just a few studies have been focused on the application of RNAi and controversy opinions appear. Some researchers held the opinion that dsRNA or siRNA could silence target gene specifically in zebrafish [23–27], whereas some other researchers found dsRNA may trigger non-specific effects in zebrafish embryo [28, 29]. Previously, we found that *in vitro* synthesized siRNA could knock down the expression level of *ntl* and phenocopy the *ntl* mutant. Nevertheless, *in vitro* synthesized siRNA cannot be viable beyond 10-somite stage and is not able to function as an inheritable fashion [24]. Here we conducted the RNAi study in zebrafish embryos by constructing a shRNA expression system including the T7RP expression vector and the T7shRNA vectors, which target the foreign *green fluorescent protein* (*gfp*) transgene and endogenous *no tail* (*ntl*) gene, respectively. The results revealed that T7 system could effectively drive the transcription of shRNA in zebrafish embryo, and subsequently knock down the expression level of specific genes.

Materials and methods

Zebrafish embryos and microinjection

Embryos from zebrafish AB line were obtained by artificial fertilization. Zebrafish embryos were incubated at 28.5 °C and the developmental stages were determined according to the standard descriptions [30]. The fertilized zebrafish eggs were injected with DNA or RNA in dose of 1–2 nl/egg. The injection concentration of pT7shGFP, pT7shNTL and pT7Bmp2b was 200 ng/μl. The chemical synthesized siRNA was introduced into the fertilized eggs with the concentration of 1 μg/μl and 2 μg/μl. The injected embryos were incubated at 28.5 °C in 0.3× Danieau's solution (10.17% NaCl, 0.156% KCl, 0.297% $MgSO_4$, 0.441% Ca $(NO_3)_2$, 3.57% HEPES [w/v], 50 IU/ml streptomycin, and 100 IU/ml ampicillin).

pCMVT7R and pT7shRNA constructs

The T7RP expression vector pCMVT7R (Figure 1A) was reconstructed from pZ NRS and pZ CEG. In this vector, *T7RP* is under the control of CMV promoter. Sense and antisense oligonucleotides of GFP and NTL, SIGT_SH, SIGB_SH, SINT_SH, SINB_SH (Table 1) used for shRNA construction were synthesized by Sangon Co. and their sequences were designed according to the published sequences [23, 31]. About 1 nmol of sense and antisense oligonucleotides (GFP or NTL) were mixed, heated to 95 °C for 10 min, and then allowed to cool down to form double-stranded DNA. The primers T7P+S and T7T−H (shown in Table 1) were designed to conduct inverse PCR to amplify the vector pZ T7T which contains a T7 promoter and a T7 terminator as well as the multiple cloning sites between the two introduced restriction endonuclease sites *Sal*I and *Hin*dIII. The cycling parameters used for PCR were as follows: a pre-denaturation of 94 °C 5 min, 30 cycles of 94 °C 30 s, 68 °C 3 min50 s and a final extension of 72 °C 5 min. The Taq polymerase used in PCR amplification was high-fidelity KOD Plus (Toyobo). The PCR products were digested by *Sal*I and *Hin*dIII, purified using the Axyprep™ DNA Gel Extraction Kit (Axygen) and ligated with GFP or NTL double-stranded oligonucleotides. Positive clones were identified by PCR amplification and verified by sequencing analysis. The detailed construction process was shown in Figure 1B.

To construct the vector pT7Bmp2b (Figure 1C), the primers T3 and SP6 were used to amplify the CDS of zebrafish *Bmp2b* from pCS2-Bmp2b vector. The PCR product was ligated with reverse PCR product amplified from pT7eGFP with primers T7+B and T7-N (Table 1) at the endonuclease sites of *Bam*HI and *Not*I.

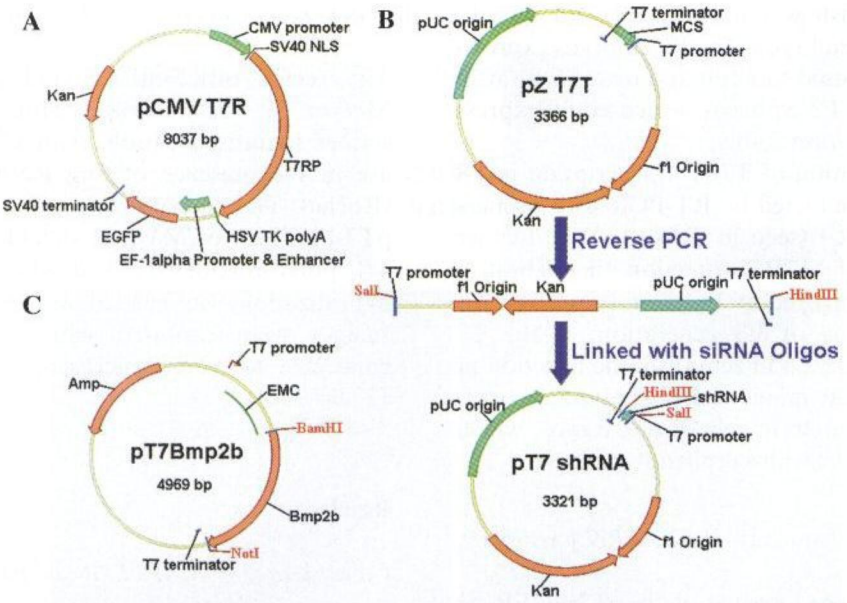

Figure 1. The structure of pCMVT7R, pCMVT7R shNTL and pT7Bmp2b. (A) Shows the plasmid pCMVT7R. T7 RNA polymerase is driven by CMV promoter and the expression of EGFP is driven by EF-1alpha promoter and enhancer as a reporter gene for selection of germline inherited individuals. (B) Shows the plasmid pT7Bmp2b. Bmp2b CDS was driven by T7 promoter. (C) Shows schematic diagram of pT7shRNA vector construction. Firstly, primers T7P+S, T7T−H were designed for inverse PCR to amplify the vector pZ T7T to introduce the restriction site *Sal*I and *Hin*dIII. GFP and NTL oligos were linked with PCR products cleaved by *Sal*I and *Hin*dIII to form the new plasmid pT7shRNA (shGFP/NTL).

Table 1. Primers used in the present study.

primers	Sequences (5′–3′)
SINT_SH	TCGACTGCAATGTACTCGGTCCTGTTCAAGAGACAGGACCGATTACATTGCATTA
SINB_SH	AGCTTAATGCAATGTACTCGGTCCTGTCTCTTGAACAGGACCGAGTACATTGCAG
SIGT_SH	TCGACGCAAGCTGACCCTGAAGTTCTTCAAGAGAGAACTTCAGGGTCAGCTTGCTTA
SIGB_SH	AGCTTAAGCAAGCTGACCCTGAAGTTCTCTCTTGAAGAACTTCAGGGTCAGCTTGCG
T7P+S	CAGGTCGACCCCTATAGTGAGTCGTATTACAATTCACTG
T7T−H	CAGAAGCTTCTAGCATAACCCCTTGGGGCCTCTAAA
T7+B	ACGGGATCCTATTATCGTGTTTTTCAAAGG
T7−N	ACGGCGGCCGCCTAGCATAACCCCTTGGGGCC
GFPA	TCCAGGAGCGCACCATCTT
GFPB	TGCTCAGGTAGTGGTTGTCGG
ActinA	CACTGTGCCCATCTACGAG
ActinB	ATTGCCAATGGTGATGAC
T7R+	CTGGTGAGGTTGCGGATAA
T7R−	TACCGAAGGAGTCGTGAAT
7G1	GTCGACGCAAGCTGACC
7N1	GTCGACTGCAATGTACT
7GN2	CCCCTCAAGACCCGTTT

Transgenic lines stably expressing T7 RNA polymerase

The embryos injected with pCMVT7R were raised to maturation and mated with wild type zebrafish. For every mating pair, at least 100 embryos were examined for GFP reporter expression. If there were some embryos showing GFP expression, these embryos were considered as pCMVT7R-transgenic embryos and sorted out for further breeding. The

F1 adult zebrafish was mated with wild type to produce the F2 embryos. The F2 embryos expressing GFP were raised to adult and mated with wild type to produce F3 embryos, which could express T7 RNA polymerase stably.

The confirmation of T7RP transcription in F3 embryos was conducted by RT-PCR with primers T7R+ and T7R− (seen in Table 1). The further confirmation of T7RP function in zebrafish embryos was approved by injection of pT7Bmp2b into the embryos of F3 generation. If the T7 system could function in zebrafish, the injection of pT7Bmp2b would mimic the phenotype of overexpression of Bmp2b in zebrafish embryos, which would result in ventralized phenotype [32].

In vivo transcription assay of pT7shRNA vectors

Every 50 embryos injected with pT7shGFP or pT7shNTL were colleted at 16-cell, dome, 30%-epiboly, 50%-epiboly, 75%-epiboly, bud and 1d stage, respectively. Total RNA was isolated with SV Total RNA Isolation Kit (Promega) and 1 μg RNA was reverse transcribed to cDNA with random 9mers primer. PCR was conducted with the primers G1, N1 and GN2 which were designed at transcription start sites and terminator respectively for detection of shGFP and shNTL transcripts. PCR conditions were as follows: 94 °C for 5 min, 30 cycles of 94 °C 30 s, 56 °C 30 s, 72 °C 30 s, and 72 °C for 5 min. The PCR products were analyzed with 1.5% agarose gel and the predicted length of PCR products was 104 bp.

Fluorescence real time RT-PCR

About 50 embryos were collected to isolate total RNA with SV Total RNA Isolation Kit (Promega). Then 1 μg total RNA was reverse transcribed to cDNA with Random 9mers primer. The cDNA could be used for the template to detect the expression level of *gfp* mRNA with *β-actin* as the internal control. The 20 μl PCR reaction system consisted of 1 μl cDNA, 0.8 uM primers, and 10 μl SYBR Green Realtime PCR Master Mix (Toyobo). PCR was conducted as follows: 94 °C for 5 min, 40 cycles of 94 °C 30 s, 58 °C 40 s, 72 °C 40 s. Every sample was repeated for three times and the results were analyzed with $2^{-\Delta\Delta Ct}$ method [33].

Whole mount in situ hybridization

The vector pBKS-ntl (a kind gift of Schulte-Merker S) was linearized with *Xho*I and transcribed to antisense probe with T7 RNA polymerase in the presence of Dig RNA Labeling Mix (Roche). The embryos injected with 200 ng/μl of pT7shNTL or pCMVT7R shNTL were fixed with 4% polyformaldehyde and whole mount *in situ* hybridization was carried out as described [34]. Images were captured with a digital camera connected to stereo microscope (Olympus SZX-12, Japan).

Results

Construction of pCMVT7R and pT7shRNA vectors

In the plasmid pCMVT7R (shown in Figure 1A), T7 RNA polymerase was driven by CMV promoter, and it was linked with EGFP reporter driven by EF-1alpha promoter and enhancer.

The shRNA expression plasmids based on T7 promoter were constructed as materials and methods. The plasmids pT7shRNA were successfully constructed (Figure 1B) after double-stranded oligonucleotides of GFP and NTL were ligated with the reverse PCR products from the vector pZ T7T.

Screening of transgenic line expressing T7 RNA polymerase

When the pCMVT7R transgenic zebrafish embryos were raised to maturation, we screened out the P0, F1 and F2 generations by the facilitation of GFP expression. As shown, the GFP proteins of P0 generation were mosaically expressed (Figure 2C, P0), while the GFP proteins of F1 and F2 generation were uniformly expressed (Figure 2C, F1/F2). The transcription of T7RP RNA in GFP-positive embryos has been validated by RT-PCR analysis with primers T7R+ and T7R− in transgenic F3 embryos expressing GFP (Figure 2A, lane 1). As a control, the T7RP transcripts were not detectable from F3 embryos that did not express GFP (Figure 2A, lane 2), supporting the evidence that T7RP was transcribed in the F3 generation of pCMVT7R transgenic zebrafish showing GFP expression.

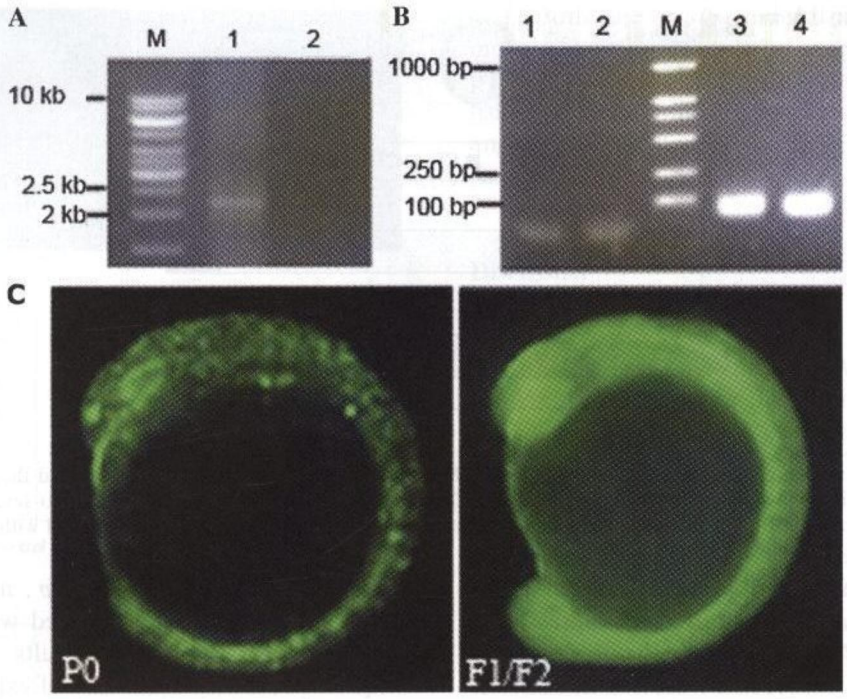

Figure 2. The confirmation of the vector pCMVT7R and pT7shRNA. (A) RT-PCR detection of T7 RNA polymerase in the pCMVT7R transgenic zebrafish F3 embryos. Lane 1 shows that GFP positive embryos could express T7 RNA polymerase mRNA, while lane 2 shows that GFP negative embryos could not express T7R. (B) The shRNA transcripts detection by RT-PCR are shown in lane 3 and 4, resembling shGFP and shNTL, with lane 1 and 2 resembling the negative control. (C) GFP expression pattern in pCMVT7R transgenic zebrafish. The left embryo shows the P0 embryo of mid-somite stage expressing GFP mosaically in the embryo. The right one shows the F1, F2 embryo of mid-somite stage expressing GFP uniformly in the whole embryo.

In order to validate the biological function of T7RP expressed in transgenic pCMVT7R zebrafish F3 embryos, the vector pT7Bmp2b was injected into the F3 embryos, with the injection into WT embryos as the negative control. The results showed that the phenotypes of Bmp2b over-expression were observed in F3 embryos (Figure 3A), with the most severe phenotype of ventralization, Class III, of 52% (Figure 3B). In contrast, there were only 7% of the embryos showing the severely ventralized phenotype in the control embryos. This confirmed that the T7RP existed in F3 generation embryos could specifically recognize the T7 promoter in the vector pT7Bmp2b and the T7 transcription system was functionally validated in zebrafish embryos.

Detection of GFP and NTL shRNA in transgenic embryos

Since T7RP mRNA in transgenic pCMVT7R zebrafish was detected and its protein was approved to be functional, the transgenic pCMVT7R F3 generation could be used as the material for microinjection for shRNA vectors. In order to analyze whether the pCMVT7R transgenic embryos could direct the transcription of shRNA in zebrafish embryos, we conducted *in vivo* transcription analysis for the pT7shGFP and pT7shNTL. The F3 generation embryos injected with the vectors of pT7shRNA were collected at seven developmental stages as materials and methods. The total RNAs of these embryos were isolated and conducted RT-PCR with primers G1, GN2 and N1, GN2, respectively. The results revealed that the PCR products in correct length were amplified from the GFP positive embryos injected with pT7shGFP and pT7shNTL (Figure 2B, lane 3 and 4) but not the negative embryos (Figure 2B, lane 1 and 2). These revealed that the functional plasmid pT7shGFP and pT7shNTL have been constructed and the pCMVT7R transgenic embryos could be applied successfully for *in vivo* transcription of shRNA. Since the transcription of

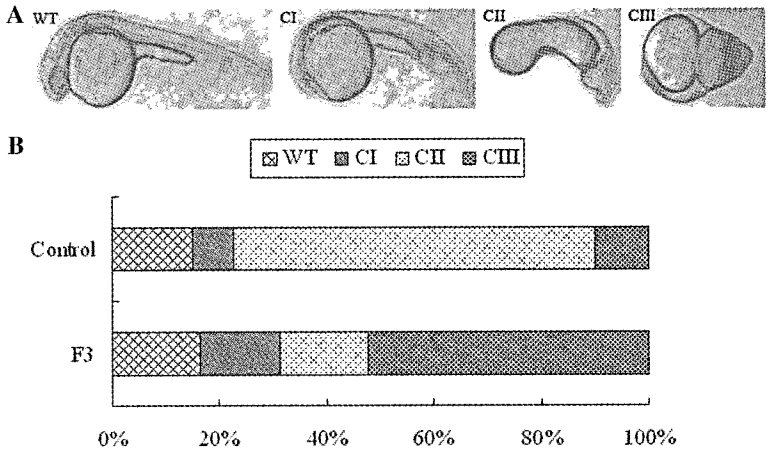

Figure 3. The validation of the function of T7RP *in vivo*. (A) Shows the wild type embryos in 1d stage and the three kinds of ventralized phenotypes injected with pT7Bmp2b, with CI, CII, CIII representing phenotypes from weakly to severely ventralization. (B) Shows the percentages of three kinds of phenotypes appeared in F3 embryos and WT embryos injected with pT7Bmp2b.

shRNA was continuously detectable from 16-cell stage to 1d stage, we only showed the expression of shRNA at one stage to resemble the production of shRNA.

Inhibition of GFP expression by shGFP transcribed by T7 system

After validation by sequencing and *in vivo* transcription, the shRNA vectors were microinjected into the F3 embryos of pCMVT7R transgenic zebrafish and the knock down efficiency of shRNA vectors was analyzed.

With regard to the embryos injected with pT7shGFP, the expression of GFP were firstly examined with fluorescent microscope and the result showed that the percentage of fluorescent embryos did not change significantly at 24 hpf, while the fluorescence intensity was remarkably reduced compared with the control embryos in the same time of exposure (Figure 4A). The further analysis of fluorescence real time RT-PCR revealed that the *gfp* mRNA of embryos injected with pT7shGFP was decreased 68% when compared with the control embryos (Figure 4B).

In order to compare the inhibition efficiency between shRNA transcribed by T7 system and chemically synthesized siRNA, siGFP was microinjected into WT embryos at 1 μg/μl and 2 μg/μl concentrations. The plasmid pCMVeGFP was taken as a control at 200 ng/μl. After injection with G (pCMVeGFP 200 ng/μl), G1 (siGFP 1 μg/μl, pCMVeGFP 200 ng/μl) and G2 (siGFP 2 μg/μl, pCMVeGFP 200 ng/μl), *gfp* mRNA relative expression levels were analyzed with fluorescence real time RT-PCR. The results were shown in Figure 4C, that the decrease of expression level of G1 and G2 were 36% and 51%, respectively. In the F3 generation embryos, siGFP at 2 μg/μl was also injected and real time RT-PCR analysis showed that the relative expression of *gfp* mRNA was 60% (Figure 4B) when the control expression was set as 100%. The results suggested that the inhibition efficiency of injected siRNA was lower than the injection of pT7shGFP into F3 generation of the transgenic pCMVT7R embryos.

Knock down of ntl expression by shNTL transcribed by T7 system

After the inhibition effect of pT7shRNA vector on foreign gene *gfp* was studied, the inhibition effect of pT7shRNA on endogenous gene *ntl* was further analyzed. *Ntl* is the zebrafish homologue of the mouse *T* (*Brachuyury*) gene [35] and the *ntl* mutant embryos lack differentiated notochord and the most posterior 11–13 of their normal 30 somites [36]. The pT7shNTL vector was injected into the transgenic F3 embryos expressing functional T7RP at a concentration of 200 ng/μl. At shield stage, these embryos were fixed and whole mount *in situ* hybridization assay revealed that 30% (36/120) embryos showed partial absence of *ntl* transcripts in germ ring (Figure 5C, D), while the *ntl* signal was detected in the whole germ ring in 100% of wild type embryos (Figure 5A, B). At 25-somite

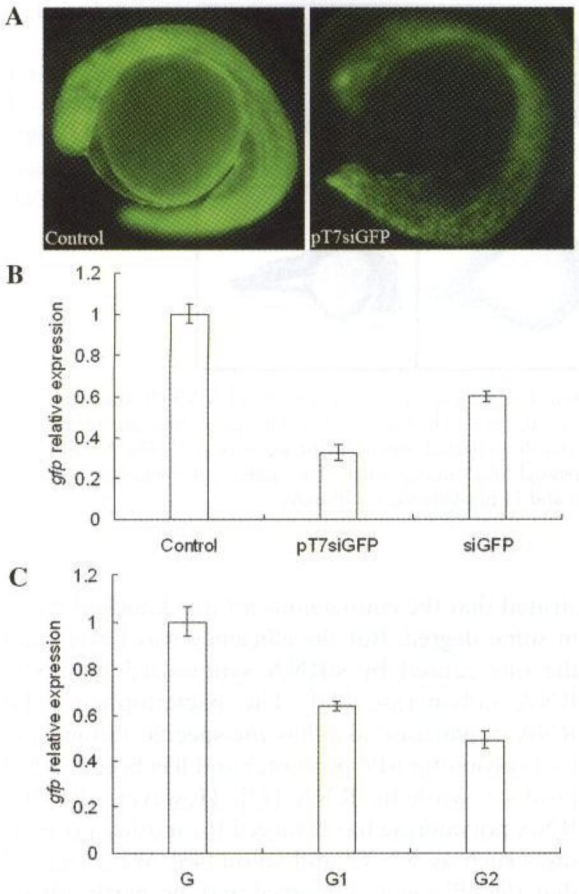

Figure 4. The inhibition effect of pT7shGFP and chemical synthesized siGFP. (A) The F3 embryo microinjected with pT7shGFP shows weaker fluorescence than the control of F3 embryo at the same exposure time. (B) The fluorescence real time RT-PCR reveals that the relative *gfp* mRNA levels of the F3 embryos injected with pT7shGFP and siGFP (200 ng/μl) are 0.32 and 0.60, respectively while the control is set as 1. (C) The real time RT-PCR analysis of *gfp* mRNA expression levels of G (pCMVeGFP 200 ng/μl), G1 (siGFP 1 μg/μl + pCMVeGFP 200 ng/μl and G2 (siGFP 2 μg/μl + pCMVeGFP 200 ng/μl) are 1, 0.64, and 0.49. The error bars show the mean ± S.D. (standard deviation).

stage, about 14% (11/77) of the injected embryos showed various extents of the phenotype of *ntl* mutant, such as lacking of the most posterior part of the notochord and somites (Figure 5F, G). Given the injected embryos were transgenic mosaics and the introduced pT7shNTL could not induce a complete inhibition of *ntl* transcription, it is reasonable that only a small proportion (less than 30%) of the injected embryos showed partial *ntl* phenotype. The wild type phenotype and typical phenotype of reported *ntl* mutant were shown in Figure 5E and Figure 5H, respectively [37]. Both the *in situ* hybridization results and morphological observation suggested that the pT7shNTL could transcribe shNTL in the zebrafish transgenic embryos stably expressing T7RP, and as a result partially knock down the expression of endogenous gene *ntl*.

Discussion

In the present work, we try to discover the possibility of T7 system in RNAi study of zebrafish embryos. Based on the specific recognition of T7RP to T7 promoter, firstly, the transgenic zebrafish line stably expressing T7RP was established; secondly, shRNA vectors which targeted foreign *gfp* gene and endogenous *ntl* gene were constructed; and finally the shRNA constructs were injected into the F3 embryos of the pCMVT7R transgenic line. Our results revealed that this T7 system based siRNA approach could function in zebrafish embryos.

As to the T7 system, although some investigators found the T7 promoter could be recognized by RNA polymerase II in cells [38, 39], the specific recognition and transcription of T7 RNA polymerase for T7 promoter has been successfully applied to shRNA synthesis [20–22]. In addition, the gene expression application of T7 system in zebrafish embryos is also approved by Verri et al. [40]. In our present study, the validation of T7RP recognition on T7 promoter was validated by injection of pT7Bmp2b into the pCMVT7R transgenic F3 embryos. Although the over-expression phenotype was observed in WT embryos, the percentage of the most severe phenotype CIII was significantly lower than that in pCMVT7R transgenic F3 embryos. This suggested that the T7RP in F3 transgenic embryos could recognize the T7 promoter in the construct pT7Bmp2b and then transcribe an elevated level of *Bmp2b* mRNA, resulting in more severely ventralized phenotype. As to the phenomena of ventralized phenotype observed in WT embryos, we doubted that it was caused by the random insertion of *Bmp2b* to the downstream of any promoters or the basal expression of T7 promoter in zebrafish cells, since even an extreme low dose (4 pg per embryo) of injection of *Bmp2b* could result in remarkably ventralized phenotype [41]. On the other hand, RT-PCR analysis confirmed that shRNA could be *in vivo*

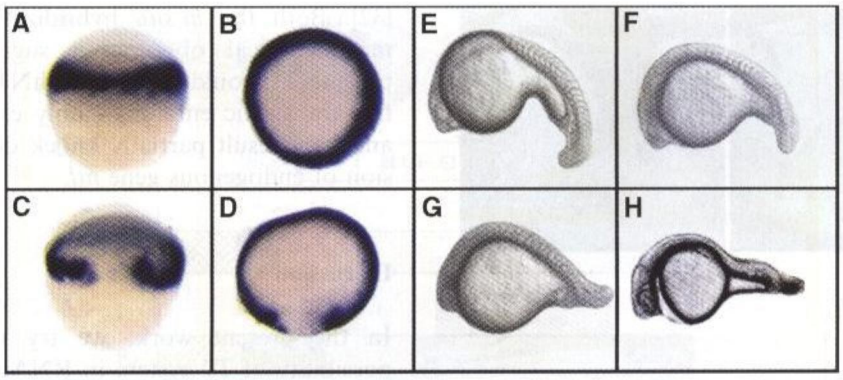

Figure 5. The inhibition effect on endogenous gene *ntl*. (A–D) Show the *ntl* expression pattern in pCMVT7R transgenic F3 embryos injected with pT7shNTL. In the control embryos, *ntl* transcripts are detected in the whole germ ring of shield stage (A, B). While in the F3 embryos injected with pT7shNTL *ntl* transcripts show partial absent in the germ ring (C, D). A and C are lateral view. B and D are animal view. E–H show the phenotype appeared at 25-somite stage. The similar *ntl* phenotype was also observed in 14% (11/77) embryos (F–G). E shows a wild type embryo and H presents the *ntl* mutant.

transcribed in pCMVT7R transgenic zebrafish embryos stably expressing T7RP.

To study the application of *in vivo* transcription directed siRNA by T7 system, both foreign *gfp* gene and endogenous *ntl* gene were chosen as the target genes. In the embryos injected with pT7shGFP, both the results of GFP fluorescence observation and fluorescence real time RT-PCR approved that GFP expression was partially inhibited. In addition, this inhibition efficiency was higher than that of the chemically synthesized siGFP either in F3 embryos or in WT embryos. As to the effect on the endogenous *ntl* gene, we chose the most effective siRNA target sequence of *ntl* as previously reported [23], and placed it between the T7 promoter and T7 terminator. In transgenic zebrafish stably expressing T7RP, shNTL RNA was transcribed as detection by RT-PCR analysis. We found that there were 30% embryos (36/120) showing partial absence of *ntl* signal in the germ ring at shield stage. As a result, at 25-somite stage, various extents of *ntl* phenotype were observed in 14% (11/77) of the injected embryos. Obviously, the percentage of embryos showing *ntl* phenotype was much lower than that showing absence of *ntl* expression. Since the *ntl* expressing cells will give rise to the formation of posterior mesoderm and notochord, our results suggest that partial inhibition of *ntl* expression at gastrula stage may not give any obvious developmental defects at later stages. Nevertheless, both results of *in situ* hybridization and morphological observation demonstrated that the endogenous *ntl* was knocked down in some degree. But the efficiency was lower than the one caused by siRNA synthesized with SP6 RNA polymerase [23]. The bacteriophage SP6 RNA polymerase also has the specific recognition to its promoter SP6 promoter and has been used to produce synthetic RNA [42]. However, the SP6 RNA polymerase has diverged from other polymerases such as T7, T3 and so on [43]. We proposed that the efficiency difference may be partly owing to the different structure of RNA transcribed with different RNA polymerases. A more obvious and reasonable interpretation is, that the shRNA transcribed by shRNA vectors in the present study was mosaically distributed in the embryo because of the transgenic mosaicism, thus it could only induce a partial inhibition effect.

As discussed above, the inhibition effect of T7 system to *gfp* and *ntl* was just the results of temporary transcription by direct microinjection. If *red fluorescence protein* (*rfp*) marker was linked with the plasmid pT7shRNA and the resulted plasmid was injected into zebrafish embryos. Then, we may screen out germline transmitted pT7shRNA transgenic zebrafish and a more effective RNAi effect could be achieved if this transgenic line was mated with pCMVT7R transgenics. Furthermore, if tissue-specific promoter is used to drive the expression of T7RP, the tissue-specific gene inhibition could be obtained, which would be more helpful in the study of gene function and gene therapy.

Acknowledgements

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