EXPRESSION OF GREEN FLUORESCENCE PROTEIN (GFP) IN ZEBRAFISH MUSCLE THROUGH INJECTION: A GENE THERAPY MODEL

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Abstract: Expression of the target gene is important for gene therapy. Presently, localized transgenesis is used for gene therapy which can be achieved by a target gene expression. Here, we have reported the plasmid mediated gene therapy to zebrafish model. For this purpose, we have chosen green fluorescent protein (GFP) as a target gene because the expression can be detected easily. GFP was inserted in a plasmid vector, pQE30 to develop the vector pQE30GFP. The plasmid pQE30GFP was constructed form plasmid, pQE30 and pEGFPC2. pQE30GFP injected directly in one group of fish into the muscle where luciferase expression was noted. In another group, after injection electroporation was performed where we have also noted luciferase expression; but, electroporation cause muscle injury to the zebrafish. In our case, the expression was very strong at the site of injection in first group in compare to electroporation group and in both the cases expression was stable more than two weeks.

Keywords: Green fluorescent protein (GFP), Zebrafish, GFP Expression, Gene therapy model

INTRODUCTION

Advancements and achievements in gene therapy continue to have a significant importance on gene medicine since 1995. Presently, the potential for new therapeutics is unlimited and there have been a number of tremendous technological up gradation that have allowed for clinical trials of gene therapy [1] .

**Both have same contribution*

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In recent years, *in vivo* gene delivery by direct injection is a convenient method to express proteins into skeletal or cardiac muscle [2,3] . Over viral and other delivery systems, plasmid-mediated gene delivery offers several advantages and it has also shown very good promise in preclinical studies [4]. The plasmid has the ability to accommodate large genes. However, skeletal muscle appears to be extremely capable to take up and express plasmids in contrast to other tissues. Direct injection of naked DNA leads to patchy expression of delivered genes among fibers in a given muscle, which is a disadvantage [4,5]. Furthermore, plasmid-mediated gene therapy will become a feasible approach for humans along with viral vectors, when systemic delivery is achieved. The major hurdles for plasmid-mediated gene therapy are active exploration [6,7]. However, some degrees of studies in zebrafish gene therapy are currently under way [8].

Due to their transparent embryos and rapid organogenesis, zebrafish were established as a tool for academic developmental biologists till date. Zebrafish was used as first vertebrate large for scale mutagenesis screen in the 1990s that produced several of mutations, some of which was used as model for human diseases. Currently, similar to the mouse, zebrafish has proven to be a useful vertebrate for research [9]. Embryogenesis is rapid and large numbers of embryos are generated due to the high fecundity of zebrafish. Under ideal conditions, the females spawn up to 300 eggs per week. The eggs also hatch rapidly. Eggs and larvae are transparent; early stages of development make zebrafish a suitable model organism to study vertebrate development [10,11] . To understand the gene function and its expression in cells, transgenic zebrafish have been consistently produced [12]. Large-scale transgenic zebrafish generation method was developed by Powers et al. [13]. Electroporation method was successfully established on different fish species eggs [14]. To investigate gene regulation in later stages of zebrafish development, electroporation was demonstrated where DNA delivery was done into the neural tube of the embryo[15]. Recently, expression methods of transgenes in adult fish have also investigated. Particle bombardment using gene gun, muscular injections of naked DNA and electroporation have been reported [16, 17] .

The green fluorescent protein (GFP) has a major impact on many areas of sciences, especially on cell biology and molecular biology. GFP can be detected in living cells without selection or staining which is the greatest advantage of GFP [18]. The potentiality of GFP has also been documented by gene therapy researchers through which one can determine the expression level, site and time course of the therapeutic gene, or the correlation between gene transfer rate and therapeutic outcome [19, 20].

We have explored the possibility of gene transfer using GFP to the level of plasmid-mediated gene expression by injection method in skeletal muscle in zebrafish. For that, we have constructed pQE30GFP

and have injected this plasmid into the muscle to develop localize transgenic zebrafish (>5 months old). These results have significant implications for the blueprint of plasmid-mediated gene therapy by injection method into skeletal or cardiac muscle.

MATERIALS AND METHODS

Zebrafish

Adult zebra fish, purchased from a local aqua shop, was maintained as per the guiding principle given in the zebrafish book [21]. The fish used in these experiments were about 4-6 months old and approximately 3 cm in length which were kept in 5-10 lit glass aquariums. The aquaria had a continuous re-circulating system, consisting of biological filters. Additional oxygen was provided by placing air-stones in the water and 1/3 of water was replaced weekly. Aquariums water temperature was maintained at 28.5°C-32°C and a constant 14/10-h light/dark cycle.

Plasmids

The vector, plasmid pQE30GFP was constructed from two original plasmids pQE30 and pEGFPC2. We have digested pEGFP-C2 with the *Hin*dIII and *Kpn*I to isolate enhance-GFP (EGFP). Then we have digested pQE30 with *Hin*dIII and *Kpn*I and inserted EGFP to obtain pQE30GFP. Details of the construction of plasmids and the composition of pQE30GFP are included in the Fig.1 (A and B).

Intramuscular Injections of GFP Plasmid

All zebrafish were injected at the age of 4-6 months old. BD ultra-fine II insulin syringe (1-2 cc capacity) was used for injection (Becton Dickinson). The volume of injection was maintained constant at 10 µl, and the amount of plasmid DNA was 10 µg per construct. The fishes were taken out of the water and provided anesthesia with MS-222 for 5-10 minutes and then an intra-muscular injection was given approximately in the end region of the dorsal fin on one flank of the fish.

Intramuscular Injections of GFP Plasmid and Electroporation

We have used another group of fish where inter muscular injection was given and after that electroporation of DNA was performed. In this zebrafish group, we have performed electroporation of the muscle with a two-needle electrode to set at a voltage of 100 V.

Expression of GFP in Zebrafish

To observe the GFP expression after two-three days**,** anesthesia provided with MS-222 and the zebrafish were placed on petri-dish and were examined under blue light (490 nm) using an Olympus fluorescent microscope, and photomicrographs were taken with a Olympus camera (C5050).

RESULTS AND DISCUSSION

Expression of GFP in Only Injection Group

Luciferase expression was noted in anesthetized zebrafish at different days after plasmid injection by using a fluorescent microscope (Fig. 2A). The levels of luciferase gene expression were achieved after 24 hours after injection which was stable for more than two weeks (Fig. 2C). We have documented 2-4 sports

in muscle in different fish after plasmid administration. The expression was very strong in the site of injection in compare to electroporation group and the expression was very stable from the very beginning.

Expression of GFP in Electroporation Group

In electroporation group, level of plasmid expression was more compared to injection group which declines within 3-4 days and then expression was stable (Fig.3A). But, electroporation results muscle injury. Recovery was noted which was very slow. Others workers has reported about muscle injury after electroporation in zebrafish model or in other animal model [5,19] . The expression was less strong in the site of injection in compare to first group and the levels of luciferase gene expression was stable after more than two weeks same as above group (Fig. 3C).

Injection of plasmid with target DNA (in our case target gene was GFP gene) into the fish muscle was sufficient for expression of transgene into muscle cells that has also shown by some other workers [22, 23]. To improve the effectiveness of transgene expression more, we have attempted electroporation after muscle injection of plasmid. Transfection efficiency is interconnected with electroporation parameters like number of pulses, pulse duration and voltage strength. As the biochemical and physical character of tissues different, so, electroporation parameters are known to be different for each tissue. Each of these parameters is required to be optimized for each tissue [24].

The GFP gene expression suggests that there is a major influence of these parameters on cell entry of plasmid like number of pulses and voltage applied. At 10 µg of plasmid DNA per fish, we have noted major increase in GFP gene expression. (Fig. 1C). Below 10 µg of plasmid DNA, we could not detect luciferase activity. A previous report on muscle injection of plasmid without electroporation recorded maximum expression at 5 µg of DNA into adult zebrafish [22].

Our studies demonstrate that plasmid incorporation offers several advantages over standard plasmid-mediated gene therapy in which gene expression is mediated by extrachromosomal plasmids. High levels of gene expression were observed in muscles in the place of plasmid integration, and the level was considerably higher. We observed decline the number of spots (transgene expression) in fish body in some fish which is due to loss of plasmid after injection. To get a stable and more expression, further work is necessary to address this issue.

In the near future, technologies to boost plasmid-mediated gene delivery by injection are likely to be at the front position as nonviral gene therapy approach. It is apparent from human clinical trials concerning retroviral vectors that integration of exogenous elements into the human genome carries real risks [25]. For skeletal muscle and cardiac muscle gene therapy, the technology of ‘injection method’ for plasmid delivery is vital. It will be vital to identify the integration sites that would be considered for use in humans. For the gene therapy strategy where integration is probable, remains a safety concern. However, the results presented here demonstrate that the possibility of enhancing plasmid-mediated gene therapy is very promising and that needs further study.

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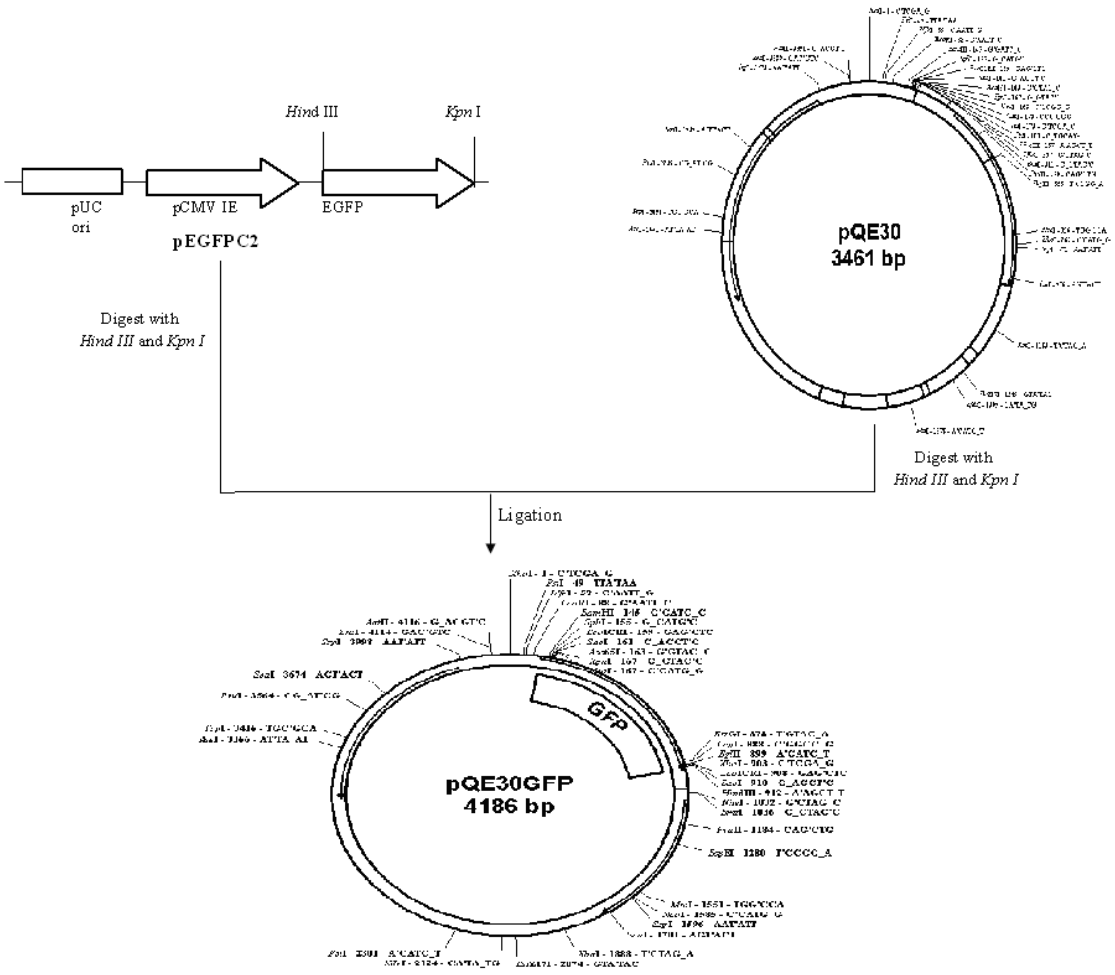
A



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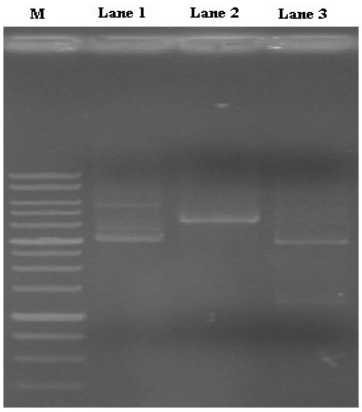
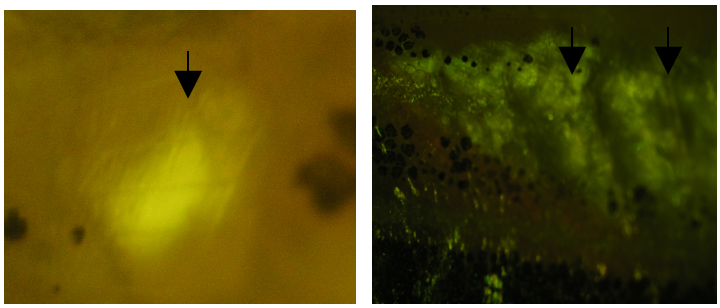


Figure 1. Schematic representation of construction and composition of plasmid, pQE30GFP, which was used for injection into zebrafish mussel. (A) For production of pQE30GFP, we have inserted GFP expression cassette into pQE30. GFP expression cassette was isolated from pEGFPC$_2$ digested by *Hin*d III and *KpnI*. (B) Electrophoresis of plasmid pQE30GFP treated with or without restriction enzyme. M: Molecular marker ; Lane 1: pQE30-EGFP; Lane 2 : pQE30-EGFP treat with *Hind*III; Lane 3:pQE30-EGFP treat with *Hind* III and *Nde* I

A **B**



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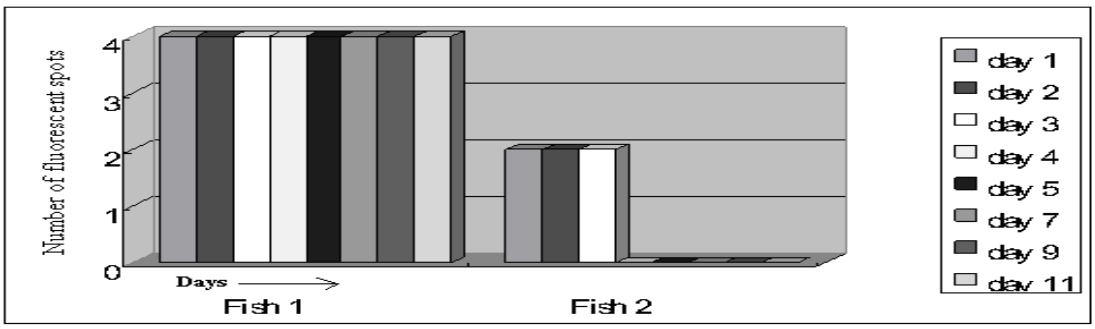
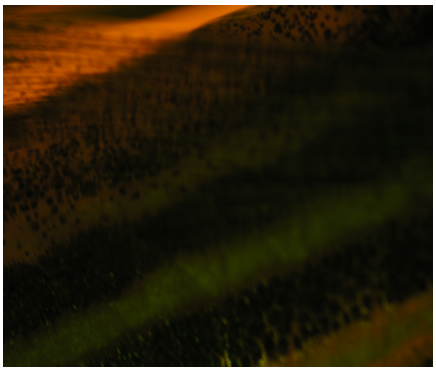


Figure 2. GFP expression into muscle after injection in zebrafish. (A) An image taken at the site of injection. (B) An image represents GFP expression other site of the body. (C) The number of sport for luciferase gene expression in respect to days (present data is from two fish).

A



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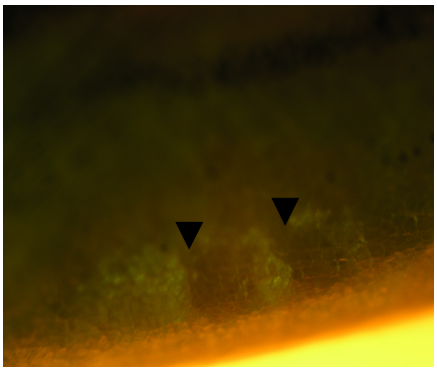

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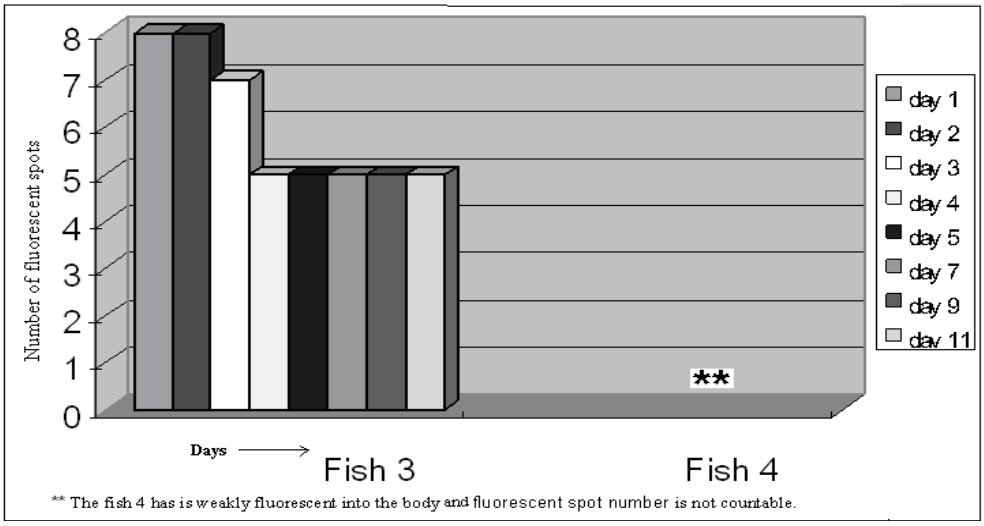

Figure 3. GFP expression into electroporation group in zebrafish. (A) an image taken at the site of injection. (B) An image represents GFP expression other site of the body; (C) The number of sport for luciferase gene expression in respect to days (present data is from two fish; in one fish fluorescence sports are countable, but in case of other fish, sports are every much scattered which can not be countable).