Biochemical and Biophysical Research Communications 430 (2013) 347–351

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effects of specific and prolonged expression of zebrafish growth factors, Fgf2 and Lif in primordial germ cells in vivo

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ARTICLE INFO

Article history:
Received 1 November 2012
Available online 21 November 2012

Keywords:
Zebrafish
nanos3 3′UTR
Lif
Fgf2
Primordial Germ Cells
PGC migration

ABSTRACT

Primordial germ cells (PGCs), specified early in development, proliferate and migrate to the developing gonad before sexual differentiation occurs in the embryo and eventually give rise to spermatogonia or oogonia. In this study, we discovered that *nanos3 3′UTR*, a common method used to label PGCs, not only directed PGC-specific expression of DsRed but also prolonged this expression up to 26 days post fertilization (dpf) when *DsRed-nanos3 3′UTR* hybrid mRNAs were introduced into 1- to 2-cell-stage embryos. As such, we employed this knowledge to express zebrafish leukemia inhibitory factor (Lif), basic fibroblast growth factor (Fgf2) and bone morphogenetic protein 4 (Bmp4) in the PGCs and evaluate their effects on PGC development in vivo for over a period of 3 weeks. The results show that expression of Fgf2 significantly increased PGC number at 14- and 21-dpf while Bmp4 resulted in severe ventralization and death of the embryos by 3 days. Expression of Lif resulted in a significant disruption of PGC migration. Morpholino knockdown experiments indicated that Lif illicited its effect on PGC migration through Lif receptor a (Lifra) but not Lifrb. The general approach described in this study could be used to achieve prolonged PGC-specific expression of other proteins to investigate their roles in germ cell and gonad development. The results also indicate that zebrafish PGCs have a mechanism to stabilize and prolong the expression of mRNA that carries *nanos3 3′UTR*. Understanding this mechanism may make it possible to achieve prolonged RNA expression in other cell types.

1. Introduction

Primordial germ cells (PGCs), the precursors of oocytes and sperm, depend on specific factors that guide their migration to the developing gonad and support their survival and proliferation [1–4]. In zebrafish, the PGCs are specified during the very early stages of embryo development by the incorporation of germ plasm that is packaged in the egg by the mother [5,6]. Following gastrulation, as the individual organs are forming in the embryo, the PGCs migrate from the region of the embryo where they originated to the area where the gonads are developing and enter the developing gonads and colonize the tissue. The major molecular cue that guides the migrating PGCs to the gonadal tissue is a protein called stromal-derived factor 1a (Sdf1a). The PGCs possess the Sdf1a receptor, Cxcr4b, on their surface which allows the cells to recognize the protein and migrate up a concentration gradient towards the developing gonad [1,2]. In addition to Sdf1a signaling, insulin-like growth factors (Igfs) and their receptors are also involved in controlling zebrafish PGC migration and survival [7,8].

The identification of zebrafish *nanos3 3′UTR* that can direct proteins of interest specifically in PGCs [4] provided a great tool for studying PGC development. The use of hybrid RNA to engineer mRNAs of the interest to carry *nanos3 3′UTR* enables PGC-specific labeling with fluorescence proteins [1,9] and expression of proteins of interest [8]. Most of the PGC-specific expression and labeling were used for studying PGCs during early development, the first 1–3 days. In an attempt to study the fate of mis-migrated PGCs at the ectopic location, we prolonged our observation for the DsRed labeled PGCs using *DsRed-nanos3 3′UTR* hybrid mRNAs. To our surprise, we found that normal-migrated PGCs continuously to express DsRed at gonadal region up to 26 dpf. To our knowledge, the globin mRNA is considered as one of the most stable mRNAs that contains a stabilizing element within its 3′UTR [10], with estimated half-lives ranging from 24 to 60 h [11]. Our finding indicated that *DsRed-nanos3 3′UTR* mRNA can be stabilized in PGCs for much longer than what we expected. Therefore, we applied this knowledge to express several protein factors that have been known to be involved in PGC development in vivo or in vitro, such as FGF2 [12,13], LIF [14,15] and BMP4 [16] and intended to evaluate their effects for over a period of 3 weeks.

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http://dx.doi.org/10.1016/j.bbrc.2012.11.014

2. Materials and methods

2.1. Animals and ethics

Zebrafish were maintained and staged as previously described [17]. All of the experimental protocols and procedures described in this study were approved by the Purdue University Animal Care and Use Committee and adhered to the National Research Council's Guide for Care and Use of Laboratory Animals.

2.2. cDNA cloning, in vitro capped RNA synthesis and antisense morpholino synthesis

cDNA of zebrafish *nanos3 3′UTR* and *bmp4* were cloned from cDNA prepared from whole embryos and *fgf2* was cloned from cDNA prepared from adult ovary using Advantage® 2 PCR Kit (Clontech). The primers used were: *nanos3 3′UTR*, Fwd1: 5′AAATCAG AATTCTGAAGCGGACATTGATGCT3′ and Rev1: 5′TTTATTAAGTCTAG AGAAAATGTTTATATTTTCC3′; *bmp4*, Fwd2: 5′GACAAACATGGGC CCTTTCCCCTTGCTTTAT3′ and Rev2: 5′GTAAGAGTCCGCGGTTAGC GGCAGCCACAC3′; *fgf2*, Fwd3: 5′ACAGACTTAGGGATGGCCACCGG3′ and Rev3: 5′TCAGCATTTGGCCGACATGGG3′. PCR program was 95 °C (1 min), 35 cycles of 94 °C (10 s)/60 °C (10 s)/68 °C (1.5 min) and 68 °C (6 min). Each PCR product was first cloned into pGEM-T-easy (Promega) and after sequence verification the cDNAs along with DsRed (pDsRed2-N1, Clontech) were ligated to *nanos3 3′UTR* to generate T7 driven and *nanos3 3′UTR* hybrid expression constructs (SFig. 1). The *EGFP-SV40 poly(A)* DNA fragment from pEGFP-N1 (Clontech) was ligated to T7 promoter to generate T7 driven *EGFP-SV40 poly(A)* construct. To generate T7 driven *lif-SV40 poly(A)* construct, the EGFP was replaced with *lif* cDNA using SacII and NotI restriction enzyme sites (SFig. 1). Capped sense mRNAs were synthesized in vitro using the T7 mMESSAGE MACHINE kit (Ambion) according to manufacturer's instructions. Antisense morpholino corresponding to *lif* nucleotides −23 to +2 (Lif-MO 5′ATGTTCCTGACATGACAGTTCATTC3′), to *lifra* nucleotides −1 to +24 (Lifra-MO 5′ AGCCAGAGTCACCCAGCCCTGCATA3′), *lifrb* nucleotides −1 to +24 (Lifrb-MO 5′AGCACTCAATAGCCAGACCGA-CATG3′), a previous identified antisense morpholino against *lifrb* [18], and a standard control morpholino (5′-CCTCTTACCTCAGTT-ACAATTTATA-3′) were obtained from Gene Tools LLC (Philomath, OR, USA).

2.3. Microinjection and fluorescence examination

In vitro synthesized capped mRNA at 100 nM was microinjected (1 to 2 nl) into the cell or the yolk just under the blastodisc of 1- to 2-cell stage zebrafish embryos. The antisense morpholino (0.2 mM) was injected or co-injected with capped mRNA using the same method. The number of DsRed-positive PGCs present in each embryo was counted at 3, 7, 14, 21 dpf using a Nikon Eclipse TE200 fluorescence microscope (Nikon, Japan). To facilitate PGC counting, each embryo or larvae was euthanized in 0.016% tricaine (Sigma–Aldrich) solution in water and partially dissociated by placing it on a slide under a coverslip to separate the PGCs before counting by fluorescence microscopy (SFig. 2).

2.4. RNA extraction and RT-PCR analysis

Total RNA was prepared from injected embryos at 3, 7, 14 and 21 dpf with Trizol reagent (Invitrogen) followed by DNase treatment (Ambion). The cDNA was synthesized using MMLV-RT (Promega) according to manufacturer's instructions. The primers used for RT-PCR analysis were *nano3 3′UTR* reverse primer Rev4 (5′GCGTATCAACCAAACAAACAATTA3′) and *fgf2* forward primer Fwd3. The PCR program was 40 cycles at 94 °C (10 s), 55 °C (10 s), and 68 °C (1 min).

2.5. Statistical Analysis

Data obtained from PGC counts were presented as the mean and standard error of mean. For statistical analysis Student's *t* tests or a one-way ANOVA were applied followed by Bonferroni (Dunn) *t* tests using a SAS program. The significance was accepted at $p < 0.05$.

3. Results

3.1. The presence of nanos3 3′UTR prolongs the expression of DsRed in the PGCs

Co-injection of *DsRed-nanos3 3′UTR* mRNA and *EGFP-SV40 poly(A)* into embryos resulted in stable DsRed expression in the PGCs for more than 3 weeks. DsRed (Fig. 1A) as well as EGFP (Fig. 1B) expression was detected throughout the embryo

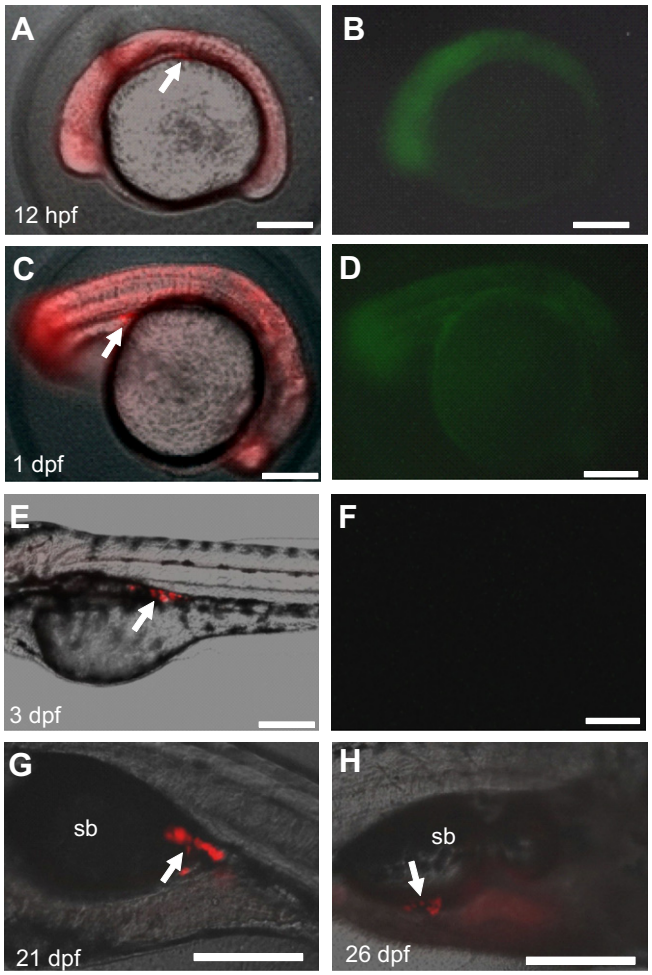

Fig. 1. The presence of *nanos3 3′UTR* prolongs DsRed expression in the PGCs for more than 3 weeks. Co-injection of *DsRed-nanos3 3′UTR* mRNA and *EGFP-SV40 poly(A)* into embryos resulted in stable DsRed expression in the PGCs for more than 3 weeks. (A) DsRed and (B) EGFP expressions are detected throughout the embryo beginning at the segmentation period with more DsRed fluorescence evident in the PGCs. (C, D) The ubiquitous expression of DsRed and EGFP remained visible at 1 dpf and gradually disappeared. (E) Only DsRed fluorescence was detected in the PGCs. (F) Expression of EGFP became invisible by 3 dpf (the same embryo as E) and (G, H) PGC-specific DsRed expression remained stable for up to 26 dpf. White arrows point to PGCs. Scale bar = 200 μm.

beginning at the segmentation period with more DsRed fluorescence evident in the PGCs (Fig. 1A). The ubiquitous expression of DsRed and EGFP remained visible at 1 dpf (Fig. 1C, D) and gradually disappeared. Only DsRed fluorescence was detected in the PGCs where it remained stable for up to 26 dpf (Fig. 1E–H). Expression of EGFP became invisible by 3 dpf (Fig. 1F).

3.2. Exogenous expression of Fgf2 significantly increased PGC number at 14 and 21 dpf

Since the presence of the *nanos3 3′UTR* resulted in stable and PGC-specific expression of mRNA introduced into the embryos, we decided to use this approach to investigate the effect of specific growth factors including Fgf2 on zebrafish PGC development. The majority (84.2 + 5.6% from 3 injection experiments) of embryos injected with mRNA encoding zebrafish Fgf2 (*fgf2-nanos3 3′UTR*) survived and developed normally. RT-PCR analysis confirmed that the *fgf2-nanos3 3′UTR* mRNA injected into 1- to 2-cell-stage embryos was continuously detected up to 21 dpf (Fig. 2A). Results of PGC counting revealed that there was no significant difference in the number of PGCs present in 3 and 7 dpf embryos that were injected with *fgf2-nanos3 3′UTR* mRNA compared to the controls (*DsRed-nanos3 3′UTR*) (Fig. 2B). At 14 dpf, however, PGC number was 35% greater in the Fgf2 expressing embryos and this significant difference was maintained through 21 dpf (Fig. 2B). Although expression of Fgf2 resulted in an increased number of PGCs, the factor had no effect on cell migration. Embryos were also injected with mRNA encoding Bmp4 (*Bmp4-nanos3 3′UTR*) which resulted in severe deformities and death of the embryos before effects on PGC development could be examined (SFig. 3).

3.3. Expression of Lif in the PGCs resulted in mis-migration of the cells

Embryos injected with *lif-nanos3 3′UTR* mRNA produced a normal number of PGCs, however, the cells were not able to migrate properly to the developing gonad. The majority of the ectopically located PGCs were observed in the junction of head and trunk region of the embryo (Fig. 3A) and along the outer surface of the yolk sac (Fig. 3B). The influence of Lif on PGC migration was dose-dependent since the percentage of gonadally located PGCs negatively correlated with the concentration of *lif-nanos3 3′UTR* mRNA introduced into the embryo (Fig. 3C). The effect on migration was also dependent on PGC-specific expression of Lif since injection of *lif-SV40 poly(A)* mRNA, resulting in ubiquitous production of Lif throughout the embryo, did not cause significant mis-migration of the PGCs (Fig. 3D).

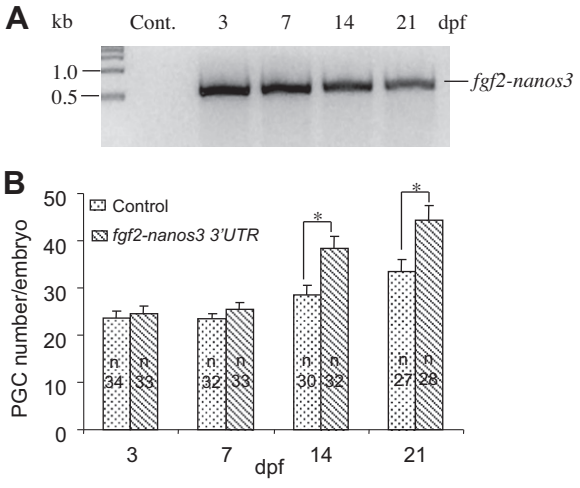

Fig. 2. Expression of Fgf2 in the PGCs significantly increased their number. (A) Results of RT-PCR showing expression of the injected *fgf2-nanos3 3′UTR* mRNA in embryos at 3, 7, 14, 21 dpf. (B) A significant increase in PGC number was observed in the Fgf2 expressing embryos beginning at 14 dpf compared to control embryos injected with only *DsRed-nanos3 3′UTR*. *n* ranging from 27 to 34 from three injection experiments. Cont.: Control RNA isolated from 3 dpf non-injected wild-type embryos. *indicates a significant difference by Student's *t* tests.

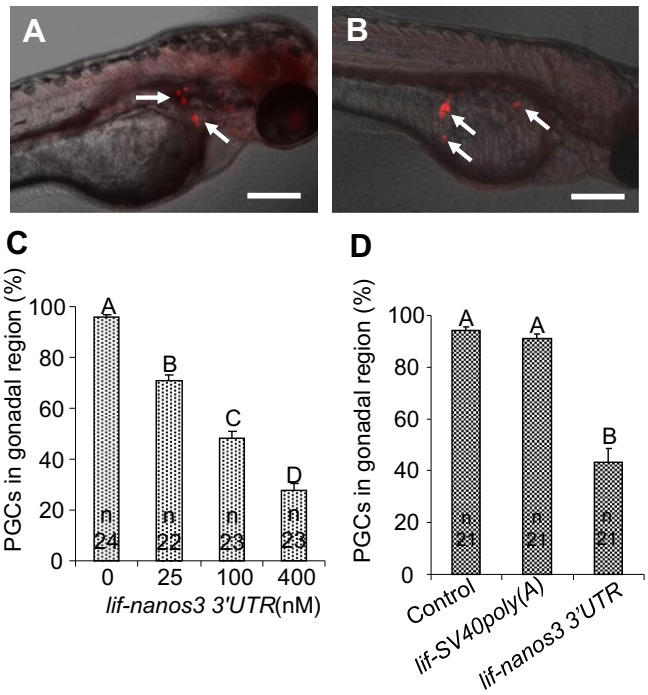

Fig. 3. Exogenous expression of Lif in the PGCs resulted in their mis-migration. Photomicrograph showing that the ectopically located PGCs (red, white arrows) were found in (A) the junction of head and trunk region and (B) outer surface of the yolk sac at 2.5 dpf. (C) The percentage of gonadal PGCs negatively correlated to the concentration of *lif-nanos3 3′UTR* mRNA used in the injection. *n* ranging from 22 to 24 from two injection experiments. (D) Ubiquitous expression of Lif by injecting *lif-SV40 poly(A)* mRNA did not cause significant mis-migration of PGCs. *n* = 21 from two injection experiments. Bars labeled with different letter indicate a significant difference from each other by Bonferroni (Dunn) *t* tests. Scale bar = 200 μm.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Lifra but not Lifrb is involved in mediating the effect of Lif overexpression on PGC migration

Morpholino-mediated inhibition of endogenous Lif expression did not cause significant mis-migration of the PGCs. Also, inhibited expression of each Lif receptor (Lifra and Lifrb), individually and together, did not alter PGC migration in the embryos (SFig. 4). However, inhibition of Lifra (Fig. 4A) but not Lifrb (Fig. 4B) expression did prevent the mis-migration of PGCs in embryos that were injected with *lif-nanos3 3′UTR* mRNA. The embryos injected with *lif-nanos3 3′UTR* and also treated with Lifra morpholino produced a normal number of PGCs that migrated properly to the developing gonad. The rescue of PGC migration in embryos that over-express Lif was only observed when Lifra expression was blocked. Inhibition of Lifrb expression did not rescue PGC migration (Fig. 4C).

4. Discussion

Previous studies have shown that the zebrafish *nanos3 3′UTR* is able to direct protein expression to the PGCs during early

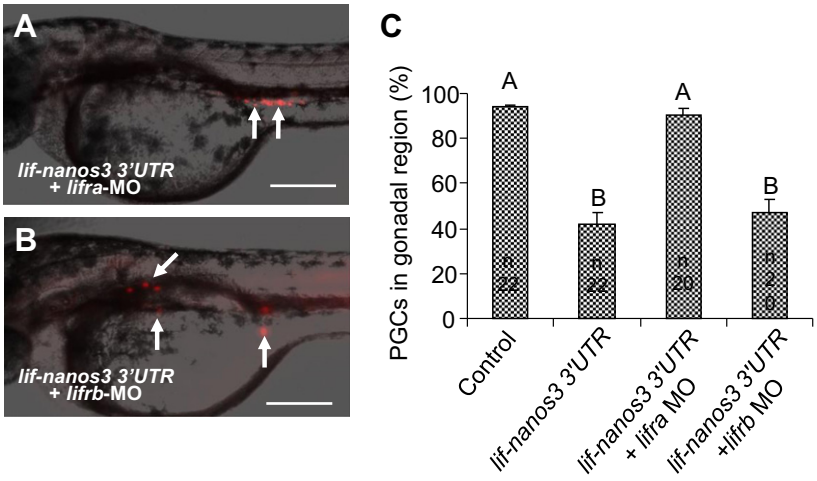

Fig. 4. Lifra signal transduction pathway is responsible for the mis-migration of PGCs that over-express Lif. (A, B) Photomicrographs of 2-dpf embryos that were injected with *lif-nanos3 3′UTR* and a morpholino directed against either (A) Lifra or (B) Lifrb. Knockdown of Lifra, but not Lifrb blocks the mis-migration of PGCs (red, white arrows) in the embryos that injected with *lif-nanos3 3′UTR*. (C) Statistical analysis showing that knockdown of Lifra significantly blocked mis-migration of PGCs caused by exogenous expression of Lif in the PGCs. *n* ranging from 20 to 22 from two injection experiments. Bars labeled with a different letter indicate a significant difference from each other by Bonferroni (Dunn) *t* tests. Scale bar = 200 μm.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

development [4]. In this study we show that the *nanos3 3′UTR* is also able to stabilize expression of the mRNA in the PGCs for more than 3 weeks. Using this method, we were able to learn that Fgf2 have a mitogenic effect on zebrafish PGCs at later stages (14 and 21 dpf). This achievement was not able to be accomplished by traditional RNA expression method since most transient RNA expression only lasts one or two days as seen in our *EGFP-SV40 poly(A)* mRNA injection. Although a miR-430 target site that can reduce mRNA stability and translation [19] has been identified within the *nanos3 3′UTR*, miR-430-mediated repression is inhibited in the PGCs by specific regions in the *nanos3 3′UTR* [19] and by the Dead end protein [20] resulting in germline-specific expression of Nanos3. Further work is needed to determine if the mechanism of germ line-specific expression also contributes to stable long-term expression in the PGCs. Identifying this mechanism and applying it to other cell types could provide a strategy to enhance cell reprogramming and/or differentiation by promoting the long-term expression of key transcription factors that regulate these processes without the need of daily or consecutive transfection of mRNAs [21].

Since Fgf2 and Lif were specifically expressed in the PGCs, the effects on PGC proliferation and migration that were observed in this study could have resulted from either an autocrine mechanism where the factor acted directly on the PGC or a paracrine effect of the factor on the surrounding somatic cells. The increase in PGC number observed in the Fgf2-expressing embryos was not evident before 7 dpf indicating that the PGCs are quiescent and not able to respond to the factor at this stage. This result is consistent with in situ hybridization studies that have shown that PGC number increases in the zebrafish embryo beginning at 10 dpf [22].

Our data demonstrated that over-expression of Lif by the PGCs results in their mis-migration which is mediated by Lifra. In mammals, Lif has been shown to serve a chemokine function by directing the migration of bone-marrow derived stem cells to damaged tissue [23]. In our study, however, since we found that morpholino-mediated knockdown of endogenous Lif and Lifra does not affect PGC migration, it is most likely that over-expression of Lif in the PGCs is disrupting another unknown signaling pathway. LIF functions by activating both the JAK-STAT [24,25] and phosphoinositide 3-kinases (PI3Ks) dependent pathways [26]. The PI3K-dependent pathway is also activated by the chemokine Sdf1a which is known to provide the directional cue required for proper zebrafish PGC migration [1,2]. Binding of Sdf1a to its receptor, Cxcr4b, activates intracellular G-protein and PI3K-dependent signaling resulting in directional PGC migration [27]. Insulin-like growth factors (Igfs) and their receptors, which can function by activation of the PI3K pathway, are also required for zebrafish primordial germ cell migration and survival [7,8]. Studies have shown that over-expression of a dominant-negative form of the regulatory subunit of PI3K (dnPI3K) results in mis-migration of zebrafish PGCs to ectopic locations outside of the gonad [27]. The effect of Lif over-expression on PGC migration may be due to disruption of the PI3Ks pathway in the PGCs resulting in the cell's inability to respond to Sdf1a and/or Igf. PGC mis-migration in *lif-nanos3 3′UTR* injected embryos can be rescued by knocking down Lifra expression indicating that Lifra is responsible for the signaling from over-expression of Lif in PGCs that disrupts their migration. Since Lifra is expressed ubiquitously in the embryo beginning at 75–90% epiboly [28], forced over-expression of Lif may result in constitutive Lifra activation in the embryo thereby limiting the availability of secondary messengers that are required by other PI3Ks-dependent factors such as Sdf1a and Igf.

Over-expression of Bmp4 in the zebrafish PGCs resulted in severe ventralization and death of the zebrafish embryos. Even though *nanos3 3′UTR* was used to direct Bmp4 expression specifically to the PGCs, the defects observed were similar to those previously reported when Bmp4 is expressed throughout the embryo [29]. Results with *DsRed-nanos3 3′UTR* injected embryos show that the *nanos3 3′UTR*-containing mRNA is initially expressed throughout the embryo as well as evidently to the PGCs. The initial ubiquitous expression of Bmp4 would be sufficient to disrupt dorsoventral patterning [29–31].

The finding that *nanos3 3′UTR* can stabilize expression of exogenously introduced mRNA for up to 3 weeks in the zebrafish embryo will provide an in vivo system to investigate the role of specific factors in regulating germ cell and gonadal development. The ability to maintain expression of the injected mRNA for up to 3 weeks will make it possible to investigate the role of factors that have a delayed effect on PGC development that is manifested late. The approach can be used to over-express factors that are normally produced by the PGCs as well as other factors that can be engineered to be secreted by the PGCs.

Acknowledgments

We thank A. Tesfamichael and J. Hooks for helping zebrafish maintenance and embryo collection.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.014.

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