High efficiencies of gene delivery and gene targeting in embryonic stem cells of the fish medaka as a lower vertebrate model

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**High Efficiencies of Gene Delivery and Gene
Targeting in Embryonic Stem Cells of the Fish
Medaka as a Lower Vertebrate Model**

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Summary

Stem cell research is one of the most fascinating areas of both fundamental biology and medical applications. Embryonic stem (ES) cells have been established in mammals [1-4] including the human [5, 6]. Considerable efforts have been undertaken towards the generation of ES cells in other mammalian and non-mammalian species because of the great potential of ES cells and ES cell technology. A small laboratory fish, medaka (*Oryzias latipes*) is an important emerging model for developmental biologists because it has external and transparent embryology, many genetic resources, and established experimental tools [7]. In this model organism, stable ES cells capable of differentiation *in vitro* [8, 9] and chimera formation *in vivo* [10], haploid ES cells capable of whole animal production by semi-cloning [11], and male germ stem cells capable of test-tube sperm production *in vitro* [12] have been established and characterized. The pluripotency genes and differentiation markers have been cloned and identified in this species [11, 13-15]. Most recently, the female germ stem cells have been identified in medaka ovary, which raises the possibility of isolating them in culture [16]. Additionally, medaka is a unique model for studying genome duplication [7] and evolution [17].

The availability of ES cells and sequenced genome data [18] provoke me to establish an efficient gene delivery system and a precise genome manipulation approach in the medaka ES model. This research focuses on the establishment of a viral vector-based gene delivery system and a homologous recombination (HR)-mediated gene targeting (GT) platform in medaka ES (MES1) cells. A baculoviral (BV) vector system was explored to significantly improve gene delivery efficiency in medaka stem cells, while the pluripotency and host integration of BV-transduced MES1 cells were assessed

for ES cell-based gene therapy *in vitro* and *in vivo*. The HR-mediated GT, an essential ES technology, was attempted by using the *p53* gene as a model to establish the medaka as an important reference for evolutionary stem cell biology.

Viral vectors hold promise and challenges in gene therapy. The BV vector system was previously evaluated as an efficient and safe gene delivery tool in human ES [19] and mesenchymal stem cells [20]. The results of BV transduction in MES1 cells demonstrate that BV vectors can give rise to 100% of transient gene delivery. BV-transduced MES1 cells reproducibly (at approximately 10^{-5}) produce GFP-expressing colonies that, upon manual isolation, develop into stable clones during 300 days of culture. Surprisingly, BV transduction can also mediate efficient gene integration in the medaka genome, as fluorescent *in situ* hybridization revealed the presence of the BV-delivered *gfp* transgene in multiple locations in nuclei and on various chromosomes of metaphase spreads. To fully evaluate the potential of BV vectors in ES-mediated gene therapy, both *in vitro* and *in vivo* differentiation assays were conducted in stable BV-transduced MES1 cells. These assays showed that BV transduction does not compromise the genome stability and pluripotency of MES1 cells. I conclude that BV can efficiently mediate gene delivery and chromosomal integration in MES1 cells. Therefore, medaka provides a powerful system for analyzing the potential of BV-mediated gene delivery in stem cells and gene therapy.

The baculovirus-permissive cell types and infectious spectrum in other piscine models were assessed in medaka adult stem cells, non-stem cells, zebrafish cells, rainbow trout cells, and medaka developing embryos. The results show that BV vectors can mediate the high efficiency of transient and stable transgene expression in medaka germ

stem cell line SG3, liver stem cell line MLA3, and a zebrafish ES-like cell line Zf. Detectable BV-mediated transgene expression was observed in medaka differentiated liver cell line MLB4, rainbow trout gonad cell line RTG, and early developing medaka embryos, suggesting the usefulness of BV vectors for both *in vitro* and *in vivo* gene delivery in different fish models. Furthermore, a recombinant BV vector containing I-*SceI* meganuclease, modifiable drug selection, and fluorescence cassettes was constructed and tested in MES1 cells, aiming at rapid production of highly homogeneous transgenic ES cells for direct differentiation and generation of rare genetic events such as GT.

GT via HR can generate precise genetic alterations in vertebrate genomes and has routinely been used in mouse ES cells [21]. Transplantation of targeted ES cells into early mouse embryos leads to the production of whole animals through germline transmission. GT technology is widely employed in functional analyses of targeted genes in developmental processes and congenital diseases. It has remained unresolved whether GT can also be developed in long-term cultivated ES cells of lower vertebrates. In this project, I successfully established HR-mediated GT in medaka ES cells. The results show that a positive-negative selection (PNS) procedure resulted in 12-fold enrichment for homologous recombinants versus random integrants. Up to 48.7% of PNS-resistant colonies were positive by PCR-based genotyping, and 46.8% of PCR-positive colonies were homologous recombinants as revealed by Southern blotting, leading to a high efficiency of up to 22.8% for the targeted *p53* disruption in PNS-resistant colonies. Furthermore, the *p53* disruption and long-term propagation under drug selection did not compromise pluripotency, as *p53*-targeted cells retained stable growth, undifferentiated ES cell phenotype, and the potential for differentiation *in vitro* and *in vivo*. These results

demonstrate that precise genetic manipulation via HR can be reproducibly obtained in medaka ES cells at high efficiency, offering a lower vertebrate model organism for GT towards knock-out animal production.

This research reveals the great potential of utilizing medaka as a complementary model for human ES cell-based gene therapy and achieving functional genomics in lower vertebrates for evolutionary biology.

Chapter 1 Introduction

1.1 Stem cell biology

In the past 30 years, several fundamental discoveries have led to a revolutionary era of stem cell biology and regenerative medicine. They are: (1) the generation of mouse [1, 2] and human [5, 6] embryonic stem (ES) cells, and recently, authentic rat ES cells [3, 4], which can be maintained in the laboratory with unlimited cell proliferation (self-renewal) and retain developmental potential for all three primary germ layers (pluripotency); (2) the establishment of genetic disease models by gene targeting (GT) in mouse ES cells [22], which is now regarded as the “gold standard” for determining the gene function of mammalian genome [21]; (3) the birth of Dolly, a cloned sheep, by reprogramming somatic cells via nuclear transplantation (NT) into enucleated eggs [23], and recently, the birth of Holly, a semi-cloned (SC) fertile female medaka [11]; and (4) the creation of induced pluripotent stem cells (iPS) by forced expression of pluripotency transcriptional factors in differentiated cells [24], which gives hope for cell therapy using patient-specific iPS [25, 26].

1.2 Medaka – an ideal lower vertebrate model for stem cell biology

To better understand stem cell biology and explore its potential for therapeutic cloning, more animal models should be introduced as complementary models to mammals. Like the widely studied zebrafish (*Danio rerio*) model, medaka (*Oryzias latipes*) is an important model organism because of its unique biological features and sophisticated research tools. First, medaka is a small freshwater fish with a short generation time. Daily produced embryos in synchronous extra-uterine development are

convenient for monitoring early embryogenesis, *in vivo* bio-imaging studies [27], and micro-manipulation procedures such as micro-injection and cell transplantation [10, 28]. Second, the completed high-quality draft genome of medaka [18] presents a small genome (700 megabases), which is only one quarter of the human genome. Together with other accessible database [29], the genetic and molecular mechanisms of organogenesis and modeling of disease [30] could be elucidated in medaka by homology gene cloning [17, 31] and gene functional analysis [28]. Third, many established transgenic fish will facilitate studies of organogenesis, such as germline development [16, 28, 32]. Furthermore, a unique see-through vertebrate model has been generated in medaka, in which all internal organs can be visualized in living animals [33], allowing for real-time observation of gene expression and cell behaviors throughout embryonic and adult life. Fourth, diploid ES cells [9, 10], haploid ES cells [11, 15], and spermatogonial stem cells [12] have been established and characterized in this species. Further, the female germ stem cells have been identified in the medaka ovary [16]. The advantages of medaka biology and the available experimental resources described above make medaka an attractive complementary model system to mammals.

1.3 Motivations

1.3.1 Establishment of medakafish as a model for stem cell-based gene therapy

Viral vectors have been extensively investigated as a powerful gene delivery system in gene therapy [34-37]. The insect baculovirus *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV)-based vectors (BV) have recently gained considerable attention [35, 38-42]. Recombinant, replication-defective BV vectors can efficiently

transduce many different types of mammalian cells [19, 43] and thus provide versatility for gene delivery. In transduced mammalian cells, BV vectors usually exist and express transgenes in a limited period of several days and do not mediate chromosomal integration [35, 38]. This feature makes BV transduction a safe gene delivery system because of a low risk of genetic instability caused by random host integration [44]. Recently, it has been demonstrated that BV vectors also give rise to high gene delivery efficiency in human ES cells [19] and mesenchymal stem cells [20] without compromising genetic stability and pluripotency. Versatility and high-efficiency gene delivery lend the BV vector system an attractive option for ES cell-based gene therapy [19]. However, the prevalent usage of other virus-based vectors, such as lentiviral, retroviral and adenoviral vectors, have led to intensive concerns about their safety in clinical trials [36, 37, 45]. By analogy, BV vectors require extensive evaluation for gene delivery, transgene integration, maintenance of pluripotency, and biosafety towards full exploitation in gene therapy.

So far, the evaluation of BV vectors has been conducted mainly in mammalian systems. Although efficiency and safety have been tested in human ES cells by using the *in vitro* differentiation model, the *in vivo* assessment of pluripotency and safety is infeasible because of ethical concerns related to human ES cells and lack of easily accessible evaluation models. Therefore, different model systems are required to obtain a comprehensive evaluation of viral vectors in gene therapy. For this, I choose the medaka (*Oryzias latipes*) as a test organism to develop a complementary model system to fully evaluate the potential of BV vectors in ES-mediated gene therapy.

1.3.2 Exploration of baculovirus as an *in vitro* and *in vivo* gene transfer tool in fish

Using a virus as a gene transfer tool has advantages over conventional physical and chemical methods because of its capability to mediate either transient or stable transgene expression in host cells with relatively high efficiency. Stem and non-stem cell lines have been derived from early embryo [9, 46-48] and adult tissues [12, 15, 49, 50] in various fish models. Additionally, the primary culture of embryonic and germ cells is a useful approach for *in vitro* cell fate tracing [28, 51]. Furthermore, fish presents easily accessible *in vivo* embryogenesis and organogenesis models for bioimaging and biomedical studies [27, 33]. However, efficient gene transfer has not been achieved in a fish cell culture system to date [49, 52]. Consequently, it is worth exploring a viral vector-mediated *in vitro* and *in vivo* gene transfer approach that can routinely be conducted without using special instruments and reagents. It is also necessary to develop a modifiable BV vector with elements that enhance gene transfer efficiency and facilitate the rapid selection of transgenic cells.

1.3.3 Establishment of gene targeting via homologous recombination in medaka embryonic stem cells

Today, GT has become a routine tool in mouse developmental genetics to generate mice with precisely designed genetic alterations to analyze gene function in whole animals (namely knock-out mice). However, this technology has long been limited in mouse, in part due to the lack of stable ES cell lines from other laboratory animals. To develop GT technology in other vertebrates like fish, particularly zebrafish and medaka, two widely investigated models in developmental biology, much effort has been made to

derive ES cells from zebrafish [46, 53], medaka blastula-stage embryos [9, 10, 47], and other fish models [48]. In particular, medaka has well characterized both ES and adult stem (AS) cell lines [54]. The availability of medaka diploid ES cell line MES1 [9, 10] and haploid ES cell line HX1 [11, 15] allows me to develop a complementary model for GT in a lower vertebrate.

To explore the activity of HR and assess the pluripotency of ES cells after genetic manipulation in fish models, the putative HR events were observed in zebrafish short-term cultivated ES cell cultures [55], and the positive-negative selection (PNS) procedure for GT has been explored in piscine non-ES cells [56]. The pluripotency of medaka ES cells has proven to be well retained after gene transfer and long-term PNS procedure [52]. Genome information and PNS vector have been available [18, 52, 56, 57]. These achievements prompt me to establish GT technology in fish.

Homologous recombination (HR) is a rare event compared to random integration in mammalian cells [21]; thus, a highly efficient gene delivery approach is demanded. Although new approaches such as zinc finger nuclease (ZFN) [58] and transcription activator-like effector (TALE)-mediated genome targeting [59] can increase the efficiency of targeted mutations, those methods also introduce deletions and random integrations into the host genome. Therefore, the precise genome manipulation approach is still highly demanded for gene functional analysis. It is also theoretically and practically feasible to achieve knock-out animal production by a combination of nuclear transplantation (NT) and ES cell-based GT technology in the medaka model.

1.4 Objective of study

The main objective of this thesis project is to establish high efficiencies of gene delivery and gene targeting in a lower vertebrate medaka fish model by applying the BV vector system and disruption of a tumor suppressor *p53* gene via HR in medaka ES cells, respectively. More specifically, the objective of this study can be divided into the following three sections.

1.4.1 Evaluation of gene delivery efficiency and potential host integration mediated by BV vectors in medaka ES cell model

I aim to investigate the medakafish as a comprehensive system to evaluate the potentials and challenges of BV vectors in stem cell-based gene therapy. Viral vectors require extensive evaluation for gene delivery, transgene integration, and biosafety towards full exploitation in gene therapy. The experimental objectives include: (1) to develop a new model system for easy experimentation by utilizing the easily accessible *in vitro* stem cells and *in vivo* embryogenesis in medaka; (2) to develop an efficient gene delivery approach that can be used for the rapid production of highly homogenous transgenic medaka ES cells, such as directed differentiation into particular cell types and generation of rare genetic events; and (3) to evaluate the safety issues associated with viral vectors in gene therapy.

1.4.2 Exploration of BV vector-permissive *in vitro* and *in vivo* fish models

Regarding the need to explore an efficient approach for *in vitro* and *in vivo* gene delivery in fish models, BV vectors may be an attractive option due to advantages including highly efficient gene delivery in mammalian cells, large cloning capacity (up to

38 kb), ease of production, lack of toxicity, replication, and preexisting immunity [35]. The objective of testing the BV-mediated transgene expression includes (1) representative medaka adult stem and differentiated cell lines and primary cell cultures *in vitro*; (2) medaka developing embryos *in vivo*; and (3) zebrafish and rainbow trout models, i.e., an ES-like Zf and rainbow trout RTG cells. Another goal is to develop a recombinant BV vector that contains I-SceI meganucleases, drug selection, and fluorescence expression cassettes and preserves useful multiple cloning sites (MCS) for further easy vector modification and application in transgenesis.

1.4.3 Establishment of targeted *p53* gene disruption in medaka ES cells

The ES cells, targeting vector, effective selection, and screening approaches are prerequisites for GT technology. I have chosen the *p53* gene as a model to establish GT technology in medaka ES cells. *p53* is present in a wide variety of organisms and is best known for its conserved role as a tumor suppressor by safeguarding genetic integrity and controlling cell proliferation or survival [60]. The specific aims are as follows: (1) optimization of procedure for delivering targeting vector into medaka ES cells, PCR-based screening of HR events, effective positive-negative selection (PNS), and Southern blotting confirmation of HR events; (2) evaluation of enhancement factor of PNS strategy; (3) validation of HR events by means of Southern blotting, fluorescent *in situ* hybridization (FISH), and sequencing; (4) evaluation of relative and absolute GT efficiencies; and (5) *in vitro* and *in vivo* pluripotency analyses of *p53*-targeted ES cells, including alkaline phosphates (AP) staining; pluripotency gene expression profiling; spontaneous, induced and directed differentiation; and chimera formation.

1.5 Organization

This dissertation is organized as follows. Chapter 1 introduces the study and provides an overview of the motivation and objective of this project. Chapter 2 reviews viral vector-based gene therapy, the classic HR-mediated GT technology originally developed in mouse, and the related knock-off and knock-down technologies used for gene functional analysis. Chapter 3 describes the experimental approaches, materials, and instruments used in this study. Chapter 4 presents the high efficiency gene delivery and host integration of BV vectors in medaka ES cells and evaluation of genome stability and pluripotency of BV-transduced ES cells. Chapter 5 reports on the exploration of BV as *in vitro* and *in vivo* gene delivery tool in different fish models and construction of a recombinant BV vector for stable gene delivery. Chapter 6 presents the generation and identification of HR-mediated *p53* gene disruption in the medaka ES model and pluripotency analyses of targeted ES cells. Chapter 7 provides a general conclusion and recommends possible avenues for future directions.

Chapter 2 Literature review

2.1 Viral vectors for gene therapy

2.1.1 The construction of viral vectors - from pathogen to medicine

The concept of gene therapy has been set up on the basis of gene delivery and genetic manipulation designed to delay the progression of certain human diseases. Among chemical-, physical- and biological-based gene delivery approaches, virus has been extensively investigated as an efficient tool in gene therapy by harnessing the naive viral infectious feature but avoiding the pathogenetic viral gene expression that is toxic to host cells. However, the prevalent usage of viral vectors has not only led to encouraging progress in terms of gene transfer, but also to intensive questions about its safety in clinical trials [34]. The occurrence of leukemia-like syndrome in patients who have been treated with virus-based gene therapy has raised serious concerns regarding the potential severe pathogenic effects of viral vectors [45]. Therefore, viral vector-based gene therapy has a history of controversy [34, 36].

An understanding of the interaction between viruses and hosts, and genetic modification of viral vectors is vital for converting the disease-causing pathogen into useful vectors in medical applications. To this end, the intact sequence that is required for virus capsid package and host chromatin integration is retained, while the viral coding sequences are removed and replaced by the expression cassette of foreign genes. The deleted viral genes encoding proteins for replication or capsid/envelope proteins are compensated by introducing a helper packaging vector. The recombinant vectors are produced in the packaging cells that co-transfected with the vector genome and a

packaging construct (Figure 2.1). After purification and quantification (titration), high-titer viral vectors can be used for human gene therapy.

Figure 2.1 Construction of viral vectors for gene therapy. a. Schematic diagram of a generic viral vector. b. A packaging (helper) construct, containing viral genes derived from the parental virus that encode structural proteins that are required for vector replication, is introduced into a packaging cell line along with a construct that contains the vector genome. Pathogenicity functions and the sequences that are required for encapsidation are eliminated from the helper construct so that it cannot be packaged into a viral particle. The vector genome contains the transgenic expression cassette and is flanked by inverted terminal repeats (ITR) and *cis*-acting sequences that are required for genome encapsidation. Some vector genomes retain viral genes that are relatively inactive, as a result of the elimination of viral early genes that are required for their transcription. Viral structural proteins and proteins that are required for replication of the vector DNA are expressed from the packaging construct and the replicated vector

genomes are packaged into virus particles (Adapted with permission from [34]. Copyright 2003 Nature Publishing Group).

2.1.2 The clinically applicable viral vectors

The clinically applicable viral vectors are adeno-associated viruses (AAVs), adenoviruses, baculoviruses, herpes simplex-1 viruses (HSV-1), lentiviruses, and retroviruses (reviewed by Thomas et al and Hu [34, 35, 38]). The main characteristics of these vectors are summarized in Table 2.1. Upon the host integration of the viral genome or the predominantly persistence as extrachromosomal episomes, these viruses are distinguished as integrating and non-integrating vectors. They are utilized for genetic alteration in dividing cells and persistent transduction in non-dividing cells under drug selection, respectively. For example, the lentiviral vector has the ability to penetrate the nuclear membrane of non-proliferating cells. This feature makes the lentiviral vector a useful tool for haematopoietic stem cell (HSC)-mediated *ex vivo* gene therapy, because the cytokine stimulation for inducing HSC proliferation is not required [34]. Recently, lentiviral vectors have proven to be an efficient gene delivery tool for generating induced pluripotent stem cells (iPS) in mouse and human cell cultures [24-26].

Several factors are crucial for viral vector applications, such as tropism and immunogenicity of the vector, strength and period of transgene expression. The ability to clone foreign genes (exogenous DNA) is also an important criterion for the choice of vector in therapeutic applications. The advantages of different viral vectors have been combined to develop better hybrid vectors. In the work of Zeng et al, a hybrid BV vector constructed by incorporation of the *rep* gene and inverted terminal repeat (ITR) from

AAV was used for prolonged BV-mediated gene delivery in human ES cells, while the BV vectors were limited for transient transgene expression [19].

2.2 The hurdles and hazards of viral vectors

Viral vectors have faced challenges in clinical applications. First, the human immunological defense system may generate immune response against introduced vectors and their gene products. Patient death in 1999 after administration of adenovirus vectors raised intensive concerns regarding their biosafety [61]. Therefore, the degree of viral-vector-induced immune and inflammatory responses is a challenge in determining the safe dose of vectors. In addition, the immune response against viral vectors may have been sensitized by previous infection with wild-type virus [62]. Similarly, complement inactivation occurring in human serum was an obstacle for baculoviral vector-mediated *in vivo* gene therapy [35].

Second, the specificity of transgene delivery is vital for the effectiveness of viral-vector-based gene therapy. Targeted gene therapy was hampered by non-specific virus uptake in different organs through systemic administration or dissemination to adjacent organs through local delivery. To solve this problem, several strategies, such as transductional targeting, pseudotyping, and molecular adaptors, have been investigated for vector improvement [34]. Li et al reported tissue-specific gene delivery to neurons for effective gene therapy of neurodegenerative diseases [39]. With a recombinant BV vector driven by a neuron-specific promoter, transgene expression was detectable in target regions remote from injection sites. Similarly, the glial fibrillary acidic protein promoter applied in BV vectors led to restricted *in vivo* gene delivery to astrocytes [42].

Third, insertional mutagenesis mediated by viral vectors is the most serious issue concerning safety. For stable gene delivery, the integrating viral vectors, such as retroviruses, have been used in clinical trials. It is believed that the randomly integrated retroviral vector into host genome raises the risk of oncogenesis, which is caused by multiple genetic lesions. In summary, the viral vectors used for gene therapy should be able to deliver therapeutic transgene expression in a specific and regulable manner for the required duration.

Table 2.1. The features of viral vectors.

Vector	Genetic material	Packaging capacity	Tropism	Vector genome forms	Transgene expression duration	Inflammatory potential	safety	advantages	limitations
AAV (adeno-associated viral vector)	ssDNA	<5 kb	Broad	Episomal ^a	Long	Unknown	High	Non-inflammatory; non-pathogenic	Small packaging capacity
Adenovirus	dsDNA	8 kb (replication defective) 30 kb (helper dependent)	Broad	Episomal	Short	High	Inflammatory response, toxicity	Extremely efficient transduction of most tissues	Capsid mediates a potent inflammatory response
Baculovirus	dsDNA	>38 kb	Broad	Episomal	Short	Unknown	High ^b	Large packaging capacity; replication-defective; easy scale up	Transient transgene expression; Inactivation by serum complement
HSV-1 (herpes simplex virus-1)	dsDNA	40 kb (replication defective) 150 kb (amplicon)	Strong for neurons	Episomal	Short	High	Inflammatory response, toxicity	Large packaging capacity	Inflammatory; transient transgene expression
Lentivirus	RNA	8 kb	Broad	Integrated	Long	Low	Integration may induce oncogenesis	Persistent gene transfer in most tissues	Integration may induce oncogenesis
Retrovirus	RNA	8 kb	Dividing cells	Integrated	Short	low	Integration may induce oncogenesis	Persistent gene transfer in dividing cells	Only transduces dividing cells; Integration may induce oncogenesis

^a AAV can mediate site-specific integration into human chromosome 19, but common AAV vectors do not contain the *rep* gene and thus cannot mediate integration. However, random integration may occur.

^b Baculoviruses are considered safe, but more studies are required to confirm the safety in *in vivo* and *ex vivo* application.

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2.3 Baculoviral vector for gene therapy

Among the widely used viral vectors, the insect baculoviral (BV) vectors have recently gained considerable attention [35, 38-42, 63, 64]. Recombinant, replication-defective BV vectors can efficiently transduce many different types of mammalian cells [19, 38, 43] and thus provide versatility for gene delivery. In transduced mammalian cells, BV vectors usually exist and express transgenes in a limited period of several days and do not mediate chromosomal integration [35, 38]. This feature makes BV transduction a safe gene delivery system because of the low risk of genetic instability caused by random host integration [44]. BV vectors have been extensively studied for both *in vitro* and *in vivo* gene therapy [38] because they are evaluated as non-pathogenic to humans and their host range is restricted to insects and invertebrates [35].

2.3.1 Cell lines permissive to baculovirus transduction

The baculovirus transduction permissive cell lines are derived from (1) human, e.g., HEK293, HeLa, HepG2, Huh-7, bone marrow fibroblast, keratinocytes, pancreatic β cells, primary foreskin fibroblast, primary neural cells, primary hepatocytes, and recently, MSC derived from human umbilical cord blood and bone marrow [20, 63, 64] and human ES cell line HES-1 [5, 19]; (2) non-human primate cells, e.g., COS-7, CV1, and Vero; (3) porcine, e.g., CPK, FS-L3, and PK15; (4) bovine, e.g., BT and MDBK; (5) rodent, e.g., CHO, PC12, rat hepatic stellate, rat articular chondrocyte, and mouse pancreatic β cells; (6) rabbit; and (7) fish, CHH-1, EPC [49], and zebrafish embryos [65].

As the spectrum of BV-permissive cell types/lines expands, the potential applications of BV vectors are receiving increasing attention. These applications include

(1) *in vitro* and *in vivo* gene therapy; (2) cell-based assays; (3) gene functional studies; (4) virology; (5) recombinant protein production in insect and mammalian systems; (6) virus production; (7) surface display; and (8) vaccine candidates [35, 38, 66-68].

2.3.2 BV transduction parameters

From the reported studies, BV-mediated transduction and transgene expression vary considerably in relation to the following: (1) cell types; (2) promoters and enhancers; (3) drugs; (4) transduction protocols; and (5) new vector design. The recombinant BV vectors containing mammalian cell-active expression cassettes referred to as BacMan viruses have been shown to lead to a high efficiency (> 90%) of gene delivery in a wide range of mammalian cells that are of liver or kidney origin, non-hepatic, osteosarcoma, and primary cells [35, 38, 43] (Figure 2.2), and BV-mediated transgene expression has been shown to persist for several to up to 20 days in host cells. Thus, BV has been utilized as a versatile vector for gene delivery and recombinant protein production in mammalian cells [35].

Leisy et al reported a BV transduction test in fish cells for the first time [49]. Among the five tested cell lines, the highest gene delivery efficiency was observed in tilapia TO cells (20-30% with or without sodium butyrate), and 0.04-2% for carp EPC cells and 0.05% for salmon CHSE-214 and CHH-1 cell lines, analyzed by β -galactosidase staining assays; whereas rainbow trout RTG-2 cells are not susceptible to baculoviruses. This study indicated that transduction efficiency may vary significantly in different model systems, even with a similar vector design and transduction protocol.

Figure 2.2 BV-mediated high efficiency of transgene expression in mammalian cells.

Transduction efficiency of these cell types is routinely greater than 90% as measured by the number of fluorescent green cells. Cell lines, BHK, baby hamster kidney; HEK 293, human embryonic kidney; U-2 OS and Saos-2, human osteosarcoma cells (Adapted with permission from [35]. Copyright 2005, Nature Publishing Group).

Regarding the effects of promoter, in human ES cells, up to 80% of cells could be transduced effectively by using the EF1 α -WPRE promoter-driven BV vector in the transient transgene expression experiment, while 25% and 40% of gene delivery efficiencies were obtained from the CMV and EF1 α promoter-driven BV vectors, respectively, analyzed by the FACS (fluorescence activated cell sorting) approach [19] (Figure 2.3B). Moreover, the efficiency of transgene expression was reported to be promoter-dependent in the zebrafish model [65]. BV transduction efficiency can also be improved with the addition of sodium butyrate, trichostatin A [43], and valproic acid [69].

The selectable marker may also be used for stable BV transduction. In the work of Condreay et al, a neomycin positive selection gene was utilized in the BV vector and derived stable transduced cell lines at high frequency by adding the drug G418 [43].

Figure 2.3 BV-mediated transient transgene expression in human ES cells. (A) A time-course observation shows one hES cell colony 1, 2, 4, and 6 days after transduction with a recombinant baculovirus harboring the elongation factor-1 α promoter-driven eGFP gene at a multiplicity of infection (MOI) of 100 pfu. Phase-contrast (left), fluorescence (middle), and merged (right) images are shown. (B) Flow cytometric analysis of eGFP-positive hES cells 2 days after baculoviral transduction at an MOI of 100 pfu. The percentages of eGFP-positive cells are indicated. (C) An embryoid body (EB) transduced with the recombinant baculovirus with the CMV promoter at an MOI of 20 pfu. Abbreviations: CMV, cytomegalovirus; EF1 α , elongation factor-1 α ; eGFP, enhanced green fluorescent protein; hES, human embryonic stem; SSC, side scatter; WPRE, woodchuck hepatitis post-transcriptional regulatory element (Adapted with permission from [19]. Copyright 2007, John Wiley and Sons).

To improve gene delivery efficiency and prolong transgene expression in host cells, the protocols for virus production and transduction have been optimized. Dilution of virus inoculum with phosphate buffered saline (PBS) rather than mammalian cell growth medium (e.g., DMEM), and prolonged incubation of cells (1 to 4-8 h) with the virus at low temperature (25 or 27 °C rather than 37 °C) can lead to higher transduction efficiency in HeLa [35] and human MSC cells [38]. Using this protocol, the ultra-centrifugation step may also be eliminated during virus harvest and titration. Introducing virus at the mid-log phase of HeLa cells, followed by addition of virus for several days may prolong transgene expression up to 10 d [69]. Additionally, a repeat transduction protocol has been used for generating stable transduced human MSC [20]. Suspension culture at low temperature during BV transduction is also required in CHO [70] and human ES cells [19], probably by increasing the cell surface exposed to virus particles. With the development of a new vector design, such as the hybrid BV vector with a FLP/*frt* excision and recombination system, transgene expression has been remarkably prolonged to 48 d without selection and the repeat transduction protocol in human MSC has been eliminated [64].

2.3.3 Establishment of medaka as a model for stem cell-based gene therapy

The versatility and high efficiency of gene delivery make the BV vector system an attractive option for ES cell-based gene therapy [19]. So far, the evaluation of BV vectors has been conducted mainly in mammalian systems. Different model systems are required to obtain the comprehensive evaluation of viral vectors in gene delivery. Therefore, the

medaka (*Oryzias latipes*), a small laboratory fish, offers us an ideal organism for experimental analysis of fundamental issues associated with gene delivery in lower vertebrates. This fish has external and transparent embryology, many genetic resources, and experimental tools [7]. The medaka ES cell lines [9, 10, 71] have been derived and well characterized, and the procedures for analyzing pluripotency and differentiation *in vitro* and *in vivo* [8-10, 52] have been established. Also, protocols for gene transfer, drug selection, and genetic analyses of ES cells have been explored and optimized [52]. In addition, medaka offers a unique see-through vertebrate model in which all internal organs can easily be visualized in living animals [33], allowing for real-time observation of gene expression and cell behaviors throughout embryonic and adult life.

2.4 Gene targeting via homologous recombination

2.4.1 Homologous recombination

Gene targeting (GT) is a versatile approach for genetic manipulation that has been widely used in functional analysis of gene and generation of animals with site-specific mutation [72]. This technology was established based on the discovery of homologous recombination (HR) reaction in mammalian cells, where the enzymatic activity catalyzing the DNA fragment exchanges between exogenous DNA molecules and host genome. Meanwhile, the parameters that influenced the frequency of HR have been determined, such as (1) the HR reaction occurs preferably on linear DNA molecules; (2) the HR activity is cell-cycle dependent, which peaks at early S-phase; (3) the vector input and the targeting allele are not the crucial factors for HR. However, the cellular machinery acting on the exogenous and endogenous DNA fragments are prerequisite to

generate the HR reaction; and (4) theoretically any gene in mammalian cells could be precisely mutated or modified by GT, based on the equal probability of all chromosomal alleles reacted with HR machinery [21].

2.4.2 Positive negative selection

One challenge people faced with establishing GT is that the ratio of functional incorporation of foreign DNA into its chromosomal homologue by HR is extremely low, at approximately one per million. The ratio of correct integration by HR versus random integration is analyzed to be 1:1,000 in mouse ES cells [22]. This has been overcome by the Capecchi group. They developed an efficient drug selection procedure to eliminate the random integrated cells and thus enrich the correctly targeted cells [73]. This selection strategy has been widely applied and referenced as positive-negative selection (PNS) (Figure 2.4).

Figure 2.4 Disruption of the endogenous gene by homologous recombination. This figure shows a replacement type vector for positive-negative selection (PNS) in ES cells.

The coding exon “b” of a target gene is disrupted by a neomycin resistance marker gene (positive selector) after homologous recombination (HR) with the linearized targeting vector. HR event between the targeting vector and the cognate chromosomal gene results in the disruption of one genomic copy of the target gene and loss of the negative selector HSV thymidine kinase gene *tk*. PCR primers P1 and P2 are used to screen for the HR events after gene delivery of targeting vector and PNS-resistant ES clones.

2.4.3 Embryonic stem cells

The choice of cells for GT technology is crucial. The candidate should fulfill the following requirements: (1) efficient genetic screening from a great number of cells; (2) long-term *in vitro* culture that allows for genetic manipulation of cells and drug selection of targeted clones; (3) a euploid karyotype and genome information of interested gene; and most importantly, (4) germline transmissibility that provides a bridge linking *in vitro* and *in vivo* genetic manipulation of the genome. Mouse ES cells have emerged as an ideal model for GT after exploring the feasibility of (1) electroporation as a gene delivery means of introducing targeting vector DNA into millions of ES cells in a single experiment; (2) effective PNS strategy that uses neomycin (neo) as a positive selectable gene to select for cells that incorporate targeting vector either at the target site by HR or at random sites by non-HR, and that uses HSV-*tk* as a negative selectable gene to eliminate the cells that contain RI of targeting vector; and (3) retention of pluripotency of ES clones after GT, which allows for the generation of germline chimeras from targeted ES cells [21] (Figure 2.5). Furthermore, the screening of targeted ES cell clones can be

robustly conducted by polymerase chain reaction (PCR) [74] and finally identified by Southern blot and DNA sequencing analysis [72].

Figure 2.5 Production of targeted ES cells by positive-negative selection and generation of knock-out mice by germline chimeras. (A) The first step is isolation of a clonal ES cell line that contains the desired mutation by PNS. (B) The second step is to use these ES cells to generate chimeric mice that are able to transmit the mutant gene to their progeny. If one allele of the target gene is mutated in the diploid ES cells, 50% of the ES cell-derived offspring are expected to be heterozygous, carrying one targeted

allele (+/-). Interbreeding of heterozygous mice results in the birth of animals homozygous for the desired mutation (-/-), if the mutation is not embryonically lethal (Adapted with permission from [21]. Copyright 2005, Nature Publishing Group).

2.5 Targeting vector design

Regardless of the GT strategy that has been used, a number of important parameters should be considered to optimize the frequency of obtaining homologous recombinant ES cells. The DNA sequence composition of the targeting vector and the optimization of ES cell growth under PNS conditions are the two most important aspects.

2.5.1 Isogenic vs non-isogenic DNA

It has been shown that the isogenic DNA of the ES cells used in GT experiments generates a higher HR frequency than that of the non-isogenic one and the strain-specific sequence polymorphisms can interfere with HR in mammalian cells [75]. Therefore, constructing the HR targeting vector from the ES cell line used in GT experiment is preferred. The most commonly used mouse ES cells are derived from different substrains of 129 strains, and ES cell lines are also derived from other inbred strains, such as C57BL/6 and BALB/c [72]. The representative genomic DNA library from the 129 strains is also commercially available. It gives us an accessible genetic resource to study the functions of gene through generating knock-out mice.

2.5.2 Replacement type targeting vector

To date, the majority of null mutants have been generated by replacement type vectors (Figure 2.4). The nature of the protein, - for example, a multidomain protein encoded by the target locus - should initially be considered when designing the type and location of the introduced mutation. A replacement vector that usually generates a deletion of the protein domain to eliminate the activity of the protein, and an insertion vector that uses the “hit and run” approach will introduce point mutations to analyze the role of individual domains. As shown in Figure 2.4, the replacement vector is linearized either at the 5’ or 3’ end and the HR is taking place between the chromosomal and vector sequences (two homology arms). By this strategy, some or all of the coding exons can be deleted. However, a small deletion may also be sufficient to eliminate one or two exons that encode a critical domain required for protein function [76].

2.5.3 Homology length

It has been shown that the frequency of HR is correlated with the length of total homology to about 10 kb, and the minimum length of the short arm is about 0.5 kb [77]. To achieve efficient PCR-based genotyping, one of the homology arms (short arm) is usually designed within 3 kb in length. Ohbayashi et al analyzed the frequency of HR in mouse ES cells in the 96-well platform by using a series of viral vectors that contained homology arms of different lengths. Their results showed a clear increase of HR rates by using long homology arms up to 16 kb in size [78]. Additionally, absolute HR frequency is also related to the genomic locus because of the different chromatin structure and accessibility of the enzymatic machinery involved [72].

2.5.4 Selection markers

The widely used positive selector is a strong promoter-driven neomycin resistance gene that confers resistance to the drug G418. Other effective drugs (e.g., puromycin, hygromycin, and histidinol) are also used as positive selectors. The endogenous hypoxanthine phosphoribosyl transferase (*Hprt1*), which is located on the X chromosome, can also be utilized as a positive selector. Only a single *Hprt1* disruption yields to a null mutation of *Hprt1*^{-/-} in the male ES cells. The drug 6-thioguanline (6TG) kills cells that contain a functional *Hprt1* gene, thus selecting for the mutant clones [22]. Regarding the negative selectors, the thymidine kinase (*tk*) gene from HSV and diphtheria toxin A (DT-A) generally achieve an enrichment of 3-10 fold and have been long used in GT experiments [21, 72]. Conditional positive selector has also been applied by making use of the endogenously expressed genes in ES cells. Take the promoterless selector for example, the expression of selector marker depends on the correct targeting events that leading to transgene expression driven by the promoter of endogenous targeted gene.

2.5.5 Cre/*loxP* recombination/excision system

The Cre/*loxP* or FLP/*frt* recombination/excision systems are widely utilized strategies to introduce tissue-specific and inducible gene disruption. It allows separate evaluation of gene function in specifically spatio-temporal pattern [79]. This conditional targeting technique overcomes difficulties in lethal effects of gene inactivation during early development, interfered phenotype by selection gene expression and compensatory mechanism by homology gene activity in the absence of targeted gene product.

In this system, the Cre recombinase can recognize and bind to a unique *loxP* site (Figure 2.6A) and efficiently excise the sequence flanked by two *loxP* sites in the same orientation by intramolecular recombination, leading to excision of introduced exogenous DNA and ligation of the chromosomal sequences (Figure 2.6B). One obvious advantage of this system is that the selection marker-introduced interference with targeted and neighbor genes can be eliminated by deleting the selection marker between two *loxP* sites. Furthermore, when the GT technology is utilized for human gene therapy (gene correction), the selection marker cassettes should be removed after HR. Therefore, the Cre/*loxP* system is a useful tool for human gene therapy, where no foreign DNA will be introduced after genetic manipulation.

Alternatively, this can be used to generate targeted transgenesis by integrating the vector carrying one *loxP* site into the host genomic *loxP* site (Figure 2.6B, reverse reaction). The equilibrium was shifted to reverse reaction by introducing the mutant *loxP* sites. Other useful applications of the Cre/*loxP* system include (1) introduction of subtle mutation (Figure 2.7A); (2) inter- and intra- chromosomal recombination and rearrangement (e.g., translocation); and (3) conditional gene targeting (Figure 2.7B) to achieve tissue- or developmental stage-specific gene targeting [72].

Figure 2.6 The Cre/*loxP* recombination/excision system. (A) Structure and sequence of a *loxP* site. The binding site of Cre recombinase, termed *loxP* consists of two 13-bp inverted repeats flanking an 8-bp spacer region that determines the orientation. (B) Cre-mediated recombination reactions. Recombination between directly repeated *loxP* sites leads to excision and recircularization of the intervening gene segment, leaving one *loxP* site on each reaction product. Note that excision (large arrow) is much more efficient than reinsertion (small arrow). Recombination between *loxP* sites of opposite orientation leads to mutation and removal of selection gene segment (Adapted with permission from [72]. Copyright 1999, Elsevier).

Figure 2.7 Generation of subtle mutation and conditional knock-out strategy by Cre/loxP system. (A) Introduction of a point mutation and removal of selection markers by Cre mediated recombination. Firstly, a replacement type targeting vector is used to introduce a point mutation into exon 2 (white line and filled triangle). The DT-A gene is lost upon HR, but is retained in cells that have integrated the vector randomly and will kill the cells. Secondly, selection markers flanked by *loxP* sites are removed by Cre-mediated recombination in ES cells, leaving one *loxP* site in the genome. Clones that lost the *tk* gene can be enriched by their resistance to FIAU. (B) Firstly, three *loxP* sites are

introduced by HR into silent regions, e.g. introns of a target gene (triangle). Secondly, upon Cre-mediated recombination in ES cells and FIAU selection, two types of deletion are obtained. Type I deletion represents non-functional alleles, whereas type II deletion should show no phenotype in mice that solely harboring two *loxP* sites in silent regions. By crossing to Cre-expression transgenic mice, the double transgenic mice will undergo Cre-mediated recombination and display the phenotype of specific mutated gene (Adapted with permission from [72]. Copyright 1999, Elsevier).

2.6 Exploration of gene targeting in other models

GT technology was first established in mouse and has revolutionized developmental genetics in this organism. Thousands of mouse genes have been disrupted by GT [80, 81]. There has been enormous interest in establishing GT in other models where stable ES cells are available, including human ES cells [82], rat ES cells [83, 84], and fish non-stem cells [56]. Compared with the mouse model, other models are still in the early stages but are worth anticipating and developing.

2.7 Feasibility of establishing gene targeting in fish

To develop GT technology in other vertebrates like fish, particularly zebrafish and medaka, two popular models in developmental biology, much effort has been given to deriving the ES cells from zebrafish [46, 53] and medaka blastula-stage embryos [9, 47], as well as from other fishes [48]. In particular, medaka has both well-characterized ES and adult stem (AS) cell lines [54]. To explore the feasibility of HR and assess the pluripotency of ES cells after GT events in fish models, putative HR events have been

reported in zebrafish ES-like cells [55], and the PNS procedure for GT has been explored in piscine non-ES cells [56]. The pluripotency of medaka ES cells has been proven to be well-retained after gene transfer and long-term PNS procedure [52]. The germline chimera has been achieved in zebrafish, where short-term cultivated blastula cells are able to contribute to the germline of the recipient [85], whereas long-term ES culture is required to carry out GT [7].

2.8 TILLING – a reverse genetics approach

The conventional functional analysis of genes is based on phenotypic mutant screening, the so-called forward genetics. However, this phenotypic screening approach limits gene candidates in terms of the detection scale. If the genes are associated with lifetime or redundancy, forward screening may not be feasible. A representative of reverse genetics, Targeting Induced Local Lesions IN Genomes (TILLING) for functional genomics, has been used to analyze the gene *in vivo*. This technology is especially useful in organisms that can produce large numbers of offspring within a short lifespan, and it has been established in plants [86], rat [87], and medakafish [88]. Requirements of this technology are that: (1) the organism can be mutagenized and (2) the mutagenized progeny can be preserved. Thanks to the well-established protocols for sperm cryopreservation and easy *in vitro* fertilization, medaka is a suitable model for TILLING (Figure 2.8).

Figure 2.8 TILLING approach in medaka. Shown is a schematic illustration of medaka TILLING library and a flow chart of generating target-selected mutants. G0 males are mutagenized using a strong mutagen ENU and crossed with wild-type females. The detection of mutant is based on the techniques of direct sequencing, Cell1 and TGCE (temperature gradient capillary electrophoresis). F1 mutant fish that include a targeted mutation are retrieved by artificial insemination using cryopreserved sperm archive. Homozygotes can be obtained in F3 by crossing the F2 heterozygotes in a Mendelian manner (Adapted from [57]. Copyright 2009, Wiley-Blackwell).

The TILLING approach consists of four main steps: (1) mutagenesis to individuals or gametes; (2) the construction of mutant library consisting of two archives, one for the screening template and the other gamete archive for recovering mutants; (3) PCR-based detection of mutation; and (4) recovery of mutant progeny [57]. The alkylating agent, *N*-ethyl-*N*-nitrosourea (ENU), is used as the mutagen to introduce point mutations. If a point mutation occurs in an exon of the target gene, the nonsense, missense, or silent mutant can be obtained in this way. A 5,000 F1 stock of library scale is estimated to achieve a null mutant (nonsense) of every gene in theory. The obvious drawbacks of TILLING are considered to be that (1) small genes in size or genes carrying large introns have a lower probability of being mutated by mutagen and (2) the phenotype of specific mutants may receive interference from unknown/unsuspected mutation(s) from the same mutant.

2.9 Gene knock-down technology in fish

There are two main strategies for studying the gene's function. They are: (1) gain of function by addition (over-expression) and phenotypic rescue methods and (2) loss of function by knock-out (KO) via HR and knock-down (KD). The KO technique has not been achieved in fish; KD is currently used as a functional genomics approach that possesses the advantage of well-established microinjection technique, rapid external embryogenesis, and availability of large amounts of embryos (zygotes) in zebrafish and medaka. Synthetic antisense oligonucleotides, morpholinos, have been shown to knock down gene activity in early developing embryos efficiently [28, 89].

When being injected into fertilized embryos, morpholinos (MO, chemically modified 25 nucleotides antisense against initiation codon ATG or block mRNA splicing) can bind to their target mRNA with high affinity and efficiently block the translation. This can be used to eliminate the function of genes acting during early embryogenesis, such as the maternal supplied mRNA at the 1-cell stage in medaka, where the homozygous mutants are not easy to obtain. Furthermore, dose-dependent phenotypes induced by morpholinos (morphant) can be obtained by controlled dosage microinjection [28], which is useful to interpret the different roles of genes during early development. As medaka has 10 days of embryogenesis compared to 3 days in zebrafish, the toxicity induced by morpholinos, especially at relatively high concentrations and its effective duration (stability), are of concern. Therefore, a well-controlled experiment such as co-injection of a MOp53 is demanded [28].

Chapter 3 Materials and methods

3.1 Materials

3.1.1 Fish and reagents

Medaka fish are maintained in fresh water at 24-28 °C with a light/dark cycle of 14/10 h. Inbred albino strain i^3 [10] and ST-II [33] are used for chimera experiments. All the experimental procedures are followed the Guidelines on the Care and Use of Animals for Scientific Purposes of the National Advisory Committee for Laboratory Animal Research in Singapore.

Embryogenesis is staged according to [90]. Medaka embryos at mid-blastula stage are used as positive control of medaka ES cell lines.

Medaka stem cell medium ESM2 is used for primary culture of embryos and adult tissues, and also for derivation of colonies after baculovirus transduction or plasmid DNA transfection; Medium ESM4 is used for stable ES cell culture and positive-negative selection in gene targeting experiment.

Medaka diploid ES cell line MES1 [9, 10], spermatogenic stem cell line SG3 [12]), putative liver stem cell line MLA3 and differentiated liver cell line MLB4, zebrafish ES-like cell line Zf, and trout cell line RTG [12] are used in the baculoviral vector-mediated gene delivery and host integration study; MES1 is used in the gene targeting experiment.

Baculoviral vectors: bvCMVgfp, bvEF1 α gfp, bvEF1 α gfp-WPRE and bvSPR are created in the Wang laboratory [19]. *Spodoptera frugiperda* Sf9 insect cells are maintained for baculovirus production by using Sf-900 II Serum-Free Medium (SFM).

Plasmid vectors: pFastBac1 (Invitrogen), pHygEGFP (Clontech), pDsRed-N1 (Clontech), pGEM T-easy (Promega) and pCVpf, pCVpr, pCVmitf, pCVmitfCVpf, and pGTP53 (p53ntk) [91] are created in the Hong laboratory. The genetic maps of these vectors are shown in Figure 3.1.

DMEM (high glucose type), HEPES, gelatin (Type A, from porcine skin), trypsin-EDTA solution, nonessential amino acids solution, L-glutamine solution, sodium pyruvate solution, penicillin/streptomycin solution, 2-mercaptoethanol and dimethyl sulfoxide are purchased from Invitrogen or Sigma.

Fetal bovine serum (PAA laboratories GmbH). Note: The quality of FBS is vital to the stable ES growth. Each batch of FBS must be pre-tested for two months of stable medaka ES cell culture. Human basic fibroblast growth factor (PetroTech). Proteinase K (For dechorionization treatment in chimera experiment, I use PK from Sigma; For molecular cloning, I use PK from Invitrogen). Fish serum (Fish serum is mitogenic for medaka ES cells in culture. Fish blood is collected from seabass (*Lates calcarifer*) or rainbow trout (*Oncorhynchus mykiss*), and a final concentration of 0.2% of seabass or 1% of trout serum is used in ESM4 medium. Medaka embryo extract (MEE, self-made. A final concentration of 400 embryos/L is used for ESM4 medium).

Figure 3.1 Genetic maps of plasmid vectors used in this study.

Polyethylene glycol 20,000, methylene blue, colchicine, ampicillin, kanamycin, SDS, trisodium citrate, Tris-Cl, EDTA, X-gal/IPTG, diethylpyrocarbonate (DEPC), methanol, acetone, isopropanol, ethanol, chloroform, paraformaldehyde, glycerol, phenol solution (pH 8.0), all-*trans*-retinoic acid, Giemsa stain, RNase A, propidium iodide (PI) and glacial acetic acid are purchased from Sigma or Merck. DNase (Stratagene), BCIP/NPT substrate solution (PerkinElmer), formamide (Molecular biology grade, Calbiochem), Phenol: Chloroform: IAA (25:24:1, Ambion, cat no. AM9732) and agarose (1st base, molecular biology grade) are purchased from indicated companies.

Geneticin (G418, Invitrogen), Gancyclovir (Gc, Cymeven, Syntex Arzneimittel GmbH, Switzerland), GeneJuice reagent (Novagen), calf thymus DNA (GibcoBRL) or sonicated salmon sperm DNA (Stratagene). tRNA (transfer ribonucleic acid, from wheat germ, Sigma) are purchased from indicated companies.

Lysis buffers for extraction of genomic DNA and Lysis buffers for mini-prep of plasmid DNA (see reagent setup). MN gel recovery and PCR purification kit (NucleoSpin Extract II, Macherey-Nagel) and MN plasmid midi kit (Nucleobond Xtra midi plus, Macherey-Nagel) are self prepared or purchased from indicated companies.

Biotin-High prime labeling kit, DIG DNA labeling kit, DIG Easy Hyb granules, blocking reagent and secondary Antibody (anti-digoxigenin-POD fab fragments) are purchased from Roche. Avidin-FITC (egg white, Sigma), Hybond N+ membrane (Amersham pharmacia biotech), Pierce ECL Western blotting substrate (Thermo Scientific), Super Signal West Femto (Thermo Scientific), BioMax Light X-ray films (chemiluminescence, Kodak) and liquid nitrogen (Cryoexpress Singapore) are purchased from indicated companies.

E. Coli competent cells (TOP10 or DH5 α strain), DH10Bac competent cells (Invitrogen), MMLV reverse transcriptase (Invitrogen) and SMART reverse transcriptase (Clontech); restriction enzymes (Promega, New England BioLabs and Fermentas); T4 DNA ligase (Invitrogen), alkaline phosphatase, calf intestinal (CIP, New England BioLabs) and Ex Taq and HS Ex Taq polymerase (TaKaRa), expand long template PCR system (Roche), GeneRuler 1-kb DNA ladder (Fermentas) and Big-Dye Terminator cycle sequencing kit (Applied Biosystems) are purchased from indicated companies.

PCR primers are synthesized by Sigma-Aldrich (Singapore) or 1st base (Singapore) and the sequences are listed in Table 3.1.

Bioinformatics softwares: Vector NTI 10.0 version (Invitrogen), BioEdit 7.0 (Tom Hall) and DNAMAN 4.5 (Lynnon Biosoft) are used for sequencing analysis, primer design, vector construction, homolog gene cloning and phylogenetic analysis. Statistical analysis software: Origin 6.1 (OriginLab Corporation, Northampton, USA).

Table 3.1. Genes and primers used for genomic PCR and RT-PCR analyses.

Category	Gene (accession)	Primers (5'→3')	Size
Genotyping	CMVgfp	CMVF :TAGTTATTAATAGTAATCAATTACGGGT gfpR :TCGGCATGGACGAGCTGTACAAG	1.3 kb
	HR events of <i>p53</i> gene targeting	1st round PCR: p53Ua (upstream of exon 1): CGCCACCAATTGAGTAGCAGT NeoRa (near ATG coden of neomycin): TGTCTGTTGTGCCAGTCATAGC	1.4 kb
		2nd round PCR (nest PCR): p53Ub (38 bp downstream of p53Ua): TGATTGGACGACACAGGACGAAA NeoRb (25 bp upstream of NeoRa): CGGAGAACCTGCGTGCAATCC	1.4 kb
	random integration of p53ntk vector	p53LU (upstream of p53 left HR arm): ACAAAAGCTGGGTACCCTCGAGAG NeoR (3' end of neomycin): CAGAGGATCCGCCGATCCCT	2.1 kb
	Endogenous <i>p53</i> allele control Access no.: AF212997.1 [92]	p53F (exon 3): TTATCCTCCGTTGGAACTTTATCAC p53R (exon 7): GTACAGCTTATTTAAAGTTTCAGAGTA	1.8 kb
Probes for Southern blotting	internal	p53INTF : ATCCGAGTGGGTCTCTAAAGTA p53INTR : TTGATTTTGAAATCCGAAGTTCTG	1.5 kb
	integration of neomycin	NeoF : ATGGGATCGGCCATTGAACAAG NeoR : TCAGAAGAACTCGTCAAGAAGG	0.8 kb
	external	p53EXTF : GACATACTGTATTTCTGAAACCTC p53EXTR : GAGATCGGTTGTGGTGCGTTC	1.5 kb
p53	p53 (full length)	p53CDSF : ATGGATCCTGTACCCGACC	1053

	U57306.1 [93]	p53CDSR: ATTGCTCTTCAGCTTCTTCCCA	bp
	flanking the replaced region by p53ntk vector	p53RTFa: TGCCCGAGAGCCAAGGTTCT p53RTRa: GTGAGGATGGTGAGGATGGG T	700 bp
	Inducible transcription by UV and H ₂ O ₂	p53ex6F: CTGTGGAGCACCGAAGCCATC p53CDSR: ATTGCTCTTCAGCTTCTTCCCA	551 bp
Stemness	<i>oct4</i> , <i>nanog</i> , <i>klf4</i> , <i>myc</i> , <i>ronin</i> , <i>sal14</i> , <i>tcf3a</i> , <i>tcf3b</i> and <i>zfp281a</i>	Sequence and RT-PCR primers refer to [11]. Supporting Online Material.	
Germ layer	Ectoderm <i>nf200</i>		
	Mesoderm <i>ntl</i> , <i>actnin2</i>		
	Endoderm <i>sox17</i>		
	Neural crest (NC) <i>Sox10</i>		
House keeping	β -actin	AM1: TTCAACAGCCCTGCCATGTA AM2: CCTCCAATCCAGACAGAGTATT	650 bp
Universal primer	M13	M13F: GTAAAACGACGGCCAGT M13R: GGAAACAGCTATGACCATG	
cDNA	Oligo dT	dT₁₈: TTTTTTTTTTTTTTTTTT	

3.1.2 Reagents and solutions

3.1.2.1 Stem cell medium

ESM2 and ESM4 are prepared according to components listed in Table 3.2.

3.1.2.2 2X Cell freezing medium

The final concentrations of FBS and DMSO are 32.5% (vol/vol) and 10% (vol/vol), in frozen cells, respectively.

3.1.2.3 10X PBS

80 g of NaCl, 2 g of KCl, 14.4 g of Na₂HPO₄ and 2.4 g of KH₂PO₄. Autoclave and store at the separated area for ES cell culture.

3.1.2.4 Medaka embryo extract (MEE) and hatching enzyme (HE)

MEE and HE are prepared using a similar protocol. High quality of MEE and HE are vital components for the ES medium preparation and dechorionization treatment in chimera experiments. Rear the embryos in a 28 °C incubator for 7 days for MEE and 10 days for HE. Collect 5,000-10,000 embryos developed at d 7 or d 10 under stereomicroscope. Homogenize the embryos in a 50-mL Falcon tube on ice using a motorized homogenizer at speed of 8,000 rpm, with an interval of 10 s/10 s homogenization and stop. After three rounds of freezing and thawing cycling, centrifuge the homogenate at 2,800 g for 45 min at 4 °C. The homogenate will be separated into three layers, the upper lipid layer in white color, the middle raw protein containing layer in green and the solid debris pellet. Adjust the concentration of MEE to 400 embryos/mL with 1X PBS and sterilize it by a 0.22-μm filter. Store the aliquots at -20 °C and each batch of MEE is subjected to test with stable ES cell culture for at least 2 weeks before use.

Table 3.2. Media for medaka ES cell culture, drug selection and differentiation.

Component (per liter)	ESM2 for primary culture	ESM4 for stable ES culture	PNS-ESM4 for colony derivation	PNS-ESM4 for maintenance	Differentia- tion medium
DMEM (high glucose)	13.37 g (magnetic stirring for 30 min)				
HEPES	4.67 g (adjust pH value to 7.8 using 10 mol/L NaOH)				
Nonessential amino acids	1 mmol/L				
Sodium pyruvate	1 mmol/L				
L-Glutamine	2 mmol/L				
Penicillin-streptomycin	100 U/mL				
2-Mercaptoethanol	100 µmol/L				
Sodium selenite	2 nmol/L				
Fetal bovine serum	15%				
Fish serum	0.2% of seabass or 1% of trout serum, respectively				
human bFGF	10 ng/mL		2 ng/mL		0
Medaka embryo extract	1,000 embryos		400 embryos		0
Geneticin (G418)	0	0	500 µg/mL	200 µg/mL	0
Gancyclovir (Gc)	0	0	5 µmol/L	5 µmol/L	0
All- <i>trans</i> -retinoic acid (RA)			0		10 µmol/L

3.1.2.5 Balanced salt saline-PEG (BSS-PEG)

BSS-PEG solution is used for rearing the chimeric embryos which are lack of the chorion. Solution A (1 L): 11 g of NaCl, 0.8 g of KCl, 0.4 g of MgSO₄·7H₂O, 0.4 g of CaCl₂·2H₂O, 10 g of PEG 20,000 and 1 mg of phenol red. Before use, add 10 mL of

100X the pen-strep, 1 mL of 10 mg/mL gentamycin sulfate and 5 mL of 5% NaHCO₃ to the solution A, sterilize by a 0.22-μm filter and store at 4 °C. Good for up to 1 month.

3.1.2.6 Lysis buffers for genomic DNA extraction

Genomic DNA of stably BV-transduced MES1 cells and ES cells that undergone gene targeting and PNS are isolated by using the following lysis buffers and purified by phenol extraction. (1) TPS buffer: 25 mmol/L Tris-Cl (pH 7.5), 133 mmol/L NaCl (8 g/L), 0.2 g/L KCl; (2) Lysis buffer: 10 mmol/L Tris-Cl (pH 8.0), 100 mmol/L EDTA, 0.5% (vol/vol) SDS; (3) prior to DNA extraction, add the proteinase K to the lysis buffer at a concentration of 200 μg/mL. The lysis buffer is then added to the cells suspended in TPS buffer with a 1:1 (vol:vol) ratio; (4) Phenol solution (Sigma), equilibrated with Tris-Cl (pH 8.0) and Phenol:Chloroform:IAA (Ambion, pH 7.9).

3.1.2.7 10X TE buffer

A 10X stock solution: 100 mmol/L Tris-Cl (pH 8.0), 10 mmol/L EDTA (pH 8.0).

3.1.2.8 Extraction/Lysis buffers for mini preparation of plasmid DNA

Alkaline Lysis solution I: 50 mmol/L glucose, 25 mmol/L Tris-Cl (pH 8.0), 10 mmol/L EDTA (pH 8.0). Alkaline Lysis solution II: 0.2 mol/L NaOH (freshly diluted from a 10 mol/L stock), 1% (weight/vol) SDS. Alkaline Lysis solution III: 60 mL of 5 mol/L potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of water.

3.1.2.9 Sodium acetate/ethanol for DNA purification

Mix the 3 mol/L sodium acetate to 100% ethanol with a ratio of 1:9 (vol:vol).

3.1.2.10 Nucleic acid transfer buffer

The 20X SSC solution: 88.23 g of Trisodium citrate, 175.32 g of NaCl. Adjust pH to 7.0. Autoclave and store at room temperature. Good for up to 1 year.

3.1.2.11 Hybridization buffers for FISH

Prepare the buffers as follows: (1) 0.5 mol/L PBS (1L): dissolve 156.01 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 358.14 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in Milli-Q water, Adjust pH to 7.0, autoclave and store at room temperature; (2) 20% dextran sulfate (1 mL): 100 μL of 20X SSC (pH 7.0), 500 μL of deionized formamide, 100 μL of 0.5 mol/L PBS (pH 7.0), 200 mg of dextran sulfate, make up to a final volume to 1 mL with Milli-Q water. Store at -20°C ; (3) Denaturing buffer (freshly prepared): 70% of formamide, 10% of 0.5 mol/L PBS (pH 7.0), 10% of 20X SSC (pH 7.0) and 10% of water; (4) Buffer I (1 L): 0.1 mol/L of Tris-Cl (pH 7.5), 0.15 mol/L of NaCl and 0.3% of Tween-20. Autoclave and store at room temperature; (5) Buffer II (Blocking buffer): dissolve 1 g of Roche Block Reagent power in 100 mL of Buffer I at 60°C for 30 min. Aliquot and store at -20°C .

3.1.2.12 Fixatives of cultured cells

Freshly prepare the followings: (1) 3% PFA, make dilution from the 16% PFA stock; (2) methanol: acetone (1:1), used for Giemsa staining of the colonies after PNS; (3) methanol: glacial acetic acid (3:1), used for chromosomes preparation within 2 h at room temperature; and (4) ethanol, used for flow cytometry, a final concentration of 70% (1 mL) is achieved drop by drop adding to the cells suspended in 300 μL of PBS on ice.

3.1.2.13 Depurination buffer for Southern blot

Mix 11 mL of HCl with 989 mL of Milli-Q water. Store it at room temperature.

3.1.2.14 Denaturization buffer and transfer buffer for Southern blot

Weight 87.66 g of NaCl and 20 g of NaOH, add approximately 800 mL of Milli-Q water. Mix to dissolve. Make up to a final volume of 1 L. Store at room temperature.

3.1.2.15 Washing buffers for FISH, Southern blot and ECL chemiluminescence

Prepare the 2X SSC with 0.1% SDS (low stringency), 0.5X SSC with 0.1% SDS, 0.1X SSC with 0.1% SDS (high stringency), 1X maleic acid (150 mmol/L of maleic acid and 100 mmol/L of NaCl) with 0.1-0.3% Tween-20.

3.2 Equipments

Cell culture plates, dishes and bottles are purchased from BD Falcon (USA) or Nunc (Denmark). Bacterial grade Petri dishes, tubes and 0.22- μ m filters, 3.5-, 5-, 10-cm dishes from Greiner are used for embryoid body (EB) formation and preparation of LB agar plates for molecular cloning. Disposable filters (Corning Bio-one) are used for sterilization of solutions. Incubators for cell culture (Sanyo, model MR-162). Inverted microscope (Carl Zeiss, model Axiovert-200) and upright microscope (Carl Zeiss, model Axioskop 2 plus) equipped with fluorescence, phase contrast, DIC objectives and imaging system (Carl Zeiss) is used for cell culture and chimera experiment; Stereomicroscope (Leica model MZFIII) and digital camera (Nikon Model E4500) is used for dissecting fish tissues, handling medaka embryos, microinjection, ES cell transplantation and primary cell culture. Cell culture hood equipped with a fluorescence stereo microscope (Nuaier) is used for isolation stable BV-transduced ES cells and PNS-resistant ES cells.

UV crosslinker (Bio-Rad), water baths and heat block, Bbrosilicate glass capillaries, glass needle puller (Φ =1 mm; Clark Electromedical Instruments), and hybridization oven (42 °C with rolling glass tubes for Southern analysis), thermal cyclers (Eppendorf, MJ PT-100, Applied Biosystems 9700), DNA sequencer (Applied Biosystems, model A3100), film developer (Kodak), bio-imaging system (for electrophoresis. Synoptics,

Cambridge) are used for Southern blotting, PCR-based genotyping, sequencing, microinjection, and chimera experiments.

3.3 Methodology

3.3.1 Construcion of plasmid vectors

The GT plasmid pGTp53 targeting the medaka *p53* gene was described [91]. This plasmid contains two cassettes respectively expressing *neo* for resistance to neomycin or G418 and the Herpes simplex thymidine kinase (*tk*) for sensitivity to gancyclovir (Gc) to enrich for the GT event by positive-negative selection (PNS). pCVpf and pCVpr express from the cytomegalovirus early gene enhancer/promoter (CV) a fusion between *pac* (the puromycin acetyl transferase gene) and *gfp* (pf) or between *pac* and *rfp* (pr). The fusion genes express both puromycin resistance for drug selection and GFP or RFP for cell labeling. The plasmid vector DNA was prepared and purified by using the Qiagen Maxi- and Midi-preps kits (Qiagen, Germany).

3.3.2 Cell culture and transfection

The medaka ES cell line MES1 was maintained at 28°C under feeder-free culture conditions on gelatin-coated substrata in culture medium ESM4 as previously described [9, 71]. Cell transfection with plasmid DNA and drug selection were performed by using the GeneJuice reagent [12, 52, 94]. Briefly, cells were seeded at $1.5\text{--}2 \times 10^6$ per well of 6-well plates. After attachment, the cells were at about 70% confluence and incubated with 2 µg of plasmid DNA in a serum-free DMEM medium for 6 h. A transfection of linearized pCMVgfp vector was setup as a positive control for gene transfer.

After medium change to ESM4, the cells were allowed to grow for 2-3 days and the trypsinization was done, 1/4 of the cells were collected for PCR detection of HR events (see PCR-genotyping section), and the other 3/4 of transfected cells ($\sim 2 \times 10^6$) were subcultured into 10-cm gelatin-coated Petri dishes (plating) for PNS. The PNS procedure was used to enrich for the GT event following transfection with pGTp53. To this, G418 (Gibco) and Gc (Cymevan, Syntex Arzneimittel GmbH, Switzerland) were added alone or in combination to cell culture at final concentrations of 500 $\mu\text{g/mL}$ and 2 $\mu\text{mol/L}$, respectively. Established transgenic cells were maintained in the presence of respective drugs at 200 $\mu\text{g/mL}$ and 2 $\mu\text{mol/L}$, respectively.

3.3.3 Cell labeling

Cells were stably labeled by transfection with pCVpf or pCVpr followed by puromycin selection (1 $\mu\text{g/mL}$) and clonal expansion of GFP- or RFP-expressing colonies [52, 71]. Alternatively, cells in culture or suspension were transiently labeled fluorescently green and red by staining with PKH 26 (Sigma) and PKH67 (Sigma) for 3 min at room temperature followed by three washes with ESM4. The fluorescent staining is stable for 2 weeks in culture.

3.3.4 Cell colony isolation

After 2-3 days of culture in 6-well plates, cells transfected with pGTp53 were seeded at clonal density in 10-cm dishes ($1.0\text{-}1.5 \times 10^6$ cells/dish). Cells were grown in the presence of G418 and/or Gc for up to 28 days. PNS-resistant colonies were picked and plated onto gelatin-coated 96-well tissue culture plates. Briefly, 10-cm dishes with ≤ 200

colonies were washed twice with PBS and submerged in 12 mL of PBS. Under a stereo microscope equipped in a cell culture hood, colonies were sucked in 40 μ L of PBS by using a p200 Eppendorf tip and transferred to in a U-bottom 96-well plate, where 200 μ L of drug-containing ESM4 medium was pre-added per well, followed by pipetting up and down several times to disaggregate the cells for attachment culture, one colony per well. One batch of picking process transferred 48-96 colonies. The ES cells formed a monolayer during 10-14 days of culture, with regular medium change every 3-5 days. After 7-12 days of culture with regular medium change, these candidate colonies were split into three 96-well plates, two plates were further used for conducting direct-Southern (plate A) and PCR screening (plate B) simultaneously (see PCR-genotyping section), and the third one was used as “archive” for expansion the HR-positive colonies.

3.3.5 Genomic PCR

PCR-based genotyping was conducted both from pooled cell populations after gene transfer and PNS-resistant colonies. The first round of PCR was performed 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s in a 25- μ L setup. The second round of PCR was performed 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s by using 2 μ L of 10 times diluted 1st round PCR products as template (nest PCR). PCR primers are listed in Table 3.1. The PCR screening of PNS-resistant clones was performed using the DNA samples from micro-extraction from 96-well plate. The PCR products were separated on a 1.0% agarose gel and documented with a bio-imaging system (Synoptics, Cambridge).

3.3.6 RT-PCR

Total RNA was isolated by using the Trizol Reagent (Invitrogen). Synthesis of cDNA templates was primed with oligo (dT)₁₈ by using M-MLV transcriptase (Invitrogen). The cDNA reaction was diluted with DEPC-treated water to 10 ng/μL. RT-PCR was carried out to analyze the gene expression of pluripotency and differentiation of targeted ES cells.

3.3.7 Southern analysis

After transfer of the enzyme digested genomic DNA, the 5×10 cm nylon membranes were assembled in hybridization bags (Roche) and submerged in 5 mL of Dig-Easy solution (Roche) containing 100 ng/mL pre-boiled calf thymus DNA, pre-hybridized overnight at a 42 °C hybridization oven. The blots were then hybridized to a DIG-labeled probe by random-primed DNA synthesis (Roche) at 42 °C for 18 h. After hybridization, the membranes were transferred into plastic container (6×12 cm) and washed with 2X SSC 0.5% SDS solution (low stringency) in a 65 °C water bath for 10 min once, followed by twice wash with 0.1X SSC 0.5% SDS solution (high stringency) at 65 °C, for 10 min each with gentle agitation by hand. The membranes were washed with 2X SSC solution twice to remove the residual SDS, assembled in hybridization bags and submerged with 5 mL of 1X melatic acid 1.5% blocking reagent (Roche) for a 2 h pre-blocking at room temperature with gentle agitation. The membranes were incubated with A 1:5000 diluted anti-DIG-POD antibody (Roche), at room temperature for 2 h or 4 °C overnight. Finally, the hybridization signals were visualized by chemiluminescent reaction with ECL reagent (Amersham Biosciences) and film development (Kodak).

3.3.8 DNA Sequencing

Cloned DNA and PCR products were subcloned into pGEM T-easy vector (Promega) and reacted with Big-Dye reagent and M13 forward and reverse primers (Applied Biosystems). After sodium acetate/ethanol purification, the samples were sequenced by ABI3100 automatic sequencer (Applied Biosystems) and data interpretation was done by BioEdit and Vector NTi softwares (Invitrogen).

3.3.9 Chromosome preparation and Fluorescent in situ hybridization

Chromosome samples from the positive clones screened by genomic PCR and Southern blot were prepared essentially as described [9, 11]. To determine transgene *neomycin* integration into the MES1 genome, one *p53*-targeted clone was subjected to FISH detection. The DNA fragment containing the ST promoter and *neomycin* was obtained from pGTp53 vector and labeled with biotinylated dUTP by using the Biotin-High Prime kit (Roche, Mannheim) as the probe for FISH on nuclei and metaphases. Chromosomes were counterstained with propidium iodide (Sigma, USA).

3.3.10 Induced cell differentiation

MES1 cells and *p53*-targeted cell clones were subjected to embryoid body (EB) formation in suspension culture in the presence of all-*trans* retinoic acid (RA; 10 $\mu\text{mol/L}$ final concentration) for induced differentiation [9, 15]. Cell aggregation and cell differentiation were monitored by phenotypes and RT-PCR analyses of expression of pluripotency and lineage-specific genes [11].

3.3.11 Chimera formation

Labeled MES1 cells and *p53*-targeted cell clones were transplanted alone or together into dechorionated STII blastula embryos and donor cells were monitored during chimeric embryogenesis [10, 95].

3.3.12 Microscopy

Observation and photography was done on Leica MZFIII stereo microscope, Zeiss Axiovertinvert and Axiovert upright microscopes with a Zeiss AxioCam M5Rc digital camera (Zeiss Corp).

3.3.13 Statistics

Statistical analyses were calculated by using Origin 6.1. Data consolidated were presented as mean $\pm$ SD and p values were calculated by using non-parametric student's t-test.

Chapter 4 Baculoviral vector-mediated high efficiency of gene delivery and integration in medaka ES cells^{*}

4.1 Introduction

Viral vectors hold promise and challenges in gene therapy [34-37]. The recombinant, replication-defective insect baculovirus *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV)-based vectors (BV) can efficiently transduce many different types of mammalian cells [19, 43] and thus provide versatility for gene delivery. In transduced mammalian cells, BV vectors usually exist and express transgenes in a limited time period of several days, and do not mediate chromosomal integration [35, 38]. This feature makes BV transduction a safe gene delivery system because of a low risk of genetic instability caused by random host integration [44]. Recently, it has been demonstrated that BV vectors also give rise to high gene delivery efficiency in human embryonic stem (ES) cells [19] and mesenchymal stem cells [20], without compromising the genetic stability and pluripotency. The versatility and high efficiency of gene delivery lend the BV vector system an attractive option for ES cell-based gene therapy [19]. However, the prevalent usage of other virus-based vectors, such as lentiviral, retroviral and adenoviral vectors, have led to intensive concerns about their safety in clinical trials [36, 37, 45]. By analogy, BV vectors require extensive evaluation for gene delivery, transgene integration, maintenance of pluripotency and biosafety towards full exploitation in gene therapy.

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So far, the evaluation of BV vectors has been conducted mainly in mammalian systems. Different model systems are required to obtain comprehensive evaluation of viral vectors in gene delivery. I am interested in developing a complementary model system to fully evaluate the potential of BV vectors in ES-mediated gene therapy. For this, I choose the medaka (*Oryzias latipes*), a small laboratory fish, as a test organism for the experimental analysis of the fundamental issues associated with gene delivery in lower vertebrates. In this model, one of the medaka ES lines, MES1, has proven to retain the ability to form chimeras where MES1 cells contribute widely to many cell lineages of the three germ layers [10, 96].

4.2 Experimental approaches

4.2.1 Plasmid and BV vectors

BV vectors and viruses were prepared by using a commercialized Bac-to-Bac protocol as described previously [19]. Briefly, virus particles were harvested from the *Sf9* insect cells, concentrated and titrated. The virus infectious ratio in transduction was calculated as multiplicity of infection ($\text{MOI} = (\text{titer of viral inoculum (pfu}/\mu\text{L}) \times \text{inoculum volume of virus } (\mu\text{L}))/\text{number of cells in transduction}$). Plasmids pCMVgfp and pDsRed-N1 (Clontech, USA) were used as positive controls and described previously [10, 12, 52]. Three kinds of recombinant baculoviruses are applied in this study. The reporter gene encoding EGFP (enhanced green fluorescent protein) is driven by the CMV, EF1 α and EF1 α -WPRE promoters and they are named as bvCMVgfp, bvEFgfp and bvEFgfpW respectively (Figure 4.1).

Figure 4.1 Schematic illustration of recombinant baculoviral vectors and plasmid.

Shown are the human cytomegalovirus early gene enhancer/promoter (CMV), elongation factor-1α promoter (EF1α), polyhedron promoter (pPolh), wood-chuck hepatitis post-transcriptional regulatory element (WPRE) and genes for green (gfp) and red (rfp) fluorescent protein. Plasmid backbone is shown in thin line. Arrows depict the extension directions of primers used for PCR-genotyping (see Table 3.1).

4.2.2 Cell culture and gene transfer

MES1 is a medaka ES cell line derived from blastula embryos of wild-type strain HB32C [9]. For baculovirus transduction, 2×10^5 MES1 cells were seeded in 24-well plate at 50% confluence and the baculovirus was suspended in pure medium DMEM, without serum, and then added baculovirus into cell at varying MOIs. After incubation at 28°C for 8 h, the complete medium ESM4 was replaced to express the transgene delivered by BV vectors.

After transduction of 2×10^5 MES1 cells with bvCMVgfp vector on 24-well plate, almost all the cells showed GFP expression and then transient transduced cells were subcultured into 10-cm petri dishes with gradient cell densities (plating). The stable BV-

transduced MES1 cells were monitored under fluorescent microscope and the medium was refreshed every week. At day 20, the uniform GFP-expressing ES colonies were picked up into 96-well plate, under a stereo fluorescent microscope equipped in the cell culture hood. After 3 rounds of clonal growth, 5 clones were obtained, namely, MC1 to 4 and MC11. The positive and negative control experiments were setup to make a comparison of gene transfer efficiency between conventional chemical reagent-based and baculovirus-based approaches. Transfection of MES1 cells with plasmid vectors were described according to [52]. Briefly, pCMVgfp was used for transfection using the GeneJuice reagent (Novagen, Darmstadt), and pDsRed-N1 (Clontech, USA) was used to produce transgenic MES1 cells expressing RFP.

Cell cultures and embryos were visualized either under a Zeiss Axiovert invert microscope by using a Zeiss AxioCam MRc digital camera or a Leica MZFIII stereo microscope and photographed using a Nikon E4500 digital camera (Nikon Corp, Japan). The statistical analysis and plotting were conducted by Original 6.1 software (OriginLab Corporation, Northampton, USA).

4.2.3 Colony formation, competitive growth and pluripotency gene expressoin

For clonal growth, cells were seeded at 10^4 cells/10-cm dish in ESM4 medium and the growth was monitored daily. Fully grown cell colonies at 20 days of culture were picked into gelatin-coated 96-well plates and expanded from 48-, 24-, 12- and 6-well plates to 10-cm dishes essentially as described [9].

A 2:1 mixture of stable BV-transduced MES1 and parental MES1 cells labeled by pDsRed-N1 (Clontech, USA) was seeded at a total of 2×10^5 cells/well in 12-well plates for co-culture under undifferentiated growth in ESM4 and daily counted following trypsinization.

Total RNA was isolated with the TRIzol reagent (Invitrogen, USA) from exponentially growing BV-integrated MES1, normal MES1 cells and mid-blastula stage embryos which MES1 cells derived from as positive control. First-strand cDNA synthesis was obtained by using M-MLV reverse transcriptase (Promega, Madison) for 2 h at 42°C and oligo-(dT)₁₈ as a primer. RT-PCR was run from 10 ng of cDNA in 25 µL reaction as described [12] for *oct4* and *nanog* (35 cycles) or *actin* (30 cycles). The PCR products were separated on a 1.5% agarose gel and documented with a bioimaging system (Synoptics, Cambridge).

4.2.4 PCR-genotyping, karyotype analysis and fluorescent *in situ* hybridization (FISH)

Genomic DNA of stably BV-transduced MES1 cells was isolated by using the lysis buffer and purified by phenol: chloroform extraction. PCR-based genotyping was done by using two primers flanking the 1.3 kb CMVgfp fragment from bvCMVgfp vector (see Figure 4.1). Chromosomes from cell cultures were prepared essentially as described [9].

To determine transgene integration into the MES1 genome, the BV transduced clone MC3 was subjected to FISH as described [97]. The DNA fragment containing the CMV promoter and gfp was obtained from pCMVgfp and labeled with biotinylated dUTP by

using the Biotin-High Prime kit (Roche, Mannheim) as the probe for FISH on nuclei and metaphases. Chromosomes were counterstained with propidium iodide (Sigma, USA).

4.2.5 Cell Differentiation *in vitro* and *in vivo*

Spontaneous and induced differentiation was done as described [9]. Directed differentiation was performed by transfection with pCMVmitf as described [8]. Cell transplantation into dechorionated mid-blastula recipients was performed essentially as described [10, 96]. Briefly, BV-transduced GFP-positive MES1 clones were microinjected alone or together with RFP-expressing MES1 cells (derived by transfection with pDsRed-N1) among the deep cells of blastula host embryos of the see-through medaka [33]. The transplanted embryos were reared at 25°C in BSS (balanced salt solution)-1% polyethylene glycol (PEG) until hatching as described [71].

4.3 BV-mediated efficient gene delivery and chromosomal integration in MES1 cells

4.3.1 BV vectors mediated efficient gene delivery in medaka ES cells

In the three BV vectors previously tested in human ES cells, the reporter gene *egfp* is driven by promoters of human cytomegalovirus early gene enhancer/promoter (CMV), human elongation factor 1 α (EF1 α) and EF1 α plus wood-chuck hepatitis post-transcriptional regulatory element (WPRE), respectively (Figure 4.1). Upon infection with these vectors in multiwell plates (24-, 12-, 6-well), the medaka ES cells (MES1) commenced transgene GFP expression as early as 8 h post transduction (data not shown).

Cell quantification led to the evaluation of gene delivery by different vectors. Using a BV vector incorporating the CMV promoter at a series multiplicity of infections (MOIs) of 2, 5, 10, 25, 50, and 100, the transduction frequency was positively correlated with the MOIs (Figure 4.2). Specifically, these BV vectors at MOI as low as 5 already gave rise to a 20.8-35.5% gene transfer efficacy. BV vectors at MOI 100 led to almost 100% GFP-expressing transgenic cells (Figure 4.2 and Figure 4.3C), compared to 7.1% for a plasmid-mediated gene delivery (Figure 4.4A) by using pCMVgfp that expresses *gfp* also from the CMV promoter [10, 52].

Similar high efficiencies were obtained also from bvEFgfp and bvEFgfpW vectors (Figure 4.2 and Figure 4.4B and C). Regular microscopic observations revealed that the BV-delivered GFP expression peaked at day 3 and weakened gradually during serial subculture until 45 days (Figure 4.3D-F and Figure 4.4D). These results suggest that medaka ES cells provide an extremely high efficiency for BV-mediated gene delivery.

Figure 4.2 Dose-dependent transduction efficiency of BV vectors. MES1 cells were incubated with individual BV vectors at indicated MOI and the gene delivery efficiency was determined by GFP expression by counting at least 1,000 cells at day 3 post transduction in triplicates. Values are means $\pm$ SD.

Figure 4.3 BV vectors-mediated high efficiency of gene delivery and expression. (A-C) Micrographs showing gene delivery and transient expression at day 3 by using bvCMVgfp at representative doses indicated. (D-F) Micrographs showing persistence of transgene expression at various days in MES1 cells after BV transduction with bvCMVgfp at MOI 100. Scale bars, 200 μ m.

Figure 4.4 Gene delivery by plasmid and BV vectors. Cells were subjected to plasmid transfection according to the Gene Juice procedure [52] or to BV transduction as described in the Materials and methods chapter. Photographs were taken at day 3 after gene delivery and merged to show the relative efficiency of transient expression (green). (A) Transfection by pCMVgfp. (B) Transduction by bvEFgfp. (C) Transduction by bvEFgfpW. Scale bars, 200 μ m. (D) Time-lapse observation of persistence and expression of BV-delivered transgene *gfp*.

4.3.2 Persistence and expression of BV-delivered transgene

Compared to an up to 3 days of BV-mediated GFP expression in human ES cells [19], the BV-mediated duration of transgene expression was much longer in medaka ES

cells. By the observation of BV-delivered transgene *gfp* persistence and expression, 6-10% proportion of GFP signal was maintained in the initially BV-transduced MES1 cells up to 30 days during subculture (Figure 4.3E and Figure 4.4D), before the GFP signal steeply disappeared in the overwhelming majority of transiently transduced MES1 cells. BV-transduced MES1 cells did not show observable cytotoxicity and compromised growth, as they exhibited continuous proliferation that is fully comparable to the parental MES1 control cells (data not shown). Upon subculture at a low density for clonal growth [10], the BV-transduced MES1 cells showed active proliferation (Figure 4.5A-D) and the colony consists of pure GFP-expressing cells (Figure 4.5E). Upon isolation and expansion, these colonies gave rise to individual cell clones capable of stable growth, continuous GFP expression and ES cell phenotype (Figure 4.6A and B). To see that BV-transduction indeed did not compromise the cell growth, clonal cells from BV-transduction were mixed with RFP-labeled MES1 cells from plasmid transfection and seeded together for competitive growth. I reasoned that the ratio at seeding would not change if two cell populations have no difference in proliferation and survival. Regular observation and quantification revealed that the initial ratio remained during various days of co-culture (Figure 4.6C-E). Moreover, the clones all retained expression of pluripotency genes *oct4* and *nanog* (Figure 4.7). Taken together, BV-vectors in medaka ES cells are able to persist for an extended period of up to several weeks to support continuous expression of the delivered transgene. This high efficiency in gene delivery and prolonged persistence make BV-transduction a powerful system in medaka for the rapid production of homogeneous transgenic ES cells.

Figure 4.5 Clonal growth and ES phenotype of BV-transduced MES1 cells. (A-E) Colony formation. Following transduction with bvCMVgfp, cells at day 4 were trypsinised and seeded into Petri dishes and monitored for growth. (A and B) Four cells. (C and D) Active divisions in a forming colony. Note that GFP-positive and negative

cells have the same phenotype characteristic of undifferentiated ES cells (E) Colonies at day 14 of clonal growth showing a colony consisting of pure GFP-expressing cells. This colony (green) has a smooth contour, and undifferentiated ES cell morphology.

Figure 4.6 Competitive growth of stable BV-transduced and parental MES1 cells. (A and B) Cell clone MC2 derived by expansion from the colony shown in (Figure 4.4E) during long-term culture (300 days). Shown are micrographs of phase-contrast (Figure 4.4A, C, F) and fluorescent optics (Figure 4.4B, D, E). (C-E) Competitive growth assay in co-culture. Clonal cells from BV transduction (green) and parental MES1 cells after pDsRed-N1 transfection (red) were mixed at a 2:1 ratio, seeded in 12-well plates, observed and counted daily. (C) Cell mixture. (D) Co-culture at day 1. (E) Relative cell numbers. Values are means $\pm$ SD from three determinations.

Figure 4.7 Pluripotency gene expression analysis of stable BV-transduced MES1 cells. RT-PCR analysis of pluripotency gene *oct4* and *nanog*. The normal MES1 cell and early developing embryo (mid-blastula stage, MB) were included for comparison.

4.3.3 High efficiency of multiple chromosomal integrations

Stable GFP expression in ES cell clones provoked me to hypothesize the stable chromosomal integration of BV vector-derived transgene. To address this, I performed PCR-based genotyping to detect the presence of BV-derived *gfp* transgene. This revealed that all of the five clones examined indeed had the transgene (Figure 4.8A). The presence and expression of BV-mediated transgene did not compromise the genetic stability, the ES cell characteristics and cell growth, as all these clones exhibited a normal karyotype of 48 chromosomes (Figure 4.8B) and a normal modal chromosome number (Figure 4.8C).

Persistence of the transgene and its continuous expression points to the stability of BV-transduced transgene in these ES clones. To determine whether the transgene was extrachromosomal or has integrated into the genome, I performed fluorescent *in situ* hybridization (FISH) on one of the five clones by using the *gfp* probe. Detailed observations on 4 metaphases revealed an average of 10 signals per metaphase. A representative metaphase having 10 signals distributing on different chromosomes is

shown in Figure 4.9A. I also found up to 9 individual signals in interface nuclei (Figure 4.9B). A closer inspection revealed the hybridization signal widely scattered in various nuclei (Figure 4.9B), making it possible to precisely count the numbers of signals per nucleus. I found that the number of signal varied and was dependent largely on the nuclear size. Small nuclei (n = 30) with an average diameter of 5.5 μm had 10.03 ± 3.73 signals, normal nuclei (n=40) with an average diameter of 7.5 μm had 13.55 ± 4.58 signals, whereas large nuclei (n = 14) with a diameter of 10 μm had 24.50 ± 3.88 signals (Figure 4.9C). Apparently, this variation in the number of FISH signal correlates well with cell cycle-associated difference in chromatin content. Therefore, BV-transduction can mediate a high efficacy of transgene integration into various chromosomes, and does not compromise the genome stability in medaka ES cells.

Figure 4.8 PCR-genotyping and karyotype analysis of BV-transduced MES1 cells.

(A) PCR analysis of BV-transduced gene. (B) Modal number of 48 chromosomes. (C)

Normal karyotype of 48 chromosomes of stable BV-transduced MES1 cells.

Figure 4.9 BV-mediated host chromosomal integration. (A-C) FISH analyses of chromosomal integration. The FISH signal is green, while whole chromosomes are red by nuclear dye propidium iodide (PI). (A) Metaphase showing ten signals on different chromosomes. Note the signal is seen scattered. (B) FISH signals in nuclei of different sizes reflecting different cell cycle phases and thus the chromatin content. (C) The FISH signals are positively correlated with nuclear size as shown in (B).

4.3.4 Retention of stable growth and pluripotency *in vitro*

The experiments described so far demonstrated the BV-transduced MES1 cell clones with multiple stable chromosomal transgene integrations retained ES cell phenotype and stable growth as plasmid labeled MES1 cells that have proven to maintain the pluripotency after long term culture and genetic engineering [52, 98]. I wanted to determine whether medaka ES cells after BV transduction also maintained this ES property. I failed to detect any difference between BV-transduced ES cell clones and the parental cell line in gene expression pattern for *oct4* [99-101], and *nanog* [102, 103](Figure 4.7), a component of the core regulatory circuit for mammalian ES cells.

Upon a clonal co-culture system of parental MES1, plasmid RFP-labeled MES1 and stable BV-transduced MES1 cells (Figure 4.10A), both of the parental MES1 (data not shown) and RFP labeled-MES1 cells were capable of spontaneous differentiation into various cell types (Figure 4.10B and C). Meanwhile the stable BV-transduced clones showed the same developmental potential *in vitro* (Figure 4.10D and E). Furthermore, by employing a well-established approach for directed MES1 differentiation [8], the stable BV-transduced MES1 cells exhibited a similar ability to their parental MES1 cells in giving rise to fully differentiated melanocytes (Figure 4.10F-H), 4 days after transfection with a vector expressing the microphthalmia transcription factor (Mitf). These results demonstrate that BV transduction does not compromise the pluripotency of medaka ES cells *in vitro*.

Figure 4.10 Differentiation of BV-transduced MES1 cells *in vitro*. (A) Undifferentiated proliferation in clonal co-culture. Parental MES1 (no fluorescence), pDsRed-transfected MES1 (pDsRed-MES1; red) and bvCMVgfp-transduced MES1 (bvCMVgfp-MES1; green) of clone MC11 were seeded at a low density and photographed at day 4 of co-culture. Similar growth by active divisions is seen for the parental and gene-delivered MES1 cells. (B-E) Spontaneous differentiation in co-culture.

(B and C) Differentiation of pDsRed-MES1 into muscles with multiple nuclei (arrowheads; B) and giant flat cell (C). (D and E) Differentiation of bvCMVgfp-MES1 cells into multinucleate (arrowheads) muscles (D) and elongated cell (E). (F-H) Directed differentiation. Cells were transfected with pCMVmitf [8] and photographed at day 4 post transfection. Introduced mitf expression from pCMVmitf is sufficient to direct differentiation into black pigmented melanocytes (arrows). (F) Parental MES1. (G and H) MC11 clone from bvCMVgfp-transduced MES1. Bars, 25 μ m.

4.3.5 Retention of pluripotency *in vivo*

Chimeric assay represents the most stringent criterion to define the pluripotency *in vivo*. In medaka, chimeric embryos can be produced at 100% efficiency by transplanting ES cells into blastula hosts, and contribution of ES derivatives to various cell lineages can easily be observed in transparent, externally developing medaka embryos [10, 96]. I took this advantage to analyze the pluripotency of BV-transduced clonal MES1 cells. I found that clones MC2 and MC11 (both were derived from bvCMVgfp transduction), retained the transplantability as described for early embryonic cells and parental MES1 cells, producing 100% chimeras (118, 54 and 32 chimeric embryos for MC2, MC11 clones and parental MES1 cells were generated and monitored, respectively). More importantly, both clones distributed widely to major compartments and organ systems including the eye, heart, trunk, head and yolk sac (Figure 4.11A). Many of these highly chimeric embryos had normal development and were able to survive the whole observation until hatching. Moreover, many transplanted clonal cells differentiated into functional cells including cardiomyocytes in the contracting heart (Figure 4.11A). The distribution pattern was not different between BV-transduced clonal cells and plasmid-transfected MES1 cells (Figure 4.11B and C). I conclude that BV-mediated gene delivery and multiple chromosomal integrations do not compromise the pluripotency of medaka ES cells *in vivo*.

Figure 4.11 Pluripotency *in vivo*. (A and B) Distribution of BV-transduced MES1 cells in chimeras of day 3 development. Wide distribution of GFP-positive donor cells to many organs is evident in host STII embryos: ey, eye; hd, head; ht, heart; od, oil droplet

(vegetal); ys, yolk sac. (C) Co-distribution of BV-transduced clone (green) and plasmid-transfected MES1 cells (red) in chimeras of day 3 development. Cells for transplantation were pre-mixed at 1:1 ratio and transplanted at approximately 200 cells/blastula. The GFP and RFP-expressing chimeric embryos were reared in BSS-PEG solutions at 28 °C until hatching, with daily solution change and fluorescent photographing.

4.4 Conclusions and discussions

In this study I have established medaka as a complementary model system to evaluate the BV vector system for gene delivery and ES cell-based gene therapy *in vitro* and *in vivo*. By exploiting this unique system for easy ES cell manipulations and analyses *in vitro* and *in vivo*, I have demonstrated three previously unidentified features of BV-mediated gene delivery.

First, I show that all the three BV vectors designed for human ES cells can reproducibly yield an almost 100% high efficiency of gene delivery, a frequency that is substantially higher than that obtained in human ES cells [19]. Two major features may account for the remarkable difference in BV transduction efficiency between human and medaka ES cell systems. On the one hand, human ES cells are usually grown on a feeder layer, and they often form aggregates in attached culture and suspension during incubation with virus particles, compromising the transduction efficiency. In contrast, medaka ES cells are maintained without a feeder layer, and single cell suspension and attached cultures are easy to obtain for gene transfer. This could provide an ideal situation for exposure to virus particles, favoring their entry into cells and thus maximal gene delivery efficiency. On the other hand, medaka ES cells can easily be maintained at

a wide range of ambient temperature from 12-32 °C in feeder-free conditions. For gene transfer, single medaka ES cells can tolerate incubation for up to 12 h at 28 °C in a protein-free basal medium containing BV vectors compared with only 2-h incubation at 25 °C for human ES cells. If the transduction efficiency is depending on the MOI and duration of incubation, then the high efficiency obtained in medaka ES cells is largely ascribed to prolonged incubation. In non-ES fish cell lines, a maximal gene delivery efficiency of up to 20% has been reported [49]. The extremely high gene delivery efficiency I obtained by BV transduction in this study may also be due to other factors such as differences in vectors, virus preparation, cell lines and experimental procedures.

Second, the BV vectors in medaka ES cells have an unusually prolonged persistence and expression for up to 30 days. This is in sharp contrast to only 3 days for human ES cells [19]. In this context, it deserves to note that BV vectors are propagated in *Sf9* insect cells that are cultured at 27 °C [19], similar to that I used for maintaining medaka ES cells. This may suggest that BV vectors are stable at this low temperature, which is consistent with the reported studies on baculovirus [35, 38]. However, the difference in culture temperature between human (37 °C) and medaka ES cells (28 °C) cannot be made fully responsible for the remarkable persistence of BV vectors in fish cells, because differentiated fish cell lines maintained at a temperature similar to, or even lower than, that used for medaka ES cells in the present study, did not exhibit the prolonged persistence and transgene expression [49]. Future work is required to elucidate the cellular factor(s) controlling the persistence/stability of BV vectors.

Finally, BV transduction exhibits a high incidence of transgene integration into multiple chromosomes in medaka ES cells. The BV vectors I used in this study are

similar to those that have been engineered to be deficient for replication in mammalian cells where BV vectors have been reported to mediate a single integration event to a particular chromosomal region under the drug selective pressure [43, 44]. Zeng et al's work in human ES cells demonstrated the stable gene integration by using a hybrid BV vector that has incorporated the *rep* gene and inverted terminal repeat sequences from adeno-associated virus (AAV) [19]. In human mesenchymal stem cells (MSC), repeated BV transduction is required to obtain transgene integration [20]. The low incidence of chromosomal integration in human cells is perhaps argued for by short persistence of BV vectors [19]. In contrast, the persistence of BV vectors in medaka ES cells is considerably longer, and may account for the observed high incidence of chromosomal integration.

Condreay et al reported the BV-mediated transient and stable gene delivery in a series of mammalian cell lines and they showed that 2 to 7 kb baculovirus fragments were integrated into the stably transduced CHO cells analyzed by restriction enzyme digestion [43]. This integration phenomenon was further investigated by the same group [44]. The discrete portions of the baculovirus DNA were present as single-copy fragments ranging from 5 to 18 kb analyzed by Southern blot and the integration occurred randomly. This may also explain the high frequency of spontaneous host integration of BV vectors in MES1 cells. Because the reporter gene (*egfp* gene driven by CMV promoter) is relatively small (1.3 kb in size), the transient gene delivery efficiency is high (100%) and the BV-delivered transgene is strongly expressed (CMV is a strong promoter which lead to high level of transgene expression in eukaryote cells), and it is easy to observe the prolonged transgene expression (GFP signal) in MES1 cells, suggesting the integration of BV vectors. Together with the FISH visualization of integration signals, the

multiple integrations happened in medaka ES cells. To further study the baculovirus integrants in lower vertebrate model, Southern blot and Genome walker approaches could be employed to detect the size of integrated baculovirus DNA fragments and identify the viral integrated genome loci in host cells.

Most importantly, BV-transduction in medaka ES cells does not abolish or compromise the genome stability and pluripotency *in vitro* and *in vivo*. This is similar to the previous observations in both medaka [10, 52, 98] and human ES cells [19] that transient and stable gene transfer mediated by either plasmid transfection (medaka) or BV transduction (human) do not change the ES cell property. However, it is surprising that medaka ES cell clones even after multiple chromosomal integrations do not show any differences in growth and pluripotency. In this study, the BV-transduced medaka ES clones with multiple chromosome integrations have resulted from colonies displaying all *in vitro* features of ES cells. It is likely that this selection have favored the establishment of pluripotent ES clones, and concurrently, excluded those cell clones with compromised pluripotency due to other chromosomal integrations, thus escaping from testing for developmental potency. It deserves to note that under the conditions used for colony formation, medaka ES cells undergo spontaneous differentiation, giving rise to three types of undifferentiated, mixed and differentiated colonies [9]. In this study, we also observed the three colony types in BV-delivered GFP-expressing and non-expressing cells. It will be interesting to determine whether differentiated cell colonies would result from either spontaneous ES differentiation or loss of pluripotency caused by integrations into multiple chromosomal sites.

The striking finding is that BV-transduction leads to a high efficiency of transgene integrations in multiple chromosomes in medaka ES cells. This is in sharp contrast to the situation in mammalian cells [44]. In the reported work in human ES cells, stable integration possibly on human chromosome 19 was obtained by using the AAV sequence in a hybrid BV vector [19]. My present finding underscores that it is necessary to reexamine this issue more extensively in human cells to fully evaluate the risk due to possible random integration of BV vectors into the human genome. The high efficiency of both gene delivery and integration in medaka ES cells make the BV system a powerful tool in the rapid production of highly homogeneous transgenic ES cells for e.g. directed differentiation into particular cell types [8] and in the generation of rare genetic events such as gene targeting by homologous recombination.

Chapter 5 Exploration of baculovirus as an *in vitro* and *in vivo* gene transfer tool in fish

5.1 Introduction

In the previous chapter, I showed that BV vectors can mediate highly efficient gene delivery and chromosomal integration in the medaka ES cell model. In fact, the high transduction efficiency of BV vectors is not restricted to MES1 cells but applies also to other cell types such as SG3 spermatogonial stem cells, non-stem cells and even primary cultures. Comparing the plasmid and BV vectors usage, plasmid DNA transfection is suitable for routine laboratory use, because of its simplicity, reproducibility, and acceptable efficiency; while the BV vectors require multi-step preparation and are more demanding for experiments where high efficiency of gene transfer is required, e.g., homogenous population of directed differentiating ES cells *in vitro* and gene delivery *in vivo* for therapy.

Adult stem and non-stem cell lines have been derived from medaka testis [12], ovary [15], and liver (Chen and Hong, unpublished). Additionally, the primary cell culture of embryonic and germ cells is a useful approach for *in vitro* cell fate tracing [28, 51]. Furthermore, fish presents an easily accessible *in vivo* embryogenesis and organogenesis model for bioimaging and biomedical studies [27, 33]. However, efficient gene transfer has not been achieved in fish cell cultures [49, 52]. Consequently, it is worth exploring a viral vector-mediated *in vitro* and *in vivo* gene delivery approach that can be routinely applied without using special facilities and reagents. It is also necessary to develop a

modifiable BV vector with elements enhancing gene transfer efficiency and facilitating rapid selection.

5.2 BV-mediated gene delivery in different fish cell lines and other fish models

5.2.1 BV-mediated efficient gene delivery in medaka adult stem and differentiated cells

In this study, I extended the observation of the BV-mediated efficient gene delivery in the medaka adult stem (AS) cell lines, the first adult testis-derived male stem cell line SG3 spermatogonia capable of test-tube sperm production [12] and a putative live stem cell line MLA3 and a differentiated live cell line MLB4.

The typical cell morphology of these cell lines in long-term cultivation is shown in Figure 5.2. The SG3 cells share some characteristics of MES1 cells, including small, round, or polygonal cell morphology; large nuclei; rapid growth; and ability to form colonies, while liver cells are large in size, branchlike, and spindle in morphology, as observed in MLA3 and MLB4 cell lines. The SG3 cells were derived from a mature testis and characterized by several lines of evidence for the spermatogonial identity, including the testicular origin, spermatogonial phenotype, expression of germ cell markers, and more convincingly, the ability of meiosis and sperm production *in vitro* [12]. The SG3 cell line offers an excellent system for genetic manipulations of the germline in vertebrates, due to the ability of long-term self-renewal, meiosis, and spermatogenesis to generate motile sperm *in vitro*. The MLA3 and MLB4 cells were isolated from liver.

MLA3 is a putative adult stem cell, and the MLB4 is a differentiated cell line and is used as a differentiated control cell line.

Figure 5.1 Typical cell morphology of medaka stem cell and differentiated cell lines.

(MES1 and SG3 showed large nuclei and round or polygonal morphology with different cell densities; MLA3 and MLB4 are liver stem and stellate cells respectively, which are branch- and spindle-like in morphology. Scale bar: 40 μm .)

The results of BV-mediated transgene expression in medaka adult stem cells (SG3 and MLA3) and differentiated cells (MLB4) showed the following (Figure 5.2): (1) GFP transgene expression mediated by BV vectors was visualized 24 h after virus transduction in SG3 and MLA3 cells at 28 °C for 8 h in DMEM medium, while 48 h in MLB4 cells, under fluorescent microscope; and the GFP signal was intense after 48 h; (2) the gene

transfer efficiency is lower in differentiated MLB4 cells (5-10%) than that in adult stem cells (80% in SG3 and MLA3 cells); (3) cell proliferation and pluripotency were not affected by BV transduction in SG3 and MLA3 cells, observed by cell growth status and typical stem cell morphology; and (4) prolonged transgene expression (30 days) was also observed in SG3 cells (data not shown).

Figure 5.2 BV-mediated transgene expression in medaka adult stem and differentiated cells. Micrographs showing gene delivery and transient expression at day 3 by using bvCMVgfp at MOI 50. Scale bars, 40 μ m.

5.2.2 BV-mediated gene delivery in medaka primary tissue cultures

As a laboratory animal, medakafish grows fast and produces eggs daily throughout the year, under good feeding and warm climate conditions. This feature offers me adequate experimental materials to routinely carry out the primary culture from early developing embryos and adult tissues. It is worth extending the application of BV in medaka primary cell culture and early developmental observation to achieve the gene transfer goal that other conventional methods cannot. To this end, early embryonic cells from the mid-blastula (MBE) stage (SOK strain) and tissue cells (liver, kidney, brain, eye, heart, and spleen) are isolated and primarily cultured for 24 h, allowing the cells to attach to the substrate, and are treated with baculovirus transduction at 28 °C for 4 h at a MOI of 100, followed by changing complete primary culture medium (ESM2) to express the transgene. The results of BV-mediated transgene in primary cell culture exhibited its ability to deliver foreign genes into primarily cultured embryonic and tissue cells, and this may facilitate special experiments that need rapid cell labeling, such as the primordial germ cell (PGC) culture [28] (Figure 5.3) and early lineage development *in vitro* tracing [51].

Figure 5.3 BV-mediated transgene expression in medaka primary tissue cultures.

The effective gene delivery was seen 1 day after the transduction in freshly isolated medaka blastocysts (A) Dividing cells are indicated by arrow. (B) Embryonic cells cultivated in attachment for 2-5 days. (C) Primarily cultivated cells from eye.

5.2.3 BV-mediated gene delivery in developing medaka embryos

Microinjection is a well-established technique in medakafish and zebrafish, and is adopted to study early developmental events by delivering artificial genetic interference into embryos. Baculovirus can be injected into embryos of zebrafish to direct gene expression in early developing embryos *in vivo* [65]. It is worth testing this in medaka embryos. For baculovirus-mediated transgene expression in early developing embryos,

microinjection is conducted to test whether baculovirus has the capability to infect and drive transgene expression in early developing embryos. Figure 5.4 shows the medaka embryo undergoing microinjection and cell transplantation.

Figure 5.4 Microinjection and cell transplantation of medaka embryos. A. Microinjection of 1-cell stage embryo using μm -class glass needle, the phenol red was injected as a tracer; B. Dechorionated mid-blastula stage embryos are ready for cell transplantation. Abbreviations: od, oil droplet; ch, chorion.

The glass needle used for microinjection and cell transplantation is prepared by a horizontal pipette puller. Micromanipulation of embryos is carried out under a stereomicroscope with a cold-lighting system. The embryos are aligned in a groove or a V-shaped ramp prepared with 1.5% agarose for microinjection and cell transplantation, respectively. Normally, 20 nL of baculovirus and 100 donor baculovirus-transduced ES cells are injected in each embryo. After that, the embryos are reared in ERM and BSS-PEG solutions for transgenic embryo and chimeric embryo development, respectively.

GFP expression in medaka embryos mediated by baculoviruses started 24 h after microinjection at the 1-cell stage, and the GFP signal was detectable for 3-5 days in the

developing embryo (Figure 5.5). This observation is consistent with BV-mediated transgene expression in zebrafish embryos [65], where the GFP signal can be observed 6 h after injection; note that zebrafish has faster embryogenesis than medaka. However, the frequency of transgene expression in medaka embryos (data not shown) is higher than that in zebrafish even by injection of 100-fold less dosage of baculoviruses. In Wagle and Jesuthasan's work, EphrinB2a was normally expressed in the posterior region of un-injected developing somites, but it was misexpressed into specific tissues induced by the BV injection, and the abnormal somite boundary was formed. In medaka embryos, the GFP signals were unevenly distributed, and embryogenesis was not affected by BV transduction. The possible reason for this different BV-delivered transgene expression needs further investigation.

Figure 5.5 BV-mediated transgene expression in early developing medaka embryos.

The microinjection delivered BV vectors led to a scattered GFP expression in early developing medaka embryos from i^3 strain. Note that the autofluorescence was evident (id) in the head and trunk area in the host. Abbreviations: ey, eye; hd, head; ht, heart; od, oil droplet; id, iridophore.

5.2.4 BV-mediated gene transfer in zebrafish and trout cell lines

BV transduction efficiency has been explored in cultured fish cells [49]. The highest transduction frequency observed was TO cells (approximately 20% and 30% without and with sodium butyrate, respectively), and 0.04-2% and 0.05% for EPC, CHSE-214, and CHH-1 cell lines, respectively. In the study, I examined the BV infection spectrum in other fish models and cell lines, including an ES-like zebrafish ZF cell line (Hong, unpublished) and rainbow trout gonad RTG cell line [12, 49]. The high efficiency of gene delivery (80-90%, Figure 5.6A) and persistence of transgene expression (Figure 5.6B) was observed in ZF cell line. The RTG cell was cultivated at a lower temperature (19 °C). The BV-delivery transgene expression was visualized in a delayed time course (4 days) and the efficiency of gene transfer was much lower than that in medaka and zebrafish cells (Figure 5.6C). RTG cells were reported to be not susceptible to BV transduction analyzed by β -galactosidase staining assay [49].

Figure 5.6 BV-mediated transgene expression in zebrafish and trout cells. (A) The efficient gene delivery was observed 24 h after the transduction in ZF cell line. (B) BV-delivered transgene expression was prolonged to 5-10 days after plating into Petri dish. (C) The BV transduction showed a delayed expression in trout RTG cell line.

5.3 Development of a useful BV vector for efficient transgenesis

The data presented so far indicate the wide application of BV vectors in different fish *in vitro* cell culture and *in vivo* development models, due to the high efficiency of gene delivery and spontaneous chromosomal integration. This provokes me to make better use of BV vectors for both transient gene delivery in rapid production of highly homologous transgenic ES cells for directed differentiation and stable gene delivery in

ES-based cell therapy, and further in precise genome manipulations such as gene targeting. To this end, I design a new BV vector that contains cassettes of *I-SceI* meganuclease, drug selection, and fluorescence expression and preserves the *I-SceI* recognition sites and unique multiple cloning sites, i.e., rare cutters for subcloning large fragments such as targeting vectors.

5.3.1 Construction of recombinant BV vector by Bac-to-Bac strategy

Bac-to-Bac is a well-established and widely used strategy for producing recombinant baculoviruses that is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli*. It contains four main steps (Figure 5.7). First, the gene to be expressed is cloned into a pFastBac1 donor plasmid as a transfer vector. Second, this transfer vector is introduced into DH10Bac competent cells that contain the parental circular wild type baculovirus genome and a helper plasmid vector that functions in Tn7-mediated transposition. This transformation usually gives 0.1-1% of recombinant progeny, and the foreign gene is inserted into the baculovirus genome by homologous recombination at the bacterial transposon Tn7 (mini-*att*Tn7) sites. Third, the high molecular weight DNA of recombinant baculovirus DNA from *E. coli* is extracted, transfected and propagated in *Sf9* insect cells. Fourth, the mature virus particles are harvested from the *Sf9* insect cells and concentrated by high-speed centrifugation (28,000 g) to certain infectious titers.

Figure 5.7 Flow chart of recombinant BV vector construction. The gene to be expressed is cloned into a pFastBac1 transfer plasmid, and the recombinant DNA is transformed into DH10Bac competent cells which contain the bacmid with a mini-*att*Tn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac1 plasmid can transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposase encoded by the helper plasmid. The positive colonies are screened by a blue color reaction of X-gal and inducer IPTG (disruption of the *lacZα* gene). High molecular weight bacmid DNA is extracted from selected *E. coli* clones, and then used to transfect insect *Sf9* cells for baculovirus production.

I designed a donor vector, pBV-ISceI vector by PCR-cloning from the backbone of the pFastBac1 vector to produce the recombinant BV vectors (Figure 5.8). This donor vector has advantages in two respects: (1) it preserves several restriction enzyme sites

(rare cutter) for large-capacity of foreign gene cloning and (2) it expresses the *I-SceI* meganuclease that will recognize and cut the unique 18-bp *I-SceI* recognition sites flanking the foreign gene(s) and facilitate the transfer of DNA fragments into the nucleus and thus improve the transgenesis efficiency [104]. The so-called pSPR vector for drug selection is then constructed based on this donor pBV-*I-SceI* vector, by adding the RFP (red fluorescence protein) and puromycin (drug selection) cassettes into BV vector. Other gene targeting vectors could be adaptable to this new BV vector system, aiming at obtaining high-efficiency gene transfer and genome integration.

Figure 5.8 Development of bvSPR vector for efficient transgenesis. P1 and P2 are PCR primers used for cloning the backbone of pFastBac1 and generating a new BV donor vector. P3 and P4 are PCR primers used for cloning *I-SceI* expressing cassette and introducing the *I-SceI* recognition sites and rare cutters for further modification. P5 and P6 are PCR primers used for cloning the fast drug selection (puromycin) and fluorescent protein (RFP) cassettes. Abbreviations: N=*NotI*; M=*MluI*; K=*KpnI*; *=*I-SceI*; E1&E2 reserved for further replacement E1=*SbfI*, E2=*SgfI*. There are *XhoI*, *HindIII*, *EcoRI* and

SpeI, *BamHI* sites between CMV and pr fragments, which can be used for further replacement.

The successful construction of bvSPR vector was confirmed by (1) PCR identification of insertion of donor pSRR vector into BV vectors, i.e., recombinant bvSPR vector (data not shown); (2) transduction of bvSPR into host insect *Sf9* cells and the visualization of RFP signal in *Sf9* cells (Figure 5.9B); and (3) efficient gene delivery in MES1 cells and generation of bvSPR-integrated ES colonies (Figure 5.9D), which can be further used to analyze the integration pattern of BV vectors in host genome.

Figure 5.9 Production of bvSPR vector in *Sf9* cells and transduction in MES1 cells.

(A) and (B) Shown are insect *Sf9* cells in suspension culture 3 days after transduction of recombinant bvSPR vectors. (B) The RFP signals are visualized in *Sf9* cells before the virus harvest. The high titers of viruses are collected at the 3rd passage (P3) infection of *Sf9* cells. (C) Test transduction was done in MES1 cells and (D) the BV-vector delivered RFP expression was observed 1 day after transduction in MES1 cells.

5.3.2 Activity of I-SceI meganuclease in MES1 cells

A molecular biology approach was employed to see whether this I-SceI enzyme and its unique recognition site system work efficiently. The testing vector pGSR expresses EGFP but not RFP protein due to the stop codon followed by EGFP, until the EGFP cassette is excised by the I-SceI enzyme at its two recognition sites that flanking the EGFP encoding sequence (Figure 5.10A). The co-transfection of these two vectors was carried out in MES1 cells, and the GFP signal was observed 24 h after transfection while the RFP signal was seen at 48 h afterwards, indicating the effective excision of the EGFP fragment and re-ligation of the RFP fragment to the CMV promoter by I-SceI meganuclease (Figure 5.10B).

Figure 5.10 Activity and efficiency test of I-SceI meganuclease system. The I-SceI-expressing vector pCVI-SceI is co-transfected with a color testing vector pGSR into MES1 cells or mico-injected into early embryos to see the excision effects of I-SceI

enzyme acts at its recognition sites, which leads to the RFP expression in transfected cells. This I-SceI meganuclease approach is aimed to improve the integration efficiency of BV-delivered transgene.

5.4 Conclusions and perspectives

This study has shown that the BV vector system can be used for both *in vitro* and *in vivo* gene delivery in different fish models. The host integration mediated by BV vectors can be used as a genetic engineering tool for stem cell-based gene therapy, as no deleterious effects were observed in terms of stable cell growth, genome stability, and pluripotency. The mechanism and pattern of chromosomal integration need to be further investigated. The construction of new recombinant BV vectors has potential for analyzing the directed differentiation of ES cells *in vitro* by producing highly synchronous differentiating cells and improving the efficiency of transgenesis by joint application of an I-SceI meganuclease system.

Chapter 6 Establishment of gene targeting in medakafish embryonic stem cells

6.1 Introduction

Gene targeting (GT) via homologous recombination (HR) can generate precise genetic alterations in vertebrate genomes and has routinely been used in mouse embryonic stem (ES) cells. Transplantation of targeted ES cells into early mouse embryos leads to the production of whole animals for analyzing the functions of targeted genes in developmental processes and congenital diseases. It remains unresolved whether GT can also be developed in long-term cultivated ES cells of lower vertebrates. There has been great interest in establishing GT in other organisms, including human, rat, and fish. In the fish medaka, attempts to develop ES cell lines [9-11], GT vectors [56], and the PNS procedure [52] have been reported. GT technology has also been attempted in ES-like cell cultures of zebrafish [55].

6.2 Vector design and selection strategy

Previously, the medaka *p53* gene has been cloned from MES1 cells [92]. Based on this sequence, a medaka *p53* GT vector (pGTp53) has been constructed [91]. The genomic structure of the medaka *p53* gene is shown in Figure 6.1. The medaka *p53* gene has a similar genomic organization as its counterparts in mouse and human. It is composed of 11 exons and 10 introns. It is 8.7 kb in size, which is smaller than that of mouse (12 kb) and human (20 kb) because of the different size of introns.

I performed a GT experiment in MES1 cells by DNA transfection in 6-well plates, using the medaka *p53* GT vector (pGTp53) containing PNS cassettes for *neo* and *tk* expression. Subculture in 10-cm dishes under drug selection with G418 and gancyclovir (Gc) gave rise to positive-negative selection (PNS)-resistant colonies, which were expanded into cell clones and analyzed for GT products. HR events were identified and characterized by (1) PCR-based genotyping; (2) Southern blot analysis; (3) DNA sequencing of 5' homology arm junction; and (4) visualization of vector integration by fluorescent *in situ* hybridization (FISH). The pluripotency of the targeted clones were examined by (1) ES phenotype; (2) embryoid body (EB) formation; (3) gene expression profiling of differentiation *in vitro*; and (4) chimera formation. The flow chart of GT process in medaka ES cells is summarized in Figure 6.2.

Figure 6.1 Comparison of the genomic organization of p53 genes from medaka, mouse and human. Black boxes, translated exon sequences; open boxes, untranslated exon sequences; thin line, intron sequences; ATG, start codon; stop, stop codon; pA, polyadenylation signal. Restriction sites used for Southern blot analysis of the medaka p53 gene are shown. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; P, *Pst*I; Sp, *Spe*I (Adapted with permission from [92]. Copyright 2001, Elsevier).

Figure 6.2 Flow chart of gene targeting in medaka ES cells. Abbreviations: EB, embryoid body; ES, embryonic stem; FISH, fluorescent *in situ* hybridization; Ge,

gancyclovir; G418, geneticin; HR, homologous recombination; *neo*, neomycin resistance gene; PBS, phosphate buffered saline; PNS, positive-negative selection; RT-PCR, reverse transcription polymerase chain reaction; *tk*, thymidine kinase gene.

The *p53* targeting vector pGTp53 used in this study contains two cassettes respectively expressing neomycin for resistance to G418 and the Herpes simplex thymidine kinase (Stk) for sensitivity to gancyclovir (Gc) to enrich the GT event by positive-negative selection (PNS) (Figure 6.3A). The STneo is inserted to replace exons 2-7 and flanked by a 0.9-kb left arm and a 4.5-kb right arm of the medaka *p53* genomic sequence, and STk is at the end of the right arm [52, 91].

Upon homologous recombination (HR), the STneo would be cointegrated, while the STk would be recombined away, with the homologous recombinant being resistant to G418 and Gc owing to the expression of neo and the absence of tk. The outcome of the HR event is that the *p53* gene fragment from exon 2 to exon 7 (including intron 2 to intron 6, in total 2,451 bp) is replaced by the simian virus-40 early gene enhancer (ST) driven neomycin (neo) gene (1,158 bp) (Figure 6.3A).

Upon random integration (RI), both the STneo and tk would be cointegrated, with random integrants being resistant to G418 but sensitive to Gc owing to the expression of both neo and tk. Therefore, the PNS by using G418 and Gc is predicted to enrich for homologous recombinants by eliminating random integrants due to their sensitivity to Gc and non-transgenic cells due to their sensitivity to G418 (Figure 6.3B).

Figure 6.3 HR-mediated p53 gene targeting in medaka ES cells. (A) Gene targeting vector pGTp53 and the *p53* locus. WT, wildtype; filled box, translated exon; open box, untranslated exon; thin line between boxes, intron; thick line, region included in pGTp53; neo, STneo cassette; tk, STk cassette; Capital letters denote restriction sites (B, *Bam*HI; E, *Eco*RI; N, *Nco*I; P, *Pst*I). Arrows depict the position and extension direction of primers (ex, external primer; in, internal primer; neo, neomycin primer). Probes used for Southern analyses are indicated. (B) Genotype and phenotype for drug selection. -,

absence; +, presence; r, resistant; s, sensitive. (C) PCR detection of HR events. Medaka ES cell lines MES1 to 3 were transfected with pGT53 (+) or pBluescript KS as mock transfection control (-), and subjected to PCR detection following 3-5 days of growth in the absence of drug selection or 14 days of growth in the presence of PNS with G418 (final 500 µg/mL) for positive selection and gancyclovir (Gc; final 5 µmol/L) for negative selection. PCR conditions: 35 cycles of 25 µL PCR reaction containing 1 µL of crude DNA for 1st PCR, and 20 cycles of 25 µL PCR reaction containing 1 µL of 1st PCR reaction.

MES1 was transfected with pGT53 to determine the GT event. To this end, genomic DNA was isolated from three medaka ES cell lines (MES1, 2 and 3 [9]) and subjected to a PCR analysis by using a pair of primers specific to the targeted *p53* locus (Figure 6.3A). This led to the detection of GT-specific PCR product in all of the six batches of pGTp53-transfected ES cells, in which PNS (500 µg/mL of G418 plus 5 µmol/L of Gc) increased the yield of GT-specific PCR product (Figure 6.3C). Therefore, all three medaka ES cell lines can mediate detectable HR at the *p53* locus. Subsequent experiments were performed in MES1 cells.

6.3 PNS enrichment for homologous recombinants

Compared to RI, HR is a rare event even in mouse ES cells [21]. I wanted to determine the efficiency of PNS for enrichment for the HR event in medaka ES cells. To do this, approximately 10^6 transfectants were seeded in a 10-cm dish and grown in the presence of G418 alone or together with Gc, and colony formation was examined after

23~28 days of culture. In a typical experiment, selection with G418 alone produced 100~300 colonies per dish, while the number of colonies decreased to 5~25 per dish upon PNS with G418 plus Gc (Figure 6.4A and C). A statistical analysis of 4 independent experiments led to an enrichment factor of 12.38 ± 3.95 for PNS in pGT53-transfected MES1 cells (Table 6.1), demonstrating the effectiveness of PNS for enriching putative homologous recombinants by eliminating possibly unwanted random integrants in medaka ES cells.

The effectiveness of PNS and cell proliferation can be observed and monitored under daily microscopic inspection (Figure 6.5). At the beginning of PNS, the transfected MES1 cells were subcultured into 10-cm dishes, and most (90%) of the cells attached to the substrate within hours (data not shown). Many floating (detached) cells were found after PBS wash and medium change from day 2 to 14 (Figure 6.5A and D, round and bright cells), suggesting the sensitivity of non-transgenic cells to the drug G418. This observation is consistent with the fact that 5-10% gene transfer efficiency can be obtained in MES1 cells for delivering linear plasmid DNA by GeneJuice reagent, and it suggests that the non-transgenic cells were eliminated by G418 at the effective concentration of 500 $\mu\text{g/mL}$ [52].

Figure 6.4 Efficiency of the PNS in medaka ES cells. Three days after transfection with linear and circular pGTp53, MES1 cells were dissociated by trypsinization and seeded onto 10-cm dishes. After 23 days of clonal growth, colonies were counted following Giemsa staining. (A and C) Colony formation in the presence of G418 alone (500 $\mu\text{g/mL}$). (B and D) Colony formation in the presence of G418 plus Gc (2 $\mu\text{mol/L}$).

Table 6.1. PNS is efficient in medaka ES cells¹⁾

Batch	Number of colonies		Enrichment factor ²⁾
	G418	G418+Gc	
1	655	46	14.2
2	490	38	12.9
3	220	14	15.7
4	275	41	6.7
total	1640	139	12.38 $\pm$ 3.95 ³⁾

¹⁾ After transfection with 2 μ g of linear pGTp53 in a 6-well, MES1 cells were grown for 2-3 days. Single cells (2×10^6) were prepared by trypsinization seeded onto 10-cm dishes each. Five dishes were grown in the presence of G418 only for single drug selection and - five dishes were subjected to for PNS with G418 plus Gc. After 23 days of culture, colonies were counted following Giemsa staining.

²⁾ Enrichment factor was as the ratio between the number of colonies formed in the presence of G418 alone and G418 plus Gc.

³⁾ Data are means $\pm$ s.d.

Figure 6.5 Effective positive-negative selection in MES1 cells. (A) Cells detachment within 2 days can be seen after plating under selection of G418. By several rounds of PBS wash and medium change, these non-transfected G418-sensitive cells will be removed and the G418-resistant cells start to proliferate and form colonies from single cell (B-D). After 14 days of double selection with G418 and Gc, the “false” Gc-resistant colonies became sensitive to Gc even the colonies already formed (E, circle), these Gc-sensitive cells that contain the tk transgene delivered by in random integration manner became tumid and will be easily removed by PBS wash (F).

The surviving cells showed active cell division (Figure 6.5B and C) and formed colonies after 23-28 days of clonal growth under constant drug stress (Figure 6.4; Figure 6.5D and E). From the continuous monitoring of fixed positions on the dishes, the effectiveness of negative selector Gc was obvious from day 14 to 28 (Gc was added throughout the PNS procedure), indicated by the donut-like colonies that formed in the first two weeks but eventually inflated, detached, and were removed by PBS wash and medium change (Figure 6.5E and F). These observations and statistics suggest that the PNS was effective and the background was reduced significantly.

6.4 Identification of homologous recombinants

I utilized four procedures to identify genuine homologous recombinants.

6.4.1 PCR screening

A PCR-based genotyping procedure was applied to screen for the targeted *p53* locus. The GT-generated mutated (MT) *p53* locus was detected by PCR amplification of the fragment between the endogenous *p53* locus and the exogenous targeting vector-introduced neomycin gene, using the external and neomycin primer (Figure 6.6A). These PCR products were subjected to further junction sequence analysis (Figure 6.6B). The successful amplification of the other wild-type (WT) *p53* locus (intron 3-4) from all the assayed samples indicated that the PCR reaction condition was optimal (Figure 6.6C). The PCR genotyping rates were 39.1% and 62.5% from two batches of individual PNS-resistant colonies (Table 6.2).

Figure 6.6 PCR screening strategy. (A) Primers for PCR screening of *p53* GT loci. HR screening PCR is performed with p53Ua (annealing endogenous *p53* allele) and NeoRa (annealing neo of vector) primers. To ensure the reliability and sensitivity of PCR, primer pair of p53F and p53R is setup as an internal PCR control which will generate a 1.8 kb PCR product from the endogenous *p53* allele (exon 3 to 5) and primer pair of p53LU and

NeoR (flanking 2.1 kb of vector fragment) is used to detect the random integration of targeting vector. (B) Genomic PCR screening for p53 HR events in MES1 cells by using primers indicated in (A). An internal control PCR was also performed using the same genomic DNA template (C) A 1.8 kb PCR product (left gel) showed efficient PCR amplification by using the genomic DNA from PNS-resistant colonies and the random integration of targeting vector (2.1 kb PCR product) is detectable from some colonies (right gel).

6.4.2 Southern blot analysis

The correct HR events were confirmed by Southern analysis using the external and neomycin probes (probe 1 and 2 in Figure 6.3A), which were designed for detection of the precise gene replacement and integration of the targeting vector, respectively. The external probe is specifically for the upstream of *p53* exon 1 (outside the short homology arm), and only the targeted/mutated *p53* locus will present the expected hybridization pattern. In the wild-type *p53* allele, the external probe is hybridized with an 8 kb *EcoRI* digested fragment, and this fragment is converted into one of 9.2 kb after replacement of exon 2 to 7 by the targeting vector (Figure 6.7A). This result conformed to the expected targeted *p53* allele and the other wild-type *p53* allele for a correct HR event in the four clones while the control MES1 DNA showed a single wild-type band (Figure 6.7B).

To statistically analyze relative GT efficiency, i.e., GT clones per analyzed sample, the PCR-positive colonies were randomly selected for Southern assay by utilizing restriction enzymes that are specifically introduced by the targeting vector, such as *NcoI* (Figure 6.8) and *XhoI* (Figure 6.9) in the *neomycin* region of the pGTp53 vector (Figure

6.3A). From three batches of Southern analysis by 5'-external probe hybridization with the *EcoRI*, *NcoI*, and *XhoI* digested DNA samples, respectively, 15 out of the 32 tested clones presented the predicted band corresponding to the HR events in these randomly selected PCR positive clones. These results led to a relative GT efficiency of 22.8% in the medaka ES cell line MES1 (Table 6.2). The re-hybridization was done in both *NcoI* and *XhoI* digested DNA samples (data not shown). The combination of these results showed convincing evidence that the correct HR events occurred with high frequency in medaka ES cells. These Southern confirmed clones are assessed for further sequencing, FISH, and pluripotency.

Figure 6.7 Confirmation of correct HR events by Southern blot using 5'- external probe hybridized with *Eco*RI diested DNA samples. (A) The external and neomycin probes were labeled with digoxigenin and used for detection of HR events and insertion of targeting vector, respectively. These two probes are specific for the endogenous *p53* locus (outside the short homology arm) and *neomycin* gene in the targeting vector, respectively. In the wild type *p53* allele, the external probe is hybridized with an 8 kb *Eco*R1 digested fragment. After replacement of exon 2 to 7 by targeting vector (the backbone and tk got lost after HR), one *Eco*R1 site (asterisk) gets lost and this fragment is converted into one of 9.2 kb. (B) Southern blot hybridization analysis of ES cell clones with putative HR events. The hybridization patterns conformed to the expected targeted *p53* allele (9.2 kb band) and the other wild type *p53* allele (8 kb band) for a correct HR event in the four clones. A control parental MES1 DNA showed a single 8 kb band. (C) The integration of targeting vector (STneo fragment) was visualized by hybridization

with neomycin probe. The targeted *p53* allele showed a 9.2 kb band corresponding to the correct HR event and a 13.3 kb band generated by *EcoR*I partial digested fragment. No hybridization signal was seen in the control MES1 cell.

Figure 6.8 Confirmation of correct HR events by Southern blot using 5'- external probe hybridized with *Nco*I diested DNA samples. (A) Design of Southern blot analysis. A 1.5 kb of external (EXT) probe was used for detection of HR event in targetting *p53* locus. (B) Positive clones detected by genomic PCR were further testified by Southern analysis with digoxigenin (DIG)-labeled external probe.

Figure 6.9 Southern blot analysis of integration of targeting vector-delivered neomycin fragment. (A) Design of Southern blot analysis. (B) Detection of insertion of neomycin (neo, 800 bp) by using *Nco*I enzyme digested genomic DNA. (C) Detection of insertion of neo by using *Xho*I enzyme digested genomic DNA. (D) Detection of HR event by 5'-external probing on the *Xho* 1 digested DNA samples.

Regarding absolute GT efficiency, i.e., the GT clones per total cells, the GT experiment was initiated from 10^6 MES1 cells; 10^5 transgenic cells were obtained with a 10% gene transfer efficiency; 100 positive drug-resistant or 10 PNS-resistant colonies were generated; and finally, 5 and 2 colonies were PCR and Southern positive, with 48.7% and 22.8% detection rates of PCR genotyping and Southern confirmation. Therefore, the relative and absolute GT efficiencies are 22.8% and 2×10^{-6} , respectively. Detailed analysis from representative reports is summarized in Table 6.3.

Table 6.2. Gene targeting efficiency at the *p53* locus in medaka ES cells.

Batch	Colonies	Colonies PCR-assayed ¹⁾ , n (%)		Colonies Southern-assayed ²⁾ , n (%)	
		Total assayed	PCR ⁺ colonies	Total assayed	Southern ⁺ colonies
1	24	23	9 (39.1%)	11 (<i>Nco</i> I)	6
2	16	16	10 (62.5%)	10 (<i>Xho</i> I)	5
3				11 (<i>Eco</i> RI)	4
total	40	39	19 (48.7%)	32	15 (46.8%)

¹⁾ PCR⁺ refers to the presence of putatively targeted p53 allele via genomic PCR by using a primer pair specific to the targeted locus (Figure 6.3A).

²⁾ Only PCR⁺ colonies were examined. Southern⁺ refers to the appearance of a new band predicted for the targeted locus. Percentage was derived by comparing Southern⁺ colonies to the total number of PCR colonies analyzed by Southern blot.

6.4.3 Junction sequence analysis

The high frequency of HR events detected from the exogenous *p53* targeting vector and endogenous *p53* locus provoked me to further detail the observation of this precise process occurring in MES1 cells. In principle, after the targeting vector has found its homologous locus and fragment exchange between vector DNA and host genome DNA at the two homology arm regions, a precise “cut-and-paste” fragment in the targeted locus will be produced (Figure 6.3A). By using the PCR primers designed for screening HR events, the PCR amplification contains the 5'-junction of the short homology arm region. Accordingly, the PCR products from the PCR-positive clones (Figure 6.6B) were subcloned into pGEM T-easy vector and then subjected to DNA sequencing. The sequencing data from 5 Southern-positive clones showed identical sequences among the 5 tested clones and the predicated 5'-junction of the endogenous *p53* locus and targeting vector (short homology arm), as well as the region flanking the short homology arm and transgene neomycin fragment (Figure 6.10). This result validated the correct HR events.

Figure 6.10 Sequencing of 5'- junction of homology arm. HR junction was confirmed by sequence analysis. (top) GT vector and targeted locus. neo and tk, cassettes expressing neomycin aminoglycoside phosphotransferase (neo) for resistance of G418 and the thymidine kinase (tk) for sensitivity to gancyclovir (Gc). Paired arrows depict the position of PCR primers on the targeted *p53* locus (bottom). Sequence of the PCR product was amplified by using the primer pair (arrows). Cell pools, transfected cell mixture survived 23 days of positive-negative selection with G418 and Gc. The *p53* sequence included in the GT vector is shown in black. Clones, cell populations expanded from single cell colonies growth under continuous drug selection. Shown here are only border sequences.

6.4.4 Fluorescent *in situ* hybridization

To determine whether the *neomycin* transgene was present in the host genome at the cellular level, I performed fluorescent *in situ* hybridization (FISH) on one of the five

Southern positive clones by using the biotin-labeled *neomycin* probe as previously described. Detailed observation on metaphase (Figure 6.11A) and interphase (Figure 6.11B) samples of GT22 clone revealed a single copy of an integrated *neomycin* gene (green dot as indicated by arrows) on the *p53* targeted ES cells.

Figure 6.11 FISH detection of targeting vector integration. Shown are integrated neomycin fragment (green signal) on the (A) metaphase and (B) interphase of MES-p53 GT22 clone.

6.5 Phenotype, differentiation and pluripotency

I analyzed the ES features of *p53*-targeted clones, in terms of phenotype; stable growth; alkaline phosphatase (AP) activity; spontaneous, induced, and direct differentiation; and chimera formation.

6.5.1 Phenotype of colonies after PNS procedure

Previously, it has been demonstrated that MES1 cell has a prominent ability for clonal growth [52, 91], even with multiple transgene integration mediated by BV vector, and retains the pluripotency after long-term drug selection [52]. By monitoring the clonal growth and morphology of single colonies, four kinds of colonies can be seen during 23-28 days of PNS culture (Figure 6.4). The PNS-resistant colonies showed active proliferation (Figure 6.5B-E) and the ideal colonies consist of morphologically identical ES cells in morphology (Figure 6.12A). After brief inspection of all colonies, i.e., ES property, compact colony, and appropriate colony diameter (~1,000 cells/colony), I decided to pick up the pure ES colonies (Figure 6.12A) or the central cells of the mixed colonies (Figure 6.12B) and give up the differentiated colonies (Figure 6.12C) or colonies that were small in size (Figure 6.12D), as these colonies were probably “false-positive” ones by PCR-genotyping. Manual colony isolation was done carefully under a stereomicroscope equipped in a cell culture hood by transferring the 50-100 drug-resistant ES colonies into a 96-well plate with preloaded drug containing ESM4 medium. After 7-12 days of culture with regular medium changes, these candidate colonies were split into three 96-well plates; two plates were further used for conducting micro-Southern and PCR screening simultaneously (see PCR-genotyping section), and the third was used as an archive for expanding HR-positive colonies.

Figure 6.12 Phenotype of colonies after positive-negative selection. Single colonies after 20 days of growth were classified as (A) type 1 (smooth, contour, and uniform ES cell phenotype), (B) type 2 (> 80% ES in central area, > 5% differentiated cells at marginal area), (C) type 3 (> 70% differentiated cells) and (D) type 4 (uniform but small colony).

6.5.2 Cell growth and differentiation of targeted clones

Compared to parental MES1 cells, the *p53*-targeted cells showed ideal ES morphology (Figure 6.13A and B), stable growth/proliferation after 300 days of serial subculture, ability of recovery from cryo-preservation (data not shown), and positive for AP staining (Figure 6.13C). RT-PCR was applied to test the gene expression of pluripotency markers that had been previously identified by phylogenetic sequence

analysis [11, 13, 15]. The transcripts of pluripotency genes *oct4*, *nanog*, *klf4*, *myc*, *ronin*, *sall4*, *tcf3a&b*, and *zfp28l* were easily detectable in all targeted clones, and the *p53* transcripts were detectable in heterozygous *p53*^{+/-} MES1 cells (Figure 6.13D).

The *in vitro* pluripotency of *p53*-targeted MES1 cells were assessed in terms of spontaneous, induced, and directed differentiation. Upon subculture at low cell density, the *p53*^{+/-} MES1 cells were capable of spontaneous differentiation into various cell types (data not shown), like the parental MES1 cells. The three-dimensional multicellular aggregates formed during suspension culture of ES cells are embryoid bodies (EBs). The EB formation recapitulates cell differentiation during early embryogenesis and has been widely used for testing *in vitro* differentiation properties of ES cells [105]. After the long-term PNS procedure, the *p53*^{+/-} cells presented ES phenotype, stable growth, and self-renewal, under the culture condition for undifferentiated growth. When these cells were cultured in suspension without anti-differentiation factors and in the presence of All-*trans*-retinoic acid (RA), they could reproducibly form the three-dimensional EBs as the parental MES1 cells (Figure 6.13E). Characterization of differentiation status of EBs has been established in mouse ES cells [106] as well as medaka ES cells [9, 11, 15], in terms of the gene expression pattern of primitive germ layer markers, including genes specific to the ectoderm (*nf200*), the mesoderm (*actinin2* and *brachyury* or *ntl*), and the endoderm (*sox17*), while the pluripotency markers *oct4* and *nanog* were down-regulated to some extent (Figure 6.13H).

Figure 6.13 Pluripotency of *p53*-targeted cells *in vitro*. (A and B) ES morphology of parental MES1 and *p53*-targeted cells. (C) The *p53* targeted ES cells are positive for AP

staining. (D) Expression profile of pluripotency genes by RT-PCR. Blastula, stage-10 embryos as positive control; MES1, parental medaka ES line 1; neg, negative control without cDNA template. *actin* was used as a control. (E) Parental MES1 and *p53*GT cells derived EB by suspension culture, in presence of RA. (F and G) Differentiated cells from 18-day-old embryoid body (EB). (H) Expression of pluripotency genes (*oct4* and *nanog*) and differentiation genes in MES1 and *p53*-GT clones after EB formation. *nf200*, neurofilament 200; *ntl*, no tail; *actn2*, actinin 2.

Furthermore, by using the *Mitf*-directed differentiation approach of MES1 cells [8], the Southern confirmed *p53*-targeted MES1 cells exhibited an ability similar to that of their parental MES1 cells to give rise to fully differentiated melanocytes (Figure 6.14A and B), 6 days after transfection with a vector expressing the microphthalmia transcription factor (*Mitf*) and GFP and puromycin fusion protein. No statistical difference was found in terms of the efficiency of producing pigment cells between parental MES1 and *p53*-targeted MES1 cells (Figure 6.14G), indicating that disruption of *p53* allele via HR reaction does not compromise the pluripotency of medaka ES cells *in vitro*. Taken together, the *p53*-targeted ES cells retained development potential *in vitro*.

Figure 6.14 Mitf-directed differentiation of *p53*-targeted ES cells. Shown is direct differentiation of MES1 *p53* targeted cells. Parental MES1 cells were transfected with

pCVmitfCVpf (a Mitf, GFP, and puromycin fusion protein expressing vector), or pCVpf vector as a control, selected by puromycin and photographed at day 6 post transfection. Introduced mitf expression from pCVmitfCVpf is efficient to direct differentiation into black pigmented melanocytes (stars) (A) and p53-targeted cells (B). The neuron-like (C), branch-like (D and E) and giant (F) cells with pigmentation in some extent can also be found during differentiation. (G) No significant difference was found in the *p53*-targeted cells in terms of efficiencies of gene transfer and Mitf expression-induced pigment cells.

6.5.3 Pluripotency *in vivo*

Chimera formation is the most stringent test for the developmental potential of stem cells *in vivo*. In medaka, the chimeric assay has been well established by transplanting non-cultivated blastomeres [96] and long-term cultivated diploid [10] and haploid [11] ES cells into the albino [10, 96] or see-through blastula host embryos[95]. Here, I tested the pluripotency of *p53*^{+/-} ES cells by chimera formation. After the derivation of a GFP-expressing *p53*^{+/-} ES clone (GT22 clone) by stable gene transfer of the pCVpf (GFP expression) vector, cell transplantation was done as previously described. I found that the *p53*^{+/-} ES cells produced 100% frequency of chimeras by using the GFP signal as an indicator (Figure 6.15A). The GFP-expressing *p53*^{+/-} ES cells were widely distributed into major compartments and organ systems including the eye, heart, trunk, head, pectoral fin, and yolk sac (Figure 6.15A and B), like the parental RFP-expressing MES1 cells transfected by pCVpr (RFP) vector in the co-transplanted embryos (Figure 6.15C). Many of the highly chimeric embryos presented uncompromised development until hatching. Moreover, the terminally differentiated cells from the transplanted GFP-

expressing cells can be found from these chimeric embryos, such as cardiomyocytes in the contracting heart (Figure 6.15C), tail (Figure 6.15D), and eye (Figure 6.15E and F). The distribution pattern was not different between the targeted and parental MES1 cells, by statistical analysis of the distribution of green and red fluorescent cells in chimeras (Figure 6.15G). Thus, the disruption of one *p53* allele in the diploid medaka ES cells by HR did not compromise the pluripotency *in vivo*.

Figure 6.15 Pluripotency of *p53*-targeted ES cells by chime. Embryos at day 3 post fertilization were photographed. GFP-labeled ES cells (GT22 clone) were seen in many

compartments (A and B) and organ systems including the eye, ear, heart, pectoral fin and trunk. (C) Distribution of parental MES1-RFP expressing and p53GT-GFP expressing cells in chimeras of day 3-7 development. (H) Co-distribution of p53GT ES (green) and plasmid-transfected MES1 cells (red) in chimeras of day 3 development. Cells were mixed at 1:1 ratio and transplanted at approximately 200 cells/blastula.

6.6 Discussions

6.6.1 HR frequency in different model organisms

Classical GT technology has long been limited in mouse because of the low frequency of HR and lack of stable ES cells in other species. Most recently, this has been achieved in the rat model [83, 84] after the establishment of authentic rat ES cells by applying the 3i culture system [3, 4]. In general, the HR occurred with a ratio of 1 in 10^6 cells, resulting in difficulty in the detection and identification of targeted clones. From previous studies that mainly conducted on mouse ES cells, the frequency of HR events depends on (1) the sequences of homologous flanking region [77]; (2) targeting vector design, i.e., isogenic versus non-isogenic DNA constructs [75, 77, 107]; (3) chromatin structure (accessibility of the enzymatic machinery, reviewed by [72]; and (4) the cell types, i.e., ES cells versus somatic cells [74, 108].

The efficiency of *p53* gene targeting achieved with the medaka ES cells in four independent gene transfers, using the PNS strategy, was significantly higher (putative HR efficiency 9.3-30.7 per 10^6 cells, or screening rate 22.8% of the PNS-resistant colonies) than those reported in mouse (putative HR efficiency 0.015-0.4 per 10^6 cells, or screening rate 2% of the PNS-resistant colonies, [73, 109-111] and rat ES cell (screening rate 2.23-

2.8%, [83, 84], human ES and induced pluripotent stem (iPS) cells (screening rate 0.14-0.24%, [112, 113] and comparable to the first HR report in human ES (detection rate 27-40%, [82]). The detailed comparison was summarized in Table 6.3. This high frequency of putative HR events in fish non-ES (6-452 per 10^6 cells, [56] and ES cells (8.6-30.7 per 10^6 cells, [52] and this study), which is calculated by the dual-selection survived clones per total number of cells undergone gene transfer, is a noticeable difference between the fish and mammalian models. Note that the gene transfer efficiency in fish cell lines by GeneJuice reagent (5-10%, BV study in this thesis) is 5-10 fold lower than that in mammalian ES cell lines by electroporation (40-60%, [22, 82-84, 111]).

Several factors may account for the remarkable difference in the HR frequency and detection rate between medaka fish and mammalian systems. First, different gene transfer and PNS protocols were utilized in mouse, rat, human, and medaka ES cells. Electroporation is widely used as gene delivery approach in GT experiments [114, 115] because physical methods of introducing DNA are in general more effective than chemical ones such as lipid and cationic reagents [21, 82]. However, the gene delivery method is not an issue for GT in medaka ES system. I used GeneJuice reagent, which is composed of a nontoxic cellular protein and a small amount of a novel polyamine, for routine transient and stable gene transfer, because of its simplicity and satisfactory efficiency. This gave rise to reproducible GT products of *p53* (this study) and *nanos3* (Guan et al., unpublished data) in medaka ES cells and putative HR events in PSM and EPC fish cell lines [56]. Other chemical reagents such as DNAfectin 2200 and electroporation [52] were also applicable for GT experiments in medaka ES cells. It

seems that high efficiency of gene delivery is not a prerequisite for HR process in the fish cell lines.

Second, I showed that PNS procedure can reproducibly enrich the targeted recombinants for an average of 12 fold relative to random integrants, which is at the range of 2-2000 fold enrichment primarily obtained in mouse ES cell model [73, 111], and it is higher than that obtained in a previous attempt in medaka ES cells (4 fold enrichment, [52]). Note that this enrichment factor may vary significantly even from a parallel experiment by targeting different genes in mouse ES cells (20 fold for *int-2* gene while 2000 fold for *hprt* gene by using the similar vectors [73]) or non-ES fish cell lines (1.4-63 fold [56]). The modification of cell plating after gene transfer and intervals of PBS wash and medium change during PNS treatment may account for this further improvement (4 to 12 fold) in MES1 cells [52, 91]. In this study, the primary cell density at seeding is much higher than the previous one ($1-2 \times 10^6$ /dish vs. $0.5-1 \times 10^5$ /dish), and the drug-containing medium refreshment was done more frequently (3-4 days vs. 7-10 days), especially during the first week of the PNS procedure. It is obvious that actively dividing cells by high density plating are more sensitive to the drugs than “quiet” ones by the low density plating, which yielded effective elimination of the non-transgenic and random integrated cells.

6.6.2 Efficient GT in medaka ES cells

One obvious technical improvement achieved in this study is the high detection rate (22.8%) for screening a rare HR event. A substantial high frequency of HR events was observed by PCR-based genotyping, from the pooled cell population after DNA

transfection and drug resistant colonies formed during the PNS procedure. This high detection rate definitely facilitated the fast screening of targeted clones and significantly reduced the scale of testing samples. Several reasons may account for the remarkable increase in HR detection rate in medaka compared to that in rodents. Theoretically, ES cells present high activity of proliferation for self-renewal and thus a high probability for gene replacement between exogenous and endogenous DNA fragments mediated by the cellular enzyme machinery for HR during mitotic division, and whether the genes involved in HR, such as orthology/homology of BRCA2, RAD51, RAD51C, RAD52, RAD54, XRCC3 are highly active in medaka ES cells needs to be further investigation [116]. Previous exploitation in fish EPC and PSM cell lines also pointed to this phenomenon [56]. Technically, careful selection of PNS-resistant colonies by ES properties may be attributed to the high PCR detection rate of HR events. The large number of small-diameter and differentiated colonies formed in PNS were shown to be “false” clones that were negative for PCR-genotyping. It is likely that this manual selection before colony picking-up has favored the detection of targeted clones, and concurrently, excluded those clones with poor clonal growth capacity due to the remainder expression of negative selector *tk* gene by random integration, thus negative for GT products. This finding demonstrates the practical development of GT approach in medaka. Final confirmation of the targeted locus came from the Southern analysis and thus led to a GT efficiency of 22.8% of the PNS-resistant clones for targeted *p53* disruption. This is substantially higher than ~2% efficiency of the *p53* targeting in mouse ES cells [73] and 2.23% of *p53* disruption in rat ES cells [83], and is in the range of 2-80% in mouse ES model [73] regarding the Southern confirmation rate. Thus, simple

gene delivery with GeneJuice reagent and effective PNS for screening the targeted ES clones allow us to simultaneously handle a large number of samples in multi-well plates and further establish the knock-out fish.

6.6.3 Retention of pluripotency

The *p53* gene encodes a tumor suppressor and plays an essential role in multiple organisms conserved from fish to mammals [60]. I assessed the pluripotency of *p53* targeted clones by the cell culture *in vitro* system and chimeric formation *in vivo* system. I found that *p53*^{+/-} cells retained all ES features in terms of ES marker gene expression profiling, inducible differentiation in cell culture, as well as differentiating into various functional cells by chimera formation. None of the six Southern-confirmed *p53*^{+/-} ES clones was found to be knocked off at two loci, and it is understandable that the two alleles of the *p53* gene in the MES1 genome may not produce a null *p53* disruption as the transcripts were still detectable by RT-PCR. The pluripotency of MES1 cells was not compromised by gene replacement, targeting vector integration and long-term PNS procedure. This result was consistent with previous exploitation aiming at establishing a fish ES cell model for GT [52, 56].

Table 6.3. Comparison of gene targeting efficiency from the representative reports.

Group	Species/cell lines	Strain	gene	Homology	Expt.	Cell No.	Single drug selection	Dual drug selection	Enrichment factor	Putative frequency HR	Main findings
Hong [56]	Epithelioma papulosum of carp (EPC) <i>Xiphophorus melanoma</i> (PSM)		<i>Xiphophorus melanoma</i> receptor kinase (<i>Xmrk^Y</i>)	6.5 kb	1. (<i>neo</i> & <i>tk</i>) 2. <i>Xmrk^Y</i> 3. <i>Xmrk^Y</i> 4. <i>Xmrk^Y</i> 5-12. <i>Xmrk^Y</i> Gene transfer by GeneJuice reagent	5.0×10 ⁵ 5.0×10 ⁵ 5.0×10 ⁵ 5.0×10 ⁵ 5.0×10 ⁵	350 424±84 502±13 188±3 85-337	0 17±6 13±2 3 55-226	-- 25 39 63 1.4-2.5	-- 20×10 ⁻⁶ 26×10 ⁻⁶ 6×10 ⁻⁶ 110-452×10 ⁻⁶ PCR screen: 0/108 Southern analyzed: 0/18	1. PNS procedure is feasible in fish cells, however, no targeted clone was obtained in non-ES fish cell lines.
Hong [52]	Medaka diploid ES cell MES1	HB32C	<i>neo</i> , <i>pac</i> , <i>hyg</i> & <i>tk</i>		1. (<i>neo</i> & <i>tk</i>) 2. (<i>pac</i> & <i>tk</i>) Gene transfer by GeneJuice reagent	1.0×10 ⁷ 1.0×10 ⁷	344 68	86 0	4 --	8.6×10 ⁻⁶ --	1. Three positive drug selectors, <i>neomycin</i> , <i>puromycin</i> , <i>hygromycin</i> and one negative selector, <i>tk</i> , were proved to be effective in medaka ES cells. 2. The pluripotency <i>in vitro</i> and <i>in vivo</i> of medaka ES cells were retained after gene transfer and long-term drug selection.
Hong (this study)	Medaka diploid ES cell MES1	HB32C	<i>p53</i>	4.8/ 0.9 kb long/short homology arm	1. 2. 3. 4. Gene transfer by GeneJuice reagent	1.5×10 ⁶ 2.25×10 ⁶ 1.5×10 ⁶ 2.25×10 ⁶	655 490 220 275	46 38 14 41	14.2 12.9 15.7 6.7	30.7×10 ⁻⁶ 16.9×10 ⁻⁶ 9.3×10 ⁻⁶ 18.2×10 ⁻⁶ PCR screen: 19/39 Southern: 15/32 % of Screening: 22.8%	1. PNS strategy gave rise to significant higher HR frequency than mammalian models; High detection rate for PCR and Southern analysis were obtained. 2. Routine gene transfer achieved successfully GT product; electroporation was not required. 3. Disruption of one allele of <i>p53</i> gene in medaka ES cells did not compromise the pluripotency <i>in vitro</i> and <i>in vivo</i> .
Bradley [111]	Mouse ES cells AB1	129/Sv	<i>p53</i>	3.7 kb	1. Gene transfer by electroporation	2.0×10 ⁸	~300	~100	3	0.015×10 ⁻⁶ PCR screen: 2/100 Southern: 2/2 % of Screening: 2%	1. A null mutation was introduced into the <i>p53</i> gene by homologous recombination in mouse ES cells. 2. Germline chimeras were obtained by blastocysts microinjection in C57BL/6 mice, and homozygous mice for a null <i>p53</i> allele were bred and 3. Tumour susceptibility was analyzed using homozygous and heterozygous <i>p53</i> disrupted mice. It showed that a normal <i>p53</i> gene is dispensable for embryonic development and an oncogenic mutant form of <i>p53</i> is not obligatory for spontaneous tumours.
Ying [83]	Rat ES cells DAc8 and DAc4 ES lines derived in 3i medium [4] and GT conducted in 2i medium	DA	<i>p53</i>	6.7/ 1.6 kb long/short homology arm	1. DAc8 2. DAc8 3. DAc8 4. DAc4 5. DAc4 Gene transfer by electroporation	N/A	N/A	N/A	N/A	PCR screen: 1/131 3/225 4/91 3/76 3/103 In total: 14/626 (2.23%) Southern: 14/14 % of Screening: 2.23%	1. Germline chimeras were obtained by blastocysts microinjection in F344 rats. However, the germline animal did not inherit the gene-targeted <i>p53</i> allele, due to the high frequency of chromosomal abnormality observed in the targeted rat ES clones. 2. By purification (subcloning) and karyotyping of the targeted ES clones, the one germline chimeric rat carrying a targeted <i>p53</i> allele was obtained from the euploid subclones. This is an historic achievement that enables targeted genetic engineering in rat ES cells (reviewed by Jacob et al., 2010).
Capecchi [22]	Mouse ES cells	Mouse C57B1/6	<i>hprt</i>	9.1/9.3 kb replacement vector (RV)/insertion vector (IV)	1 (RV) 2 (IV) Gene transfer by electroporation	7.8×10 ⁷ 4.1×10 ⁷	3.0×10 ⁴ 2.25×10 ⁴	32 21	937 1,071	0.4×10 ⁻⁶ 0.5×10 ⁻⁶ Southern analyzed: RV: 23/23 IV: 3/12 HR, 9/12 random integratoin	1. The PNS efficiencies obtained from replacement and insertion targeting vectors indicate that the ratio of HR to non-HR is approximately 1 to 1,000 after introducing the targeting vector into host ES cells. 2. The HR efficiencies of both replacement and insertion vectors are strongly dependent upon the extent of homology between exogenous and endogenous sequences.

Table 6.3. Comparison of gene targeting efficiency from the representative reports (continued).

Group	Species/cell lines	Strain	gene	Homology	Expt.	Cell No.	Single drug selection	Dual drug selection	Enrichment factor	Putative HR frequency	Main findings
Burdon [84]	Rat ES cells RIF5.2 RISD10 2i culture medium	F344 SD	<i>hprt</i>	4.7 kb	1. RIF5.2 2. RIF5.2 3. RISD10 4. RISD10 electroporation	2.7×10 ⁶ 2.7×10 ⁶ 3.0×10 ⁶ 3.0×10 ⁶	58 53 43 60	1 1 3 1	58 53 14.3 60	0.4×10 ⁻⁶ 0.4×10 ⁻⁶ 1.0×10 ⁻⁶ 0.3×10 ⁻⁶ % of Screening: 6/214=2.8%	1.Genome modification via homologous recombination in rat ES cells is effieicnt by using <i>hprt</i> gene as a model. Germline chimera was not reported in this study. 2.Pluripotency in vitro in targeted rat ES cells was retained.
Thomson [82]	Human ES cells		<i>HPRT1</i> <i>Oct4</i> (<i>POU5 F1</i>)	10/ 1.9 kb long/ short homology arm	1. ExGen 50 (cationic) 2. FuGene-6 (lipiod) 3. <i>HPRT1</i> 4. <i>Oct4</i> 5. <i>Oct4</i> (another vector with longer 3' homology)	N/A N/A 1.5×10 ⁷ 1.5×10 ⁷ 	130(G418 ^r)/ 35 (6-TG ^r) 261 (G418 ^r)/ 61 (6-TG ^r) 350 (G418 ^r)/ 50 (G418 ^r +Gc ^r) 103 (G418 ^r) 56 (G418 ^r) by electropora- tion 11 (G418 ^r) by FuGene-6	0 0 7 (G418 ^r + Gc ^r + 6- TG ^r) 	-- -- 50 -- --	-- -- 0.4×10 ⁻⁶ PCR: 7/7 for <i>HPRT1</i> knockout Southern: 28/103 (27%) Southern: 22/56 (40%) 0 HR clone	1. First attempts of GT in human ES cells by employing the chemical and physical gene transfer protocols. 2. The targeting ratios for both two genes in test are comparable to those reported in mouse ES cells, and suggest that although successful transfection strategies differ between human and mouse ES cells, the frequency of homologous recombination itself may be similar.

Chapter 7 General conclusions and Outlook

7.1 General conclusions

The motivations of this study are raised for the establishment of high efficiencies of gene delivery and gene targeting in the lower vertebrate medaka fish model. First, I presented original work on the medakafish as a complementary system to evaluate the potential and challenge of baculoviral (BV) vectors in stem cell-based gene therapy. I showed that the small laboratory fish medaka provides an excellent system for *in vitro* and *in vivo* analyses. BV vectors can give rise to almost 100% of gene delivery in medaka embryonic stem (ES) cells. Surprisingly, BV transduction can also mediate high frequency of gene integration into various chromosomes. Importantly, BV transduction does not compromise genome stability and pluripotency. Therefore, medaka represents a powerful system for analyzing the potential of BV-mediated gene delivery in stem cell biology and gene therapy.

The significance of our work is threefold: (1) a new model system for easy experimentation; (2) a new ability to obtain 100% gene delivery, pointing to the possibility of considerably improving efficiency in mammals as well; and (3) most importantly, my finding that BV can also mediate high efficiency of integration into various chromosomes is in striking contrast to rare integration in the human genome, underscoring the safety issues associated with viral vectors used in human gene therapy.

Second, I showed the feasibility of using BV vector as efficient gene delivery tool in other widely investigated zebrafish and rainbow trout models, indicating its potential for (1) virology for fish disease; (2) cell-based assays; and (3) gene functional studies.

Furthermore, the construction of a bvSPR vector provides the an easily modifiable vector system for (1) directed differentiation of ES cells; (2) stable gene delivery and integration for ES-based gene therapy; and (3) efficient transgenesis for rare genetic events.

Third, I showed that the successful homologous recombination (HR) in medaka as a test organism and the production of ES clones harboring a targeted locus for *p53*, a gene essential for tumor suppression and genome stability. The positive-negative selection (PNS) procedure is an effective approach for enrichment of homologous recombinants versus random integrants in medaka ES model. By PCR-based genotyping, Southern analysis, and DNA sequencing, the relative and absolute GT efficiencies are 22.8% and 2×10^{-6} , respectively, which provide us a highly efficient ES model for precise genome manipulation. I also showed that *p53* disruption and long-term propagation under drug selection do not compromise pluripotency, as *p53*-targeted ES cells retained stable growth, undifferentiated ES cell phenotype and potential of differentiation *in vitro* and *in vivo*. These results demonstrated that medaka ES cell is a versatile lower vertebrate model organism for GT towards knock-out animal production in species outside rodents.

7.2 Outlook

7.2.1 Direct phenotypic analysis of targeted null mutation in haploid cell

Thanks to the generation of medaka haploid ES cells [11], it offers an excellent yeast-like system for direct genetic analyses of molecular events in various cell lineages *in vitro*. Any recessive mutation of essential genes, e.g., tumor suppression and pluripotency, will show a clear phenotype in the absence of a second gene copy. Currently, I am working on the possibility of establishing efficient GT and direct

phenotypic analysis in medaka haploid ES cell model. The phenotypic analysis of loss-of-function disruption can be demonstrated by *p53* gene replacement in medaka haploid ES cell line HX1. *p53* is present in a wide variety of organisms and is best known for its conserved role as a tumor suppressor by safeguarding genetic integrity and controlling cell proliferation or survival. *p53* is expressed in both medaka diploid and haploid ES cells. By performing HR-mediated GT in HX1 cells and screening individual cell clones using PCR assays, I can see that wild-type HX1 cells reproducibly give rise to a *p53*-specific cDNA PCR (cPCR) product, whereas homologous recombinants provide a genomic PCR (gPCR) product specific to the targeted locus and become negative for cPCR. This produces 38% GT efficiency. This correct targeted mutation is validated by Southern blot analyses and sequencing.

Haploid ES cells with a single GT event are equivalent to diploid ES cells with both alleles targeted and thus allow for direct analysis of the null phenotype. The phenotypes of *p53* null mutation can be directly analyzed in cell culture *in vitro* through the loss-of-function and gain-of-function approaches. For this, a series concentration of 50, 100 and 200 $\mu\text{mol/L}$ hydrogen peroxide (H_2O_2) is used to induce cellular senescence in HX1 cells. In the presence of H_2O_2 , control HX1 cells exhibit cell growth arrest and senescence, whereas *p53*-null cells present continuous growth and lack of senescence. Moreover, the introduction of an expression plasmid containing the wild-type medaka *p53* gene into *p53*-null ES cells can restore growth arrest and senescence in response to H_2O_2 treatment.

In this on-going project, I have established the high efficiency of GT and clear null phenotypes by making use of haploid ES cells in medaka as a first organism for haploid genetic modifications and analyses. This approach features yeast-like simplicity and

speed. Starting with GT vectors, a whole GT experiment from cell transfection to phenotypic analyses can be achieved within 3 months. Homologous recombination activity exists from bacteria to human [21]. I anticipate that this one-step knockout approach will find its application in several other organisms including *Drosophila*, frog, and human, where haploid cell cultures are available [117-119].

7.2.2 Generation of knock-out fish by combination of nuclear transplantation and HR-mediated GT in ES cells

In fact, GT is a long-desired technology in fish. The ES cells accessible for genetic manipulation and long-term drug selection have been available in medaka, although the germline transmission by ES cell transplantation has not been achieved in this species. Nevertheless, the breakthrough of nuclear transplantation (NT) gave us an alternative to the germline transmission problem in the medaka model. The successful production of clonal medakafish by NT was reported from freshly isolated embryonic cells into enucleated oocyte [120], or nonnucleated and diploidized egg [121] and most recently, by direct NT of haploid ES cell into normal oocyte [11]. The future of knock-out technology by a combination of NT and ES technology in medaka is promising, when the HR of targeting locus in ES cells is accomplished [7, 122].

Abbreviations

aa	amino acid
AAV	adeno-associated virus
AP	alkaline phosphatase
BCIP/NBT	5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium salt
bFGF	basic fibroblast growth factor
bp	base pair
BV	baculoviral
cDNA	complementary deoxyribonucleic acid
CDS	coding sequence
CMV	cytomegalovirus
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DMEM	Dulbecco's modified essential media
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EB	embryoid body
EDTA	ethylene diamine tetraacetic acid
EF1 α	human elongation factor 1 α promoter
ES	embryonic stem
FBS	fetal bovine serum

FISH	fluorescent <i>in situ</i> hybridization
GFP	green fluorescent protein
Gc	ganciclovior
G418	geneticin, derivative from gentamicin
GT	gene targeting
hfp	hours post fertilization
HR	homologous recombination
HX1	haploid X-chromosome-carrying cell line 1
iPS	induced pluripotent stem cells
IPTG	isopropylthio- β -D-galactoside
ITR	inverted terminal repeat
kb	kilo base
LB	Luria-bertani medium
MES1	medaka embryonic stem cell line 1
Mitf	microphthalmia transcription factor
MMLV-RT	moloney murine leukemia virus reverse transcriptase
MO	morpholino oligo
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
nt	nucleotide
NT	nuclear transplantation
<i>Ol</i>	<i>Oryzias latipes</i>
ORF	open reading frame

PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
PI	propidium iodide
PNS	positive negative selection
POD	peroxidase enzyme
RA	<i>All-trans</i> -retinoic acid
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
SC	semi-cloning
SDS	sodium dodecyl sulfate
SG3	spermatogonial cell line from i ³ strain
SSC	sodium chloride tri-sodium citrate solution
tk	thymidine kinase
TILLING	<u>T</u> argeting <u>I</u> nduced <u>L</u> ocal <u>L</u> esions <u>I</u> N <u>G</u> enomes
UV	ultraviolet
WPRE	wood-chuck hepatitis post-transcriptional regulatory element
X-gal	5-bromo-4-chloro-3-indoyl- β -D-galactoside

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