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Transgenic Manipulation in Zebra Fish by Combination of Cre-loxP Recombinant System, Tol2 Transposon System, and RNAi Technique

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ABSTRACT

The overall goal of this research is to make precisely controlled transgene constructs to target genes in zebrafish and facilitate their functional studies. Many of the previous transgenic techniques (e.g. morpholino) can only produce transient gene expression or inhibition, which is not good for long-term studies. Also, transgenes that randomly integrate into the chromosomes are typically difficult to control and are poorly regulated. Thus, it would be of great benefit to develop a reversibly controlled transgenic tool to help study gene function. Here, we propose to combine three distinct techniques to generate a stable transgenic tool to facilitate gene functional study in zebrafish, including Cre-loxP recombinant system, Tol2 transposon system, and RNAi technique. The first objective is to combine both Cre-loxP and Tol2 systems to make a convertible and movable transgene construct for insertional inactivation assays in zebrafish. The generated transheterozygous *rfp*/*gfp* fragments can be translocated to other loci in the fish genome upon the introduction of Tol2 transposase, which may result in insertional mutations and/or new patterns of interest. The second objective is to make a precisely controlled shRNA transgene construct to inhibit the *gfp* and/or *fli1* expression in zebrafish. Once the target gene is silenced, the zebrafish should show reduced green fluorescence, or some altered phenotypes (in the case of an endogenous blood cell specific target) that can be easily observed.

The results showed that we successfully established a stable homozygous pBa/RFP/loxP2/GFP/SBIR transgenic zebrafish line. The conversion from *rfp* to *gfp* expression was performed efficiently by heatshock-activated Cre recombinase *in vivo*. And the subsequent

germline transmission of the converted *gfp* expression was observed in heatshocked pTol-EF1α-RFP-loxP2-GFP transgenic fish outcrosses. The newly generated red/green transheterozygous fish from the cross of non-converted red fish and converted green fish are ready for further insertional mutation assay using Tol2 transgenic fish (Fuji70) or *Tol2* mRNA. The construction of the shRNA plasmid was completed and the F0 microinjected zebrafish showed mosaic *rfp* expression in a few blood vessel cells as well as muscle cells, which indicated the potential success of *fli1* driven shGFP transcription *in vivo*. Future goals include an examination of the efficiency of shGFP anti-*gfp* expression in pTol-Fli1-EGFP transgenic fish, and Cre recombinase mediated shGFP deletion and target gene expression recovery *in vivo*.

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CHAPTER 1

INTRODUCTION

Zebrafish as a Model Organism

In the post-genomic era, gene functional studies have become a major goal in cell and molecular biology. The mouse is traditionally the vertebrate model of choice for these investigations, mainly because of well-established transgenic knock-in and knock-out techniques. However the application of these techniques in mice suffers from several limitations, such as long generation time to sexual maturity, high cost, and animal care issues. Therefore, an alternative, affordable vertebrate animal model has been sought.

The zebrafish (*Danio rerio*) developed as an excellent vertebrate model system provides unique advantages over other organisms for identifying novel genes and elucidating their functions in developing animals. Zebrafish can be housed and maintained in large quantities. They develop rapidly and take less than 4 months for maturation. Several hundred eggs can be produced from each mating, providing a great benefit for transgenic screens. The big and optically transparent embryos can be easily manipulated, especially for DNA and RNA microinjections (Dahm *et al*., 2005). Unlike other vertebrate models, such as the mouse or *Xenopus*, the organs in zebrafish larvae have fewer cells, which make them easier to study. However, zebrafish still share most of the same organs and cell types as other vertebrates,

including humans (Veldman *et al*., 2008). In addition, the zebrafish genome sequence is about to complete. The sequenced genome consists of ~$1.9x10^9$ bp of DNA presently (http://www.sanger.ac.uk/Projects/D_rerio/), which is organized into 25 pairs of chromosomes (Wen *et al*., 2008). A complete genome sequence is expected to be finished within the next few years, which will greatly facilitate the systematic analysis of genetic functions regulating vertebrate development and differentiation (Dahm *et al*., 2005).

Transgenesis and Mutagenesis in Zebrafish

A large-scale ENU (N-ethy-N-nitrosourea) mutagenesis was performed in zebrafish to identify mutated genes in the past several years (Driever *et al*., 1996; Haffter *et al*., 1996). In these experiments, male zebrafish (spermatogonial cells) were induced with ENU mutagen and mutant phenotypes were screened in F3 progenies. Approximately six thousand mutants were identified using this method, including those involved in the abnormal development of fin, retina, brain, blood, and notochord, as well as deficiencies in epiboly, gastrulation, and axial patterning in early development (Driever *et al*., 1996; Haffter *et al*., 1996). Due to the laborious work required to identify mutant genes via positional cloning in this traditional method, an alternative technique using retrovirus based insertional mutagenesis was developed and successfully applied to identify 315 essential genes responsible for early zebrafish development (Amsterdam *et al*., 2004). Using this method, screens for the mutated genes are greatly facilitated by the presence of a molecular tag at the site of the mutations where the genomic DNA sequence flanking the inserted element can be easily cloned and sequenced (Amsterdam, 2006).

Recently, some of researchers found that they could induce targeted frame-shift mutations efficiently in zebrafish with customized zinc-finger nucleases (ZFNs) (Meng *et al*., 2008; Doyon *et al*., 2008; Ekker, 2008; Foley *et al*., 2009). With the help of ZFN expression

vectors, they can generate and introduce ZFN-encoding RNAs into zebrafish embryos to identify ZFN-induced mutations in targeted genomic sites (Meng *et al*., 2008; Doyon *et al*., 2008). ZFNs consist of a chimeric fusion between a Cys_2-His_2 zinc finger protein (ZFP) and the nonspecific cleavage domain of FokI endonuclease, in which the ZFP is responsible for DNA-binding specificity and the nuclease domain is in charge of cleavage activity. Because dimerization is required for nuclease domain activity, two ZFNs must be assembled on the target DNA for efficient DNA cleavage (Foley *et al*., 2009). ZFNs can be used to introduce double-strand DNA breaks (DSBs) in targeted genome loci and the repairs of these breaks often result in the introduction of insertions and deletions at these sites (Foley *et al*., 2009). It has been shown that the customized ZFNs can introduce targeted knockout mutations in cultured cells as well as model organisms including zebrafish (Wilson, 2003; Porteus *et al*., 2005). However, improvements are still needed to circumvent several limitations using ZFNs for target gene mutations. First, some of the genes may not contain the desired regions as required for the ZFN target sites. Second, a few of the engineered ZFNs cannot induce desired mutations in the target sites *in vivo* because of their deficiencies of the target binding affinities. Third, mutations introduced by ZFN are mostly random insertions or deletions. Therefore, it is hard to predict the mutations at the target sites using this method (Foley *et al*., 2009).

Other techniques, such as modified antisense oligomers (morpholinos, MOs) and targeting induced local lesions in genomes (TILLING) have been developed recently for specific mRNA knockdown and point mutations in a targeted locus for reverse genetic approaches in zebrafish (Heasman, 2002). MOs usually have 25 bases in length which can bind to complementary sequences of target RNAs. Unlike siRNAs, MOs do not degrade the target RNA molecules. Instead, they bind to a target sequence of RNA to interfere with the interactions

between other molecules and the target RNA. Therefore, they can block translation by binding to the 5'-untranslated region of target mRNA to hinder ribosome assembly. Also, they are capable to block splicing by binding and inhibiting pre-mRNA processing via inhibition of the splicesome components (Sumanas *et al*., 2002; Bill *et al*., 2009; Heasman, 2002).

Unlike MOs, the method of TILLING combines a standard mutagenesis technique with the chemical mutagens such as ENU and Ethyl methanesulfonate (EMS) and a sensitive DNA screening-technique to identify single base mutation (or point mutation) in a target gene. The genomic DNAs isolated from a large population for PCR amplifications of selected target genes are pre-required for performing TILLING (Sreelakshmi *et al*., 2010). Dr. Edwin Cuppen and his colleges found 255 mutations by using this TILLING method, which demonstrated that TILLING can be used for detections of ENU-induced mutations in zebrafish genome (Wienholds *et al*., 2003). Although both Morpholinos and TILLING techniques have been widely applied in the gene functional studies in many different organisms, there are still limitations in these two methods. Morpholinos are active only for a short period after introduction, thus they can only induce a transient effect and fail to impact on long-term phenotypes. Meanwhile, TILLING is not only time-consuming, but also inefficient for intron-rich genes with small exons due to the low effectiveness for obtaining null alleles (Doyon *et al*., 2008).

The generation of transgenic zebrafish by microinjection of plasmid DNA into fresh fertilized eggs was established and standardized over the past 20 years (Stuart *et al*., 1988 and 1990). However, early procedures generally relied on the random integration of transgenes into the recipient genome which can result in concatemeric DNA integration of hundreds of copies (Stuart *et al*., 1988), and it is usually difficult to achieve a stable and reproducible expression level among transgenic lines (Udvadia *et al*., 2003). It was observed that the transgenes in

zebrafish were usually silenced by some mechanisms when they were passed to subsequent generations (Stuart *et al*., 1988; Udvadia *et al*., 2003). Currently, the reason for the low efficiency of recombination and germline transmission is unknown, but the copy numbers of transgenes in the transgenic genome might be important (Hans *et al*., 2009). Therefore, an improved transgenic model system in which the transgene can be well controlled both spatially and temporally would be very valuable. There are already many genetic tools available for this purpose, including the use of site-specific recombinases (SSRs) such as Cre, Flp, and Φc31 in transgenic mouse and zebrafish studies (Dymecki *et al*., 2007; Hans *et al*., 2009); Transposon-mediated single copy insertion for transgenesis studies, such as Tol2 and *Sleeping Beauty* (SB) transposon systems (Hans *et al*., 2009; Urasaki *et al*., 2008; Takeda *et al*., 2007). These nonviral integration systems are widely used genetic tools for genome transgenesis and mutagenesis, because they have advantages of simple and inexpensive production, no induction of immunological responses, and improved safety (Sharma *et al*., 2008).

RNA interference (RNAi) is another new and promising technique for transgenesis studies, especially for understanding eukaryotic gene regulation and function. Currently, it has been applied to study gene function in plants (Travella *et al*., 2006), worms (Hoffmann *et al*., 2009), flies (Preall et al., 2006; Kavi *et al*., 2009), fish (Liu *et al*., 2005), and mammalian systems, e.g. mice (Dickins *et al*., 2007) and humans (Li *et al*., 2006).

Site-specific Recombination: Cre-loxP

Site-specific recombinases (SSRs) can cause deletion, insertion or inversion of target DNA sequences by breaking and rejoining DNA molecules at specific sites (Branda *et al*., 2004). The Cre-loxP recombinant system mediates site specific DNA recombination and was originally described in bacteriophage P1 (Sternberg *et al*., 1981). Cre (cyclization recombination) is a

38Kda protein that can recognize loxP (locus of crossover in P1) sites to facilitate the site-specific recombination of DNA. Bacteriophage P1 uses this Cre-loxP recombination system to maintain the proper partition of P1 plasmid into the daughter cells (Sternberg *et al*., 1981).

The loxP site is a 34 bp DNA sequence, including two 13bp inverted repeats (Cre recognition sites) and an 8 bp asymmetrical spacer (in the middle of two IRs; Fig. 1). During DNA recombination, two molecules of Cre monomers bind to two loxP sites (one Cre molecule per inverted repeat) to form a DNA synaptic complex for recombination. Then the intervening DNA fragment will be excised or inverted, depending on the orientation of the two loxP sites (Branda *et al*., 2004). The asymmetric spacer sequence determines the orientation of the loxP sites (Hoess *et al*., 1982). If the two loxP sites are present in opposite orientation, it will lead to inversion; while a direct orientation results in deletion of target genes (Fig. 2). The deletion event can result in loxP-flanked gene sequence forming a circular DNA and leaving behind one loxP site at the excision site. The loxP-containing circular DNA molecule will be degraded and have little chance to integrate back into its original excision site (Branda *et al*., 2004). Additionally, a reciprocal exchange of the loxP flanking sequences could be generated if two loxP sites reside in separate linear DNA sequence upon Cre recombinase activity (Branda *et al*., 2004). Some recent papers described the chromosome loss resulted by the inverted loxP sites (Lewandoski *et al*., 1997; Gregoire *et al*., 2008; Otsuji *et al*., 2008). After the chromosome duplication, the sister chromatid carrying the inverted loxP sites may be equally exchanged or unequally exchanged by Cre activity. Equal exchange between the two chromatids will produce two normal chromosomes. But unequal exchange will generate an acentric chromosome and a dicentric chromosome, which will be lost during cell division (Lewandoski *et al*., 1997, Fig. 45). Thus, the deletion activity is preferred than the inversion activity by Cre-loxP function.

The Cre-loxP system has been successfully applied for transgenesis studies in mouse (Bedea *et al*., 2009; Ludes-Meyers *et al*., 2009; Bailey *et al*., 2009) and human cells (Kowolik et al., 2004; Kijima *et al*., 1999) as well as zebrafish (Thummel *et al*., 2005; Seok *et al*., 2010; Hans *et al*., 2009; Yoshikawa *et al*., 2008). The site-specific recombination mediated by Cre-loxP has allowed researchers to control not only site-specific deletion, integration and copy numbers of transgenes, but also to temporally regulate recombinase action in a target genome. There are some inducible Cre recombinase systems developed recently for spatial and temporal regulation, such as the tetracycline-inducible system (Belteki *et al*., 2005 ; Rao *et al*., 2009), tamoxifen-inducible system (Speck *et al*., 2009; Weber *et al*., 2009; Hans *et al*., 2009), and the temperature controlled heatshock promoter (*Hsp70*) activating Cre-loxP system (Thummel *et al*., 2005).

For tamoxifen-regulated Cre recombinase system, Cre-ER^{T}, Cre is fused to a mutated human estrogen receptor at the C terminus that can bind tamoxifen (TM) or 4-hydroxy-tamoxifen (4-OHT), but not endogenous estrogens. With the introduction of TM or 4-OHT, the Cre-ER^{T} is released from the mutated human estrogen receptor and localizes to the nucleus where it is active (Loonstra *et al*., 2001; Hans *et al*., 2009). The tetracycline-inducible model is based on the regulatory elements of the transposon Tn*10*-encoded tetracycline resistance operon of *Escherichia coli*. Three essential components are required for the spatial and temporal regulation of transgenes. First is the tetracycline-regulated transactivator (tTA: Tet off) or the reverse tetracycline transactivation (rtTA: Tet on). Depending on the promoters used, either transactivator can be expressed spatially. Second is the tetracycline response element (TRE), which is linked to the transgene to control its expression. Generally, a minimal cytomegalovirus (CMV) enhancer is included. Third is the tetracycline analog, doxycycline (Dox), which can

activate or inhibit target gene expression based on which of the transactivator is used. In the Tet off system, Dox binds tTA and renders it incapable of binding to TRE, thus inhibiting target gene expression. In the Tet on system, Dox binds rtTA and activates its binding with TRE, thus targeting gene expression (Belteki *et al*., 2005; Rao *et al*., 2009; Bailey *et al*., 2009). For the temperature controlled Cre recombinase system, Cre gene expression is regulated by a heatshock protein promoter, *Hsp70*, which can be activated by environmental temperature above 37℃. This system was developed by Dr. Ryan Thummel and has been applied in many transgenic zebrafish studies because of its extreme convenience (Yoshikawa *et al*., 2008; Hans *et al*., 2009; Feng *et al*., 2007). These improved Cre recombinase systems have shown the possibility of inducible Cre-mediated target DNA excision and the goal for their applications is to get minimal or preferably no excision in the non-induced situations.

Figure 1.

Sequence of the loxP Site That Can be Recognized by Cre (Sternberg et al., 1981).

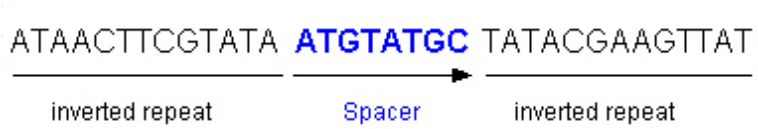
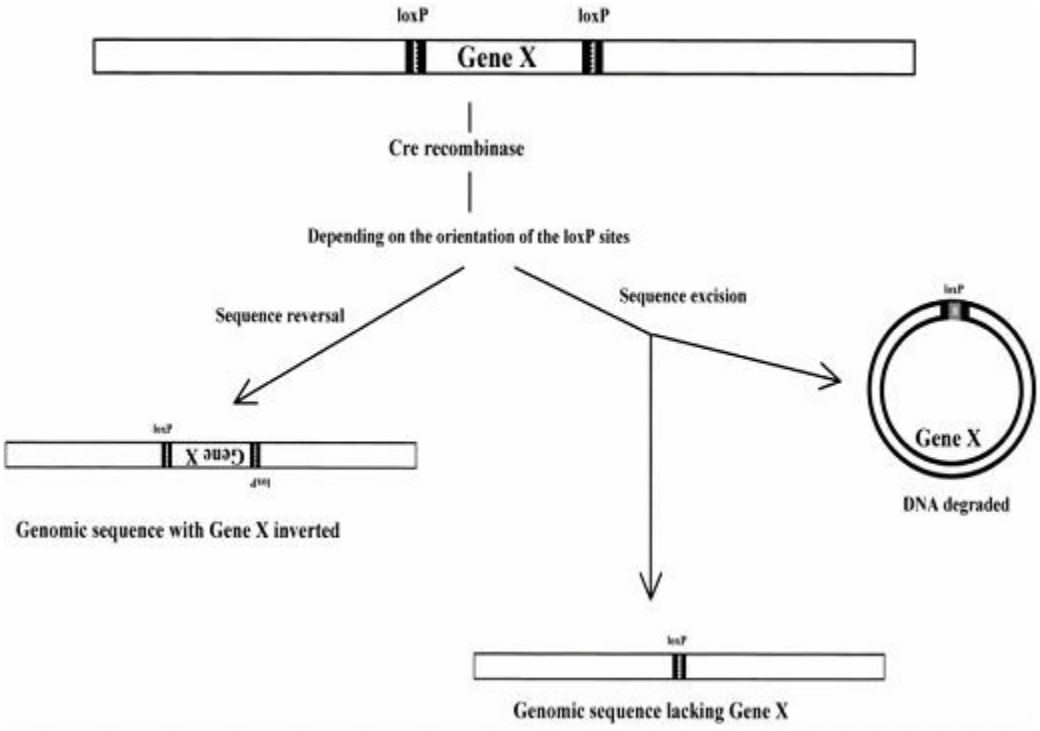


Figure 2.

Schematic of Cre-loxP Mediated DNA Recombination.

Gene X is either excised or inverted depending on the same or opposite orientations of the two loxP sites (Stricklett *et al*., 1999).



Transposon Systems: Tol2 and Sleeping Beauty (SB)

Transposons (initially known as "jumping genes") are mobile or transportable elements that can move around from one place to another on the same chromosome or different chromosome in a single cell. They perform copy-and-paste or cut-and-paste actions during translocation and cause inversion mutations in target cells. DNA transposons are mostly found in bacteria, but some exist in the genomes of insect, fish, worm and human. Generally, DNA transposons contain a transposase gene required for transposition and flanking sequences that can be recognized by the transposase (Ivics *et al*., 2010; Ni *et al*., 2008). The transposition process

mainly consists of four steps: first, transposase recognizes and binds to its target sites (transposon inverted repeats, TIRs); second, a synaptic complex is formed upon the binding of transposase subunits; third, the synaptic complex will be released from the donor sites; finally, this synaptic complex reintegrates into another target site (Ivics *et al*., 2010; Ni *et al*., 2008).

The Tol2 transposon system was discovered as the first active DNA-based transposon in vertebrates and it has been shown to be active in all vertebrate cells tested thus far, including zebrafish, mouse, chicken, *Xenopus* and human (Kawakami, 2005, 2007). Tol2 was found firstly in the medaka fish genome (Koga *et al*., 1996). It belongs to the *hAT* family of transposons and utilizes a cut-and-paste mechanism for DNA transposition (Koga *et al*., 1996). Studies have demonstrated that the minimal recognition site sequences required for Tol2-mediated transposition are the 200bp left end (5'-end) and the 150bp right end (3'-end) regions (Urasaki *et al*., 2006). Several advantages, such as wide host range, high cargo capacity (more than 10kb transgene), and single-copy integration of transgene, has made Tol2 an ideal transgenic tool for gene functional studies (Kawakami, 2005). To date, many reports have shown the successful application of the Tol2 transposon system for gene transfer studies in zebrafish (Balciunas *et al*., 2006; Kawakami *et al*., 2004), mice (Kawakami *et al*., 2004) and human cells (Balciunas *et al*., 2006). Other than the Tol2 transposon, another *hAT* family member, Tol1, was recently found in the genome of the medaka fish *Oryzias latipes* (Koga *et al*., 1995, 2007). It has been tested actively in many organisms, such as medaka fish, zebrafish, frog, mouse, and human (Kodama *et al*., 2008). However, its transgenic capabilities remain unclear.

Sleeping Beauty (SB) is the first active member of the Tc1/mariner family of transposons discovered in vertebrates (Ivics *et al*., 1997). SB uses cut-and-paste activity during translocation and results in the excision of the element from its original position to a secondary locus. It has

three components, the transposase, inverted repeats (IRs), and TA base pairs which are the potential integration sites of SB. The SB transposase has an N-terminal DNA-binding domain, a nuclear localization signal, and a C-terminal catalytic domain containing the DDE motif (composed of 2 invariable aspartic acid residues and a glutamic acid residue) that functions in excision and subsequent integration of transposon DNA (Huang *et al*., 2006). The advantages of SB transposon system in transgenic studies are a wide range of utilization in vertebrates and the short IRs (230bp) for recognition and binding of SB. It has been used in many different organisms (Ivics *et al*., 2007; Dupuy *et al*., 2006). Recently, Partow Kebriaei and Laurence Cooper (Williams, 2008; Singh *et al.*, 2008) proposed a clinical gene transfer trial that would introduce the SB-mediated genome altered T cells into the patients with CD19+ B-lymphoid malignancies to improve their health. This was the first human gene transfer trial utilizing the SB transposon system. Additionally, SB was found to be able to promote tumor formation by insertional mutagenesis of oncogenes, without causing wide-scale genomic instability (Collier *et al*., 2009).

Target Gene Silencing with RNAi

In order to examine our gene-specific inhibition and recombination system, we chose the fish blood vessel specific gene (*fli1*) as our study target. The *fli1* gene is well studied by some research groups (Truong *et al*., 2000), and more importantly, its promoter has been successfully cloned and applied to drive gene expression in the tissues of blood and blood vessels (Kamer *et al*., 2006; Lawson *et al*., 2002). Thus we choose the *fli1* promoter to test our Pol II promoter driven stable shRNA expression design.

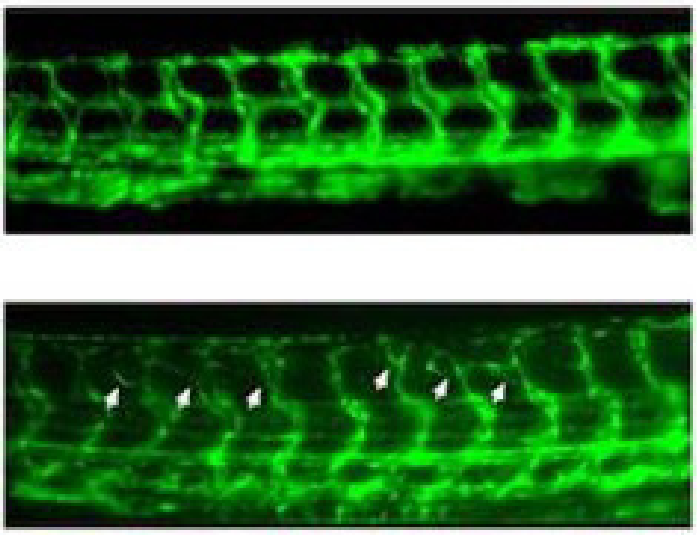
Fli1 (Friend leukemia virus integration 1) was first identified as a common site for retroviral integration in Friend virus-induced erythroleukemias. Fli1 is a member of the Ets

family of transcription factors. Ets factors have over 40 members which are characterized by an 85 a.a. region of homology termed the Ets domain, which can bind to the Ets recognition element 5'-GGA(A/T)-3' (Karim *et al*., 1990; Eisbacher *et al*., 2003). Fli1 is the earliest known endothelial cell marker in zebrafish and persists in blood vessels until at least 2 dpf (Lawson *et al*., 2002). The *fli1* promoter driving marker gene expression (e.g. *gfp*) has been applied in many transgenic zebrafish studies. For example, in the transgenic fish line Tg(fli1a-efgp), *egfp* expression can be observed in blood, blood vessels and pharyngeal arches, giving a visible demonstration of embryonic vascular development in zebrafish (Otsuji *et al*., 2008) (Fig. 3).

Figure 3.

Tg(pfli1a-egfp) Transgenic Zebrafish (Dr. Albig's lab).

Arrows indicate the *fli1a* promoter driving *egfp* expression in specific blood vessel cells of fish.



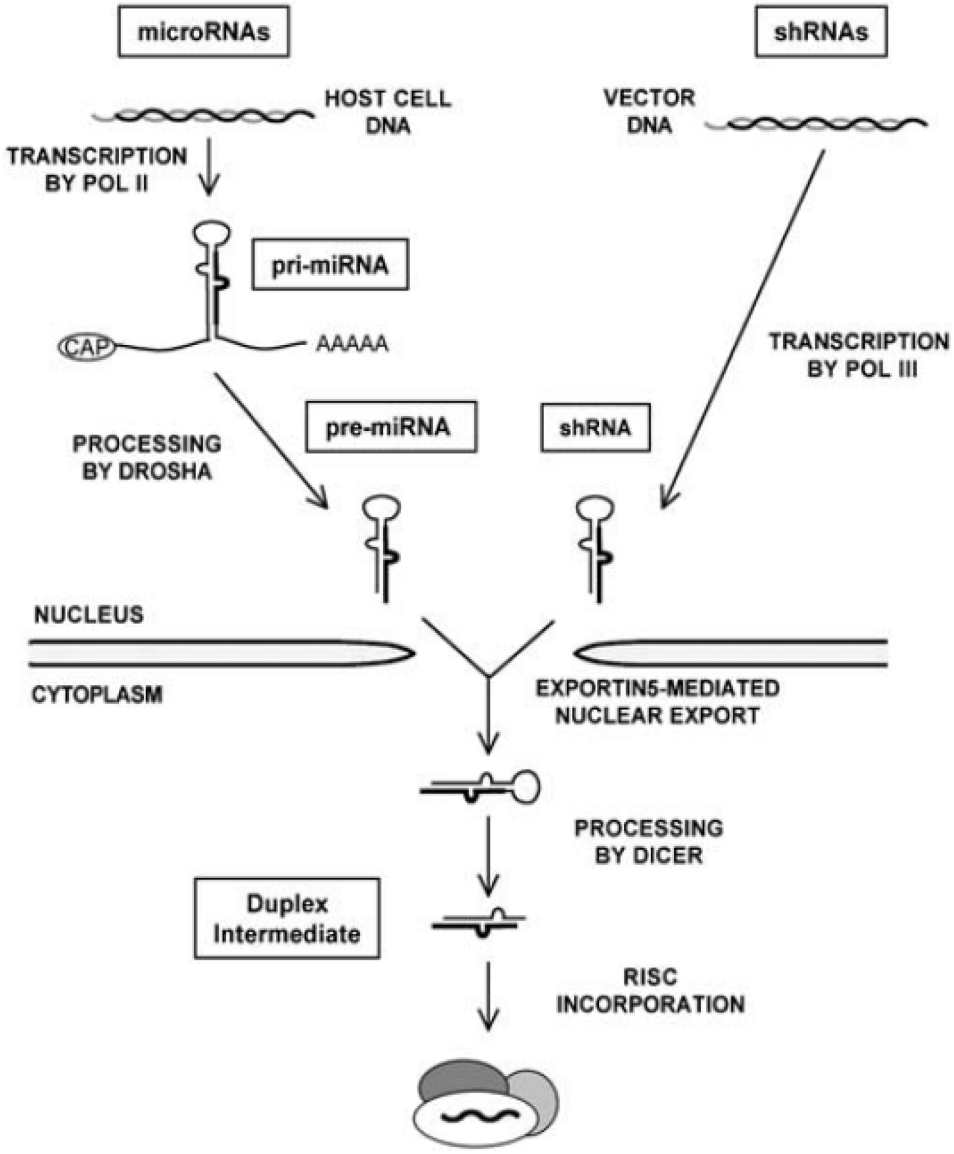
RNA interference (RNAi) is an evolutionarily conserved mechanism that suppresses the expression of genes that determine fundamental cell fate decisions of differentiation and survival. In RNAi, small double-stranded RNAs (dsRNA) that are cut from long double-stranded RNAs or from transcripts can form stem-loops and silence gene expression by several

mechanisms: 1) targeting mRNA for degradation; 2) preventing mRNA translation; and 3) establishing regions of silenced chromatin (Brennecke *et al*., 2003; Sandy *et al*., 2005). Target mRNA degradation occurs when one strand (the antisense or guide strand) of the small interfering RNA (siRNA) directs the RNA-induced silencing complex (RISC) that contains the RNA endonuclease Argonaut2 to cleave its target mRNA containing a complementary sequence (Fig. 4). Certainly, siRNA-mediated mRNA degradation has become a powerful technique for silencing gene expression due to its high specificity.

Figure 4.

Schematic Diagram of microRNAs and shRNAs Processing.

miRNAs are transcribed by polII as pre-miRNAs and then processed by nuclear RNase III enzyme Drosha to give a hairpin intermediate. shRNAs are directly transcribed by polIII to generate a similar hairpin intermediate. (Cullen, 2006)



In mammalian cells, RNAi inhibition can be accomplished by introducing small interfering RNA (siRNA) or short hairpin RNA (shRNA) into cells. The siRNAs are generally introduced directly as short RNA fragment containing two 19 nt duplexes and 2 nt overhangs at the 3' ends. The shRNAs are usually transcribed from a plasmid construct which consists of a promoter, a short DNA (encoding a shRNA) and a terminator sequence (Sandy *et al*., 2005). The artificial structure of shRNA mimics pre-micro RNAs (pre-miRNA), which could be processed endogenously into single stranded miRNAs. Then, these miRNAs would further incorporate with RNA endonuclease Argonaute proteins to form RISC, which is capable of regulating both RNAi and translational repression (Sandy *et al*., 2005; Zhou *et al*., 2005).

For *in vivo* gene silencing, shRNAs have been delivered using viral vectors or microinjections into fertilized embryos. Many studies have used Pol III promoters, such as U6, H1 and tRNA promoters, to drive shRNA expression (Xia *et al*., 2002; Coumoul *et al*., 2005). The advantages of Pol III promoters include, 1) they can introduce high levels of shRNA expression, which in turn induces efficient silencing; 2) the small size of gene construct can be easily inserted into viral vectors. However, the drawbacks of non-cell specificity and off-target silencing have limited its applications (Chang *et al*., 2004; Zhou *et al*., 2005). An alternative way to deliver RNAi is to use Pol II driving shRNA expression. Compared with Pol III promoters, Pol II promoter-directed synthesis of shRNAs has the unique advantages of direct cell- or tissue-specific gene silencing. In fact, some miRNAs are known to be expressed by Pol II promoters (Xia *et al*., 2006; Lee *et al*., 2004; Lin *et al*., 2006; Zhou *et al*., 2005). Therefore, using Pol II directed sysnthesis of shRNA to mimic natural miRNA sysnthesis could be an efficient RNAi strategy *in vivo*.

Only limited tests have been made to examine the potential of shRNA-mediated silencing using Pol II promoters, especially in zebrafish. Thus we plan to utilize a cell-specific promoter (*fli1* promoter) to synthesize shRNA and silence target gene expression in transgenic zebrafish.

CHAPTER 2

OVERALL OBJECTIVE AND SPECIFIC GOALS

Overall Objective

The overall objective of this research is to create mobile and easily controlled transgenic constructs to generate insertion mutations or target gene expression inhibition in zebrafish so as to facilitate gene functional studies. There are several transgenic and recombinant tools available to study gene function. However, many of them can only introduce transient gene expression or inhibition, which are not good for long-term study. Also the genes that randomly integrate into the chromosome are, in general, poorly controlled and sometimes poorly expressed depending on the inserted locations. Thus, it is necessary to develop a reversibly controlled transgenic tool to help study gene function. The efficiency, specificity, and flexibility of this method should make it promising for wide applications in many other organisms.

Research Objective

As described above, a site-specific recombinant system like Cre-loxP can ensure precisely targeted gene insertion or deletion, and the Tol2 transposon system may generate single copy integrations into chromosomes. Furthermore, the RNAi technique can allow target gene silencing at the posttranslational stage. Thus, our first objective is to make an exchangeable and mobile transgene construct combining both the Cre-loxP and Tol2 transposon systems. Any gene

flanked by two loxP sites (same orientation) will be deleted by Cre recombinase and result in altered gene expression. This is handy for gene functional study. Furthermore, the Tol2 transposase can help mobilize the whole construct to other locations on the chromosomes to cause new insertions and expression patterns of interest. The second objective is to construct a powerful vector that contains a cell specific promoter driving the expression of a shRNA complementary to *gfp* and/or *fli1* in an attempt to silence the *gfp* and/or *fli1* gene expression. In addition, we propose to flank this shRNA fragment with loxP sites in order to make it removable. Subsequent introduction of Cre recombinase will delete the shRNA fragment allowing recovery of target gene expression in the next generation. The combination of Cre-loxP, Tol2, and RNAi will allow specific and efficient genomic modifications that will help to clarify specific gene functions *in vivo* and *in vitro* in the future. Furthermore, because there are no exogenous factors needed in these systems, the combined systems should be useful in virtually any organism.

Specific Goals

Primary Goal: Combine Cre-loxP recombinant system and Tol2 transposon system to make a convertible and movable transgene construct for establishing an easy transposition assay in zebrafish.

a) Establish and characterize pBa/RFP/loxP2/GFP/SBIR transgenic zebrafish models. The transgenes in these fish can convert from *rfp* to *gfp* expression after exposure to Cre recombinase.

b) Establish homozygous transgenic zebrafish containing these “red to green” convertible transgenes pBa/RFP/loxP2/GFP/SBIR.

c) Cross homozygous convertible transgenic zebrafish with pHsp70-Cre-egfp transgenic zebrafish (gift from Dr. Ryan Thummel, Notre Dame) to produce transheterozygotes.

Heatshock the offspring to activate Hsp70 promoter and drive the expression of Cre to cause the conversion from *rfp* to *gfp* expression. Outcross this converted transgenic zebrafish with the wild type zebrafish to test for converted transgene germline transmission.

d) PCR verification of *rfp* and/or *gfp* fragments in different transgenic strains. Use PCR as an alternative tool to test the existence of converted or unconverted fragments in transgenic progeny.

e) Cross the transheterozygous *rfp*/*gfp* zebrafish with Tol2 transposase transgenic zebrafish to mobilize the *rfp* or *gfp* constructs from one location to another. The transposition may insert the *rfp* or *gfp* constructs into new locations and cause insertion mutations.

Second Goal: Determine if RNAi constructs against *gfp or fli1* can be used as a dominant marker and as an example of gene function assay. Combine the Cre-loxP recombinant system and the RNAi technique to create a precisely controlled transgene construct to target the GFP marker gene and/or blood vessel cell in zebrafish. The goal of the study is to inhibit ("knock out") target gene expression through RNAi function, and then cross the RNAi transgenic fish with Cre transgenic fish (or introduce Cre mRNA) to alter the RNAi expression so as to recover the target gene expression in the next generation.

a) Establish and examine pTol2/fli1/IT1/shGFP/loxP2/RFP transgenic zebrafish. Design shGFP construct to block *gfp* expression (pTol2/Fli1/EGFP transgenic zebrafish obtained from Dr. Ekker's lab). This is a preliminary test to determine if the RNAi construct can be used as a dominant marker for gene function study.

b) Examine the efficiency of Cre recombinase mediated shRNA deletion and target gene expression recovery assay, by using pHsp70-Cre-egfp fish or Cre mRNA.

CHAPTER 3

MATERIALS AND METHODS

Recombinant DNA Techniques

Plasmid DNA Extraction and Purification

The methods for plasmid DNA extraction and purification in this project were based on the alkaline lysis method developed by Sambrook and Russell (2001) with slight modifications. Briefly, 3 ml LB broth with antibiotic (Ampicilin or Kanamycin) was inoculated with desired E.coli colony and incubated in shaker overnight at 37℃. 1.4 ml of the overnight culture was transferred into a 1.5 ml microcentrifuge tube and spun for 20 seconds at high speed (14,000 g). The supernatant was discarded. Then another 1.4 ml of the same culture was transferred into the same tube and spun again to collect the maximum of the cell pellet from the E.coli culture. The cell pellet was re-suspended thoroughly in 0.1 ml cell resuspension solution (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 2 μl RNase A) with pipetting. Then 0.2 ml cell lysis solution (0.2 M NaOH, 1.0% SDS) was added and mixed gently by inversion. After 2 min incubation at RT (room temperature), 0.15 ml neutralization solution (3 M KOAc, pH 4.8) was added into the mixture and inverted 6 to 8 times. After 2 min incubation at RT, the sample was microfuged at high speed (14,000 g) for 5 min. The supernatant which contained plasmid was transferred carefully to a fresh tube for phenol/chloroform/isoamylalcohol (mixture of 25:24:1, v/v/v)

purification. An equal volume of the phenol/chloroform/isoamylalcohol (500 µl) was added to the supernatant and vortexed for 1 min at RT. After 20 sec span, the aqueous layer was transferred to another fresh tube and mixed with 1 ml of 95% ethanol. The mixture was incubated at -70℃ for 30 min and microfuged again for 10 min at high speed. The supernatant was discarded and the pellet was rinsed with 0.1 ml of 95% ethanol. The plasmid DNA pellet was air dried and dissolved in 50 µl water or TE buffer for store at -20℃.

Agarose Gel Electrophoresis

1% (w/v) agarose was melted with 1x TAE buffer (40 ml) in microwave for 2 min. The gel solution was poured in horizontal minigel box after it cooled down to about 55℃. A comb was inserted to make wells for holding DNA samples (10 – 20 µl/well). After the gel was solidified, the comb was removed gently and the gel box chamber was filled with 1x TAE buffer to just cover the gel. Then DNA samples mixed with loading buffer (40% Glycerol, 1.0% SDS, 0.5x TBE, 0.1 M Na-EDTA, pH 8.0, 0.25% Bromophenol blue) were loaded into each well. The gel was run at 90 volts until the blue dye reached 3/4ths gel (about one hour). The gel was then transferred to a staining box in which it was stained by the EtBr (ethidium bromide) solution (10 mg/ml in TAE buffer) for 10 min. dH_2O was used to de-stain the gel for 10 min and a gel picture was taken by a digital camera under UV light.

Restriction Endonuclease Digestion

Multiple restriction enzymes were used in this project according to the different DNA sequences. The combinations of the appropriate enzymes, buffers, and BSA were optimized according to the New England Biolabs Manual. A 20 µl system was used for analytical digestion and 60 µl system for preparative digestion. The amount of DNA used for analytical digestion

was less than 200 ng and 5-10 ug for preparative digestion. All digestions were kept in 37℃ water bath for 1-6 hours.

DNA Restriction Fragment Purification

After restriction enzyme digestion, the DNA fragments were differentiated by agarose gel electrophoresis and the appropriate bands were cut, dissolved, and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA). Briefly, the DNA band excised from the gel was transferred to a pre-weighed 1.5 ml microfuge tube containing 3 volumes of ADB buffer (agarose dissolving buffer, w/v). The tube was then incubated at 55℃ for approximately 5-10 min until the gel was completely dissolved. The melted agarose solution was transferred into a Zymo-Spin column which was placed in a 2 ml collection tube. The tube together with the spin column was centrifuged at 14,000 g for 10 sec. The flow through was discarded. 200 µl of wash buffer was added to the column which was inserted into the collection tube again and centrifuged at 14,000 g for 10 sec. Another 200 µl of wash buffer was added to the column and spun again for 30 sec. The flow through was discarded and the column was transferred to a fresh 1.5 ml tube. 8-10 µl of dH_2O was added to the column that was then centrifuged at 14,000 g for 1 min to elute the DNA.

Fluorometric Measurement of DNA

DNA concentration was measured by a fluorometric machine (ND-3300, NanoDrop, DE). The dye Hochst 33258 (0.2 ng/µl) was used for DNA staining and a standard curve was created before testing the sample DNA concentrations according to the factory manual (DNA standard, 70 ng/µl). 2 µl of TNE buffer (10X, 100mM Tris; 2.0M NaCl; 10mM EDTA; pH 7.4) was first loaded into the DNA detecting well to “blank” the machine and then a 1:1 ratio of DNA: dye was loaded to measure DNA concentration.

DNA Ligation

The DNA vector and insert were ligated (1:2 ratio, 20 ng/µl) in a total of 20 µl ligation mixture containing 2 µl 10X ligase buffer and 1 µl T4 DNA ligase (400 u/µl, New England Biolabs, Ipswich, MA). The mixture was incubated at 16℃ for 6 hours or overnight. After incubation, 1 µl of 5 M NaCl was added and the DNA was precipitated with 60 µl 95% Ethanol at -70℃ for 30 min. Followed by a 10 min centrifuge, the DNA pellet was resuspended in 15 µl dH_2O and stored at -20℃.

Plasmid Transformation and Plating

The Competent Cells such as One-shot TOP10 Competent E.coli cells and One-shot ccdB Survival2 TIR Competent cells (Invitrogen, Carlsbad, CA) were used in all plasmid transformations. Briefly, the competent cells (50 µl aliquot) were thawed on ice and 2 µl (up to 5 µl) of DNA ligation mixture was added to the vial with gentle flip. The mixture was incubated on ice for 30 min and then followed by heat shock at 42℃ for 30 sec. After incubation on ice for 2 min, 0.25 ml of preheated (42℃) SOC medium was added to the vial which was incubated in the shaker at 37℃ for 1 hour with spin speed of 220 rpm. After the incubation, 80 µl of transformed cells were spread on to each LB plate containing Amp or Kan antibiotics (50-100 µg/ml). The plates were incubated inversely in the incubator at 37℃ overnight. The colonies grown on the plates were picked for sub-culture and plasmid mini-prep analysis next day.

Hybridization

The complementary oligonucleotides were mixed at equimolar concentration in a 0.5 ml microfuge tube (e.g. 100 µl system) containing 10 µl annealing buffer (10 mM Tris, pH 7.5-8.0, 50 mM NaCl, 1 mM EDTA or NEB Buffer 2). The tube was placed in a boiling water bath

(beaker) for 5 min and then cooled down slowly to RT. The hybridized oligo fragments were ready for ligation.

Bulk Plasmids Prep

The Bulk Plasmids Prep method (similar to the protocol of mini-prep) was used to store large quantities (0.3-0.5 mg/ml) of cloned plasmids. Briefly, 50 µl of an overnight E.coli culture was inoculated into a one liter flask containing 250 ml LB broth and incubated at 37℃ in shaker overnight. The culture was centrifuged at 7,000 rpm for 10 min in a 250 ml GSA bottles. The supernatant was discarded and the pellet was re-suspended in 10 ml cell resuspension solution. The solution was then split equally into two separate 30 ml screw-cap centrifuge tubes and mixed with 12 ml fresh cell lysis solution separately. After briefly vortexing, the tubes were incubated on ice for 10 min and centrifuged at 11,000 rpm (4℃) for 15 min. The supernatants were transferred to fresh 250 ml bottles. 9 ml of 8 M NH_4OAc and 80 ml of cold 95% Ethanol were added into each bottle. After incubation at -20℃ for 1 hour, the mixtures were centrifuged again at 8,000 rpm (4℃) for 15 min and the supernatants were poured off. The pellets were rinsed with cold 95% Ethanol and air dried. The DNA pellet was re-dissolved in 500 µl TE buffer or sterile water and stored at -70℃.

Genomic DNA Isolation

3-5 days old embryos (20-30 embryos) or cut fins from adult fish were collected and rinsed twice with PBS in a 1.5 ml microfuge tube. The embryos were drained thoroughly and the fins were mashed with a plastic stick, then 1 ml cell digestion buffer (10 mM Tris, pH 8.0; 2.5 mM EDTA, pH 8.0; 100 mM NaCl; 0.5% SDS) was added to the tube. The cells were completely dissolved by pipeting. Then, 10 mg/ml Proteinase K was added to a final concentration of 0.2 mg/ml (2 µl per 100 µl) to the cell mixture. The tube was incubated at 50 ℃

for 2 hrs or overnight for digestion. Phenol/Chloroform/Isoamy extraction was performed to purify the genomic DNA as with plasmid purification (please see plasmid mini-prep for details). The isolated genomic DNA was air dried and resuspended in 100 µl dH_2O for future use (store at -20℃).

Polymerase Chain Reactions

PCR reactions were performed using a Bio-Rad ALD1233 Peltier Thermal Cycler (Hercules, CA). The general procedures for PCR were optimized as follows: for a 100 µl reaction system, 10 µl of 10X PCR buffer (Pfu, Strategene, Wilmington, DE or Taq, New England Biolab, Ipswich, MA) was mixed with 2 µl dNTPs (10 mM), 5 µl 5'-primer (20 µM), 5 µl 3'-primer, 50-100 ng template DNA, and appropriate dH_2O. The reaction solution was covered with 1 drop of mineral oil and then placed in Thermal Cycler for PCR reaction. The PCR tube was incubated at 95℃ for 5 min and followed by a pause at 80℃. 1 µl of Pfu polymerase (2.5 u/µl) was added into the tube and then the program was started again for a repeat of 30 cycles: denaturation at 95℃ for 1 min, annealing at 58℃ for 1 min, and extension at 72℃ for 2 min. A final extension of 8 min at 72℃ was included after the 30 cycles in the program and the products were stored at 4℃. The PCR products were analyzed on 1.5% agarose gel for verification.

Plasmid Construction

Exchangeable and Mobile *rfp*/*gfp* Transgene Plasmid

The exchangeable and mobile *rfp*/*gfp* transgene plasmid was first created by Dr. Jie Dong in our lab. This plasmid contained two loxP recognition sites flanking the *rfp* gene and SV40 poly (A) signal for performing Cre-loxP deletion. The *gfp* coding sequence was placed immediately downstream of the *rfp* reporter gene, allowing exchange from *rfp* expression to *gfp*

expression under Cre activity. Two SBIR (Sleeping Beauty Inverted Repeats) sites were introduced for mobilizing the entire expression cassette by Sleeping Beauty Transposase and therefore be used to test the ability of introduced *SB*-transposase to catalyze *de novo* integration of the coinjected transgene (Fig. 5a and 5b). Subsequent SB-mediated deletions could also possibly result in the mobilization of the expression cassette to randomly chosen alternative sites in the genome. In addition, the presence of the loxA site downstram to the *gfp* should eventually allow Cre-mediated specific gene replacement. This plasmid represented our prototype vector with the combination of Cre-loxP recombinant system and SB transposon system. (For details of the plasmid construction please refer to Dong, 2005)

Figure 5a.

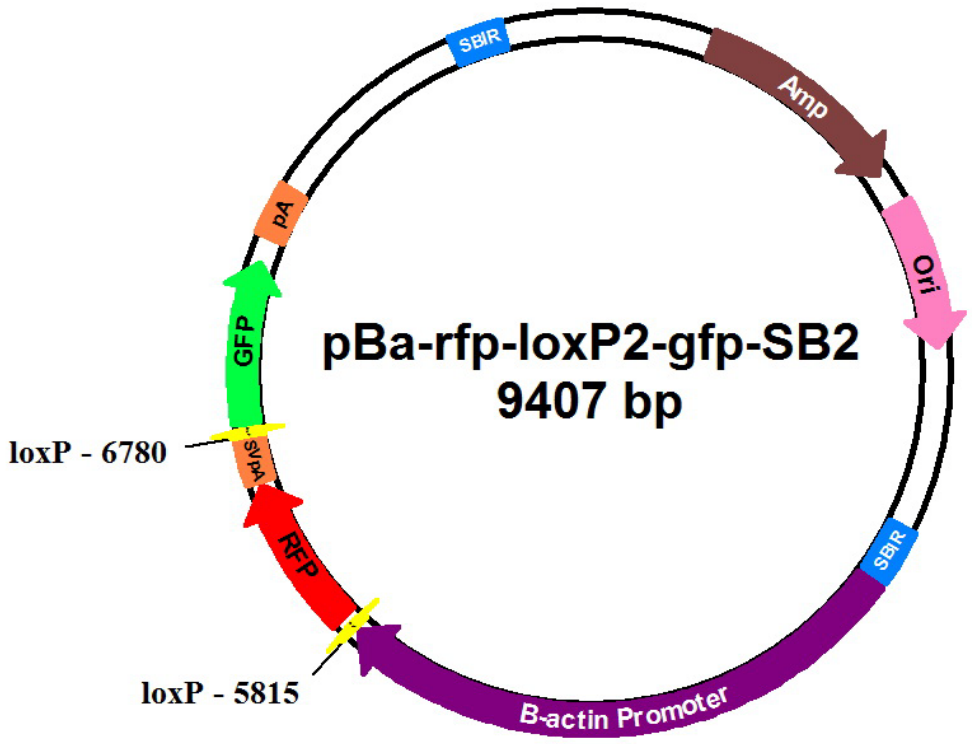
Basic Components of Plasmid pBa-rfp-loxP$_2$-gfp-SB$_2$

pBa-rfp-loxP$_2$-gfp-SB$_2$

SBIR	β-actin P	loxP	*rfp*-pA	loxP	gfp-pA	loxA	SBIR

Figure 5b.

Schematic Representation of Plasmid pBa-rfp-loxP$_2$-gfp-SB$_2$ (Dong, 2005)



Alternative Exchangeable and Mobile *rfp*/*gfp* Construct

Because of the design problems of the prototype plasmid pBa-rfp-loxP$_2$-gfp-SB$_2$, the exchanged *gfp* gene showed mosaic expression patterns and failed to pass through the germline in transgenic zebrafish. Therefore, we switched to the alternative construct pTol-EF1α-loxP-DsRed2-loxP-EGFP (Hans *et al*., 2009). This was similar to our vector, but provided a more sustained transgene expression pattern (Fig. 6). This plasmid also included two loxP sites flanking the DsRed2 reporter gene (that could be deleted by *Cre* recombinase activity) and following this, an EGFP reporter gene. However, it included the *Tol2* transposon system instead of the *SB* transposon system to mobilize the expression cassette, which has been demonstrated to

produce single-copy integrations in transgenic zebrafish. The β-actin promoter in our plasmid was replaced by EF1α promoter (Kawakami *et al*, 2004). This construct was kindly provided by Dr. Michael Brand (Dresden, Germany) as an integrated single-copy transgene in a stable line of transgenic zebrafish that we called "Dresden red fish".

Figure 6.

Basic Components of Plasmid pTol-EF1α-loxP-dsRed2-loxP-EGFP

pTol-EF1α-loxP-dsRed2-loxP-EGFP

TIR	EF1α P	loxP	dsRed2-pA	loxP	EGFP-pA	TIR

Cell Specific shRNA Plasmid

To create a cell specific shRNA vector, several components were assembled together: a blood vessel specific *fli1* promoter, an intron with SD (Splice Donor) and SA (Splice Acceptor), a shRNA fragment, two loxP sites, two TIR sites (Tol2 recognition sites) and a *rfp* reporter gene. The backbone plasmid of pTolfli1epEGFPDest was chosen and it was developed in Dr. Lawson's lab (University of Massachusetts Medical School, MA), which contains the blood vessel specific *fli1* promoter, two TIR sites and one *egfp* reporter gene. Therefore, we had to insert an intron fragment, two loxP sites, a DNA encoding a shRNA fragment, and replace the *egfp* with *rfp* reporter gene in the backbone plasmid. There were two other intermediary vectors, pTopo and pUC19, used during the construction.

In general, the intron fragment (including the first exon and the splice donor) of *fli1* was cloned into Topo Vector (Invitrogen, CA) by PCR from zebrafish genome using ZeroBlunt TOPO Cloning Kit for Sequencing (Invitrogen, CA) to generate pTopo-IT1 (IT1: intron1) vector (Fig. 7). The PCR program was designed as following: 94℃ 10 min; 80℃ 0 min; 35 cycles for

94℃ 1 min, 61 ℃ 1 min, and 72℃ 2 min; 72℃ 8 min; 4℃ 0 min. Three enzyme (NheI, KpnI, and NcoI, New England Biolabs, Ipswich, MA) restriction sites were introduced into the primers for further translocation of the intron fragment.

Intron PCR primers:

5' – primer: 5'-GACGCTAGCACTGGTACCGAATATTGTCGGGCTCCACTGAA-3'

3' – primer: 5'-CAGCCATGGGAGTGTTTGCATTTGTAGCCCTCCC-3'

Both pTopo-IT1 and pUC19 were digested with EcoRI and PstI to generate the intermediary vector pUC19-IT1 (Fig. 8). Then, the synthesized loxP oligonucleotides (upstream) was inserted into pUC19-IT1 vector ahead of the first exon, using two restriction sites NheI and KpnI (New England Biolabs, Ipswich, MA). Thus the vector of pUC19-IT1-loxP was generated.

Upstream loxP oligonucleotides:

5' - CTAGCATAACTTCGTATA**ATGTATGC**TATACGAAGTTATCAGGTAC

3' - CTGATAACTTCGTATA**GCATACAT**TATACGAAGTTATG

The next step was to insert the synthesized SA (Splice Acceptor) oligo into pUC19-IT1-loxP vector to make pUC19-IT1-loxP-SA. Two unique restriction sites were introduced into the short SA oligonucleotides: AflII and NcoI (New England Biolabs, MA).

SA oligonucleotides:

5' – TTAAGACGTTTTTTTTTTTGCAGGC

3' – CATGGCCTGCAAAAAAAAAAAACGTC

The downstream loxP oligonucleotides (same orientation as the upstream loxP) was synthesized and inserted into pUC19-IT1-loxP-SA using enzymes AflII and EcoRV (New England Biolabs, Ipswich, MA). This was located just upstream of the SA oligo in the newly formed vector of pUC19-IT1-loxP2-SA (Fig. 9).

Downstream loxP oligonucleotides:

5’-ATCATAACTTCGTATA**ATGTATGC**TATACGAAGTTATCAC

3’-TTAAGTGATAACTTCGTATA**GCATACAT**TATACGAAGTTATGAT

The assembled intron fragment of IT1-loxP2-SA was translocated from pUC19-IT1-loxP2-SA vector back into the pTopo vector to generate another intermediary construct of pTopo-IT1-loxP2-SA (Fig. 10) using EcoRI and PstI (New England Biolabs, Ipswich, MA). The next step was to translocate the intron fragment loxP-IT1-loxP-SA into the backbone plasmid of pTolfli1epEGFPDest to generate the negative control plasmid pTol-fli1-loxP-IT1-lxoP-SA-EGFP lacking the shRNA fragment. The restriction enzymes NheI and NcoI were used for this translocation. Meanwhile, the synthesized preliminary anti-*gfp* shRNA oligonucleotides (shGFP1, shGFP2, shGFP3, and shGFP4, which were designed to target GFP coding sequence in pTolfli1epCherryDest transgenic zebrafish) were inserted into the intron region of pTopo-loxP-IT1-loxP-SA to make the shGFP intermediary vector pTopo-loxP-IT1-shGFP-loxP-SA (Fig. 11). Two unique restriction sites (HindIII and MfeI, New England Biolabs, Ipswich, MA) were introduced into the shGFP oligonucleotides for their easy translocations. After constructing the vector pTopo-loxP-IT1-shGFP-loxP-SA, the intron fragment loxP-IT1-shGFP-loxP-SA was again translocated into the pTolfli1epEGFPDest vector to generate the microRNA plasmid pTol-fli1-IT1-loxP-shGFP-loxP-SA-EGFP (Fig. 12), using two enzymes NheI and NcoI. A microRNA plasmid with EGFP reporter gene was thusly created.

shGFP oligonucleotides:

shGFP1: mimic human mir-30 structure designed by IDTDNA ddsiRNA - site1

5’ –AGCTTTGCTGTTGACAGTGAGCGAGGGCGATGCCACCTACGGCAAGTGAAGCCA

CAGATGTTGCCGTAGGTGGCATCGCCCCTGCCTACTGCCTCGGACTTCAAGGGC

3’ –AATTGCCCTTGAAGTCCGAGGCAGTAGGCAGGGGCGATGCCACCTACGGCAA

CATCTGTGGCTTCACTTGCCGTAGGTGGCATCGCCCTCGCTCACTGTCAACAGCAA

shGFP2: mimic human mir-30 structure designed by IDTDNA ddsiRNA – site7

5’ –AGCTTTGCTGTTGACAGTGAGCGAGCACAAGCTGGTCAGTACGACTAGTGAAG

CCACAGATGTAGTTGTACTCCAGCTTGTGCCTGCCTACTGCCTCGGACTTCAAGGGC

3’ –AATTGCCCTTGAAGTCCGAGGCAGTAGGCAGGCACAAGCTGGAGTACAACTAC

ATCTGTGGCTTCACTAGTCGTACTGACCAGCTTGTGCTCGCTCACTGTCAACAGCAA

shGFP3: mimic Danio rerio mir-30a structure designed by IDTDNA ddsiRNA – no mutation

5’ –AGCTTGGCTCCTTGCAGTTGGGCGATGCCACCTACGGCAATTGTAATGCAGAAA

ATCTCAGTTGCCGTAGGTGGCATCGCCCTGCTACTGGTGGCCC

3’ –AATTGGGCCACCAGTAGCAGGGCGATGCCACCTACGGCAACTGAGATTTTCTGC

ATTACAATTGCCGTAGGTGGCATCGCCCAACTGCAAGGAGCCA

shGFP4: mimic Danio rerio mir-30a structure designed by IDTDNA ddsiRNA – mutation

5’ –AGCTTGGCTCCTTGCAGTTGGGCGATGCCACCTACGGCAATTGTAATGCAGAAA

ATCTCAGTTGTCGTAGTGCATCGCCCTGCTACTGGTGGCCC

3’ –AATTGGGCCACCAGTAGCAGGGCGATGCAGTACGACAACTGAGATTTTCTGCAT

TACAATTGCCGTAGGTGGCATCGCCCAACTGCAAGGAGCCA

The last step was to replace the EGFP reporter gene with RFP reporter gene from the pTolfli1epCherryDest plasmid (Dr. Lawson’s lab) to generate the ultimate plasmid pTol-fli1-IT1-loxP-shGFP-loxP-SA-RFP. The restriction enzymes used for this last step were NcoI and BamHI (New England Biolabs, Ipswich, MA). But, before the replacement of EGFP to RFP, the plasmid pTolfli1epCherryDest was digested by BamHI and re-ligated to itself to produce a single BamHI restriction site vector. The same process was done for plasmid

pTolfli1epEGFPDest before the insertion of the intron fragment.

Figure 7.

Schematic Representation of pTopo-IT1 (intron1) Vector

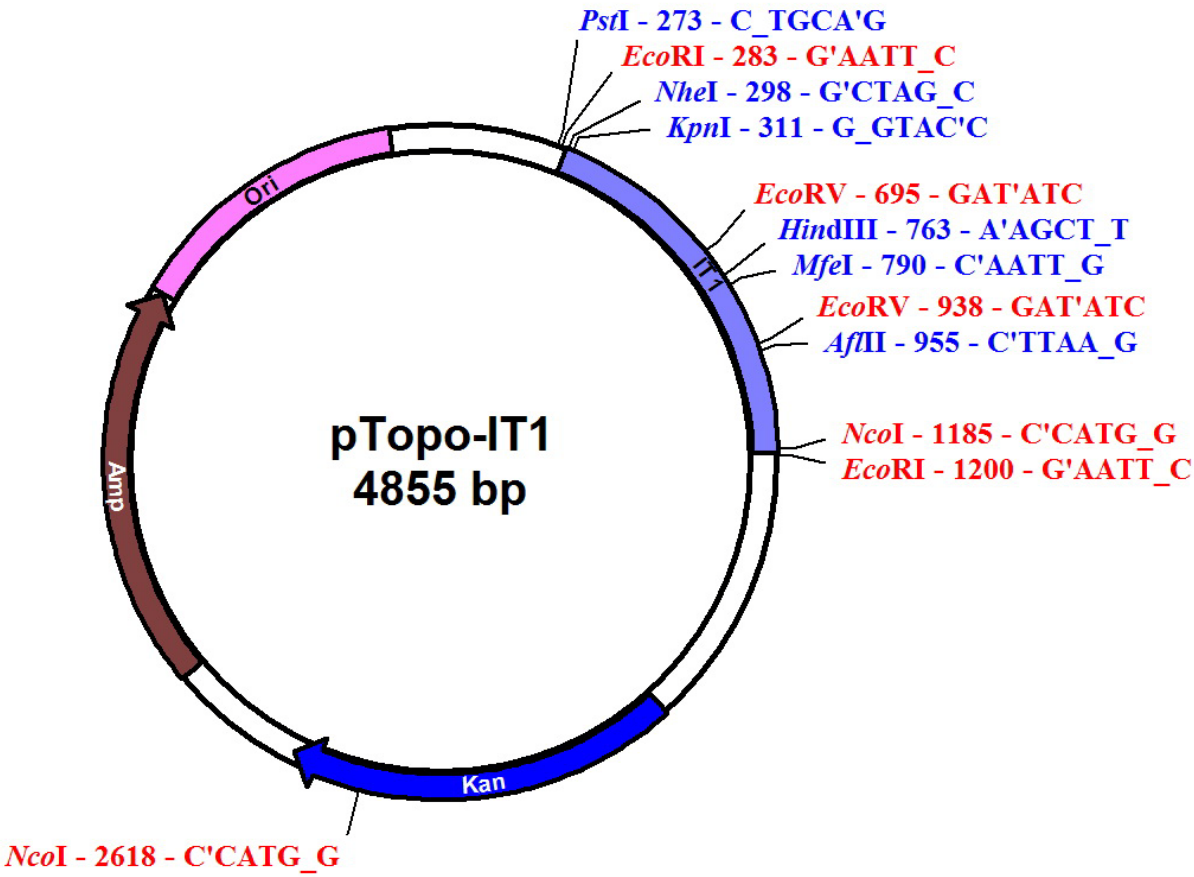


Figure 8.

Schematic Representation of pUC19-IT1 Vector

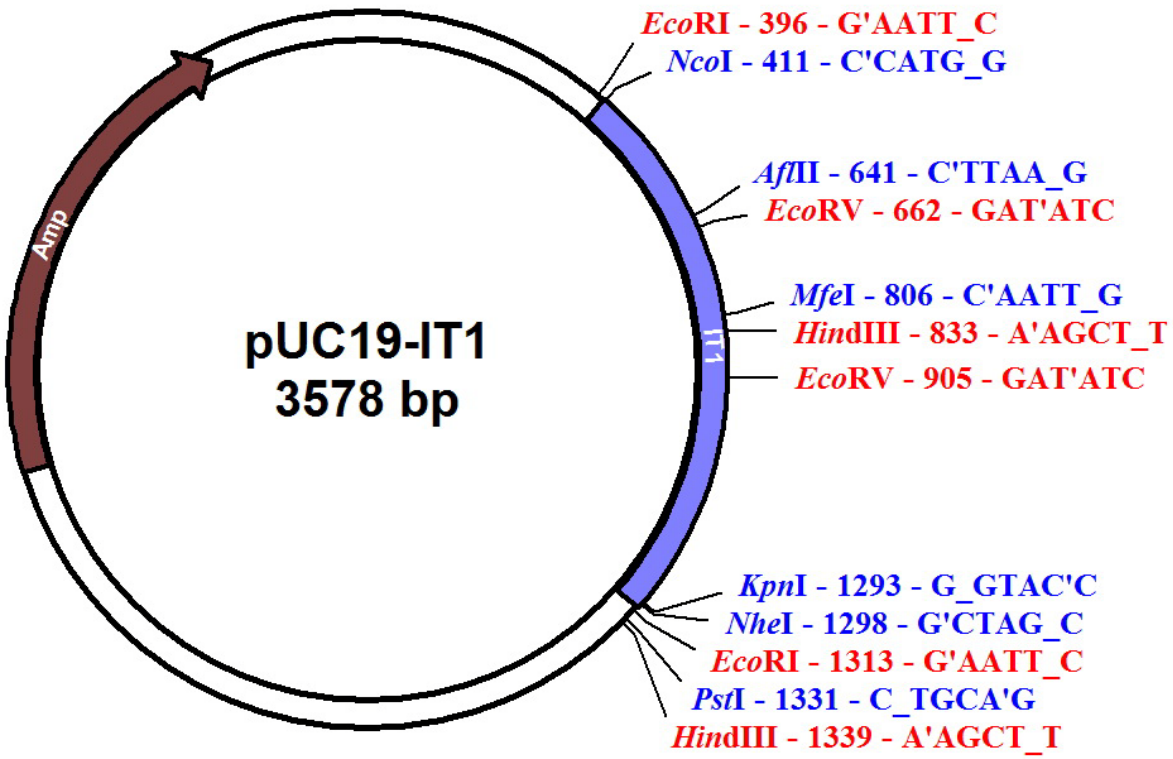


Figure 9.

Schematic Representation of pUC19-IT1-loxP2-SA Vector

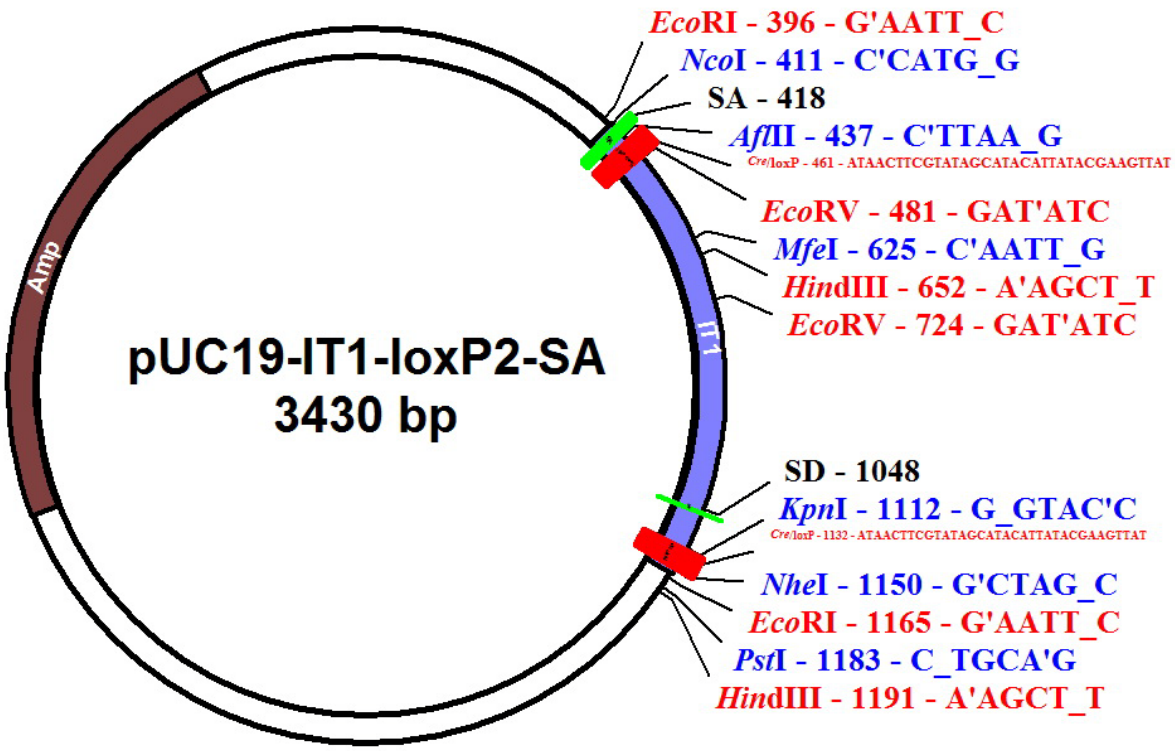


Figure 10.

Schematic Representation of pTopo-IT1-loxP2-SA Vector

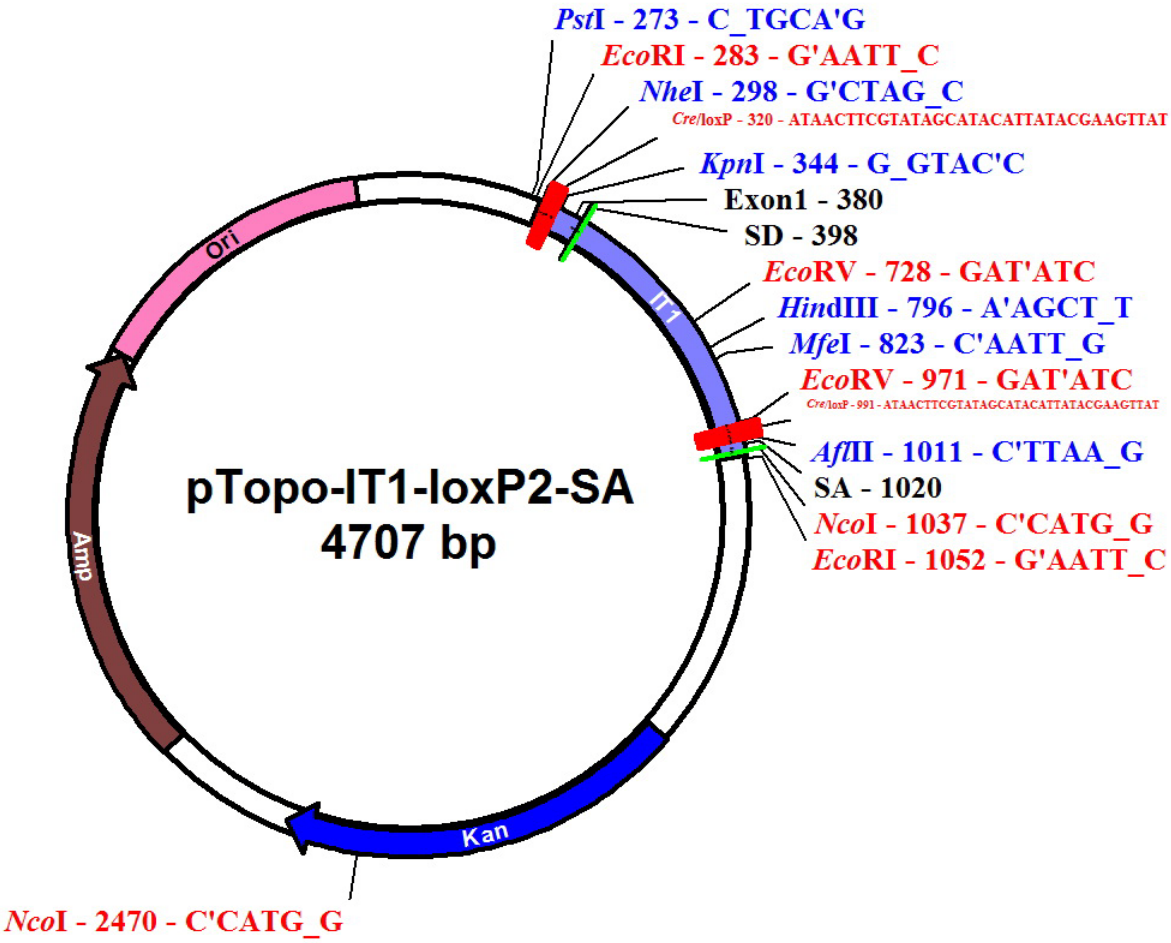


Figure 11.

Schematic Representation of pTopo-IT1-loxP-shGFP-loxP-SA Vector

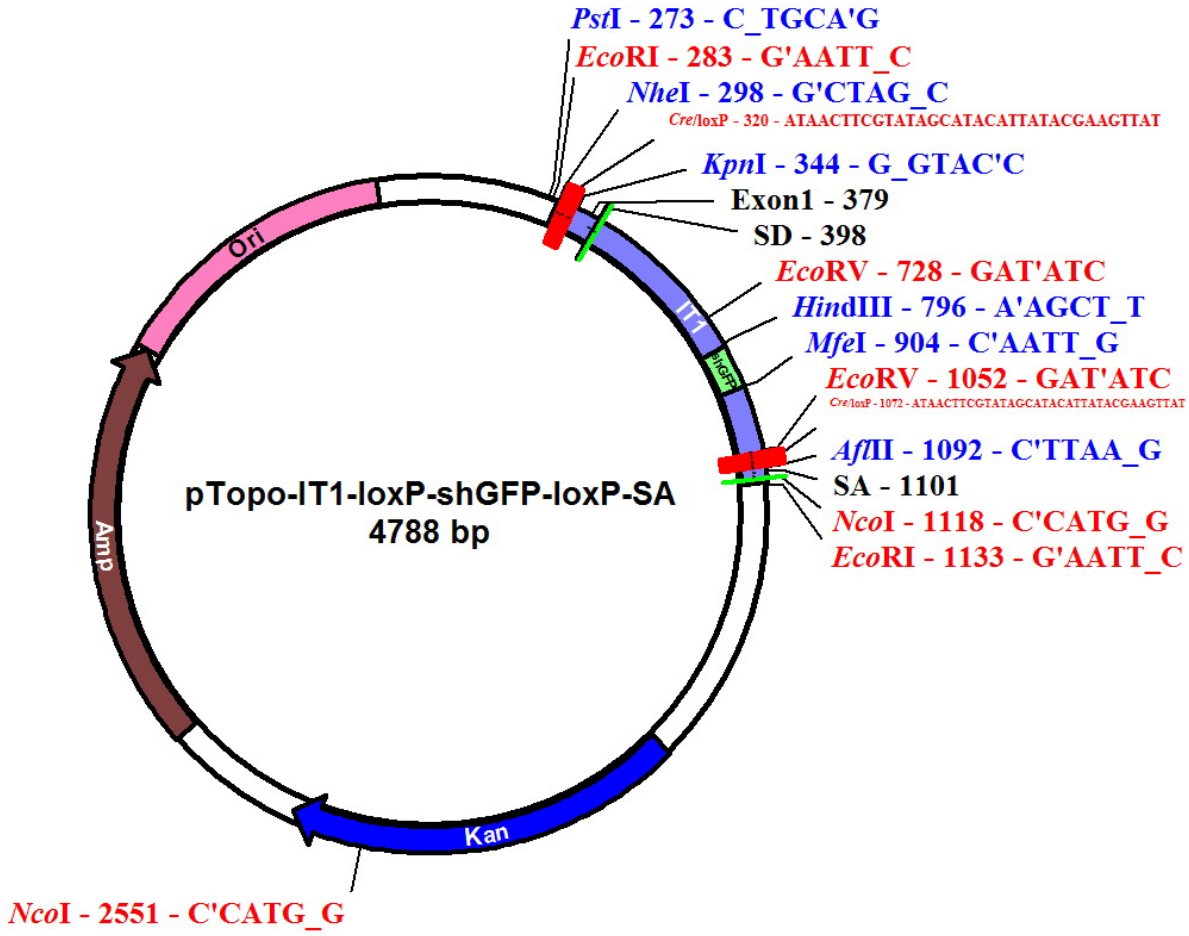
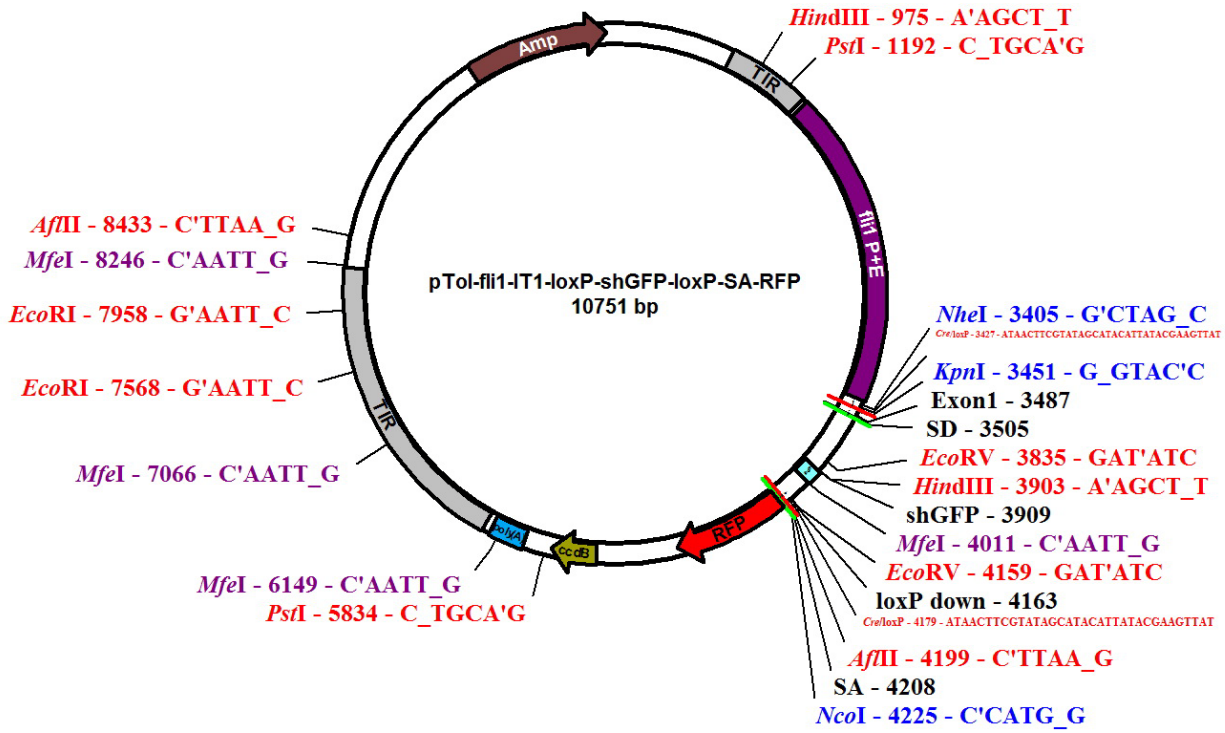


Figure 12.

Schematic Representation of pTol-fli1-IT1-loxP-shGFP-loxP-SA-RFP Vector



Production of Transgenic Zebrafish

Zebrafish Maintenance

All fish including the WT (wild-type) fish, Tg (transgenic) fish, and baby fish were maintained at 28.5℃ in the fish room on a 14h light/10h dark cycle. The general procedures for zebrafish care and maintenance are found in Dr. Westerfield's paper (Westerfield, 2000). The experiments conducted on zebrafish were conformed to the rules of the IACUC Animal Care Protocols. Briefly, for breeding zebrafish, 1-2 males and 1-2 females were placed together in a breeding tank which had a 2 mm mesh barrier to protect the eggs from being eaten by the adult fish. Fertilized eggs were collected on the following day in a finger bowl. Unfertilized or dead

embryos were removed. The eggs were kept in the finger bowl for 5-6 days and then transferred into a small plastic tank for breed up to 30 days. Paramecia were provided to the baby fish between day 5 and day 14 and freshly hatched brine shrimp were provided from day 15 to day 30. Fish older than one month would be transferred to a bigger tank and fed with a mixture of fresh brine shrimp egg and dry flakes twice a day.

Microinjection

Fresh fertilized zebrafish eggs were collected and placed on a depression slide. The one or two cell stage eggs were injected with DNA using a micromanipulator (PV830, Pneumatic Pico Pump, WPI, FL) under a dissecting microscope. The micropipettes/needles were produced with horizontal puller (Flaming Brown Micropipette Puller P80/PC, Sutter Instrument Co., Novado, CA) using 1.2 mm fibre-filled glass capillary tubing (Frederick Haer & Co., Bowdoinham, ME). The DNA containing 0.2% phenol red was injected through a pressure controlled flowing pipette and the flow rate was controlled by application of 1-5 psi (1 psi ≈6.9 kPa) of pressurized air (Dong, 2005). The volume of the injected DNA was estimated to be about 300 pl. Generally, 25-50ng/µl circular plasmid DNA alone or co-injected with 30-60ng/µl *Cre* or *Tol2* mRNA were efficient for making transgenic zebrafish (Stuart *et al*., 1988).

Recombination Assay in Zebrafish

In Vitro mRNA Synthesis

Cre mRNA was transcribed in vitro from plasmid pGEM4z-Cre-A64 that was gifted from Dr. Joseph Merregaert (Van den Plas *et al*., 2003). The plasmid pGEM4z-Cre-A64 was linearized with SpeI (New England Biolabs, Ipswich, MA) digestion and purified with a DNA column (Promega, Madison, WI) before in vitro transcription. A T7 mMessage mMachine Kit (Ambion, Austin, TX) was used to generate 5’ capped mRNA from the template DNA in a 20-

100 µl reaction mix incubating at 37℃ for 1 hr. The transcribed mRNA was purified by DnaseI digestion followed by LiCl precipitation and 70% ethanol wash, according to the manufacturer's protocol. The concentration of the mRNA was measured by the UV light absorbance at 260nm and the RNAs were stored at -70℃ in small aliquots.

The same procedures were applied to produce Tol2 mRNA from plasmid pCS-TP (gifts by Dr. Allen Albig, Indiana State University, IN) in vitro. The pCS-TP vector was linearized by NotI (New England Biolabs, Ipswich, MA) digestion. The steps for in vitro transcription and purification were the same as above.

In Vivo Heatshock Assay

In order to test the *in vivo* Cre recombinase activity, pHsp70-Cre-egfp transgenic zebrafish (gifts by Dr. Ryan Thummel, Notre Dame, IN) were crossed with pBa-rfp-loxP$_2$-gfp-SB$_2$ transgenic fish and pTol-EF1α-loxP-dsRed2-loxP-EGFP transgenic fish separately. The 24 hpf fertilized eggs were collected and heatshocked at 40℃ for 5 min using a water bath, which could produce enough Cre recombinase for Cre-mediated deletions (Bai *et al.*, 2009). The efficiency of the Cre-loxP induced deletion after heatshock could be observed by color exchange using a fluorescent microscope (Nikon SMZ1500, Melville, NY) and/or by designing appropriate PCR primers to query the genes in the fish genome.

Photography and Imaging

Embryos and baby fish from the transgenic zebrafish were kept on a depression slide and anesthetized with 0.005% tricanine (3-aminobenzoic acid ethyl ester, Sigma, St. Louis, MO) before photography. Images were captured using Nikon software (NIS-Elements D 3.0) and a digital camera (CoolSNAP cf, Roper Scientific Photometrics, Tucson, AZ) attached to a Nikon fluorescent microscope (SMZ1500). A GFP filter with excitation (470 mm) and emission (500

mm) was used to detect GFP expression. A rhodamine filter with excitation and emission wavelengths of 540 nm and 570 nm was applied to detect RFP expression.

CHAPTER 4

RESULTS

Project I: Exchangeable and Mobile RFP/GFP Transgene Assay

Aim: Establish and characterize transgenic zebrafish with exchangeable and mobile *rfp*/*gfp* marker genes. a) Cre-loxP induced conversion of *rfp* to *gfp* expression assay; b) Germline transmission assay of converted *gfp* zebrafish; c) Generation of *rfp*/*gfp* transheterozygous zebrafish; d) Tol2 induced *rfp*/*gfp* translocation and insertional mutation assay.

A. RFP/GFP Conversion Test with Original Construct pBa-rfp-loxP$_2$-gfp-SB$_2$

1. Plasmid Construction and Establishment of Stable Transgenic Fish

The plasmid of pBa-rfp-loxP$_2$-gfp-SB$_2$ (Fig. 13) was constructed and injected into the zebrafish (F0) by Dr. Jie Dong in our lab. Not long after her graduation, I took over this project and started to screen the positive transgenic zebrafish which showed red (*rfp* expression) color under the fluorescent microscope. The pHsp70-Cre-egfp (Fig. 13) transgenic fish was gifted from Dr. Ryan Thummel (University of Notre Dame, Notre Dame, IN) which would express Cre recombinase after heatshock stimulation. When the temperature was raised over 37℃, the Hsp70 promoter would be activated to promote Cre expression. The *gfp* fused to Cre worked as a marker gene to indicate Cre expression with very weak green fluorescence which would

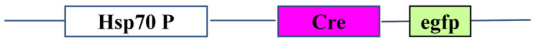
disappear in a short time. It would not interfere with the *gfp* expression in the *rfp-gfp* converted fish after heatshock.

Figure 13.

Schematic Graph of pHsp70-Cre-egfp and pBa/RFP/loxP2/GFP/SBIR Constructs

P – Promoter; SB – Sleeping Beauty (transposonase); SBIR – Sleeping Beauty Inverted Repeats (SB recognition site)

pHsp70-Cre-egfp



pBa-rfp-loxP2-gfp-SB2

2. Establishment of Homozygous pBa-rfp-loxP$_2$-gfp-SB$_2$ Transgenic Fish

There were 20 F0 fish found to have mosaic red in their body and they were outcrossed with wild-type (wt) fish to generate one or more stable transgenic line. Three out of 20 fish were discovered to be able to transmit their RFP gene into the next generation (F1). The F1 red fish obtained in one of these crosses were then intercrossed to themselves to produce possible homozygous red fish (F2) which would greatly facilitate our future Cre-loxP recombinant assays. In total, 149 F2 fish were examined under the fluorescent microscope and 46 of them were found to show brighter red color then the others. These red fish were separated and crossed to themselves again to generate additional homozygous red fish (F3). The very bright red F3 fish (11 out of 255) were picked to outcross with wt fish to test their homozygosity. 5/11 fish were found to be the homozygous red fish, because all of the offpring were red. After four generations

breeding, the homozygous pBa-rfp-loxP$_2$-gfp-SB$_2$ transgenic fish were successfully generated (Fig. 14, 15). Intercrosses of F3 fish were continued to produce F4 and even F5 generations for generating more stable homozygous transgenic fish. (Table 1)

Figure 14.

Flowchart of Establishing Homozygous pBa-rfp-loxP$_2$-gfp-SB$_2$ Transgenic Fish

WT – wildtype; BR – bright red; HR – homozygous red; VBR – very bright red

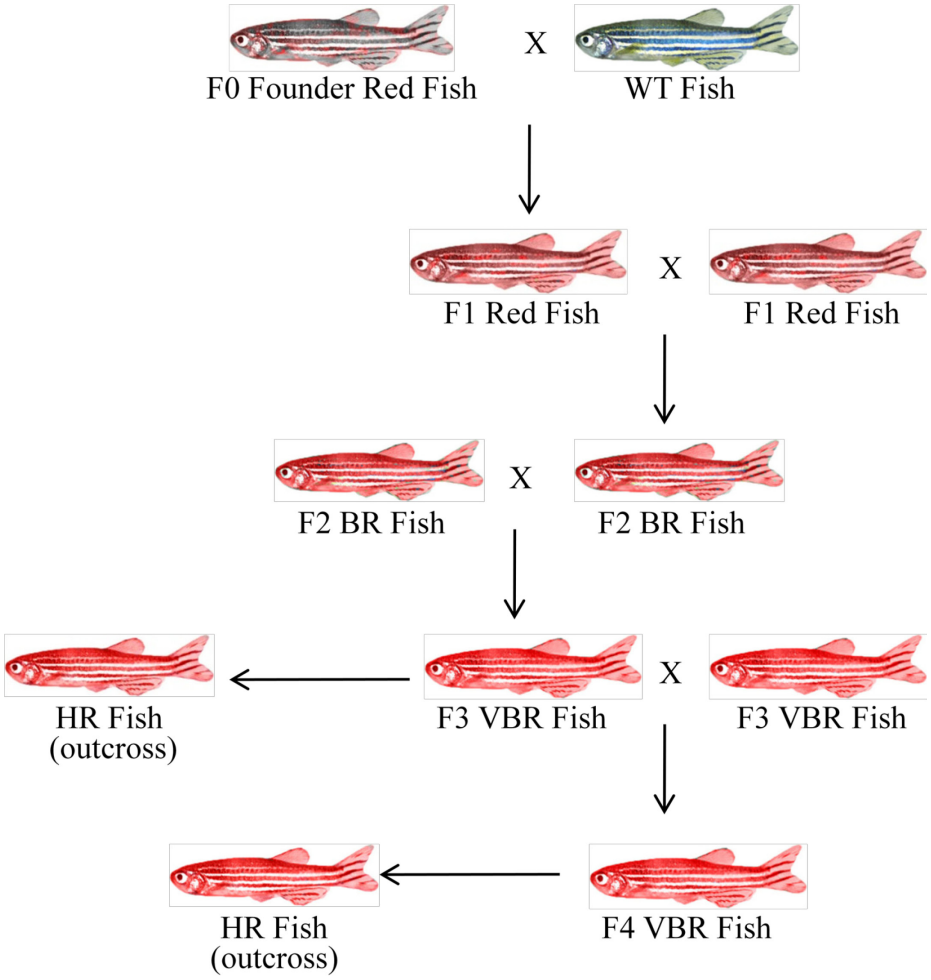


Table 1.

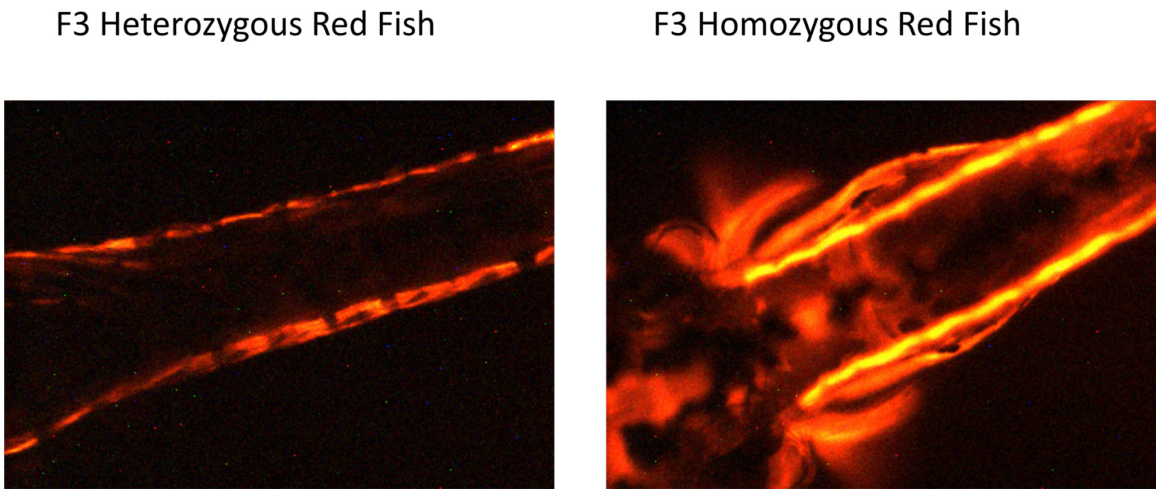
Summary of Fish Examined During Establishing Homozygous Transgenic Fish

BR – bright red; HR – homozygous red; VBR – very bright red

	Red	BR	VBR
F0	20		
F1	25/58	3/58	
F2	73/149	46/149	
F3	114/255	67/255	11/255 (5 HR)
F4	180/288	93/288	15/288 (7 HR)

Figure 15.

Heterozygous vs Homozygous pBa-rfp-loxP$_2$-gfp-SB$_2$ Fish



3. Cre-loxP Mediated rfp/gfp Conversion Assay

3.1 Preliminary tests in which F1 heterozygous pBa-rfp-loxP$_2$-gfp-SB$_2$ zebrafish crossed with pHsp70-Cre-egfp transgenic zebrfish showed that the converted green-expressing construct

(Heatshock @40℃, 30min) failed to be passed through the germ-line. Some of the cells which inherited both of the pBa-rfp-loxP_2-gfp-SB_2 and pHsp70-Cre-egfp constructs showed an alteration from red to green in the offspring after Cre activity induced by the Hsp70 promoter. Also, most of the green expressing cells showed red color as well. These green cells would disappear in the following 2-3 weeks. Outcross of these transgene converted zebrafish to wild type fish failed to pass the green color into the next generation.

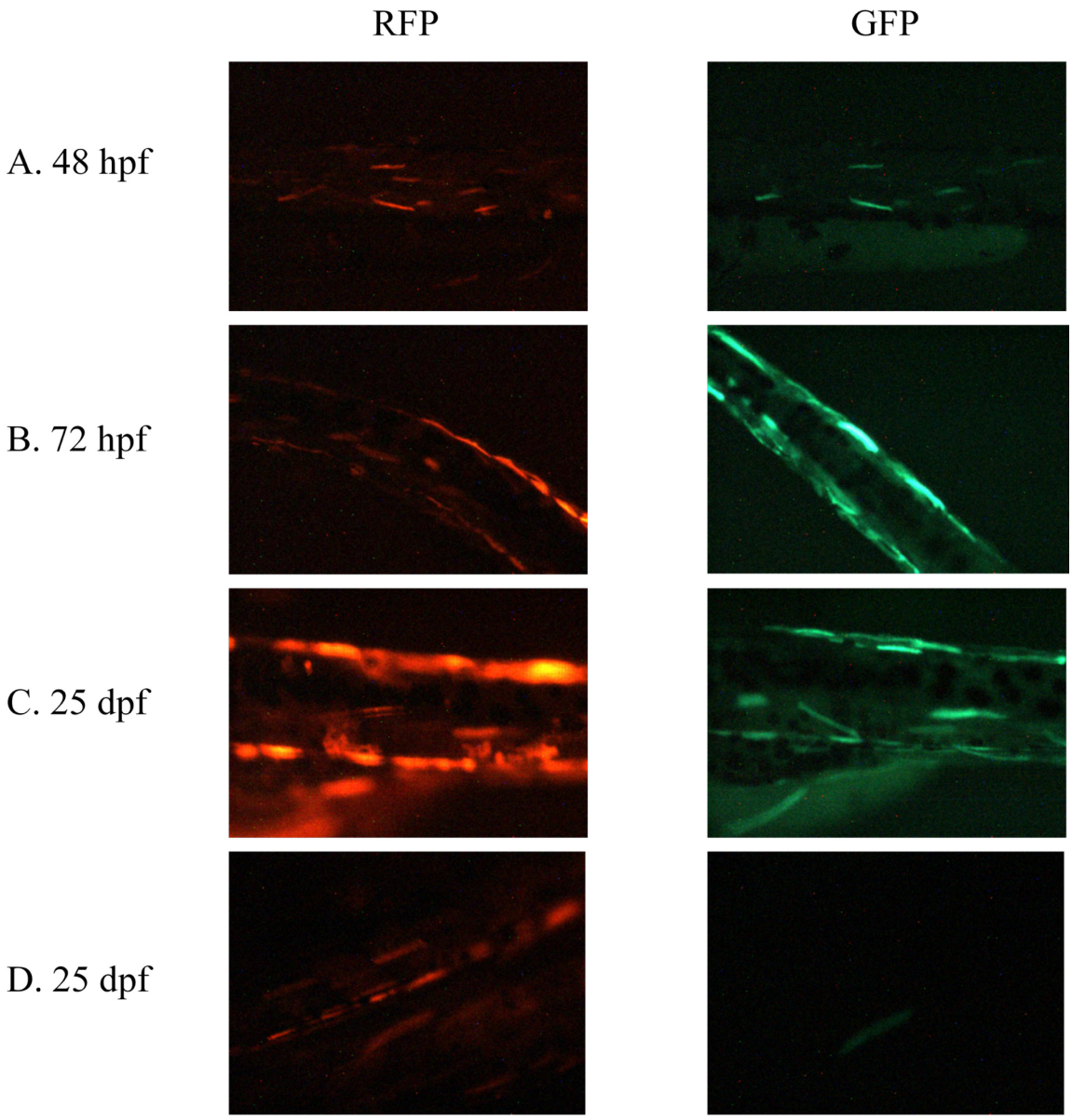
3.2 Crossing F3/F4 homozygous red pBa-rfp-loxP_2-gfp-SB_2 transgenic zebrafish with pHsp70-Cre-egfp transgenic zebrafish were expected to have a better chance to pass the converted construct through the germline (Fig. 16). Various versions of these tests are summarized below.

Figure 16.

Converted RFP/GFP Gene Expression in Different Crosses

A, B, and C: Cre female crossed with H-red male; D: Cre male crossed with H-red female. 24 hrs old embryos were heatshocked at 40℃ for 5 min to activate Cre recombinase.

H-red: homozygous red; hpf: hours of post-fertilization; dpf: days of post-fertilization.



3.2.1 Test of crossing homozygous pBa-rfp-loxP_2-gfp-SB_2 male fish with pHsp70-Cre-egfp female fish (Table 2a). For one test: the 24hr old embyos were heatshocked at 40℃ for 15min and 30min (100 embryos for 30min group, 60 embryos for 15min group, and 25 non-heatshock controls). Heatshocks were repeated for 2 more times at 48hrs and 72hrs to ensure complete induction of Hsp70 promoter and Cre recombinase. Embryos were examined under the fluorescent microscope after each heatshock. The results showed that all the offspring were expressing red throughout the body and most of the few green cells were also red. This might be due to the presence of several copies of the construct integrations in the chromosomes where only some of them were converted by Cre. Alternatively, the red color may decay slowly due to the stability of DsRed protein. Most of the control embryos showed red color only, but still some of them had some green cells as well. This could be explained by "leaky" maternal expression of Cre from the female pHsp70-Cre-egfp fish. Some baby fish still showed green in their body (under fluorescent microscope) after 2 months of age.

3.2.2 Test of crossing pHsp70-Cre-egfp male fish with homozygous pBa-rfp-loxP_2-gfp-SB_2 female fish (Table 2b). Since we had problems with the maternal expression of *Cre* in female pHsp70-Cre-egfp fish, we tried to test the reciprocal cross to verify our results. For one test: 24hrs old embyos were heatshocked at 40℃ for 5 min (47 embryos in heatshock group and 20 in non-heatshock control group). *rfp*/*gfp* expression was examined under fluorescent microscopy after 48 hrs. The results showed that 23 offspring had red and weak green colors in their bodies and 17 offspring showed red color only. The green color was much weaker than the comparable babies from male homozygous pBa-rfp-loxP_2-gfp-SB_2 cross. This might be explained by the less or delayed expression of Cre from male pHsp70-Cre-egfp fish.

Table 2

Summary of the Conversion from rfp to gfp Expression after Heatshock.

Red/Green: offspring showed red and/or green colors in the body; HS: heatshock; Red only: offspring showed red color only.

a. Female pHsp70-Cre-egfp crossed with male pBa-rfp-loxP_2-gfp-SB_2

	Total	Red/Green	Red only
Heatshock	495	303 (61%)	192 (39%)
Non - Heatshock	102	17 (17%)	85 (83%)

b. Male pHsp70-Cre-egfp crossed with female pBa-rfp-loxP_2-gfp-SB_2

	Total	Red/Green	Red only
Heatshock	171	78 (46%)	93 (54%)
Non - Heatshock	153	0	153 (100%)

3.3 Outcrosses of the red/green converted fish with wild type fish failed to pass the converted red/green into the next generation. A total of 29 testcrosses were made to examine if the converted red/green (red/green fish after heatshock) could be transmitted through the germline. About 2195 baby fish were examined under fluorescent microscopy after 48 hrs, but only one red fish was observed. Since the frequency of germline transmission was low (almost none) this work needed to be redesigned. (Table 3)

Table 3

Germline Transmission Test of Red/Green Converted Fish

Red/Green – babies showed red and/or green color; Red only – babies showed red only

	Testcrosses	Offspring	Red/Green	Red Only
Fish Num.	29	2195	None	1

4. PCR Verification of rfp/gfp Existence in Transgenic Lines

Genomic DNA isolated from tail fin of adult fish or 5 dpf embryos was used for PCR to test the existence of the *rfp* and/or *gfp* fragments in different transgenic lines. PCR primers were designed based on the sequence of plamid pBa/RFP/loxP2/GFP/SB2, and the PCR program was set as follows: 30 cycles of 94℃ 1 min, 52℃ 1 min, 72℃ 2 min and followed by 72℃ 5 min.

Primers used for *rfp*/*gfp* PCR tests:

rfp Detection (860bp)

5’ primer: 5’-ATCTGAGGCGCGCATTGTCACACTA-3’

3’ primer: 5’-TAGTCCTCGTTGTGGGAGGTGATGT-3’

gfp Detection (890bp)

5’ primer: 5’-CCGCGACTCTAGATCATAATCAGCCA-3’

3’ primer: 5’-AGGGCAGATTGTGTGGACAGGTAA-3’

Figure 17.

PCR Tests for *rfp*/*gfp* Fragments in Different Strains.

WT: wildtype fish; Red outcross: red pBa-rfp-loxP$_2$-gfp-SB$_2$ fish outcross with wt; Heatshock: offspring of red pBa-rfp-loxP$_2$-gfp-SB$_2$ fish crossing with pHsp70-Cre-egfp fish heatshocked at 40℃, 5 min; Red/Green outcross: fish that showed red and green after heatshock crossed with wt fish (germline transmission of *rfp*/*gfp* test); M: DNA Marker; NHSC: Non-Heatshock Control fish; Red: red only fish after heatshock; R/G: red/green fish after heatshock.

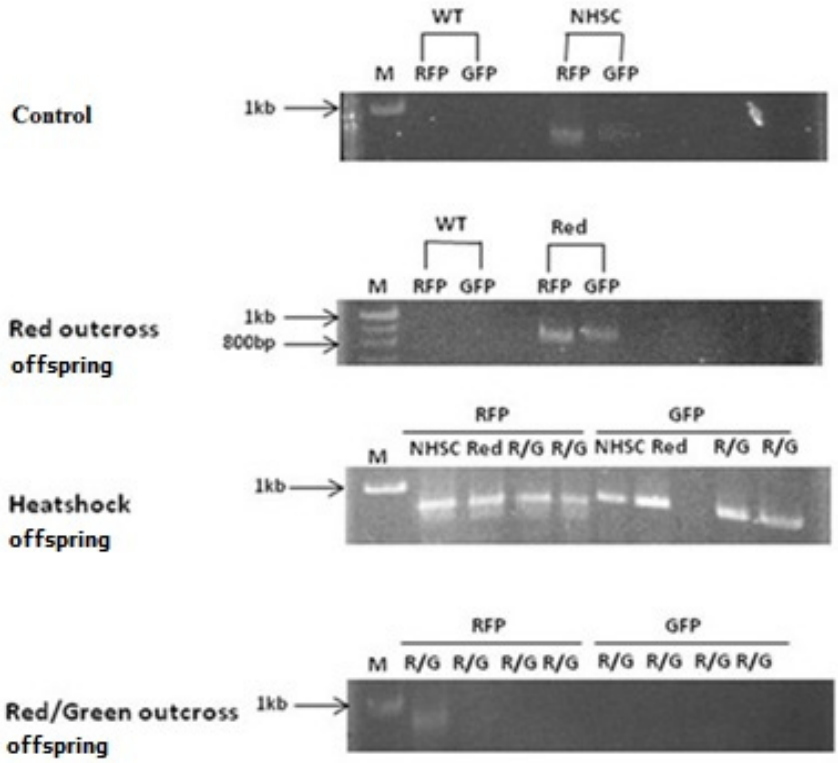


Table 4

PCR Results for Detection of rfp/gfp in Different Strains.

WT: wild type; Red outcross: red pBa-rfp-loxP$_2$-gfp-SB$_2$ fish outcross with wt; HS: heatshock fish (40 ℃, 5 min); Non-HS Control: Non-Heatshock Control fish; Red only: fish showed red only after heatshock; Red/Green: fish showed both red and green after heatshock; Red/Green outcross: fish that showed red and green after heatshock crossed with wt fish.

Strain	Transgene	Number of tested fish	*rfp* Positive	*gfp* Positive
WT control	No	2	0	0
Non-HS control	Yes	4	4	4
HS Red only	Yes	5	2	2
HS Red/Green	Yes	10	2	2
Offsrping of Red outcross	Yes	4	4	4
Offspring of Red/Green outcross	Yes	16	1	0

PCR results from controls showed that there was no *rfp* or *gfp* fragment inside the wildtype genome; the red pBa-rfp-loxP$_2$-gfp-SB$_2$ fish which was outcrossed with wildtype fish had both *rfp* and *gfp* in the genome. However, when tested with the heatshock group was tested (homozygous red fish crossed with Cre fish), some of the fish showed existence of *rfp* or *gfp*.

This might be explained by a multiple copy of integration of our plasmid pBa-rfp-loxP$_2$-gfp-SB$_2$, in the chromosome. After heatshock, *rfps* as well as *gfps* may have both been deleted (see discussion). For the germline transmission tests of *rfp*/*gfp* converted fish, we examined the offspring from the cross of heatshocked red/green fish with wildtype fish. Only 1 out of 2195 tested fish showed *rfp* positive band (Fig 17.). This reinforced our hypothesis that the plasmid sequences in these fish might get partially or completely deleted due to the multiple copies of integration of pBa-rfp-loxP$_2$-gfp-SB$_2$, which could not be predicted as of our original design. Therefore, an improvement of the pBa-rfp-loxP$_2$-gfp-SB$_2$ transgene was required. (Table 4)

5. Use of Cre mRNA as an alternative source of recombinase.

Cre mRNA was transcribed *in vitro* from a plasmid pGEM4z-Cre-A64 gifted from Dr. Joseph Merregaert. Then *Cre* mRNA was injected into one or two cell stage embryos produced from the cross of homo-red pBa-rfp-loxP$_2$-gfp-SB$_2$ zebrafish and wild type fish. About 300 embryos were injected with Cre mRNA (30 ng/μl). The embryos were examined after 24 hours using the fluorescent microscopy. The results showed that a few of the offspring that survived (about 30%) had one or two cells in the body expressing green color, and these green cells also contained red color. The efficiency of Red/Green conversion was not comparable with the pHsp70-Cre-egfp test. The reasons might lie in the poor quality and low translation efficiency of Cre mRNA injected in the fish. Further attempts to optimize the concentration and quality of Cre mRNA might be helpful, but the decision was made to rely on DNA-based source of Cre recbombinase.

B. rfp/gfp Conversion Test with pTol-EF1α-dsRed2-loxP2-EGFP Transgenic Fish

Because of the failure of passing the converted *rfp*/*gfp* through germline with our pBa-rfp-loxP$_2$-gfp-SB$_2$ transgenic fish, we decided to switch to an equivalent vector pTol-EF1α-loxP-

dsRed2-loxP-EGFP. This construct was already provided within a stable line of transgenic zebrafish (gifted from Dr. Michael Brand, Dresden, Germany), which we refer to hereafter as "Dresden red fish". This plasmid was designed similarly and had been demonstrated to undergo efficient *rfp*/*gfp* conversion (Hans *et al*., 2009). It also contained two loxP sites flanking the Red (DsRed2) gene and followed by a Green (*egfp*) gene. However, it used the Tol2 transposon system rather than the SB transposon system for DNA cassette translocation. Tol2 was demonstrated to have the advantages of higher cargo capacity and single copy of transgene integration (Balciunas *et al*., 2006; Urasaki *et al*., 2006).

1. Cre-loxP Recombination Assay

The pTol-EF1α-loxP-dsRed2-loxP-EGFP fish (Dresden red fish) were crossed with pHsp70-Cre-egfp fish (Cre fish) to perform the Cre-loxP recombinant test. Efficient *rfp*/*gfp* conversion was observed after heatshock activation of Cre recombinase (24 hpf embryos were heatshocked at 40℃ for 5 min; longer incubation for 10 min, 20 min, and 30 min showed no significant difference. Some of the Non-HS control fish also showed red to green conversion which could result from the "leakage" of Cre recombinase from the uninduced pHsp70-Cre-egfp transgene. However, this would not diminish the efficiency of *Cre-loxP* mediated *rfp*/*gfp* conversion for further germline transmission tests (Fig. 18a, 18b).

Figure 18a.

Cre-loxP Mediated Recombination in the Cross of Female Dresden Fish with Male Cre Fish.

HS: 24 hpf embryos were heatshock at 40℃ 5 min; Non-HS: no heatshock

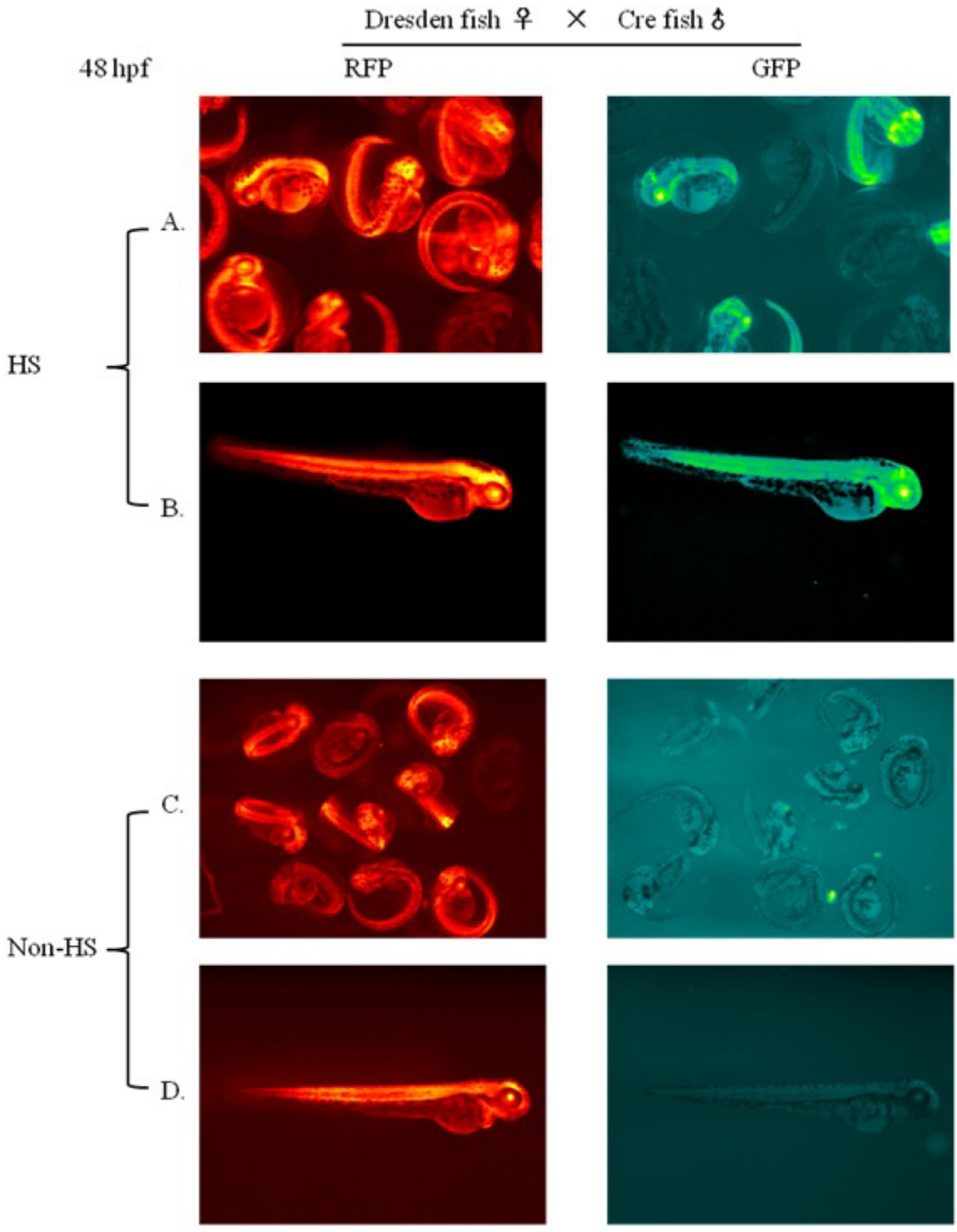
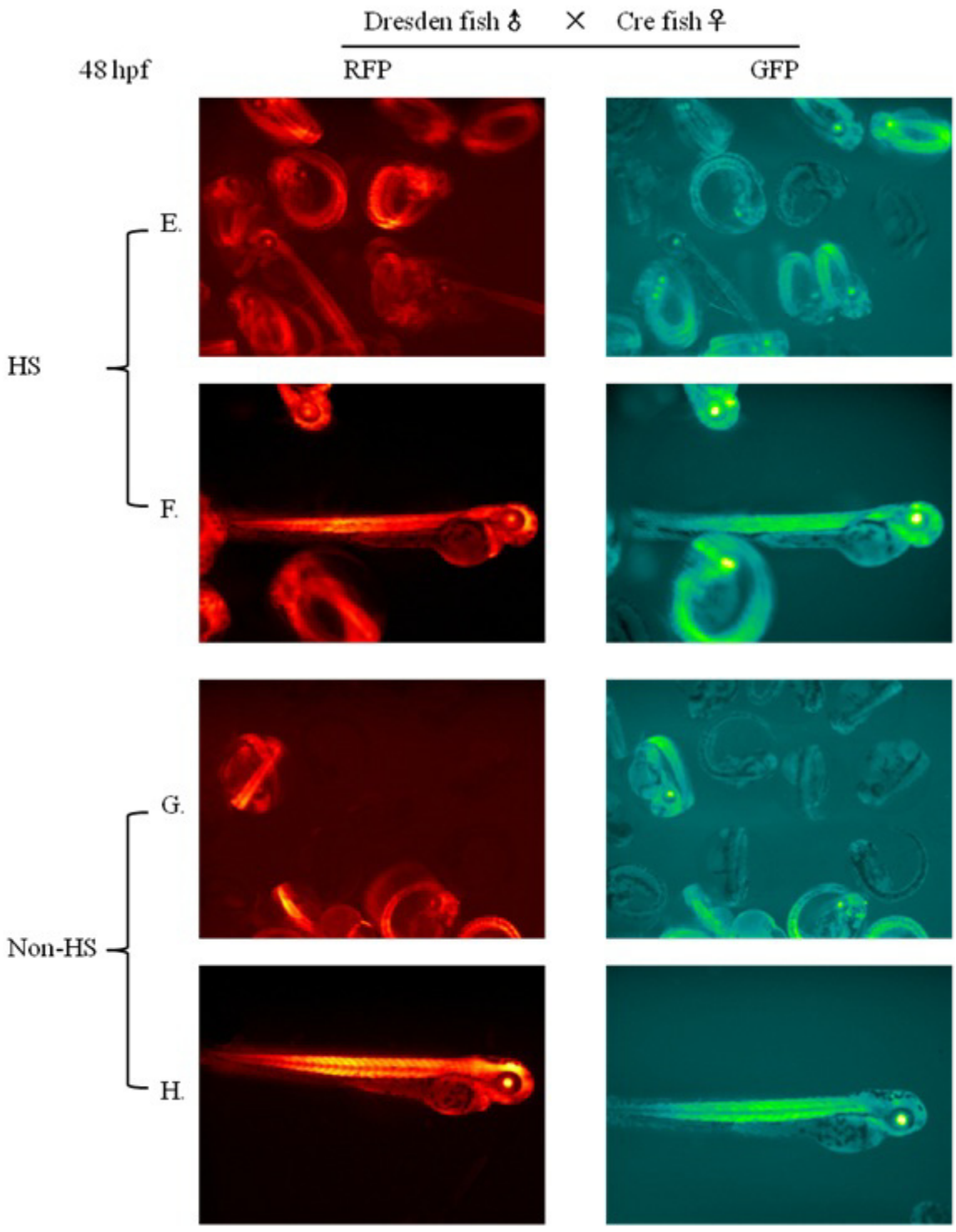


Figure 18b.

Cre-loxP Mediated Recombination in the Cross of Male Dresden Fish with Female Cre Fish.

HS: 24 hpf embryos were heatshock at 40℃ 5 min; Non-HS: no heatshock



2. Germline Transmission Assay

To test if the converted *rfp*/*gfp* transgene could be passed through the germline in the transgenic zebrafish, we outcrossed the mature heatshock fish that showed conversion from red to green at an early age to wildtype fish. The fertilized eggs were collected and examined for fluorescence using fluorescent microscopy after 24 hrs. The results showed that the converted *rfp*/*gfp* transgene could be transmitted through the germline (Fig. 19a, 19b). All three expression patterns: red only (*rfp* expression), green only (*gfp* expression), and red/green (both *rfp* and *gfp* expression), were detected under microscopy in both of the standard cross (heatshock female fish cross with wildtype male fish) and the reciprocal cross (heatshock male fish cross with wildtype female fish). Most of the offspring (311 out of 602) expressed green color only which demonstrated that they retained the converted GFP gene only. Whereas, a few of the offspring showed red color only (19/602) or both red and green colors (7/602) throughout the body, this demonstrated that the recombination mediated by Cre-loxP activity could be successfully passed through the germline so as to be inherited by their offspring to generate a stable transgenic line. (Table 5)

Table 5

Summary of Converted rfp/gfp Germline Transmission Assay.

HS: heatshock; WT: wildtype; I – IV: different strains

	HS ♀ × WT ♂				HS ♂ × WT ♀				Total
	I	II	III	IV	I	II	III	IV	
Green Only	19	74	21	20	58	72	42	5	311
Red Only	0	0	0	0	0	16	0	3	19
Red + Green	2	0	0	0	0	0	0	5	7
No Red/Green	6	47	19	22	47	95	27	2	265
Total	27	121	40	42	105	193	69	15	602

Figure 19a.

Converted *rfp*/*gfp* Germline Transmission Assay with Forward Cross (heatshock female fish cross with wildtype male fish)

HS: heatshock; WT: wildtype.

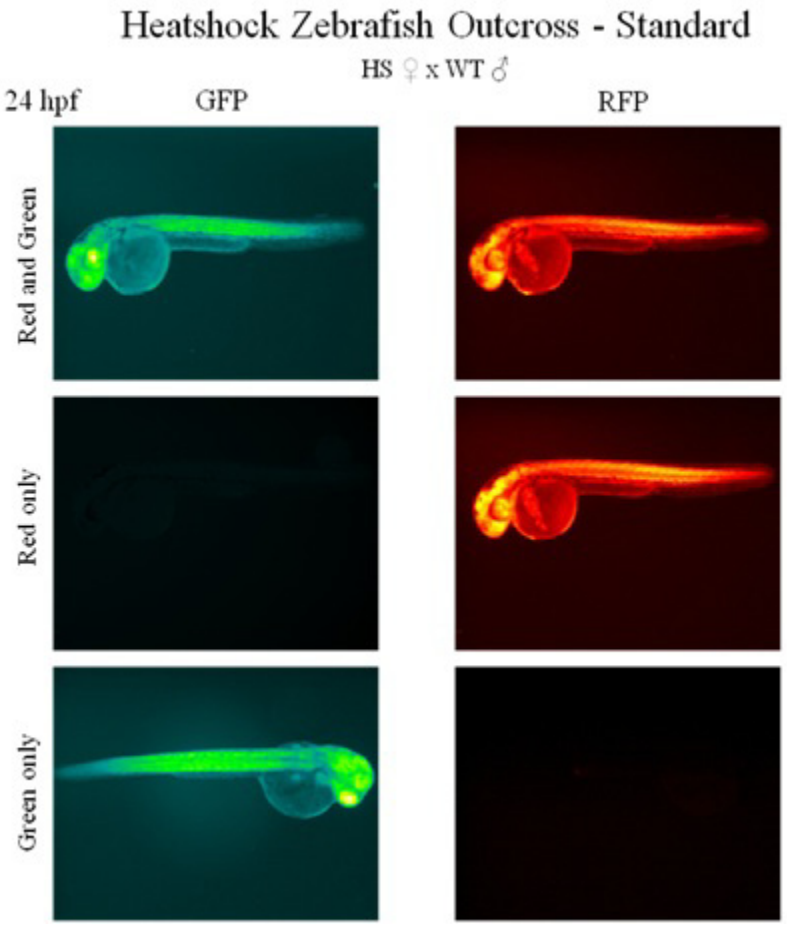
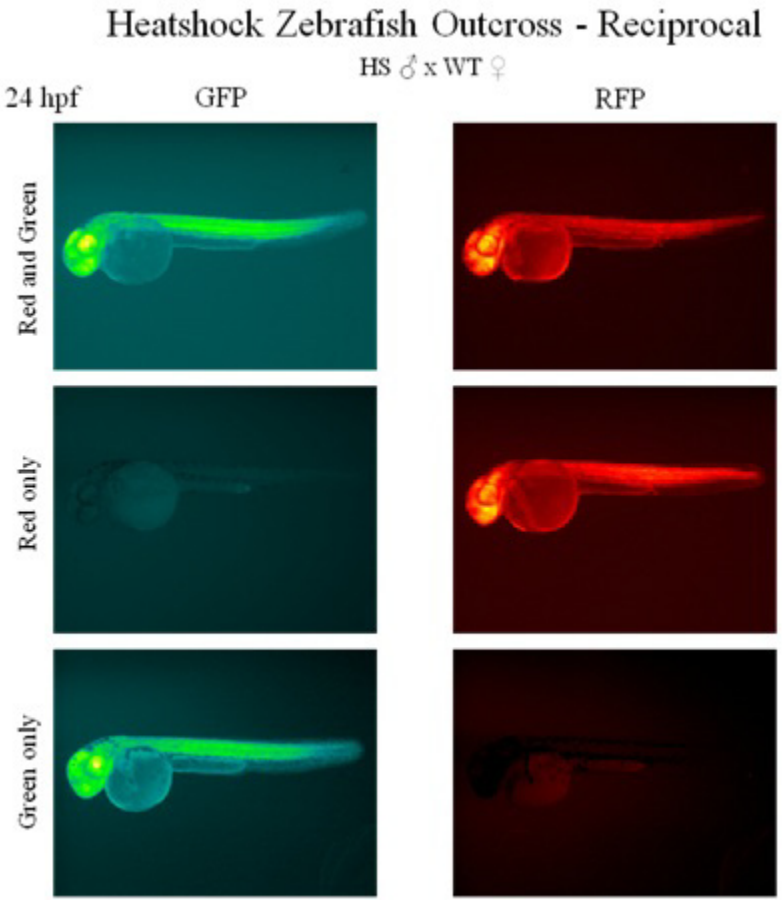


Figure 19b.

Converted *rfp*/*gfp* Germline Transmission Assay with Reciprocal Cross (heatshock male fish cross with wildtype female fish).

HS: heatshock; WT: wildtype.



3. Production of *rfp*/*gfp* Transheterozygous Zebrafish

Since we successfully obtained green fluorescent fish (pTol-EF1α-loxP-EGFP) from heatshocked red fluorescent fish (pTol-EF1α-loxP-dsRed2-loxP-EGFP, Dresden red fish), we decided to cross these two transgenic lines to produce the *rfp*/*gfp* transheterozygous (transhet) fish which contained the *rfp* gene and *gfp* gene at the same locus (allele) in homologous chromosomes. The transheterozygous fish should pass either *rfp* or *gfp* into the next generation,

but never both. This could be verified by a transheterozygous outcross test: the offspring should show either red or green fluorescence. The generation of transheterozygous fish will be useful for genetic studies of allele expression and also be beneficial for the further *Tol2*-mediated transgene mobilization assay.

The offspring examined by fluorescent microscopy showed two patterns from crossing homozygous Dresden red fish with heterozygous heatshock green fish: half expressed red only and half expressed both red and green (Fig. 20a). In a cross of heterozygous Dresden red fish with heterozygous heatshock green fish, the offspring showed four patterns: ¼ red only, ¼ green only, ¼ red and green, and ¼ no color (Fig. 20b).

Figure 20a.

Production of Transheterozygous Zebrafish.

Homo-red fish: homozygous Dresden fish; Het-green fish: heterozygous heatshock green fish.

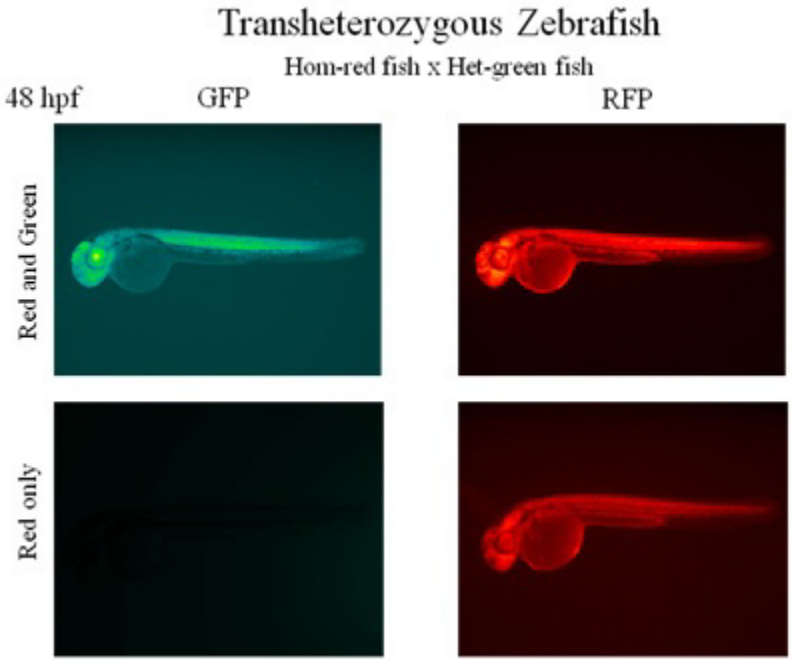
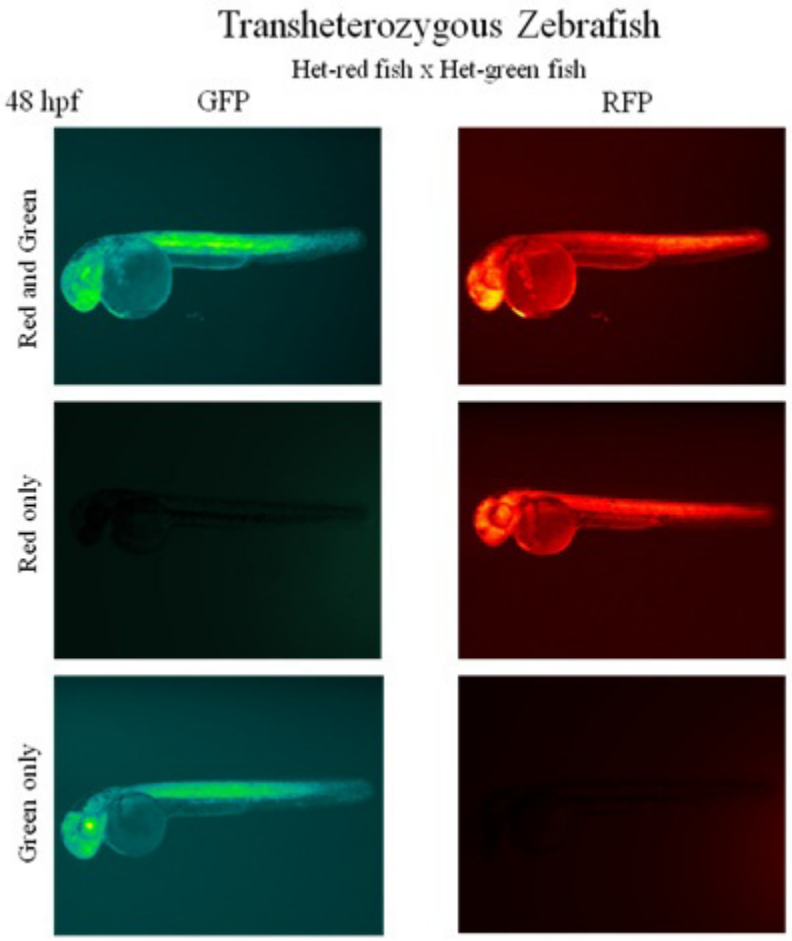


Figure 20b.

Production of Transheterozygous Zebrafish.

Het-red fish: heterozygous Dresden fish; Het-green fish: heterozygous heatshock green fish.



4. Tol2-Mediated Transgene Mobilization Assay

To test for Tol2-mediated transgene mobilization *in vivo*, we obtained a transgenic line of pHsp70-Tol2/EF1α-DsRed2 (Fuji70) from Dr. Koichi Kawakami (Japan) which could express Tol2 transposase (with an EF1α regulating RFP marker gene) under control of the temperature sensitive heatshock promoter Hsp70. The red color indicated the successful integration of the entire expression cassette into the fish genome, and then Tol2 transposase would be activated by the induction of increased temperature (Hsp70 promoter activation). Unfortunately, both of the

Fuji70 fish and Dresden red fish used DsRed2 as the marker and EF1α as the promoter. Therefore, these fish showed same expression patterns under the fluorescent microscopy which make it difficult to distinguish their transgene expressing activity *in vivo*. We propose despite this obstacle, a pathway to generate the transheterozygous *rfp*/*gfp* fish which also contains Tol2 transgene for characterizing Tol2 activity (Fig. 21). This procedure would be used when a Tol2 expressing fish lacking *rfp* becomes available in the future.

Another method we could use to test the Tol2 activity is to inject Tol2 mRNA into the *rfp*/*gfp* transheterozygous fish embryos. This experiment is ongoing and the possible results will be similar to the results predicted in Fig. 21.

Tol2 mRNA was transcribed from pCS-TP plasmid in vitro using T7 mMessage mMachine Kit (Ambion, Austin, TX). The transcribed Tol2 mRNA fragment was analyzed on the native agarose gel (Fig. 22). There were two distinct bands shown on the gel which indicated that the RNA was running with its secondary structure. The Tol2 mRNA fragment is about 2 kb in length.

Figure 21.

Schematic of Tol2-Mediated Transgene Mobilization Assay.

Transhet fish: Transheterozygous pTol-EF1α-loxP-dsRed2-loxP-EGFP fish with both red and green colors; Fuji70 Tol2 fish: pEF1α-dsRed2/Hsp70-Tol2 fish; HS: heatshock; het-: heterozygous; hom-: homozygous; red fish: Dresden red fish; orange fish: Fuji70 Tol2 fish; green fish: heatshocked Dresden green fish; red-green/Tol2 fish: potential red or green translocated fish of interest.

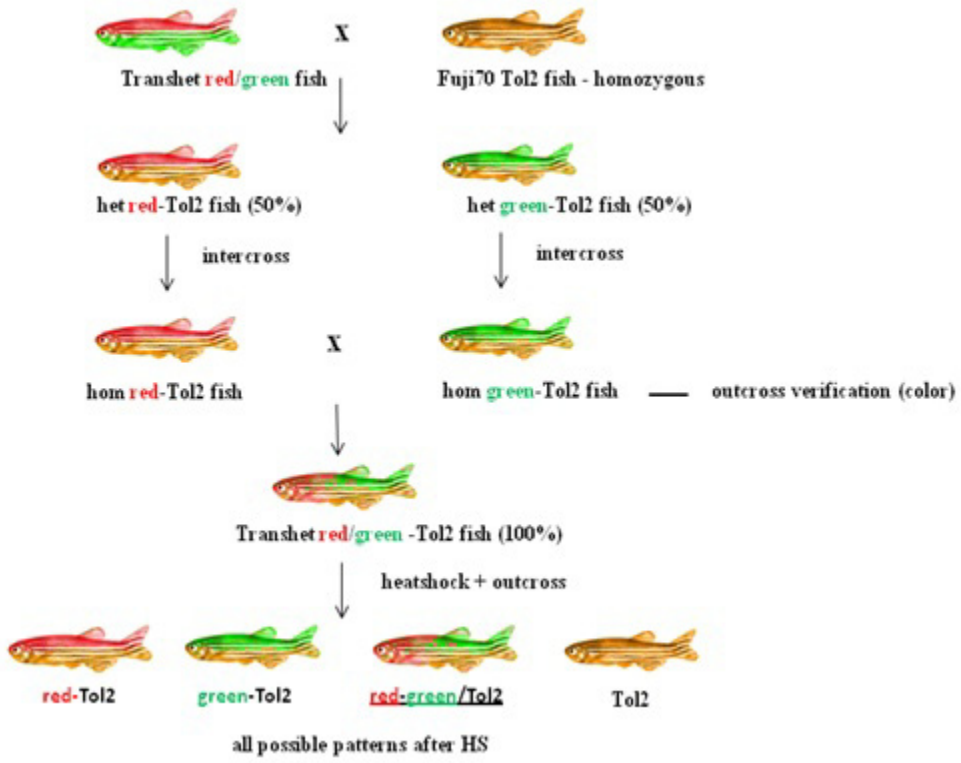
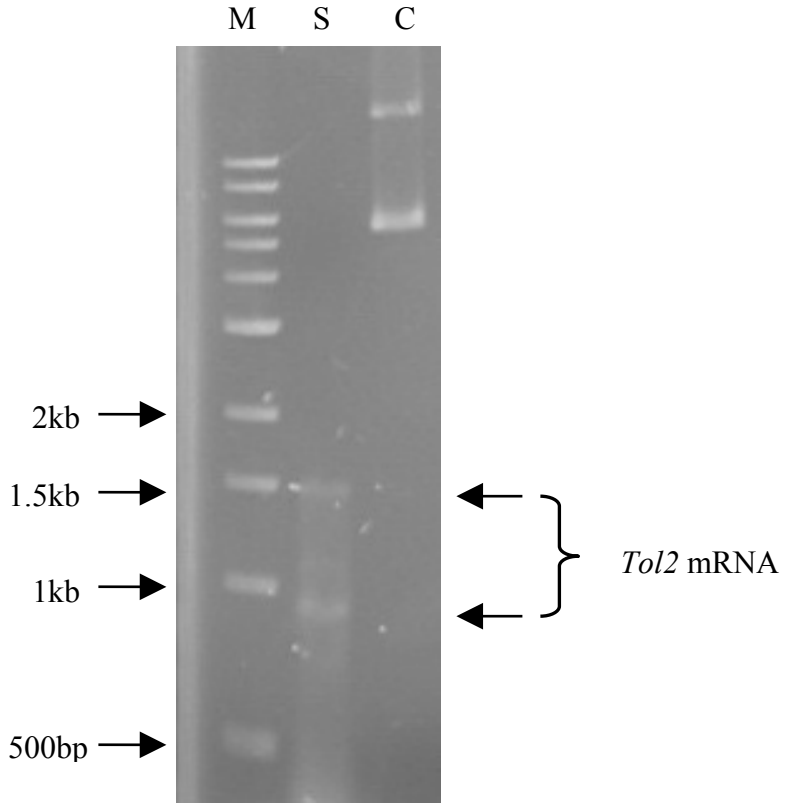


Figure 22.

Native Gel Analysis of *Tol2* mRNA Transcript.

M: 1kb marker; S: *Tol2* mRNA sample; C: control DNA.



Project II: Cell Specific shRNA Plasmid Construction and Characterization

Aim: Determine if RNAi constructs driven by Pol II promoter against *gfp* and/or *fli1* can be used as dominant marker and as example of gene function assay. a) Construct shRNA plasmid. The first exon-intron fragment was cloned from *fli1*gene into the vector to insert the shRNA. Splice donor (SD) and splice accepter (SA) were introduced to splice out the intron to express *rfp* marker gene. Two loxP sites (same orientation) were inserted to flank the whole first exon-intron-shRNA cassette for reverse inhibition assay; b) microinject and characterize the pTol-fli1-IT1-loxP-shRNA-loxP-SA-RFP transgenic zebrafish; c) perform transgene expression

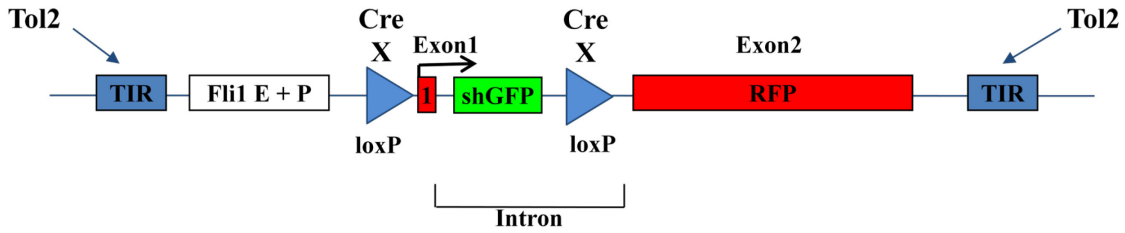
inhibition test; d) perform shRNA reverse inhibition assay using Cre-loxP activity. (A completed plasmid pTol-fli1-IT1-loxP-shGFP-loxP-SA-RFP map is shown in Fig. 12; a schematic vetor is shown below Fig. 23)

Figure 23.

Schematic of pTol-fli1-IT1-loxP-shGFP-loxP-SA-RFP Vector.

Fli1 E+P: Fli1 enhancer + promoter; Tol2: Tol2 transposonase; TIR: Tol2 Inverted Repeats (Tol2 recognition site); loxP: Cre recognition site; Exon1 and Intron: cloned from *fli1* gene; shGFP: shRNA anti-GFP; RFP: red fluorescent marker gene.

pTol-fli1-IT1-loxP-shGFP-loxP-SA-RFP



Cloning *fli1* Intron Fragment into Topo Vector

The *fli1* intron fragment (IT1) was cloned from the zebrafish genome into the Topo Vector (Invitrogen, CA) to generate pTopo-IT1 by PCR. The PCR product was 899bp (Fig. 24), containing the first exon of *fli1* and a splice donor site. EcoRI and other restriction enzymes were used to verify the correct insertion of the PCR product and Topo vector (Fig. 25 A and B). Since the IT1 was amplified with Pfu polymerase which would generate blunt end fragment, the insertion of IT1 into the TOPO vector (3,956bp) could be in either orientation. However, using a PstI and KpnI double digestion, the IT1 was found to be inserted in the forward orientation (5' to 3' of PCR).

Figure 24.

PCR of *fli1* Intron

M: 1kb marker; S: IT1 PCR sample; C: positive control, RFP PCR sample

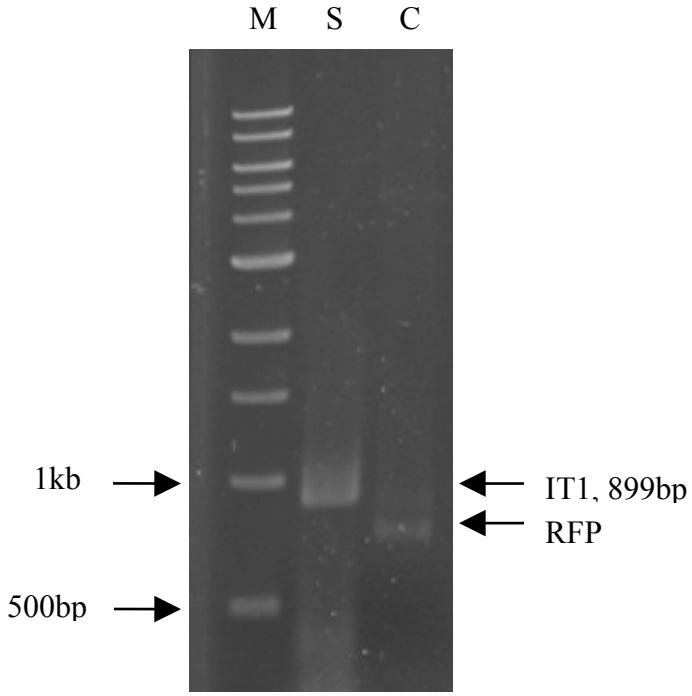
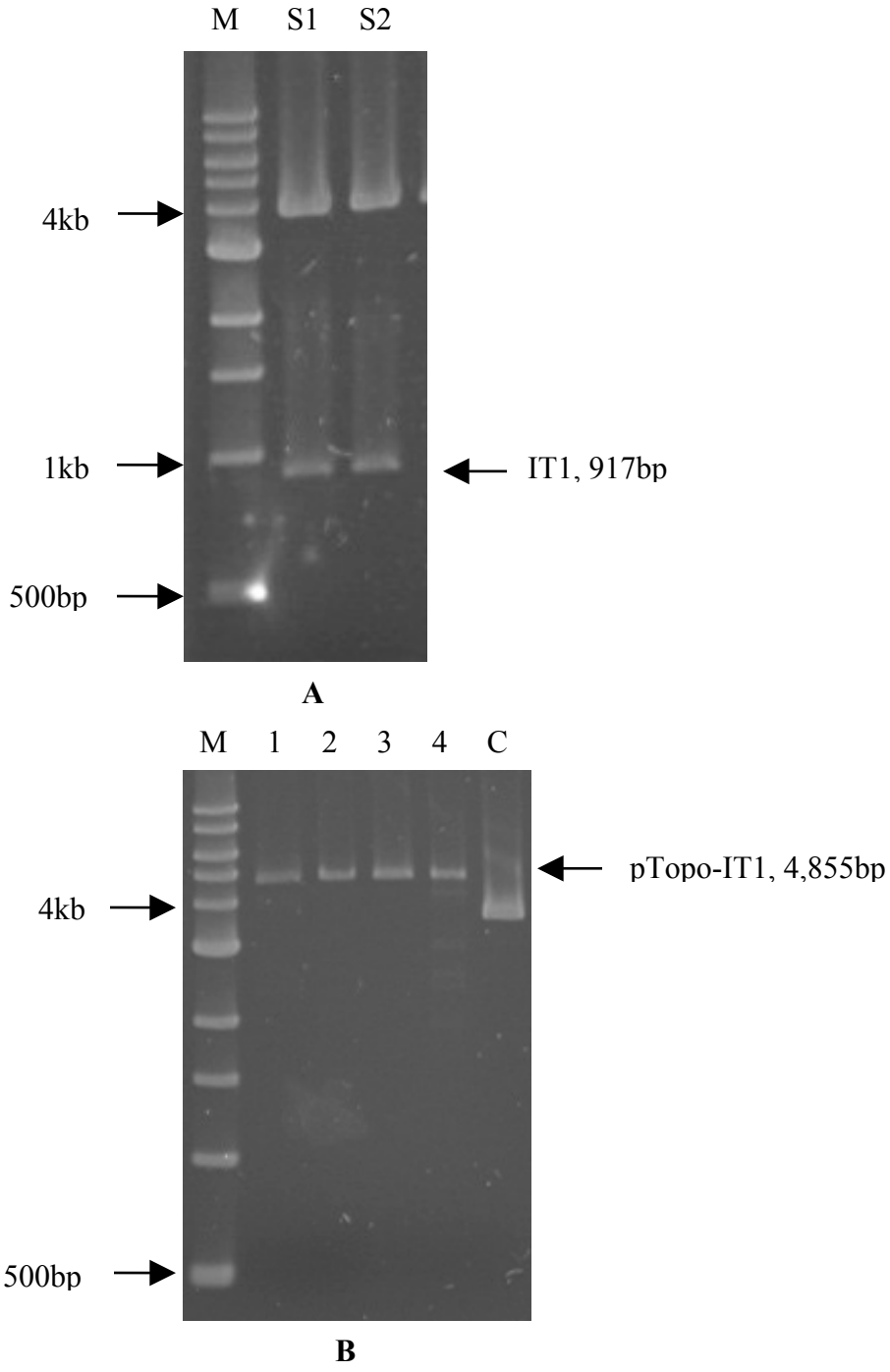


Figure 25.

Verification of pTopo-IT1 Vector

M: 1kb marker; S1, S2: EcoRI digest; 1-4: further digestion with S1 sample; 1: NheI; 2: KpnI; 3: PstI; 4: PstI + KpnI; C: uncut negative control.



Construction of pUC19-IT1 Vector

In order to add other elements into the IT1 fragment, another intermediate vector, pUC19, was used. Both pTopo-IT1 and pUC19 were cut with EcoRI and PstI to generate 927bp and 43bp fragments respectively. Then the 927bp fragment (IT1) was ligated into pUC19 to make the pUC19-IT1 vector. HindIII was used to verify the ligation, which should produce a 512 bp fragment (Fig.26). Sample 1 and 3 showed the correct bands, and sample 1 was digested with other enzymes for further verification (Fig. 27a, 27b). Enzymes AflII, NcoI, PstI, NheI, and KpnI should have only one band on the gel because of their single restriction site in the vector, but AflII (with or without NcoI and EcoRV) produced two more weak bands, which indicated that the AflII was contaminated. Other enzymes except EcoRV showed the correct bands. EcoRV was predicted to have two restriction sites in the IT1 sequence. Apparently, one of the two restriction sites of EcoRV was missing or mutated. For sample v and vi which were digested with NheI + PstI and KpnI + PstI (Fig. 27b), each produced only one band on the gel, indicating that the orientation of IT1 inserted in pUC19 was inverted (backward 3' to 5' compared to the original PCR design 5' to 3').

Figure 26.

Verification of pUC19-IT1 with HindIII

M: 1kb marker; S1-S3: plasmid samples

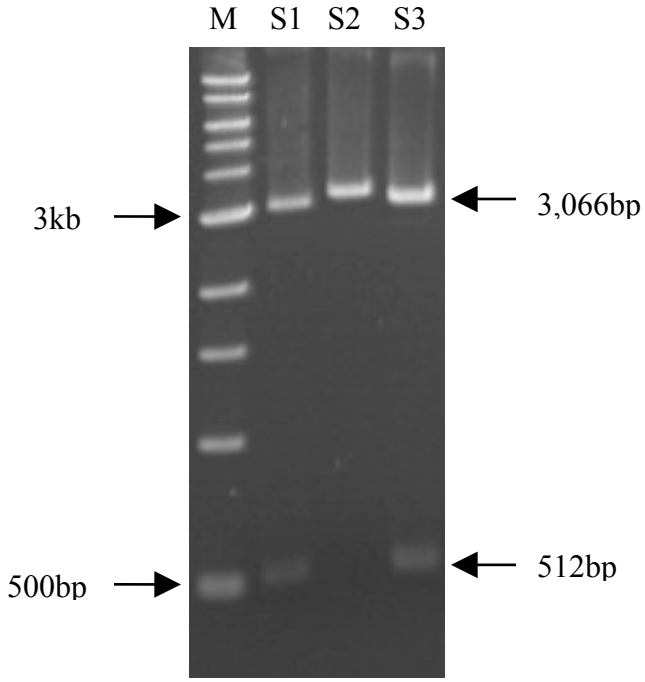


Figure 27a.

Further Verification of pUC19-IT1

M: 1kb marker; 1-6: further digestion with S1 sample; 1: AflII; 2: NcoI; 3: EcoRV;

4: AflII + NcoI; 5: EcoRV + PstI; 6: AflII + EcoRV; C: uncut negative control

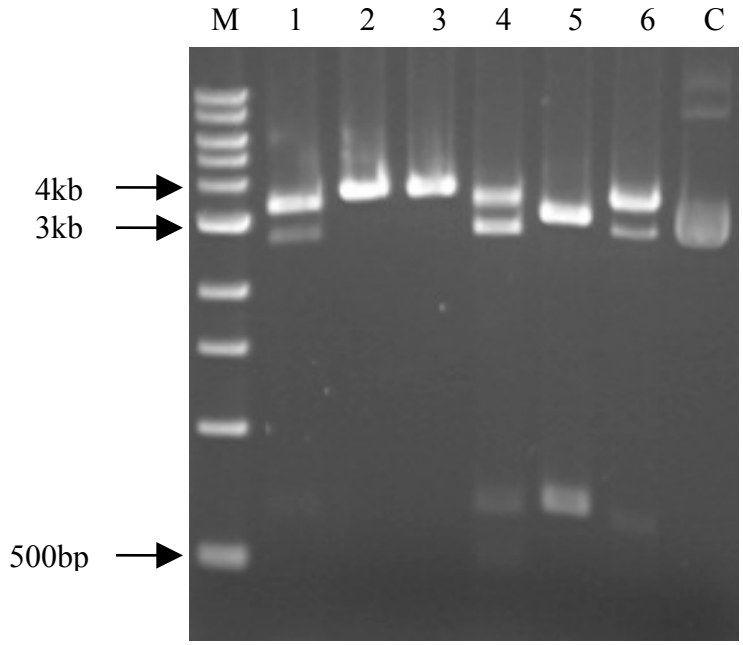
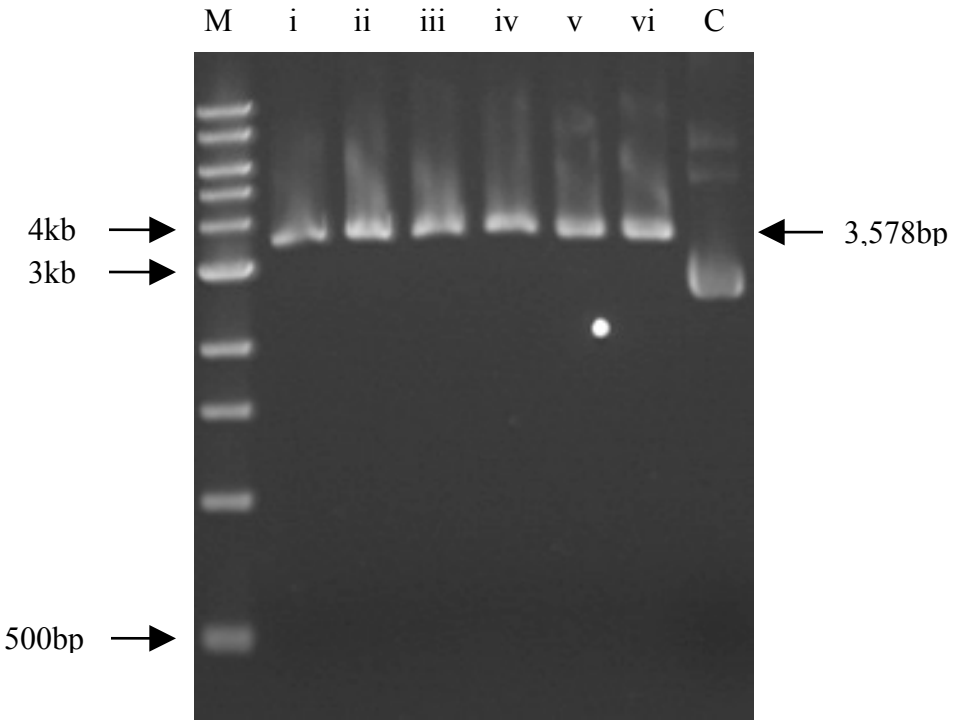


Figure 27b.

Further Verification of pUC19-IT1

M: 1kb marker; a-f: further digestion with S1 sample; i: NheI; ii: KpnI; iii: EcoRV; iv: PstI; v: NheI + PstI; vi: KpnI + PstI; C: uncut negative control



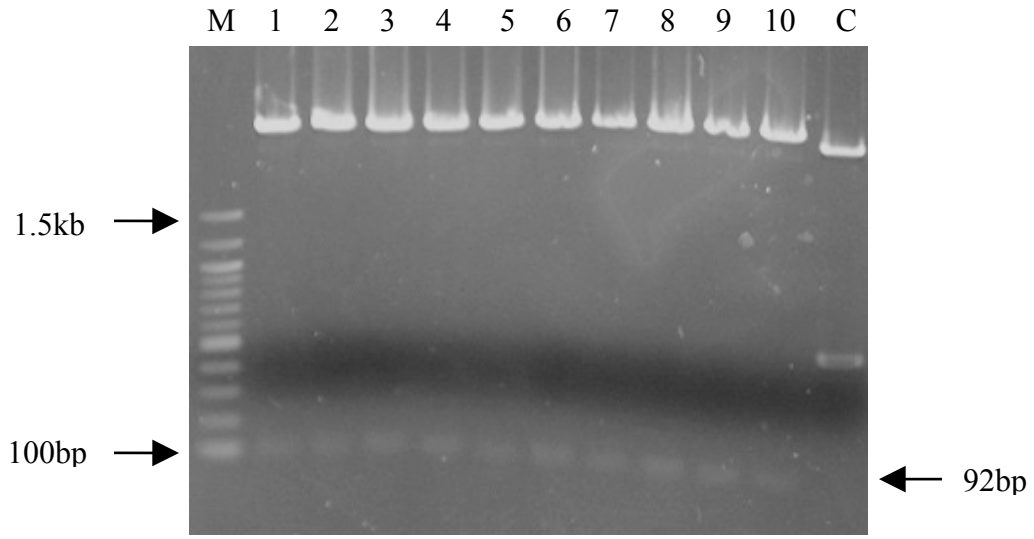
Insertion of Upstream loxP Site into pUC19-IT1 Vector

The synthesized upstream loxP site was inserted using NheI and KpnI double digestion of the pUC19-IT1 vector. The ligated plasmid was then isolated and verified by restriction enzymes KpnI and PstI, which should produce a 92bp fragment. The small bands very close to the 100bp marker were identified as the correct cut fragments (Fig. 28).

Figure 28.

Verification of pUC19-IT1-loxP

M: 100bp marker; 1-10: samples cut with KpnI + PstI; C: positive control, pUC19-IT1 cut with HindIII.



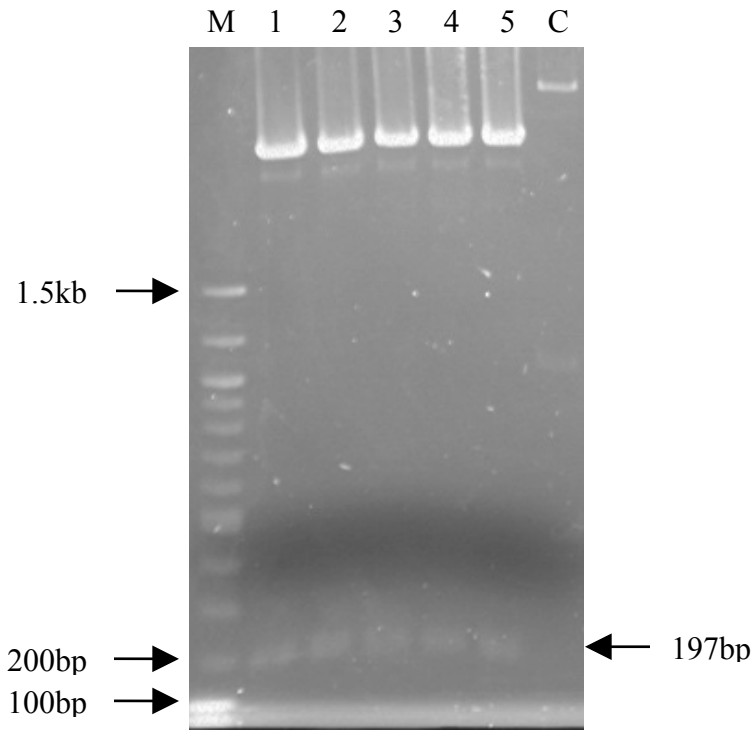
Insertion of Splice Acceptor (SA) into pUC19-IT1-loxP Vector

The SA oligo was inserted using restriction enzymes AflII and NcoI. A 197bp band was expected on the gel with digestion by MfeI and NcoI for verification. A band near the 200bp marker was found on the gel as expected (Fig. 29).

Figure 29.

Verification of pUC19-IT1-loxP-SA

M: 100bp marker, 1-5: samples cut with MfeI and NcoI; C: positive control.



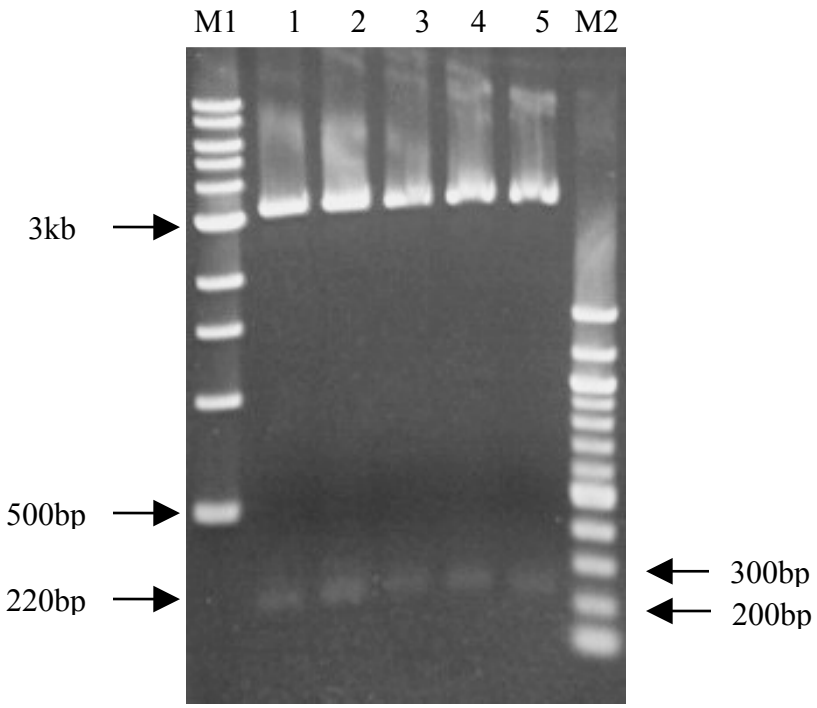
Insertion of Downstream loxP Site into pUC19-IT1-loxP-SA Vector

The same orientation downstream loxP fragment was inserted into pUC19-loxP-IT1-SA by restriction digestion with AflII and EcoRV followed by verification with MfeI and NcoI. The expected fragment on the gel was a 220bp band, which was observed near the 200bp maker in Fig. 30. This completed the construction of pUC19-IT1-loxP$_2$ -SA.

Figure 30.

Verification of pUC19-IT1-loxP$_2$-SA

M1: 1kb marker; M2: 100bp marker; 1-5: samples cut with MfeI and NcoI.



Translocation of *fli1* Intron Back to Topo Vector

The modified *fli1* intron of IT1-loxP$_2$-SA was translocated back to the Topo vector, using the restriction sites EcoRI and PstI. Enzymes PstI and EcoRV were applied for primary verification (Fig. 31a) and other restriction enzymes were also used for further verification, such as EcoRI, AflII, MfeI, HindIII, KpnI etc (Fig. 31b). A 706bp band was expected to be seen on the gel for samples cut with PstI and EcoRV. Single cut band was observed on the gel for enzymes such like AflII, MfeI, HindIII, and KpnI. Two bands were shown for the EcoRI cut sample, which was consistent with the designed sequence. All of the other combined digestions produced correct bands, e.g. AflII + HindIII, 215bp; NheI + NcoI, 1.5kb and 700bp; MfeI +

HindIII, 33bp. Therefore, the intermediate vector of pTopo-IT1-loxP$_2$-SA was successfully generated.

Figure 31a.

Verification of pTopo-IT1-loxP$_2$-SA

M: 1kb marker; S1-S5: samples cut with PstI and EcoRV

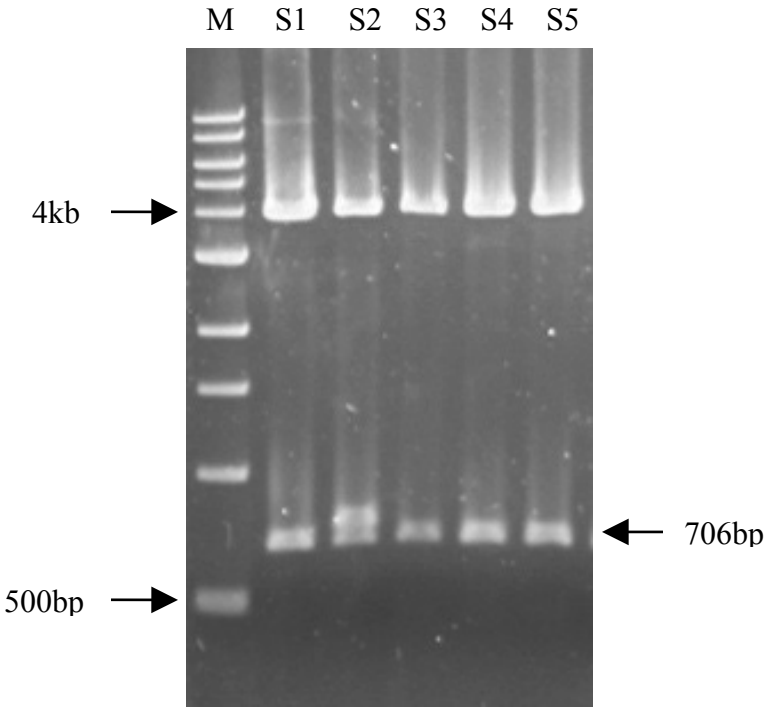
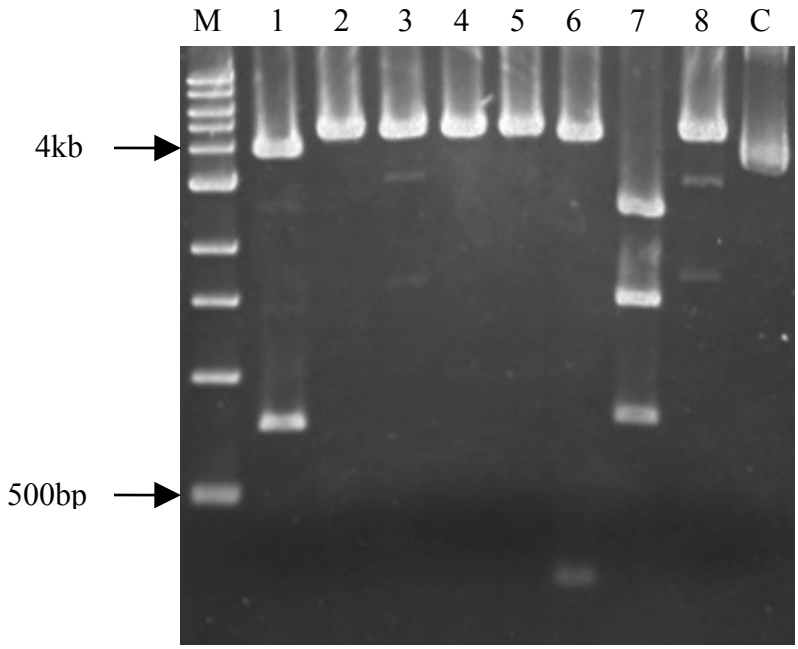


Figure 31b.

Further Verification of pTopo-IT1-loxP$_2$-SA

M: 1kb markder; 1-8: further digestion with S1 sample; 1: EcoRI; 2: AflII; 3: MfeI; 4: HindIII; 5: KpnI; 6: AflII and HindIII; 7: NheI and NcoI; 8: MfeI and HindIII; C: uncut negative control.



Construction of pTol-fli1-IT1-loxP$_2$-SA-EGFP Vector

Before the shRNA was introduced into the IT1 intron module, the fragment of IT1-loxP$_2$-SA was transferred from pTopo-IT1-loxP$_2$-SA into the pTolfli1epEGFPDest plasmid using enzymes NheI + NcoI to make pTol-fli1-IT1-loxP$_2$-SA-EGFP vector as a negative control for fish microinjection. The destination vector pTolfli1epEGFPDest was processed with BamHI digestion and religation which removed the CAM (chloramphenicol) gene including a second cut site for NcoI. Therefore, the destination vector was shortened by 703bp and contained a single NcoI site. The constructed pTol-fli1-IT1-loxP$_2$-SA-EGFP vector (without CAM gene) was

digested by NheI + NcoI (719bp), AflII (4.2kb, 6.4kb), HindIII (2.9kb), and KpnI + EcoRV (635bp) for verification (Fig. 32a, 32b).

Figure 32a.

Verification of pTol-fli1-IT1-loxP_2-SA-EGFP

M: 1kb marker; S1-S3: samples cut with NheI and NcoI.

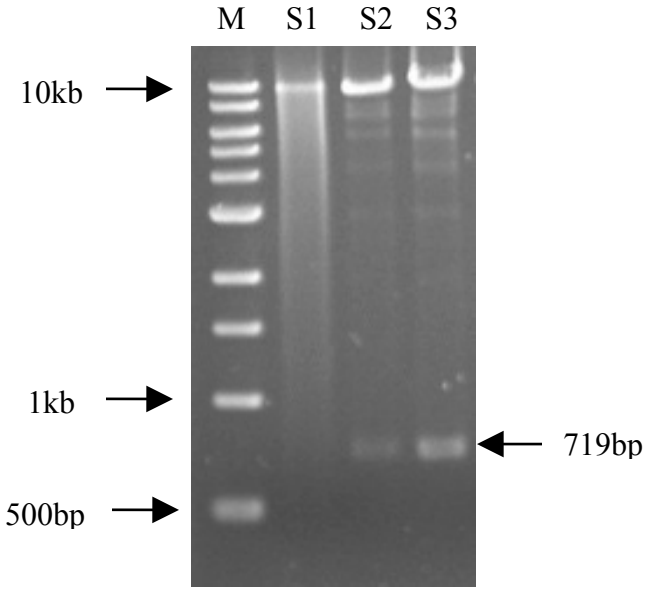
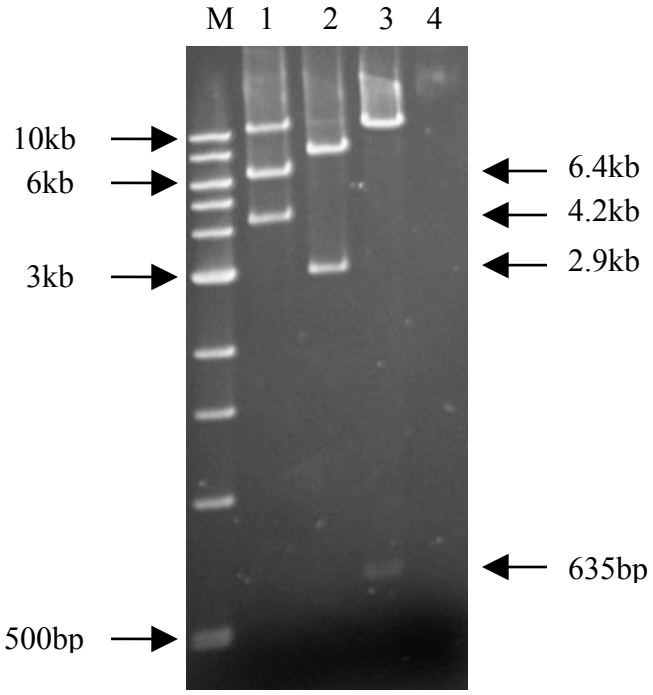


Figure 32b.

Further Verification of pTol-fli1-IT1-loxP$_2$-SA-EGFP

M: 1kb marker; 1-3: further digestion with S2 sample; 1: AflII; 2: HindIII;

3: KpnI and EcoRV; 4: uncut control.



Insertion of shRNA into pTopo-IT1-loxP$_2$-SA

The shRNAs oligos designed to mimick the intracellular miroRNA were synthesized and ligated into the pTopo-IT1-loxP$_2$-SA vector, using HindIII and MfeI. Two shGFPs which were targeting different sequences of the GFP coding region (site1 and site7) were inserted separately to make constructs of pTopo-IT1-loxP-shGFP1-loxP-SA and pTopo-IT1-loxP-shGFP7-loxP-SA. Restriction enzymes of AflII and HindIII were used to verify the constructs (Fig. 33, 34) and an 110bp band was expected on the gel. Sample 1 and 3 showed the right bands in Fig. 33, as did

samples 3, 8, 9 in Fig. 34. Thus, the construction of the *fli1* intron module was completed and ready to be translocated into the destination vector.

Figure 33.

Verification of pTopo-IT1-loxP-shGFP1-loxP-SA

M: 1kb marker; S1-S3: samples cut with AflII and HindIII.

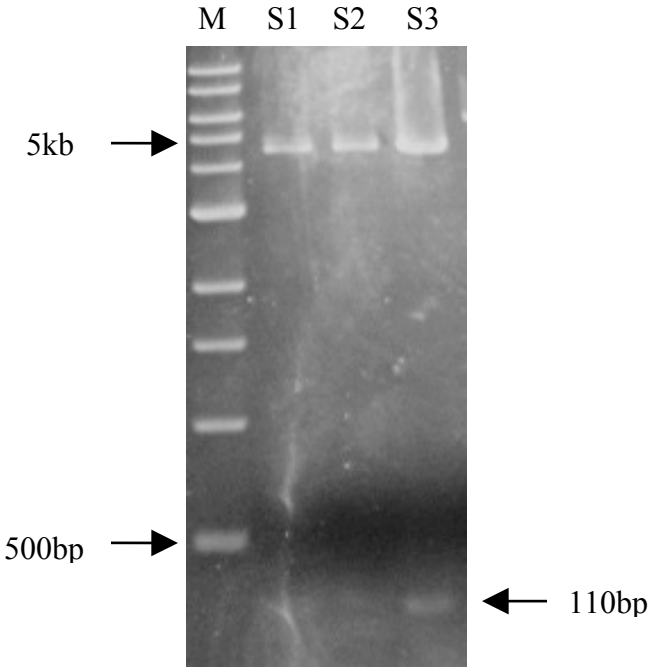
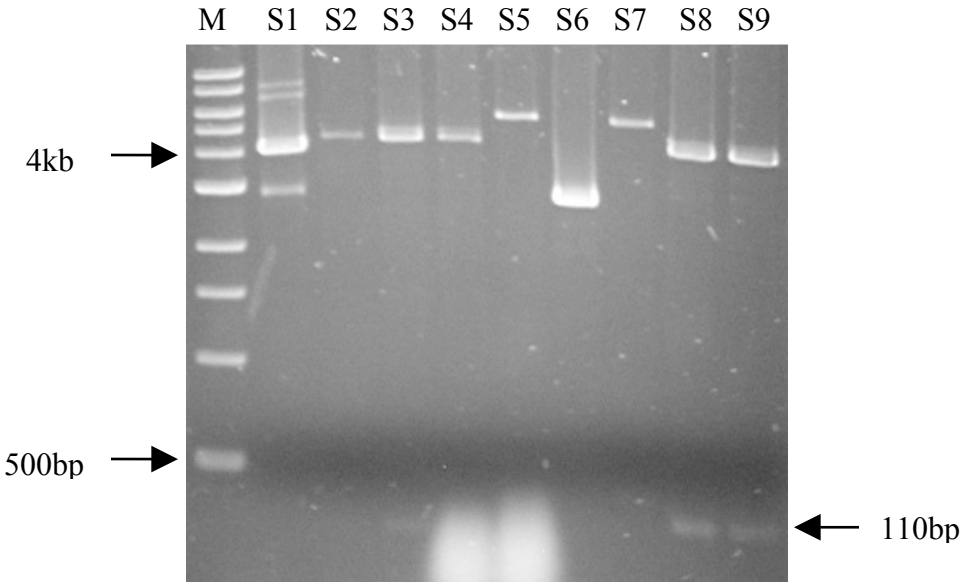


Figure 34.

Verification of pTopo-IT1-loxP-shGFP7-loxP-SA

M: 1kb marker; S1-S9: samples cut with AflII and HindIII.



Translocation of IT1-loxP-shGFP7-loxP-SA into Destination Vector

The *fli1* intron modules IT1-loxP-shGFP1-loxP-SA and IT1-loxP-shGFP7-loxP-SA were cut out with NheI and NcoI from the Topo vector and inserted in the processed destination vector to make pTol-fli1-IT1-loxP-shGFP1-loxP-SA-EGFP and pTol-fli1-IT1-loxP-shGFP7-loxP-SA-EGFP shRNA constructs. The HindIII digest was used to ensure the correct ligations and a band of 2,934bp was expected on both of the gels (Fig. 35, 36). The samples S5, S7 in Fig. 35 and S9 in Fig. 36 were believed to be the right bands.

Figure 35.

Verification of pTol-fli1-IT1-loxP-shGFP1-loxP-SA-EGFP

M: 1kb marker; S1-S7: samples cut with HindIII.

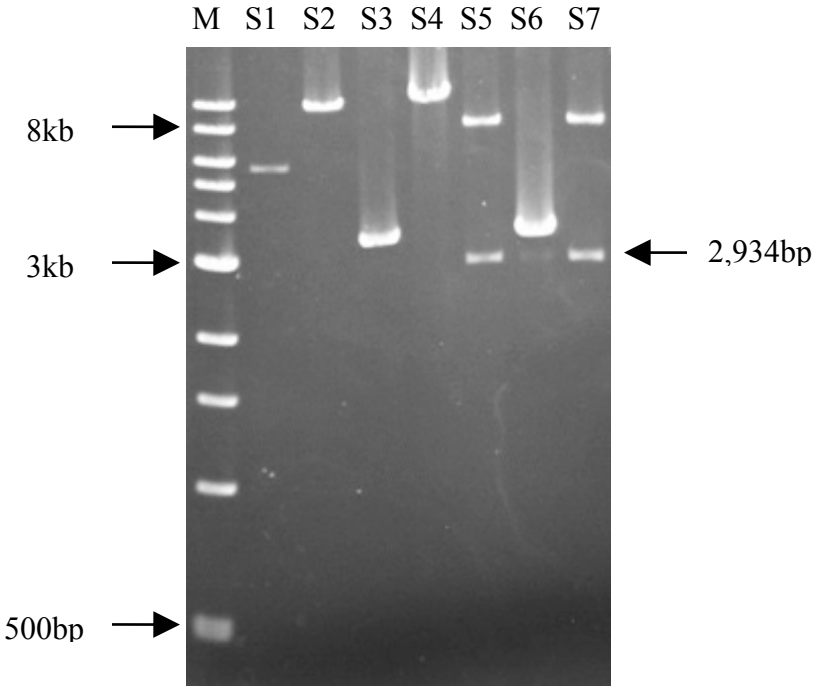
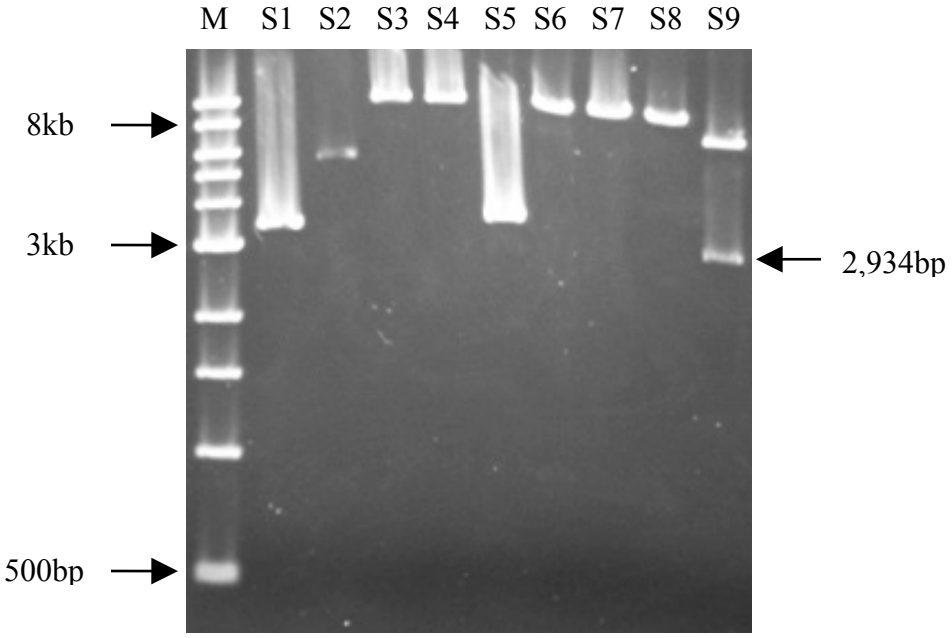


Figure 36.

Verification of pTol-fli1-IT1-loxP-shGFP7-loxP-SA-EGFP

M: 1kb marker; S1-S9: samples cut with HindIII.



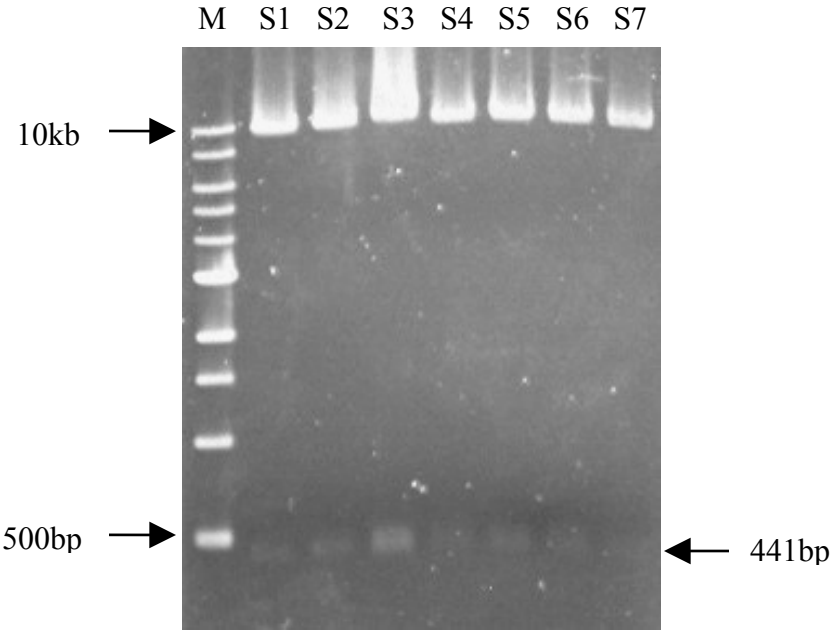
Construction of Final microRNA Vector

The last step to finish construction of the shRNA vector was to replace EGFP marker gene with RFP marker gene in the pTol-fli1-IT1-loxP-shGFP-loxP-SA-EGFP plasmid. To do this, the RFP gene was cut out from the vector of pTolfli1epCherryDest with NcoI and BamHI and put into pTol-fli1-IT1-loxP-shGFP-loxP-SA-EGFP to replace the EGFP (digested with NcoI and BamHI). Since the RFP had another NcoI restriction site in its sequence, the two cut sites of NcoI (441bp) in the final construct were used to verify the successful replacement (Fig. 37). The plasmids pTol-fli1-IT1-loxP-shGFP1-loxP-SA-RFP and pTol-fli1-IT1-loxP-shGFP7-loxP-SA-RFP were successfully constructed.

Figure 37.

Verification of pTol-fli1-IT1-loxP-shGFP-loxP-SA-RFP

M: 1kb marker; S1-S3: shGFP1 samples cut with NcoI; S4-S7: shGFP7 samples cut with NcoI.



Production of Transgenic Zebrafish with shRNA Constructs

shRNA constructs were injected with or without Tol2 mRNA to generate transgenic zebrafish. Briefly, 25 ng/µl shRNA constructs were injected into one or two cell stage fish embryos with or without Tol2 mRNA (35 ng/µl). Tol2 transposase translated from the mRNA could promote the transgene integration efficiency via single copy integration into the chromosomes. The expression of the marker genes RFP or GFP were examined under the fluorescent microscope at 24 hpf, 48 hpf, and 72 hpf. Other plasmids, such as pTolfli1epEGFPDest, pTol-fli1-IT1-loxP$_2$-SA-EGFP, and pTolfli1epCherryDest were also injected as positive controls.

For establishing pTol-fli1-IT1-loxP-shGFP-loxP-SA-RFP transgenic fish lines, 97 wildtype eggs were injected with 25 ng/µl shGFP1 plasmid and 41 of them showed red color in a

few blood vessel cells and/or muscle cells (Fig. 38). In addition, the same mosaic expression patterns were found in 10 out of 29 shGFP7 (25 ng/μl) injected embryos (Fig. 39). Similar expression patterns were observed in the pTolfli1epEGFPDest injected fish (3/70; Fig. 40), pTol-fli1-IT1-loxP_2-SA-EGFP injected fish (2/51; Fig. 41), and pTolfli1epCherryDest injected fish (2/30, Fig. 42). The mosaic expression of transgene is very common in the F0 generation, which can later be resolved in outcrosses to generate uniformly expressing lines.

Preliminary test for the shRNA constructs against GFP gene expression in the pfli1-EGFP transgenic fish showed some potential inhibition activities in blood vessel cells, in which the green color was weakened or blocked in a few individual cells (Fig. 43, 44). But the overall inhibition efficiency for the *gfp* expression was not good: 5 out of 90 shGFP1 injected fish and 2 out of 70 shGFP7 injected fish showed the potential inhibition effects.

Figure 38.

pTol-fli1-IT1-loxP-shGFP1-loxP-SA-RFP Transgenic Fish.

Tg1-Tg3: three different transgenic fish with shGFP1 construct; Arrow: potential transgene expressing cells.

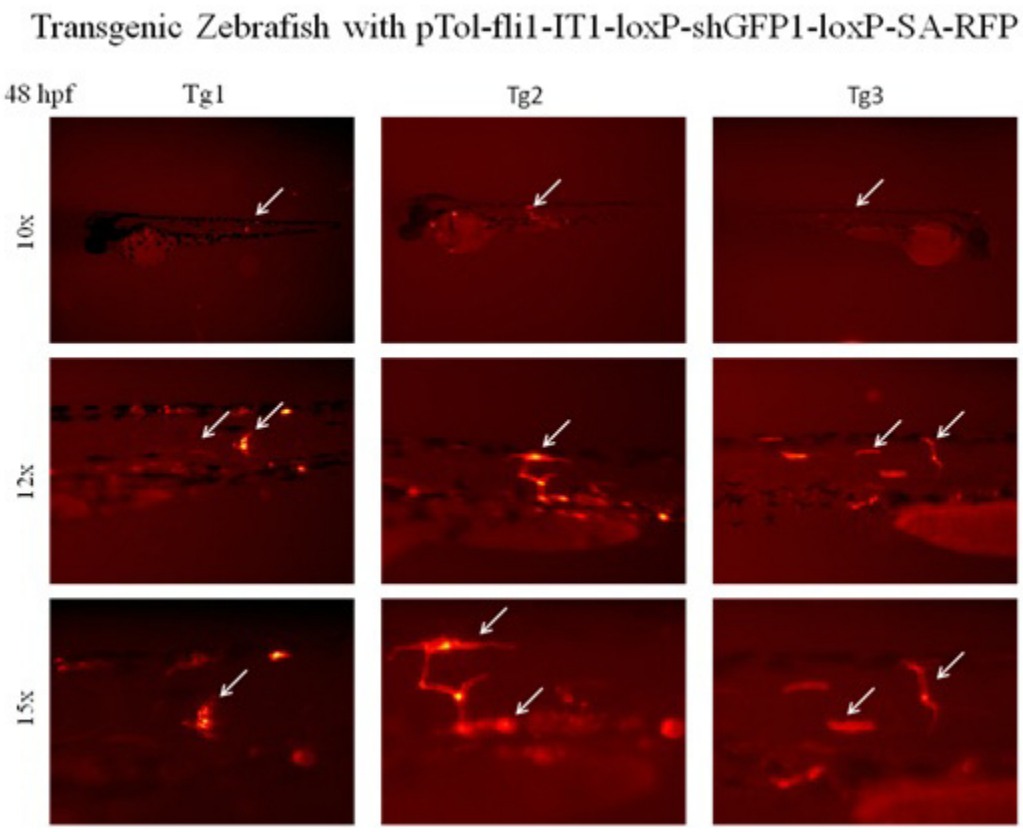


Figure 39.

pTol-fli1-IT1-loxP-shGFP7-loxP-SA-RFP Transgenic Fish.

Tg4-Tg6: three different transgenic fish with shGFP7 construct; Arrow: potential transgene expressing cells.

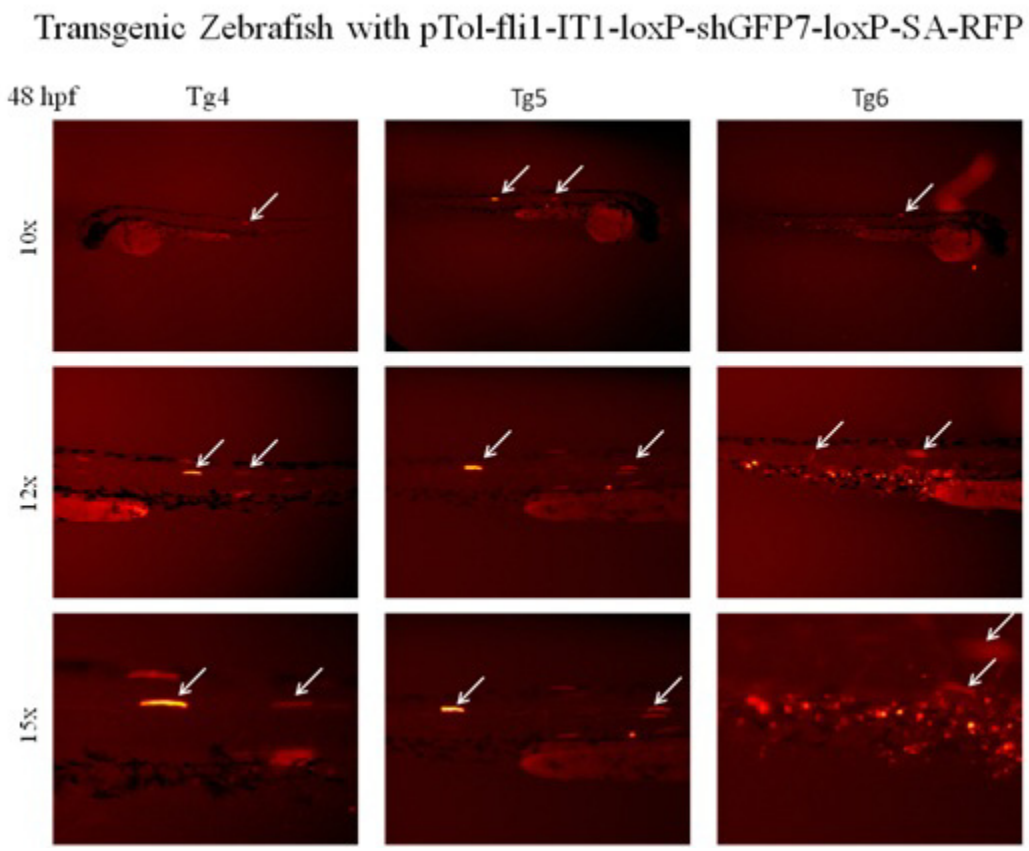


Figure 40.

pTolfli1epEGFPDest Transgenic Fish.

Tg1-Tg2: two different transgenic fish with destination construct; Arrow: potential transgene expressing cells.

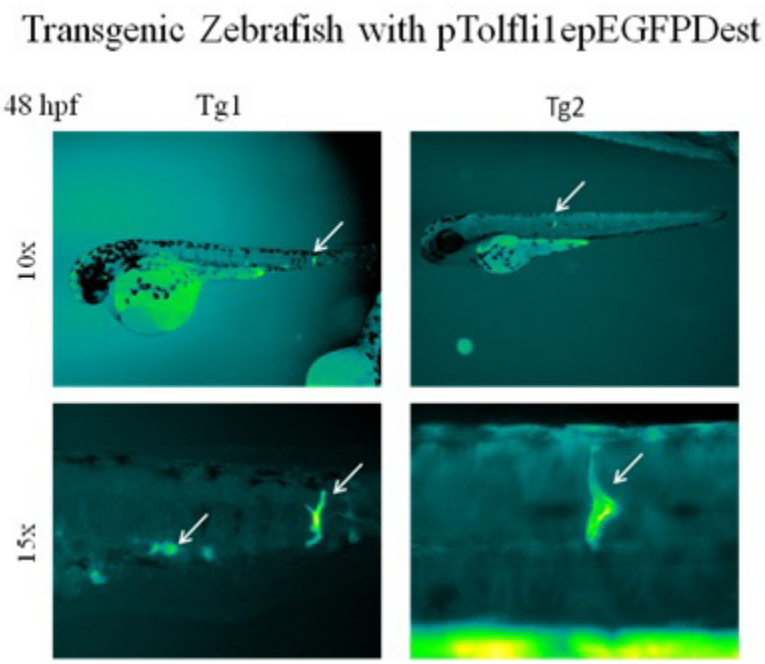


Figure 41.

pTol-fli1-IT1-loxP$_2$-SA-EGFP Transgenic Fish.

Tg1-Tg2: two different transgenic fish with processed destination construct (no shRNA); Arrow: potential transgene expressing cells.

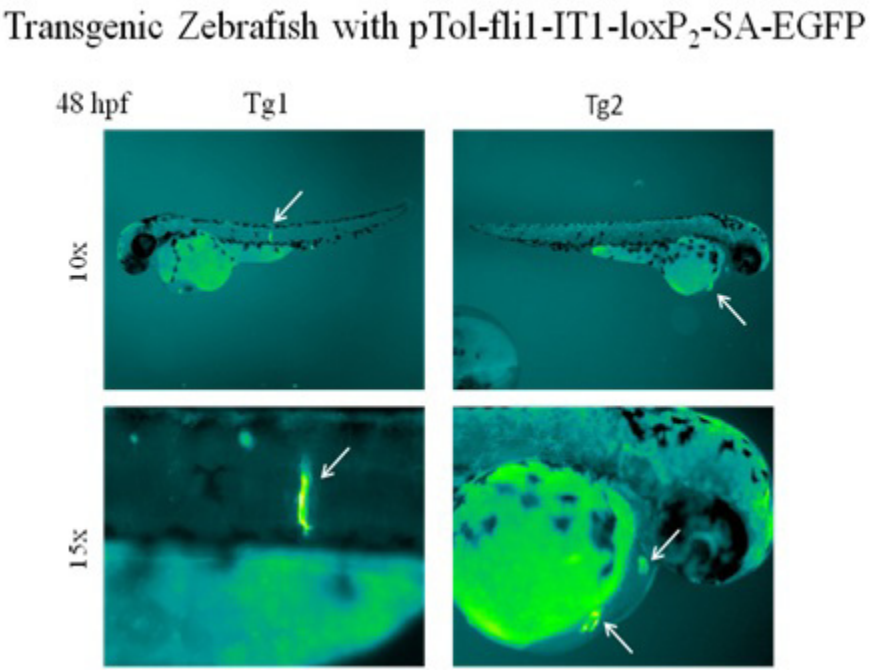


Figure 42.

pTolfli1epCherryDest Trangenic Fish.

Tg1-Tg2: two different transgenic fish with Cherry destination construct; Arrow: potential transgene expressing cells.

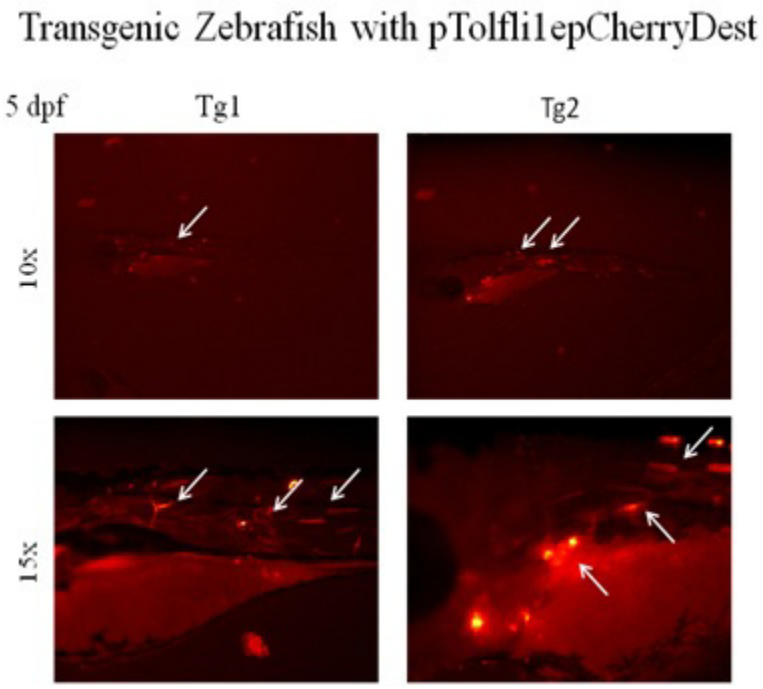


Figure 43.

pTol-fli1-IT1-loxP-shGFP1-loxP-SA-RFP Injection in pfli1-EGFP Transgenic Fish.

Control: un-inj fish; Tg1-Tg2: two different transgenic fish with shGFP1 construct.

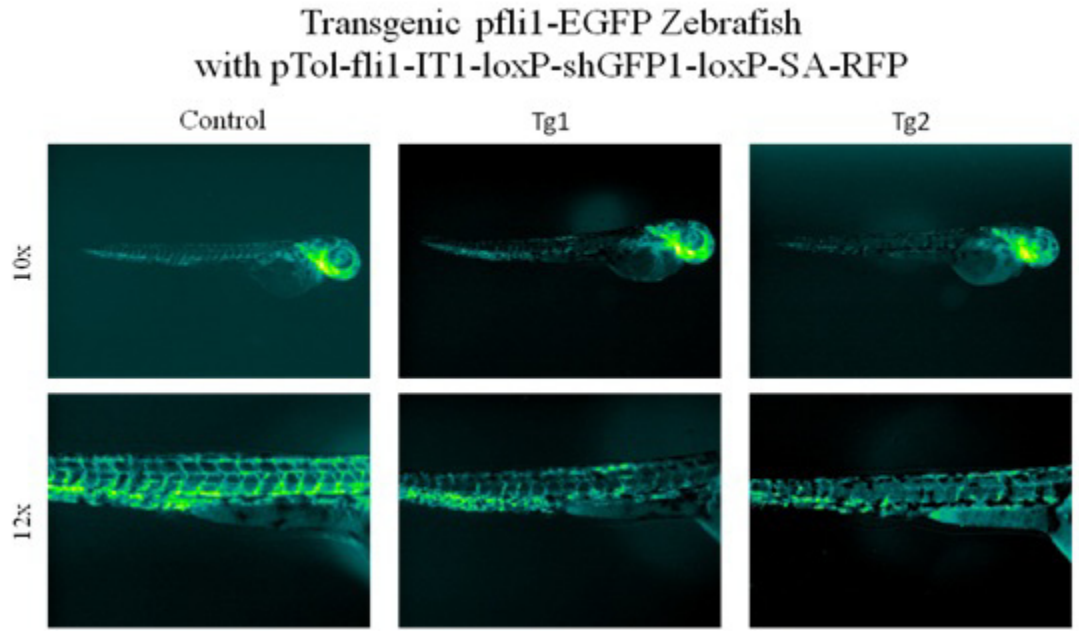
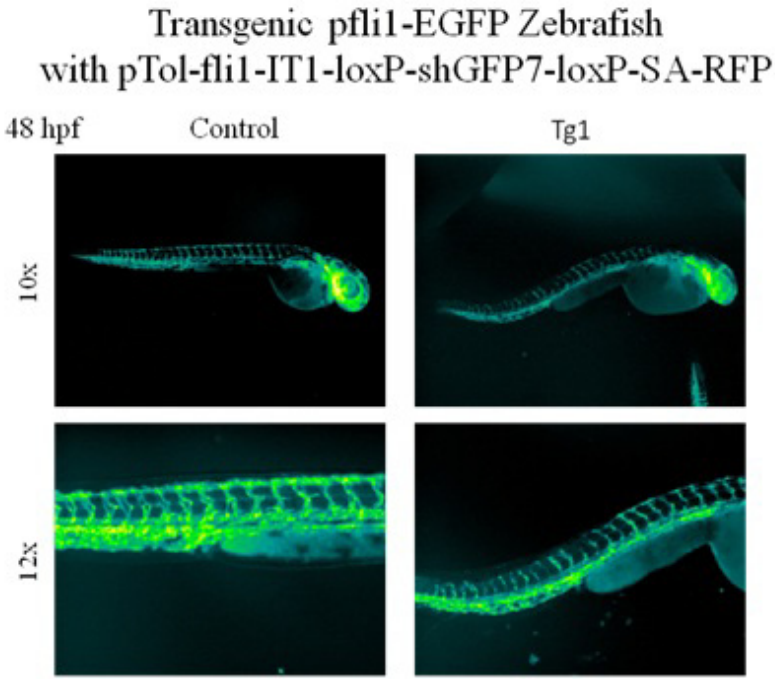


Figure 44.

pTol-fli1-IT1-loxP-shGFP7-loxP-SA-RFP Injection in pfli1-EGFP Transgenic Fish.

Control: un-inj fish; Tg1: a transgenic fish with shGFP7 construct.



CHAPTER 5

DISCUSSION

Establishment of Homozygous pBa/RFP/loxP$_2$/GFP/SB$_2$ Transgenic Fish

The construction of plasmid pBa/RFP/loxP$_2$/GFP/SB$_2$ and F0 founder fish microinjection were done by Dr. Jie Dong. I started with screening the positive *rfp* expression fish and then outcrossing them to establish stable transgenic line as well as the subsequent homozygous transgenic line. The F0 *rfp* positive fish showed mosaic red fluorescence throughout body, which is common for founder transgenic fish. After three generations of outcrosses and intercrosses, five fish that showed very bright red color were estimated as homozygous red fish, because all of their offspring were red in an outcross. Using the homozygous red fish was most convenient for performing the Cre recombinase induced *rfp* to *gfp* conversion assay. Because all the offspring would inherit the pBa/RFP/loxP2/GFP/SBIR construct, we just needed to screen for converted *gfp* expression.

Cre-loxP Recombination Assay *in vivo*

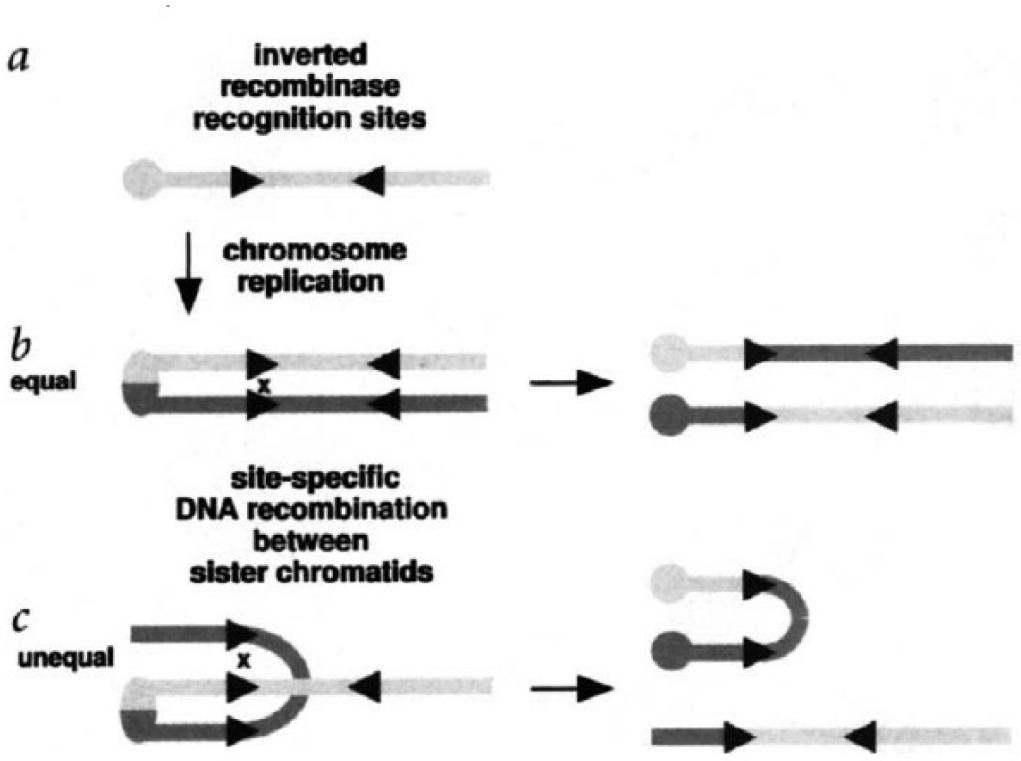
To perform the Cre-loxP recombination assay, the homozygous red fish were crossed with Cre fish (pHsp70-Cre-egfp) and their 24 hpf offspring were collected and heatshocked at 40 ℃ for 5 min in a water bath. A fluorescent microscope was used to detect *rfp* and *gfp* expression. The results showed that we succeeded in replacing *rfp* expression with *gfp* expression via Cre-loxP recombination in the first generation of heatshocked offspring. The efficiency of the

conversion was a little different in the offspring from a cross of female pBa/RFP/loxP2/GFP/SBIR fish with male Cre fish compared with the reciprocal cross of male pBa/RFP/loxP2/GFP/SBIR fish with female Cre fish. The reciprocal cross generated a better conversion from *rfp* expression to *gfp* expression (61% vs 46%). Surprisingly, the offspring from the control group using female Cre fish also showed some *gfp* expression without heatshock activation. In contrast, there is no converted *gfp* expression observed in the control using male Cre fish. This phenomenon could be explained by the maternal expression of Cre in female Cre crosses, which would appear in the germline cells during meiosis. Thus, the offspring from the female Cre parent would get more activated Cre recombinase than the offspring from the male Cre parent. Similar Cre leakage results were confirmed by Dr. Stefan Hans (Hans *et al*., 2009). Besides the different *rfp* and *gfp* expression patterns in the heatshocked offspring, the converted *gfp* failed to pass through the germline into the next generation. The reason may lie in the multiple complex tandem insertions of our construct into the chromosomes. When Cre is activated, it's possible that the whole construct including both *rfp* and *gfp* is deleted. This would happen if transgene integration resulted in an inverted loxP sites flanking the inserts. In this case, the unequal exchange between the duplicated loxP sites may generate an acentric chromosome and a dicentric chromosome, which would be lost during cell division causing cell death (Fig. 45, Lewandoski *et al*., 1997; Gregoire *et al*., 2008; Otsuji *et al*., 2008). To solve this problem, a single copy transgene integration into the chromosome is needed (e.g. Tol2 transposon system). This would be very helpful in reducing the complexity of Cre-loxP recombinant activity.

Figure 45.

Schematic Mechanism of Cre-loxP Mediated Chromosome Loss.

a. Chromosome that may get lost (light grey) after Cre-mediated recombination. Circle: centromere; Arrows: two loxP sites in inverted orientation. After chromosome replication, sister chromatids are shown as one light grey and one dark grey. b. Equal sister chromatid exchange produces two normal chromosomes, when the recombination takes place using the two loxP sites that in the same orientation on the two sister chromatids. c. Unequal sister chromatid exchange generates a dicentric chromosome and an acentric chromosome, when the recombination occurs using a proximal loxP site on one sister chromatid (light grey) and a distal loxP site on the other sister chromatid (dark grey) (Lewandoski *et al*., 1997).



An alternative verification of *rfp*/*gfp* fragments using PCR indicated that the fish that contained the plasmid pBa/RFP/loxP2/GFP/SBIR had intact *rfp* and *gfp*. However, when Cre was induced by heatshock to initiate recombination, fish with pBa/RFP/loxP2/GFP/SBIR suffered frequent deletion and loss of *rfp* and *gfp*. A few of the offspring still had the right size *rfp* or *gfp* fragments, but most of them lost both *rfp* and *gfp*. This is contrary to the intended design. We believe that this must be facilitated by a complex multi-copy transgene structure allowing complete deletions of both genes via Cre-loxP activity.

Attempts to demonstrate germline transmission of the converted *gfp* into the next generation failed for all pBa/RFP/loxP2/GFP/SBIR transgenic fish. We chose red/green progeny (showing both *rfp* and *gfp* expression at an early age after heatshock) to cross with wildtype fish to examine the efficiency of *gfp* germline transmission. From a total of 29 red/green fish crosses, only one individual out of 2,000 offspring showed red color, whereas none of them could pass *gfp* into the next generation. Presumably, following heatshock, *rfp*/*gfp* genes were either lost (due to gene deletion) or inhibited by some mechanism in the next generation. Current results suggested that virtually complete deletion of both *rfp* and *gfp* genes was a strong possibility.

Alternative Plasmid pTol-EF1α- RFP-loxP2-GFP Assays

Since our original plasmid failed to pass the converted *gfp* through germline, we decided to switch to an alternative plasmid pTol-EF1α- RFP-loxP2-GFP that shares high similarity with our plasmid for further studies. The transgenic zebrafish line (generated by Tol2 transposition) containing pTol-EF1α-RFP-loxP2-GFP was obtained from Dr. Michael Brand's lab. It has been shown to convert with high efficiency from *rfp* to *gfp* expression following exposure to Cre recombinase (Hans *et al*., 2009). Our Cre-loxP recombinant results also confirmed this efficient conversion and again the Cre recombinase leakage from female Cre fish was observed in the

non-heatshocked offspring. Furthermore, the heatshocked *gfp* germline transmission test succeeded in allowing the *gfp* gene to pass into the next generation. The offspring showed a green only pattern as well as both red and green in combination, which indicated that not all *rfp* got converted to *gfp* or that the red color was derived from the translated RFP protein retained in the fish germline.

Because we successfully generated the *gfp* germline transmission fish, we then crossed the converted *gfp* fish back with the Dresden red fish to create transheterozygous *rfp*/*gfp* fish, in which the *rfp* and *gfp* genes are located at the same locus. Nineteen fish that showed both red and green at early age were crossed with wildtype fish to screen for potential *rfp*/*gfp* transheterozygous fish. If the fish is transheterozygous, then there should be half red and half green fish in its progeny. Luckily, we got one parental fish that produced this pattern in its offspring. The next step is to introduce Tol2 transposase into the transheterozygous fish to facilitate the easy transposition assay and possible gene function studies via insertional mutagenesis. The principle behind the Tol2 assay is that if no transposition occurs, there should be no expression of both *rfp* and *gfp* in individual fish. If there are both red and green expressions, the translocation of either *rfp* or *gfp* occurred. Subsequently, we can perform PCR test to locate the deletion or insertion location. Unfortunately, the Tol2 fish (Fuji70, pHsp70-Tol2/EF1α-DsRed2), we planned to use as the source of Tol2 transposase, contains the same promoter and *rfp* gene as the Dresden red fish. These genes showed no obvious difference in *rfp* expression. So, we have to use Tol2 mRNA or make another Tol2 fish having a different maker gene for further tests.

Construction of shRNA Plasmid and Microinjection

The second project was to determine if RNAi constructs against *gfp* and/or *fli1* can be used as dominant marker and as an example of gene function assay. The construction of an shRNA expressing plasmid was completed. We cloned the first exon and first intron sequences from zebrafish *fli1* gene into the backbone of the pTolfli1epEGFPDest vector (the *egfp* was later replaced with *rfp*) and fused the shRNA fragment into the intron region between the *SD* (splice donor) and *SA* (splice acceptor) elements for its transcription. Then we introduced the two loxP sites to flank the whole inserted first exon and intron cassette for later Cre-loxP recombination, which should delete the entire cassette, including the shRNA, and result in the recovery of the inhibited target gene re-expression (but *rfp* marker gene expression should now be absent).

Microinjected zebrafish (25 ng/µl) showed mosaic *rfp* expression in a few blood vessel cells as well as muscle cells, which indicated the potential success of *fli1* driven shGFP transcription *in vivo*. The preliminary injection of the shGFP construct into the pTol-Fli1-EGFP green fish showed weak anti-*gfp* expression in some blood vessel cells. But, there was no *rfp* expression detected. This indicated that the weak *gfp* expression could be artifactual in these fish. Further tests to examine the efficiency of shGFP anti-*gfp* expression in pTol-Fli1-EGFP stable germline transformation are needed.

Conclusion

Although there is much to be done, our results to date demonstrate that efficient and precise transgenic manipulation in the zebrafish genome will be possible and applicable using combined DNA recombinant techniques, such as Cre-loxP and Tol2, in which the transgene could be specifically converted by Cre recombinase and transmitted into the next generation with high efficiency. Subsequent Tol2 induced DNA translocation is believed to be able to facilitate

insertional mutation analysis by simple *rfp* and *gfp* tags (transheterozygous *rfp*/*gfp* fish). Additionally, the introduction of the RNAi technique into the Cre-loxP and Tol2 system will facilitate the specific inhibition of target gene expression, followed by re-expression upon Cre activity. The efficiency and specificity of this combined system will make it ideal for gene functional studies. Future work will focus on characterization of Tol2 induced insertion mutation assays and examination of shRNA introduced target gene inhibition in zebrafish.

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APPENDIX A

Sequence of plasmid pTol-fli1-IT1-loxP-shGFP1-loxP-SA-RFP

CCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAA
TCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAA
GAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATT
AAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGC
CCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGC
ACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCG
GCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCG
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