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Cautious use of *fli1a:EGFP* transgenic zebrafish in vascular research

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ABSTRACT

Integration of exogenous sequence into an intact genome may cause some artificial phenotype or unspecific observations. We noticed that there is unspecific vascular expression when using *fli1a:EGFP* transgenic embryos for whole-mount *in situ* hybridization (WISH) experiments. We therefore tested whether the residual vector sequence contained in the *fli1a:EGFP* transgene or the integration of transgene into the genome may cause this expression 'noise' and/or deregulation of gene expression at a genome-wide level. RNA probes were synthesized using two different methods, i.e. vector-based and PCR-based. The vector-based *dnmt3* probe showed unspecific vascular expression in *fli1a:EGFP* embryos, but not in wildtype embryos, by WISH. Moreover, we also found that compared to that in wildtype, there were alterations in gene expression at whole-genome level in the *fli1a:EGFP* embryos. Our finding that the vector sequence contained in the *fli1a:EGFP* genome causes unspecific vascular expression by WISH and the genome-wide expression profiling is altered in *fli1a:EGFP* embryos strongly argue that extra caution should be taken for data interpretation when using transgenics, such as *fli1a:EGFP*, in developmental biology studies.

1. Introduction

The zebrafish, *Danio rerio*, has been demonstrated as an ideal model organism for vertebrate developmental biology in last two decades due to the following features: *in vitro* fertilization, rapid development of early embryos, and availability of genetic tools including forward genetics and reverse genetics. In particular, zebrafish embryo has been widely used for optical imaging due to its transparency combined with easy access to many kinds of transgenic lines expressing fluorescent proteins in a cell-type specific manner [1]. Among them, the *fli1a:EGFP* transgenic line has become a very useful tool for examining vascular development in zebrafish [2]. Application of this vascular-specific transgenic zebrafish in conjunction with time-lapse confocal microscopy allows continuous *in vivo* observation of embryonic vasculature for the first time in vertebrates [2]. For example, *fli1a:EGFP* transgenic line has helped characterize the detailed angiogenesis processes in wildtype zebrafish embryos as well as in mutant embryos including some disease models, such as tumor angiogenesis [3].

In addition to their wide application in live imaging, the *fli1a:EGFP* embryos have also been often used to further define the mechanism of vascular development. For such endeavors, whole-mount *in situ* hybridization (WISH), is one of the most popular techniques to examine gene expression during development and adulthood. Usually, RNA probes labeled with DIG or Fluorescein were hybridized to the target mRNA in the embryos to form a RNA–RNA hybrid. Then the hybrid was detected by the anti-DIG or anti-Fluorescein antibodies conjugated with a fluorochrome or an enzyme that converts a substrate to a visible dye to show the expression pattern of target mRNA. Surprisingly, when we performed WISH experiments with *fli1a:EGFP* embryos, we often observed a vascular-specific expression pattern for genes which have not been reported or anticipated in wildtype embryos previously. This leads us to pursue the underlying causes for these inconsistent expression pattern between wildtype and *fli1a:EGFP* embryos and how 'ectopic' vascular expression of these genes in *fli1a:EGFP* embryos arises.

The *fli1a:EGFP* transgenic line was generated with a 15-kb genomic fragment encompassing the first exon and upstream sequence of zebrafish *fli1a* which is sufficient to drive EGFP expression specifically in vascular endothelial cells [2]. The linearized p*fli1a*5EGFP DNA, which is injected into one-cell-stage embryos to get germ line transmission, contains some sequence from the backbone vector, pGEM3zf [2]. For WISH experiments, a linearized plasmid DNA which contains the gene of interest and a short sequence from the vector itself, is used as template for probe synthesis. The vector sequence included in the RNA probe may interact with the residual vector sequence integrated into the genome and cause the background or noisy expression in transgenic embryos generated with similar vectors. In the case of *fli1a:EGFP*, we speculate that the residual vector sequence in the *fli1a:EGFP* transgene may confer the 'artificial' ectopic gene expression in vascular tissues vessels of *fli1a:EGFP* embryos.

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Here, we report that when using two different methods to synthesize RNA probe for WISH experiments, the unspecific vascular expression of *dnmt3* in *fli1a:EGFP* line was observed and this was caused by cross reaction with the remaining vector sequence in the *fli1a:EGFP* transgene. Unexpectedly, the integration of this *fli1a:EGFP* transgene into the fish genome also caused expression changes at a genome-wide level.

2. Materials and methods

2.1. Fish strains and embryos

Embryos were obtained by natural spawning of adult zebrafish wide-type AB strain and *fli1a:EGFP* transgenic zebrafish. The *fli1a:EGFP* transgenic line was kindly provided by Steve Wilson (UCL, UK) and outcrossed with AB strain for more than 10 generations. Zebrafish embryos were raised and maintained at 28.5 °C in system water and staged as described previously [4].

2.2. RNA probe synthesis

RNA probes were synthesized using two different methods. Vector-based probe synthesis: the *dnm3* fragment was amplified from cDNA of zebrafish embryos at 24 hpf, using regular gene-specific primers dnmt3F and dnmt3R. The PCR product was cloned into pGEM-T vector and linearized with NotI for antisense probe synthesis as described previously [5]. PCR-based probe synthesis: we designed a primer with the T7 promoter sequence included 5′ upstream of the reverse primer (T7-dnmt3R), and then used this primer to amplify the *dnmt3* gene from zebrafish cDNA of 24 hpf. After purification, the PCR product was directly used as template to synthesize RNA probes with T7 RNA polymerase (Roche). All the PCR-based probes in this work were synthesized using this method. The detailed information for the primer used in this study is provided in Table S1.

2.3. Whole-mount in situ hybridization

Whole-mount *in situ* hybridization (WISH) was performed as described previously [5]. DIG-labeled probes including *dnmt3*, *grb2*, *kdelr2*, *rtkn2*,*egfp* and 240-bp were used in this study.

2.4. Southern blotting and LM-PCR

Southern blotting was performed as described previously [6]. LM-PCR was performed essentially as described previously [7].

2.5. Microarray analysis

Total RNA of wildtype and *fli1a:EGFP* embryos at 24 and 36 hpf were extracted using Trizol. cDNAs were labeled with Cy5- or Cy3 and hybridized to Agilent Zebrafish Oligo Microarrays (Shanghai Biotechnology Co. Ltd., Shanghai). Expression data were subjected to hierarchical clustering analysis and subsequently depicted in heatmap format. The lists of up- or downregulated genes are provided in Table S2 and S3.

3. Results

3.1. RNA probe synthesized using regular plasmid method gave a vascular expression in fli1a:EGFP embryos

To demonstrate whether the ectopic vascular expression in *fli1a:EGFP* embryos was conferred by the residual vector sequence in the *fli1a:EGFP* transgene, we performed WISH with RNA probes synthesized using two different methods. One is the routine method using plasmid DNA as template. After linearization, antisense RNA probe was synthesized with T7 or SP6 RNA polymerase, which was referred as plasmid-based method hereon. The other is PCR-based method. Namely, we designed the reversed primer containing a T7 promoter sequence to amplify the gene of interest using PCR, which is used as the template for probe synthesis. The synthesized RNA probe, thus, does not contain any sequence from the vector. We chose *dnmt3* as an example, since no expression of *dnmt3* is reported in vessels [8]. As shown in Fig. 1, the *dnmt3* probe synthesized using the pGEMT vector (pGEM-*dnmt3*) as template detected expression in neural tube as well as in the axial vessel region in *fli1a:EGFP* embryos at 24 and 36 hpf. In contrast, the same probe only detected expression in the neural tube, but not in the vessels, in wildtype embryos. This suggests that the vector-based probe may detect unspecific background expression in vessels of *fli1a:EGFP* embryos. To clear this out, we synthesized the *dnmt3* probe using PCR-based method (PCR-*dnmt3*). PCR-*dnmt3* probe only detected dnmt 3 expression in neural tube, but not in vessels in both *fli1a:EGFP* embryos and wildtype embryos. Since the only difference between the vector-based probe and PCR-based probe is the presence of residual vector sequence, it is possible that the vector sequence retained in the vector-based probe may bind to some sequence present only in *fli1a:EGFP* transgenic line but not in wildtype, thus causing this discrepancy. One of the candidates is the residual vector sequence that is incorporated into the genome together with transgene.

3.2. The 240-bp sequence within the fli1a:EGFP transgene is the cause of the unspecific vessel expression

To verify the remaining vector sequence in the transgene, we performed linker-mediated PCR (LM-PCR) to isolate the flanking genomic sequence of *fli1a:EGFP* transgene. As shown in Fig. 2A, we obtained a 240-bp fragment immediately downstream of EGFP coding sequence. Blast analysis showed that 123 out of 240-bp is identical to the SV40, and two short sequences are identical to the pGEM-T vector (Fig. 2B). To examine whether this 240-bp fragment is linked with EGFP gene on the chromosome, we did Southern blotting experiment with EGFP gene and this 240-bp fragment as probes, respectively. The results showed that both EGFP and 240-bp probes produced the similar patterns and there is only one single-copy of this transgene in the genome of this *fli1a:EGFP* transgenic line (Fig. 2C). These data strongly suggest that the 240-bp is linked with the EGFP gene.

To further test whether this 240-bp fragment cause the 'artificial' vascular expression in WISH experiments, we synthesized RNA probes with the EGFP gene and the 240-bp fragment, respectively, using PCR-based method (Fig. 2D). WISH showed that both probes gave the same expression patterns in vessels in *fli1a:EGFP* embryos, although to different extent, probably due to the different length of these two probes used.

3.3. Expression profiling was disrupted in fli1a:EGFP embryos by microarray analysis

The integration of exogenous sequence into the intact genome might have some influences on the expression pattern of the whole genome. To test this hypothesis, we performed expression profiling at a genome-scale by microarray analysis. We compared the expression profiling of wildtype and *fli1a:EGFP* embryos at two different stages, 24 and 36 hpf, respectively. As shown in Fig. 3A and B, there is an obvious gene dysregulation. Compared to the control wildtype embryos, expression of 107 genes was upregulated while expression of 128 genes was downregulated in *fli1a:EGFP* embryos. GO analysis indicated that a number of biological processes were

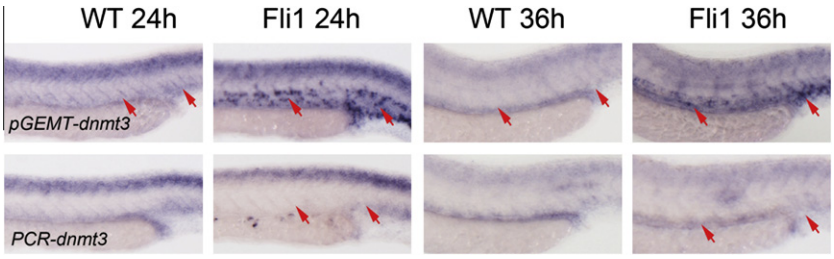

Fig. 1. Vector-based *dnmt3* probe showed vascular expression of *dnmt3* in *fli1a:EGFP* embryos, but not in wildtype embryos, whereas PCR-based *dnmt3* probe did not detect vascular expression of *dnmt3* in both types of embryos. Arrows indicate vascular expression.

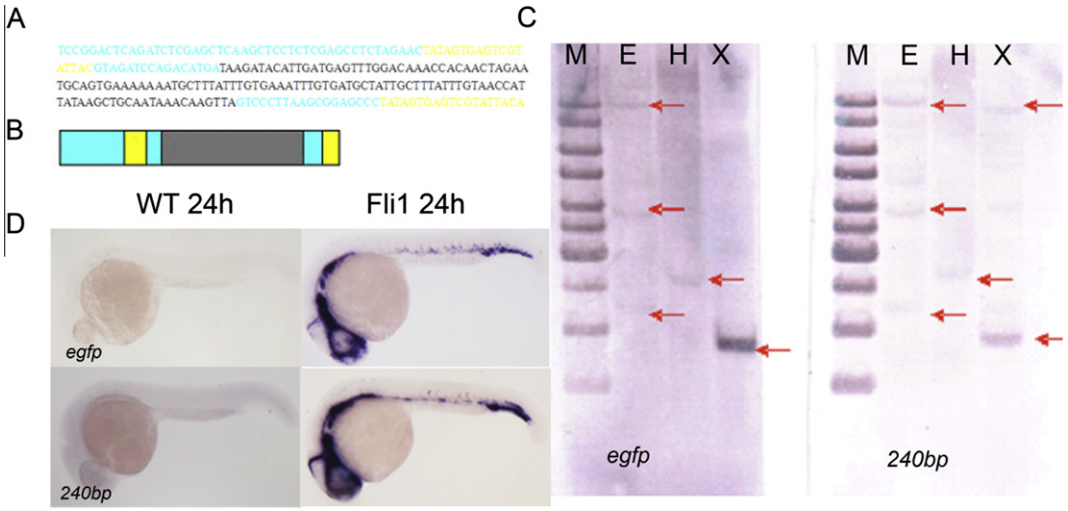

Fig. 2. Characterization of a 240-bp fragment in the *fli1a:EGFP* transgene. (A) The flanking sequence of *fli1a:EGFP* transgene in the genome. (B) Structure analysis of 240-bp sequence showed that it contains some vector-based sequence. Blue, 240-bp unique sequence; yellow, pGEM-T sequence; grey, SV40 sequence. (C) Southern blotting showed that the 240-bp fragment is tightly linked with the GFP gene and there is single-copy of *fli1a:EGFP* transgene in the genome. M, marker; E, EcoRI; H, HindIII; X, XhoI. (D) Both EGFP and 240-bp probes generated by a PCR-based method demonstrated the same expression pattern in blood vessels in *fli1a:EGFP* embryos, but not in wildtype.

affected in the *fli1a:EGFP* embryos (Fig. 3C). To validate the microarray data, we randomly chose three genes for WISH experiment, including *grb2* (upregulated), *kdelr2* (downregulated) and *rtkn2* (downregulated) (Fig. 3D). WISH experiments with PCR-based RNA probes showed that, consistent with the microarray data, *grb2* expression was indeed, increased in *fli1a:EGFP* embryos, compared to that in wildtype. Similarly, *kdelr2* and *rtkn2* expression was downregulated in *fli1a:EGFP* embryos, which agrees well with the microarray data.

4. Discussion

Here, we report that the well-known and one of the most popular transgenic lines in zebrafish research, *fli1a:EGFP*, displays unwanted vascular expression pattern by WISH when using regular plasmid-based RNA probe, likely due to the reaction with the residual vector sequences incorporated in the genome together with transgene. We identified a 240-bp fragment in the *fli1a:EGFP* genome, which is tightly linked with the EGFP gene, and confers this vascular expression 'noise'. Using PCR-based probes could avoid such vascular expression pattern caused by this 240-bp vector sequence in *fli1a:EGFP* fish. Moreover, the integration of *fli1a:EGFP* transgene into the fish genome caused alterations in expression profiling at a genome-scale level.

Application of transgenics in zebrafish combined with time-lapse confocal microscopy has greatly facilitated in vivo live imaging of any cell-type of interest. Direct visualization of such cells in vivo helped us decipher the detailed cellular process from cell migration, growth to differentiation during embryogenesis and adulthood. For example, the ability to visualize vascular development in vivo with the *fli1a:EGFP* embryos has advanced our understanding of the cellular and molecular mechanisms of vessel development in vertebrates [9]. More importantly, it provides a great tool to analyze the angiogenesis-related diseases, such as excessive tumor angiogenesis [3]. However, this man-made transgenics involves exogenous transgene construct containing some vector sequence, which will cause 'ectopic' expression pattern in the fluorescence reporter-positive cells when using vector-based probes. Although this could be avoided by using PCR-based probes which does not contain any vector sequence, the integration of transgene into an otherwise intact genome of an organism may cause some worries about the gene expression changes at a genome-wide level, as reported here. Our results indicate that extra caution should be taken when using *fli1a:EGFP* and other man-made transgenics for developmental biology studies, and data from the transgenics may not be directly applied into normal or pathological conditions.

Authors' contributions

F.L. conceived the project and designed the experiments; Z.L. carried out the experiments; Z.L. and F.L., analyzed the data; and F.L prepared the manuscript. Both authors read and approved the final manuscript.

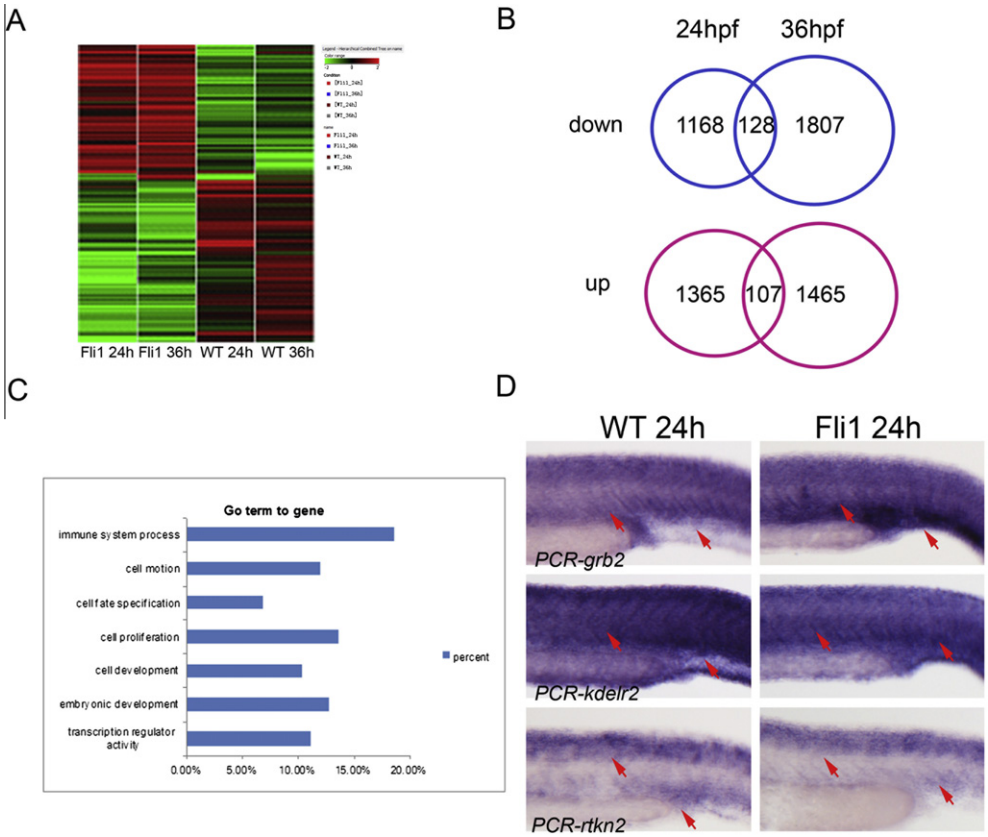

Fig. 3. Expression profiling changes in *fli1a:EGFP* embryos as detected by microarray experiments (A) Heatmap analysis of expression profiling between wildtype and *fli1a:EGFP* embryos at two different stages, 24 and 36 hpf, by microarray. (B) Overlap of up- or downregulated genes in *fli1a:EGFP* embryos at 24- and 36 hpf, compared to wildtype. Blue circles, downregulated genes; Red circles, upregulated genes. (C) GO analysis showed that a number of genes were affected in the *fli1a:EGFP* embryos. (D) Validation of microarray data by WISH. Three probes, *grb2*, *kdelr2* and *rtkn2*, were synthesized using PCR-based approach. Arrows showing the different gene expression levels in two types of embryos.

Competing interests

The authors declare that no competing interests exist.

Note added in proof

While the manuscript was in preparation, Cha and Weinstein also reported the similar finding as we described here [10].

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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.2012.09.064.

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