Developing a Gene-trapping Approach for Gene Identification Using Nuclear Transfer in Zebrafish

K. Y. Lee

Department of Animal Science, Michigan State University, MI 48824

Dept. of Aquaculture & Biotechnology, Faculty of Marine Life Science, Kunsan National University, Korea

지브라물고기 복제방법에 의한 유전자 동정 및 유전자트랩법 개발

이 기 영

미시건대학교 동물자원과학과, MI 48824 U.S.A.

적 요

이 연구는 gene-trap construct를 가지고 있는 배양세포로부터 trap gene을 확인하고 클로닝한 다음 이러한 세포를 이용하여 복제 지브라물고기를 만들기 위해 수행되어졌다. 본 연구에서 gene-trap과 연관된 복제 지브라물고기가 성공적으로 만들어졌다. 본 실험에서 두 종류의 백터(SA/GFP-TP와 Neo-TP)가 사용되었다. 이들 벡터에 의해 전이된 모든 종류의 세포는 항생제에 의해 선별을 하여 분석에 이용하였다. SA/GFP-TP에 의해 전이된 세포의 경우, 단일세포상에서 GFP 발현도가 낮아 본 연구에서 동물복제에 사용되지 않았으며, Neo-TP에 의해 전이된 세포주가 복제실험에 이용되었다. Neo-TP 세포에 의한 복제실험 결과, 총 1179개의 핵치환 난으로부터 44(3.7%) 개의 배자가 포배기에 도달하였으며, 8(0.8%) 개의 배자가 부화시기에 이르렀다. 그리고 3마리는 성숙단계에 이르렀으며, 이중 1마리에서 정상적으로 gene-trap 전이가 이루어짐을 Southern blot 분석을 통해 확인되었다.

(**주요어** : 핵치환, 유전자트랩, 지브라물고기)

I INTRODUCTION

Zebrafish, *Danio rerio* has increasingly become an important and a superb model organism for cellular and developmental studies, and functional genomics, as evidenced by the ongoing effort to sequence its whole genome. The significance of zebrafish functional genomics has been further enhanced by the finding of synteny conservation between zebrafish and human(Gates et al., 1999; Postlethwait and Talbot, 1997) and by the fact that zebrafish has produced a significant number of mutations that model human diseases(Dooley and Zon, 2000; Penberthy et al., 2002; Zon, 1999).

Studies utilizing zebrafish have provided us with a unique opportunity to integrate the tools of forward genetics, experimental embryology and molecular biology towards understanding vertebrate development. A genome sequencing project is expected to be completed soon to understand the genetic makeup of this model system. The challenge has now shifted to understanding functions of the more than 30,000 predicated

Corresponding author : Ki-Young Lee, Address: Cellular Reprogramming Laboratory, Department of Animal Science, Michigan State University, 1230 Anthony Hall, East Lansing, MI 48824 Tel : 1-517-432-7065, E-mail : leekiy@hanmail.net

genes present in a typical vertebrate genome. At present, two forward genetic approaches are adopted for functional studies of zebrafish genes, i.e. chemical mutagenesis followed by positional or candidate gene cloning and retroviral insertional mutagenesis methods (Amsterdam et al., 1999; Golling et al., 2002; Haffter et al., 1996).

The ES cells are amenable to various genetic manipulations, such as gene-trapping and homologous recombination, yet are still able to participate in normal embryonic development and contribute to germ line. Taking advantage of the ES cells, it is feasible to create libraries of murine ES cells in which each individual clone has one gene tagged or disrupted. The functions of these genes can then be studied when the cells are used to produce living animals harboring the tagged or disrupted genes. Unfortunately, successful development and utility of ES cells in other animal organisms are yet to be demonstrated. For nearly 10 years, at least three laboratories have been trying to develop ES cells equivalent to its mouse counterpart in zebrafish(Sun et al., 1995) and medaka(Wakamatsu et al., 1994; Hong et al., 1998). Although these ES-like cells exhibited many characteristics of their mouse counterpart, only short time cultures are shown to contribute to germ line(Ma et al., 2001). It remains unknown whether these cells are still able to contribute to germ line after elongated period of genetic manipulation.

With the advances made in the field of nuclear transfer, it has become a reality that, at least in mammals, many cell types including adult somatic cells can be genetically manipulated and their nuclei are still able to promote normal development when transplanted into enucleated eggs(Lai et al., 2002); (McCreath et al., 2000). This finding offers opportunities to create gene-tagged or “knockout” animals in virtually any organisms in which nuclear transfer can be performed, including zebrafish.

Gene-trap is a method of random insertional mutagenesis that uses a fragment of DNA coding for a reporter or selectable marker gene as a mutagen(Friedrich and Soriano, 1993). Gene-trap has become a powerful tool for a genome-wide gene discovery and functional analysis in various model organisms, such as *C. elegans* (Zwaal et al., 1993), *Drosophila melanogaster* (Spradling et al., 1999) and mice [Hicks, 1997 #82; Durick, 1999 #83]. In zebrafish, retroviral insertional mutagenesis has been successfully developed by Nancy Hopkins’ laboratory at MIT (Amsterdam et al., 1999; Golling et al., 2002) by direct injection of virus into blastula-stage embryos. The power of this approach has been clearly demonstrated by the isolation of more than 500 insertional mutants and cloning of 75 mutant genes in a 2-year period. Though many tagged genes can be isolated in short time, creating mutants takes much longer as a mutant can only be analyzed in F3 homozygous fish, which requires more than a year to obtain. Therefore this approach alone is not sufficient to isolate and assign functions to approximately 30,000 genes anticipated in the zebrafish genome. Additionally, the method used by Hopkins’ lab does not involve pre-selection for gene-traps, which should improve efficiency for gene identification and function characterization.

Therefore, this studiy aims to develop alternative strategies for both forward and reverse genetic studies for defining gene functions in zebrafish, i.e. the gene-trap approaches, based on the nuclear transplantation technique developed(Lee et al., 2002; Huang et al., 2003).

Ⅱ MATERIALS AND METHOS

1. Design for gene trap constructs : SA/GFP-trap vector and Neo-trap vector

Two kind of retroviral gene-trap constructs

were adopted. The first one(SA/GFP-TP), constructed in my laboratory, carries a GFP reporter gene containing a splicing acceptor and an internal neo gene(Fig. 1). The second one(Neo-TP), obtained from Dr. Hicks(Hicks et al., 1997), contains a promoter-less neo gene located in the LTR sequence of a retroviral vector (Fig. 2).

2. Cell culture

For culturing zebrafish cells, the DMEM (Dulbecco's modification of Eagles's medium) was used as the basic medium. It contains 3mM L-glutamine, 4.5gm/L glucose, but lacks sodium pyruvate(Gibco BRL, Rockville, MD and Cellgro,

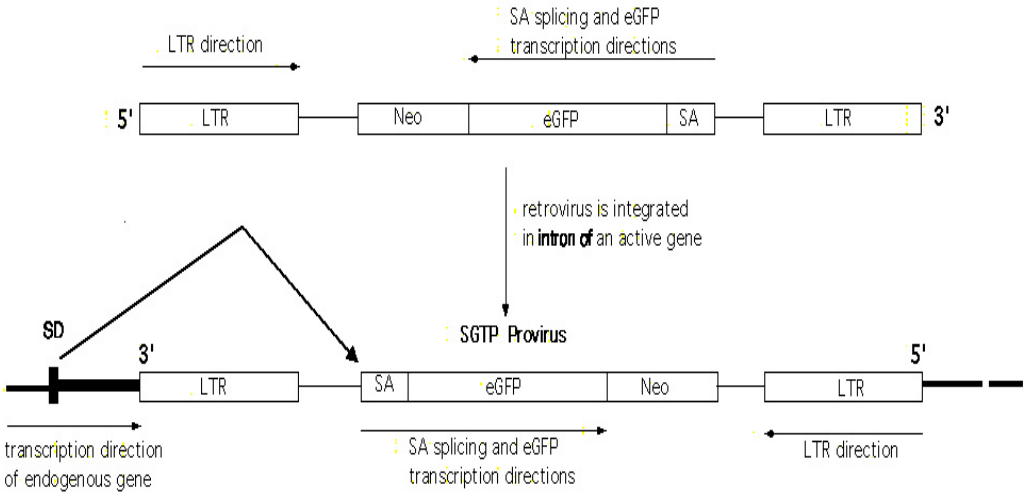

Fig. 1. SA/GFP-trap vector. SA/GFP retroviral gene-trap construct contains a splicing acceptor (SA) with a partial peptide fused in frame with eGFP.

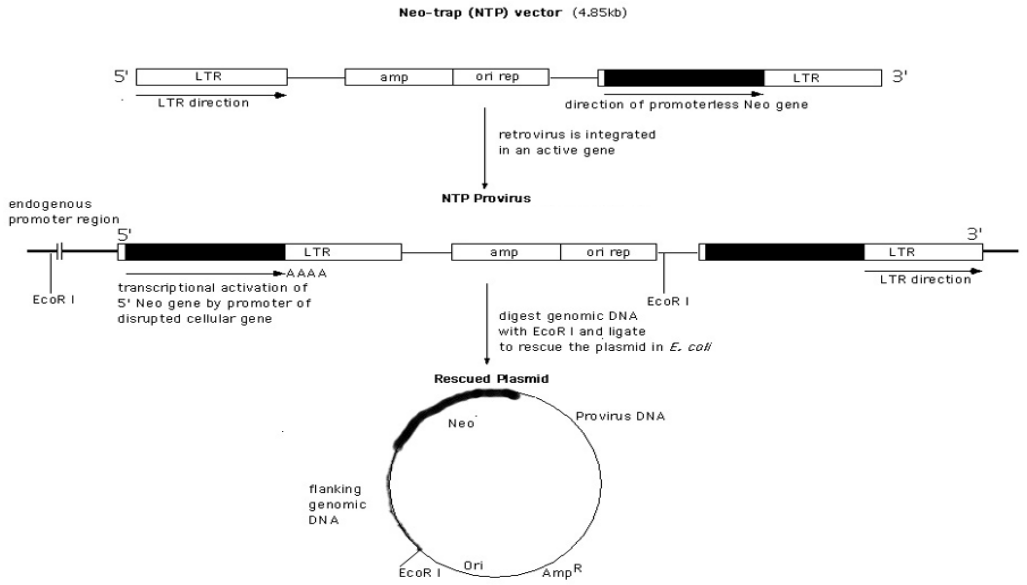

Fig. 2. Neo-trap vector. Neo-TP retroviral vector carries a promoterless neo gene in its 3' LTR.

Mediatech, Inc.). The medium was supplemented with 15% fetal bovine serum(vol/vol, Gibco BRL, Rockville, MD), 1% trout serum(SeaGrow, East Coast Biologics, Inc.), bovine insulin(10 μg/ml, Sigma) and 0.5%(vol/vol) zebrafish embryo extracts.

To establish long-term cultured cells, Primary cells collected from 5-15 somite embryos of the wild-type Tubingen strain of Zebrafish were cultured in DMEM based medium at 28 ~ 29℃ with 5% CO_2. bFGF(Sigma, 20 ℃ 50 ng/ml) was included in the medium for the first two weeks to inhibit pigment cell formation. After in continuous culture for eight weeks and about 13 subcultures, the cells were considered as long-term cultured cells. These cells were infected with retroviruses with exogenous DNA constructs. These infected cells were also cultured at a lower density to generate individual clones (Fig. 4-a), expand them. Again, each clonal cells (Fig. 4-b) were used for DNA extracts and a plasmid rescue experiment to identify trapped sequences.

For nuclear transfer, infected cells, as a donor, are subjected to serum starvation by culturing them in DMEM medium supplemented with 0.5% FBS for four days, then they were dissociated with trypsin/EDTA(0.25% trypsin/ 1mM EDTA in PBS), washed once with PBS and centrifugation at 1000 rpm for 5 minutes. Cells are suspended in DMEM containing 0.5% FBS and kept on ice until nuclear transfer. Infected cells were also grown in the drug G418 (800 mg/l) for 14 days after the infection according to the preliminary study(data not shown).

3. Preparation of recipient eggs

Zebrafish were kept on a 14 hour light/10 hour dark cycle. Pairs of male and female fish were placed in a mating cage, separated by a divider. The next morning, the divider in one mating cage was removed to allow the male to chase the female. After the male fish touched the female two or three times, the female was immediately removed from the cage, anesthetized for approximately 1 minute in 0.1% tricaine solution(Sigma). The fish was gently squeezed from the urogenital opening to obtain unfertilized eggs. Good quality eggs were directly placed in Holtfreter's solution and dechorionated with protease. After brief washing with Holtfreter's solution for 4 times, the eggs were immediately transferred into pre-cooled(4℃ Hank's solution supplemented with 1.5% BSA(wt/vol, fraction V, heat shocked, Roche). These eggs were used as recipients for nuclear transfer up to an hour.

4. Nuclear Transfer

To remove the egg pronuclei, recipient eggs were placed in a drop of Hank's solution(~100 μl) containing 1.5% BSA and then covered with mineral oil(Sigma). After activation, the cytoplasm of the fish egg coalesces, moves toward the animal pole, and forms the blastodisc. The blastodisc of the zebrafish takes ~12 min to form at 24℃ and after 40 min becomes a full-sized one-cell egg. Each dechorionated egg was oriented using the holding or injection needle to determine the position of the pronucleus, which is located at the animal pole just underneath the egg surface against the second polar body, which could be identified as a transparent ball of ~8 μm diameter. The nucleus was sucked out from the egg with a fine glass needle by aspirating a small amount of cytoplasm just underneath the polar body.

All nuclear transfers were carried out using a Narishige system(NT-188NE, Leeds Precision Instruments, Minneapolis, MN) with an Axiovert 200 microscope(Carl Zeiss).

Transplanted eggs are transferred from Hank's

solution to small containers like 60-mm or 100-mm Falcon tissue culture dishes containing Holtfreter's solution and maintained in a 28 ~ 29℃ ncubator. Since the freshly transplanted eggs are extremely delicate, unnecessary movements should be avoided for the first 24 hours. After first 3 days, they were transferred to a mouse cage filled with fish water and fed with paramecia for 10 days, then switched to both paramecia and live brine shrimps. After another 10-15 days, they were transferred to regular fish tanks until they reached sexual maturity.

5. Southern blotting analysis.

The genomic DNA from each fish was digested with restriction enzyme Hind Ⅲ and Hind Ⅲ / EcoR I, separated by electrophoresis, and blotted on nylon membrane. A internal neo fragment of was used for probe and was labeled with ^{32}P, and hybridized to the membrane using the QuickHyb solution(Stratagene, La Jolla, CA). To reveal the size of the hybridization bands, 1kb marker DNA was also labeled and used for hybridization.

Ⅲ RESULTS AND DISCUSSION

1. Identification and expression of gene-trap events

To utilize the cloning technique for characterization of gene functions in zebrafish, I performed genetic manipulation on the cultured cells using the gene-trap strategy.

As a first step, cultured cells in various ages were infected by the provirus carrying the two gene-trapping constructs. The infected cells were subjected to drug selection(neomycin treatment) because the two constructs carry the neomycin resistant gene. All those cells survived the neomycin treatment should carry the proviral insertions. SA/GFP retroviral gene-trap construct contains a splicing acceptor(SA) with a partial peptide fused in frame with eGFP(Fig. 1) If the viral vector is inserted in an intron and a fusion protein is generated from its upstream coding exon serving as splicing donor(SD). For SA/GFP-TP construct carrying the GFP reporter gene, the infected cells were expressed(Fig. 3) under the fluorescent microscope.

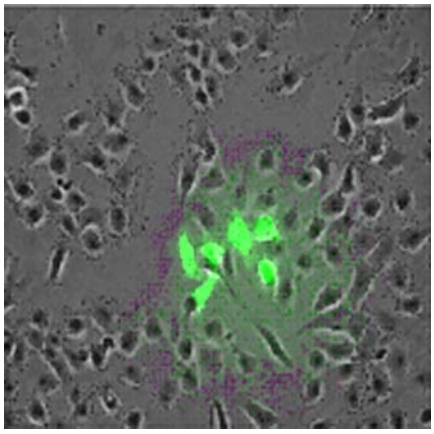

Fig. 3. GFP expression on the infected cells infected with SA/GFP-trap vector.

The second construct Neo-TP contains a promoter-less neo gene located in the LTR sequence of a retroviral vector(Fig. 2). Since the neo gene lacks a functional promoter, all of the neo-resistant cells should carry viral insertions immediately downstream of an active promoter. the cells were infected with a lower titer so that most of the individual cells resistant to the neo-selection may carry a single insertion, which simplifies gene identification process later. Neo-TP retroviral vector carries a promoterless neo gene in its 3' LTR. When a virus is made, the neo will be duplicated into 5' LTR. When used to infect cultured cell, neo-resistant cells were grow out and that means it was integrated near a gene and activated by an endogenous trapped promoter(Fig. 4).

Pseudotyped retrovirus(Lin et al., 1994) for each

gene-trapping construct was generated to infect cultured zebrafish cells that had been successfully used in nuclear transplantation experiments. In each case, neo-resistant clones were obtained after G418 drug selection. For SA/GFP-TP, approximately 0.2% cells of the neo-resistant population appeared positive for GFP expression, indicating generation of a GFP fusion protein(s) by splicing(Fig. 3). However, GFP expression of these cells was not strong enough for picking out on the fluorescent microscope when I expanded them into individuals. That's why I couldn't use these cells for nuclear transfer.

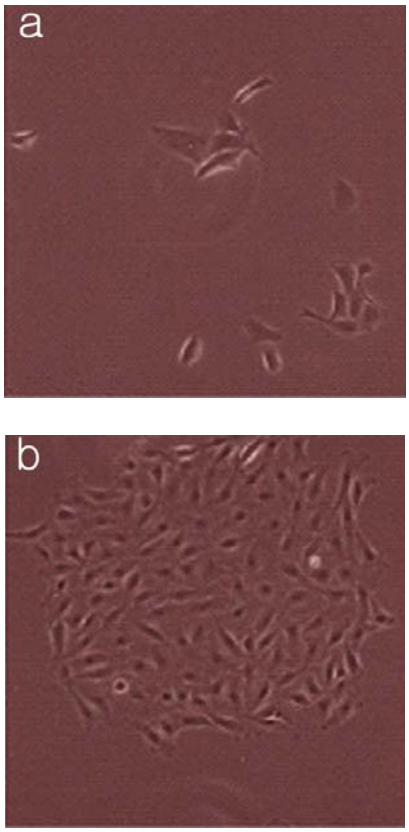

Fig. 4. Neo-resistant cells.
Independent clones of neo-resistant cells carrying a trapped promoter. (a) Individual neo-resistant cells, (b) Proliferated clonal cells from individual cells.

For Neo-TP, since the neo gene lacks a functional promoter, all of the neo-resistant cells should carry viral insertions downstream of an active promoter(Hicks, 1997). This construct contains an internal ampicilin resistant plasmid DNA fragment with bacterial replication origin, which facilitates the isolation of trapped flanking genomic DNA by a simple plasmid rescue procedure. Isolated DNA from the neomycin-resistant fibroblast cells infected by Neo-TP, was digested with EcoR1 restriction enzyme and transformed into bacteria after ligation. This procedure led to the isolation of seven clones carrying flanking cellular DNA with a typical retroviral integration signature sequence(Fig. 5).

These clones contained genomic DNA ranging from 1kb to 7kb and sequences of 300-600 bp were obtained from each of the rescued plasmids. Database searching showed that all of them share high homology to zebrafish sequences(Table 1).

Table 1. BLASTN search results of zebrafish DNA sequences identified by Neo-TP selection

Trapped clones	Species	Sequence ID/Scores
Trap 1	Zebrafish	AL590146/5e-42
Trap 11	Zebrafish	AL772279/2e-26
Trap 19	Zebrafish	AL590151/2e-69
Trap 31	Zebrafish	AL596027/6e-91
Trap 36	Zebrafish	AL732436/4e-18
Trap A3	Zebrafish	AL807389/5e-24
Trap A4	Zebrafish	AL590148/2e-38

Interestingly, none of the 7 sequences matched any previously known genes but three of them showed significant match to zebrafish ESTs. The relatively short DNA sequences we acquired do not allow us to determine how the structures of the genes have been disrupted. However, with the genome sequencing project actively going on and sequences being annotated, it will be easier in future for us to define the disrupted genes.

Clones	Genomic Seq.	LTR	Proviral Seq.
Trap 1	ATGTAACTAA	ACTTTCTGGGGTGG	ACATCC
Trap 11	GTGAACCATT	ACTTTCTGGGGTGG	ACATCC
Trap 19	GGAGAGTTTC	ACTTTCTGGGGTGG	ACATCC
Trap 31	CTAACTACNG	ACTTTCTGGGGTGG	ACATCC
Trap 36	ATATATTTCAG	ACTTTCTGGGGTGG	ACATCC
Trap A3	GATTTGTGAT	ACTTTCTGGGGTGG	ACATCC
Trap A4	ATAGAAACGG	ACTTTCTGGGGTGG	ACATCC

Fig. 5. Junction sequences of isolated proviral insertions by Neo-TP.
Sequences in red are retrovirus sequences and in black are cellular DNA sequences.

Table 2. Nuclear transplants generated using gene-traped cultured fibroblast cells

	No. of individuals			
Donor cells	Eggs operated	Blastula	Hatched	Adult
Neo-TP cells	1179	44(3.7)	8(0.8)	3(0.3)

* Numbers in parentheses represent the percentage of the total number of transplants.

Cloned fish were produced from these cells.

For fish cloning using tagged cells, initially, nucleus donors directly selected from a mixture of cells(Neo-TP cells) were used. A total of 44 embryos(3.7%) out of 1179 transplants were reached blastula stage; 8 of these embryos (0.8%) hatched and 3(0.3%) of them survived to adulthood. Overall, ~90% of operations did not yield any developing embryos and The overall success rate from Neo-TP cells was about 0.3%, probably because of poor egg quality(Table 2).

Three cloned fish(KCRun) were maintained and analyzed for the trapping events by the Southern blot after reaching adult stage(Fig. 6). The genomic DNA from each fish was digested with restriction enzyme Hind Ⅲ Ⅲ / EcoR I, separated by electrophoresis, and blotted on nylon membrane. A internal neo fragment of Neo-TP vector was used for probe and was labeled with ^{32}P. As a result, Neo-TP cells have two different hybridization bands, suggesting that there are two kinds of insertions in the cell

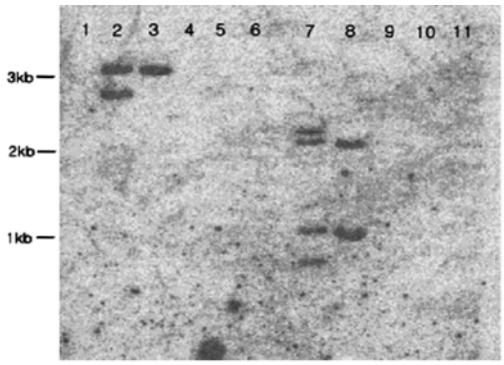

Fig. 6. Southern blot analysis of cloned zebrafish.
Genomic DNA was digested with Hind Ⅲ Ⅲ / EcoR I(7~11). Lane 1, negative control using wild-type zebrafish cell. Lane 2(about 3kb) and 7 (Neo-TP cell DNA, mixed). Lane 3 and 8, KCRun1. Lane 4 and 9, KCRun 2. Lane 5 and 10, KCRun 3. Lane 6 and 11, negative control using wild-type zebrafish.

mixtures. One(KCRun1) out of three cloned Zebra-fish(KCRun1-3) has an amplified fragment and

was labeled with ^{32}P, indicating that this carries Neo-TP gene in the genomic DNA and the cloning of Zebrafish from gene-trapped cells were succeeded.

The gene-trapping constructs have been successfully used in mouse ES cells to isolate over 100 genes(Hicks, 1997). This study in cultured cells also led to the isolation of zebrafish cellular DNA sequences flanking actively transcribed ESTs

A major limitation with using provirus is that insertions are probably non-random. This means that we cannot tag every single gene present in zebrafish using this approach. Another concern is that this approach only tags genes that are expressed in culture conditions. It is possible that genes expressed in the fibroblasts are less interesting in the context of developmental studies of specific organs or cell lineage. That is why this study was focused on cell cycle and proliferation genes that may be related to cancer models.

Gene-trapping has been shown to be a powerful approach for gene isolation and sequence identification in mouse ES cells, and a potential powerful approach for *in vivo* gene function study. In zebrafish, equivalence of the mouse ES cell is not yet available, but I find our cultured fibroblast cells are amenable to proviral insertion, DNA transfection, and can also be used to produce fertile zebrafish by animal cloning. The goal of this study, therefore, is to isolate and identify genes by gene-trap in our cultured fish cells, with the ultimate aim to tag all possible genes that are expressed in the culture conditions and to use the tagged cells to generate animals for *in vivo* gene function studies. In future, It should be able to trap genes in a special cell line such as pancreatic progenitor cells or beta cells. In this way, *in vitro* gene-trap would still be useful for tissue specific developmental studies. Also zebrafish will have all the genetic tools available to the mouse system, and fully realize its potential to study vertebrate gene function and to model human diseases.

Ⅳ ABSTRACT

This involves identifying and cloning trapped genes from cultured cells carrying the gene-trap constructs and generating cloned zebrafish using these cells for functional study. Gene-trapping studies in gene-trapped cells were carried out in initial and cloned zebrafish carrying gene-trap events were successfully produced based on the nuclear transplantation technique. Two kind of retroviral gene-trap constructs were adopted. The first one(SA/GFP-TP), constructed in my laboratory, carries a GFP reporter gene containing a splicing acceptor and an internal neo gene. The second one(Neo-TP), obtained from Dr. Hicks (Hicks et al., 1997), contains a promoter-less neo gene located in the LTR sequence of a retroviral vector. The infected cells were subjected to drug selection(neomycin treatment) because the two constructs carry the neomycin resistant gene. All those cells survived the neomycin treatment should carry the proviral insertions. For Neo-TP, Isolated DNA from the neomycin-resistant fibroblast cells infected by Neo-TP, was digested with EcoR1 restriction enzyme and transformed into bacteria after ligation. This procedure led to the isolation of seven clones carrying flanking cellular DNA with a typical retroviral integration signature sequence. These clones contained genomic DNA ranging from 1kb to 7kb and sequences of 300-600 bp were obtained from each of the rescued plasmids. Database searching showed that all of them share high homology to zebrafish sequences. For fish cloning using tagged cells, initially, nucleus donors directly selected from a mixture of cells(Neo-TP cells) were used. A total of 44 embryos(3.7%) out of 1179 transplants were reached blastula stage; 8 of

these embryos(0.8%) hatched and 3(0.3%) of them survived to adulthood. One out of three lived cloned zebrafish has an amplified fragment and was labeled with ^{32}P.

(**Key words** : Nuclear Transfer, Gene-trap, Mutagenesis, Zebrafish)

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