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SOMATIC CELL NUCLEAR TRANSFER IN ZEBRAFISH

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SOMATIC CELL NUCLEAR TRANSFER IN ZEBRAFISH

By

Kannika Siripattarapravat

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ABSTRACT

SOMATIC CELL NUCLEAR TRANSFER IN ZEBRAFISH

By

Kannika Siripattarapravat

Zebrafish (*Danio rerio*) have been recognized as one of the best model organisms for the study of developmental biology and human diseases. As of yet, the utilization of zebrafish has not reached its promise. To fulfill this potential, a methodology to generate conditional knock-in/ -out must be developed. Somatic cell nuclear transfer (SCNT) is a potential approach to produce genetically-modified zebrafish. This can be accomplished by transferring gene-targeted cells into enucleated eggs. The primary focus of this dissertation is to improve the efficiency of SCNT. Subsequently, the use of SCNT technology may be extended to enhance the use of zebrafish as a vertebrate animal model. Three important parameters have been characterized and optimized to meet such a goal: recipient eggs, nuclear transfer technique, and cultured donor cells. While the zebrafish cloning technique has been published, it is highly inefficient. Moreover, the existing protocol is difficult to replicate, likely due to poor characterization of zebrafish egg physiology at the time of nuclear transfer. We have demonstrated that, following egg activation, eggs undergo dynamic changes in cell cycle stages and that is likely to affect cloning efficiency. To improve upon this, we implemented a technique in which recipient eggs can be maintained *in vitro* at metaphase II of meiosis (MII) stage in Chinook salmon ovarian fluid. This should provide a uniform source of recipient eggs for SCNT. Accordingly, we have developed a reliable

SCNT protocol that overcomes the challenge of using zebrafish MII eggs with intact chorion as recipient cells for SCNT, by using laser-assisted inactivation of egg genome and micropyle for transfer of the nucleus. This technique has been validated by using phenotypic screening, karyotyping, and genotyping of cloned zebrafish produced. Cloned zebrafish are normal healthy individuals, and go on to produce thousands of healthy offspring. The SCNT technique can be used to produce clones from the major strains of zebrafish used in the research community. Additionally, we have showed that zebrafish SCNT can be used to investigate the influence of donor cell sources on cloning efficiency. By using transgenic fish that express tissue specific green fluorescence protein (GFP) as sources of donor cells, we have found that the type of donor cells used in SCNT influences the developmental capacity of the cloned fish from the blastula stage up to 4 days. In parallel, we have done extensive work to optimize the *in vitro* culture conditions for zebrafish cells, and described new cell culture and DNA transfection protocols for cultured cells. We explored the possibility of increasing SCNT efficiency by modifying the donor nuclei using histone deacetylase (HDAC) inhibitors. Our SCNT model can be further implemented in combination with existing technology to facilitate gene knock-in/ -out experiments in zebrafish. The ultimate goal is to enhance its prominent role as an animal model for human diseases.

DEDICATION

To my beloved family: my parents, sisters and brother.

To my father, Pichit Siripattarapravat, who has always believed the best thing he can give me is his support toward achieving the highest level of education. As a consequence, this has been my primary motivation for earning a PhD.

To my mother, Viyada Siripattarapravat, who has always given me strength from her unreserved love and encouragement.

To my sisters and brother whom always have stood by me.

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LIST OF ABBREVIATIONS

AB	Zebrafish strain AB
AF	Adult caudal fin fibroblasts
A2P	L-ascorbic acid 2-phosphate sesquimagnesium salt
BAPTA	1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid
Cdc2p	Phosphorylated cell division cycle 2 (Cyclin dependent kinase)
ClnB	Cyclin B
CSOF	Chinook salmon ovarian fluid
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dpNT	day post-nuclear transfer
EB	Zebrafish embryo at 90% epiboly stage
EE	Zebrafish embryonic extracts
ENU	N-Ethyl-N-Nitrosourea
ES cells	Embryonic stem cells
ET	Embryonic tailbud
FBS	Fetal bovine serum
GFP	Green fluorescence protein
GR	Zebrafish embryo at germ ring stage
H-BSA	Hank's balance salt solution with 0.5% bovine serum albumin
HDAC	Histone deacetylase
hpf	hour post-fertilization

hpC	hour post-collection
hpNT	hour post-nuclear transfer
HR	Homologous recombination
H2AzGFP	Histone H2A tagged with GFP
IVF	*In vitro* fertilization
K	Keratinocyte SFM
MII	mature-arrested egg at metaphase II of meiosis
MO	Morpholinos
MPF	Maturation promoting factor
NAC	N-Acetyl-L-Cysteine
pA	Polyadenylation signal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RACE	Random amplification of complementary DNA ends
RNA	Ribonucleic acid
SA	Splice acceptor
SCNT	Somatic cell nuclear transfer
SD	Splice donor
SNP	Single-nucleotide polymorphism
TAB	Outcrossing Tuebingen and AB strains
TS	Trout serum
TSA	Trichostatin A

Tu	Zebrafish strain tuebingen
TuLF	Zebrafish strain tuebingen – long fin
VPA	Valproic acid
WT	Wild type zebrafish
ZFN	Zinc finger nuclease

INTRODUCTION

During embryonic development, cells get progressively committed to a specific tissue without taking a reverse path or switching between cell lineages (de-differentiation or trans-differentiation, respectively). However, with somatic cell nuclear transfer–cloning (Gurdon and Uehlinger, 1966; Wilmut et al., 1997), the committed cells can be reprogrammed to reacquire the epigenetic signature of a pluripotent cell. The donor cell subjected to somatic cell nuclear transfer will regain its ability to self-renew and differentiate into every cell type, ultimately developing into a whole organism in a process known as nuclear reprogramming. The results of nuclear reprogramming experiments have confirmed the hypothesis that as a cell differentiates its epigenetic status changes while its genomic DNA remains unaltered.

The nuclear reprogramming process involves substantial chromatin remodeling including, but not limited to, histone methylation, acetylation and DNA demethylation, culminating with alteration of gene expression pattern, characteristic of tissue-specific ones, to that of an embryonic cell. Since this is probably the most challenging adaptive assignment for a cell, it is not successful in most cases, accounting for the low efficiency of cloned animal production, approximately 1-5%, across species (Cibelli, 2007). A few of the factors that can influence the success of nuclear reprogramming include factors present in the oocyte, the type of somatic cells used as nuclear donors and the *in vitro* micro-manipulation of both donor and recipient cells.

Due to its many favorable characteristics, the zebrafish is a widely used model for studies of human diseases and developmental biology. In addition to being

phylogenetically closer to humans in comparison to flies and worms, the zebrafish also possesses a short generation interval, high fecundity, transparent embryos, low husbandry cost and amenability to large-scale phenotypic screening. In addition, zebrafish share organ similarities to those of mammals, and mutant phenotypes that resemble human genetic diseases.

In comparison to the mouse-model, however, homologous recombination has not been successfully implemented in zebrafish, in part, due to the lack of a robust system for derivation of embryonic stem cells – a practical approach for generating knock-in/ -out mice. To utilize zebrafish model systems at parity with more labor-intensive but more common rodent model systems, a reliable and simple method for reverse genetics is necessary. Up until now, only one group has reported the production of germline-competent embryonic stem cells with the capacity to undergo gene targeting in zebrafish (Fan et al., 2004b; Ma et al., 2001); however nearly a decade after their publication, there is no report yet on the generation of germline-competent founder fish using the aforementioned approach.

Somatic cell nuclear transfer (SCNT) has the potential to become the method of choice for germ-line genetic modification in zebrafish. The first successful report of cloned zebrafish was published in 2002 using cultured cells derived from embryos at 5-15 somite stages (Lee et al., 2002). However, the efficiency of cloned fish production over the total number of eggs manipulated has remained at 2% or less. Since the publication, no cloned fish has been produced by the described technique.

In this dissertation, current approaches for genetic modification of zebrafish including the generation of knock-in and knock-out fish are discussed. A novel approach for somatic cell nuclear transfer in zebrafish is described. Also the major parameters that influence the success of cloning such as the condition of donor nuclei and recipient oocytes including pretreatment of oocytes with compounds to maintain and/or enhance their reprogramming capacity are investigated. Finally, the use of donor cells isolated from different cell lineages is explored.

Chapter 1 is a literature review where we justify zebrafish use as a model organism. We review current approaches and tools for transgenesis and mutagenesis. More specifically to SCNT, we discuss the different recipient cells used in the procedure.

Chapter 2 focuses on *in vitro* handling of zebrafish cultured donor-cells and the optimization of *in vitro* culture conditions and DNA transfection. This chapter includes culture medium and supplements for zebrafish cells, major characteristics of the cultured cells, and transfection efficiency. I describe our attempts to alter the epigenetic configuration of donor nuclei to facilitate nuclear reprogramming. The epigenetic status of zebrafish cells derived from both embryo and adult tissue is also addressed. Trichostatin A and Valproic acid, the small molecules, were evaluated for their effects on epigenetic modification to improve nuclear reprogramming efficiency.

Chapter 3 focuses on characterization of maturation promoting factor as well as modulation of its activity in zebrafish eggs. An ideal cell cycle stage of the recipient egg for somatic cell nuclear transfer is described. This chapter also addresses the differences of zebrafish eggs before and after activation and ultimately provides a key success of the

cloning technique described in Chapter 4.

Chapter 4 describes a novel methodology for somatic cell nuclear transfer in zebrafish that is able to produce cloned fish in a routine basis. The approach employs non-activated recipient eggs, laser-firing enucleation, and nuclear transfer through a micropyle. This novel approach overcomes challenges of the first cloning technique reported that include extending time of manipulation, ensuring removal of egg genome, reducing technical difficulties due to handling de-chorinated eggs and fragile reconstructed embryos.

Chapter 5 describes the type of donor cell that provides high efficiency of SCNT in zebrafish. In this chapter we address the hypothesis that the capacity of a cell to be reprogrammed depends upon the type of cell used as a nuclear donor. Zebrafish SCNT efficiency was evaluated using different donor cells isolated from 5 different tissues. In doing so, the rate of cloned fish production from embryonic donors derived from 5 different transgenic strains were compared; HGn62A–skin, HGn28A–skin, HGn8E–heart, HG21C–fin and notochord, and HGn30A–hatch gland.

The last chapter is the conclusion and future direction of our work. We summarize the work done by us and others, and discuss the possible next steps for this area of study.

The multitude of favorable attributes held by zebrafish, coupled with the recent successful nuclear transfer using long-term-cultured cells make this vertebrate the prime candidate for gene loss-of-function studies. The work described in this dissertation includes the development of an efficient system for nuclear transfer of

zebrafish using somatic cells, a technology that may enable the creation of gene knock-out/ -in models. Taken together the studies help provide a more clear direction that should be taken in working to improve efficiency of nuclear transfer cloning in zebrafish.

CHAPTER 1

LITERATURE REVIEW

A. Zebrafish as a model organism

Zebrafish, *Danio rerio,* belong to the Cyprinidae family, the same as carps and minnows. Adult zebrafish are relatively small fresh water fish, approximately 3-4 centimeters in length, allowing them to be kept in a simple aquarium system in almost any laboratory. The embryos are transparent and develop *ex vivo,* making it possible to study them in detail from fertilization to hatching. Zebrafish are native to the tropical climate of Asian countries, and were first identified in India (Engeszer et al., 2007). They were extensively promoted as a model organism by George Streisinger at the University of Oregon and quickly embraced by the world's community of developmental biologists. As the zebrafish model gained in popularity, the zebrafish research community created the 'Zebrafish Information Network' (ZFIN) that contains a variety of essential information on this model organism. There are many fish strains that are utilized in the research community, the more popular ones being Tuebingen, AB, and wildtype.

As a laboratory animal the zebrafish possesses many distinct advantages over the mouse and can be considered one of the best animal models for developmental studies, particularly if gene loss of function studies were available. Zebrafish represents a vertebrate that can serves as a convenient, relatively inexpensive, and useful model for the study of normal and pathological animal development, physiology, aging, cell death, and disease (Beis and Stainier, 2006; Berghmans et al., 2005; Kishi S, 2002; Pyati et al., 2007). Desirable characteristics possessed by zebrafish include its fecundity,

external fertilization, rapid embryonic development, and a short generation interval (Nüsslein-Volhard and Dahm, 2002; Zon, 1999). Furthermore, zebrafish eggs are large and transparent, facilitating DNA injection, cell labeling, and transplantation experiments (Lee et al., 2002). Despite the lack of a reliable reverse-genetic system, the aforementioned beneficial traits found in zebrafish have promoted the extension of large-scale mutagenesis screening of this organism. Taken together, these features make zebrafish a great complement to *D.melanogaster* and *C.elegans* for developmental biology studies (Amsterdam et al., 2004; Driever et al., 1996; Haffter P, 1996) and become the widely used vertebrate.

The overarching theme of this dissertation is the development of new tools and reagents that can make zebrafish a more powerful animal model to understand human disease. One of the current shortcomings of the zebrafish as a model is the inability to perform reverse genetic studies. Our objective is to demonstrate that by performing homologous recombination in cultured somatic cells and later using somatic cell nuclear transfer (SCNT) with those cells, fertile cloned offspring can be generated.

A careful review of the data involving nuclear transfer experiments of all the species cloned to date revealed that small improvement has occurred since the original reports of SCNT (Thuan et al., 2010). The most important impediment to the development of new protocols is the lack of a suitable system that allows SCNT experiments on a large scale, at low cost, with a reasonably short endpoint, and readily-available reagents. Considering all these requirements, it is not surprising that mammals with long gestation periods or high per diem costs have not contributed to rapid

advancement of the field. Zebrafish can be the ideal model to investigate factors that influence efficiency of nuclear transfer-cloning.

B. Methods for transgenesis, forward, and reverse genetics in zebrafish

Since the successful introduction of zebrafish as a model system, the number of transgenic zebrafish has grown exponentially in the past decades. According to the zebrafish information network (ZFIN), to date, there are thousands of transgenic zebrafish expressing fluorescence proteins (Sprague et al., 2008). Zebrafish embryos are transparent, enabling delivery of transgenes easily by direct injection. The approaches reviewed below are some of the most utilized by the research community.

i. Transgenesis

- **Plasmid DNA injection and Meganuclease *I-SceI***

Delivery of plasmid DNA to one-cell embryos has become a common approach to generating transgenic zebrafish (Nüsslein-Volhard and Dahm, 2002). It consists of simply injecting the plasmid DNA into embryos and later identifying the transgenic animals. However the rate of transgenesis is low, and its expression is unpredictable. Transgenesis rate can be improved by using *I-SceI* meganuclease-mediated transgenesis (Grabher et al., 2004; Thermes et al., 2002). This is done by modification of the plasmid construct: having the transgene flanked by insulators and *I-SceI* recognition sequences (18 bp), and delivery by injection of the plasmid DNA together with *I-SceI* enzyme to one-cell embryos. It is still unclear how *I-SceI* improves transgenesis, yet it facilitates functional integration of the transgene and stable germline integration.

- **mRNA transfer for transient expression and caged mRNA**

The injection of mRNA to produce transient expression of transgene in embryos is well described (Nüsslein-Volhard and Dahm, 2002). The transgene can be expressed instantly once delivered. The mRNA is commonly produced by *in vitro* transcription (the most widely used is Mmessage Mmachine SP6 kit, from Ambion), as capped mRNA. This transient effect of transgenes can be used for many purposes such as the study of transient effect of the transgene at a particular developmental stage, rescue of the phenotype of mutant embryos, delivery of functional genes, i.e., transposase enzyme for transposon-mediated mutagenesis and direct gene targeting by zinc finger nucleases, among others.

The recent development known as photo-mediated gene activation using caged mRNA, allows for more control over time as well as site of gene activation following mRNA transfer (Ando et al., 2001). In this technique, the translation of caged RNA is inhibited by a photo-removable protecting group. Depending on the types of caging molecules, the caged RNA can be reactivated by a specific wavelength of UV light following a photolysis of caging molecules (Kao, 2006). When the UV light is introduced at a specific location/tissue, mRNA is released for translation. Thus, it allows for studies of gain-of-function in a time and specific place. Note that the caged RNA is more stable than mRNA, and can be maintained up to 17 hours after RNA transfer, facilitating cell tracing studies (Ando et al., 2001).

ii. ***Random mutagenesis***

Forward genetics is routinely performed in zebrafish and consists of studying phenotypes prior to the identification of the responsible gene. This is facilitated, in part, by many of the desirable characteristics possessed by zebrafish, including its fecundity, external fertilization, rapid embryonic development and a short generation interval. Despite the lack of a reliable reverse-genetic approaches – those in which a mutation of a specific gene is induced and later its phenotype studied – forward genetics coupled with the aforementioned beneficial traits found in zebrafish, have promoted the study of large-scale mutagenesis in this organism, making zebrafish the first vertebrate used in these types of studies, and therefore, a great complement to *D.melanogaster* and *C.elegans* models.

Random mutagenesis approaches, while successful in generating mutant fish, usually calls for large-scale screenings that are laborious, costly and time consuming. In addition, not all mutants produced display a phenotype, as the mutagenesis often causes null phenotypes.

- **Random point mutation by ENU**

Germ line mutagenesis in zebrafish can be done by chemical means. N-Ethyl-N-Nitrosourea (ENU) is an alkylating agent that generates random mutations in the germline. This chemical mutagen is widely used in the zebrafish research community (Grunwald and Streisinger, 1992; Solnica-Krezel et al., 1994). The mutagenesis process calls for consecutive treatment of adult-male zebrafish with ENU to generate random point mutations in spermatogonia. The treated fish, carrying mutated sperm, is then

bred to produce mutant offspring. The mutated genes can be later identified in the offspring by positional cloning, candidate gene approach, or target-selected screening as described below. Because founder fish contain mosaic mutations among their spermatogonia, thousands of mutant lines can be generated using this approach (Haffter et al., 1996). The sperm of treated male fish can be stored frozen in a library prior to the retrieval of affected gene(s) information following the phenotypic screening. Unfortunately the mutated genes are not easily identified, demanding extensive molecular screenings that are laborious and time consuming (Driever et al., 1996; Haffter et al., 1996). Most of the point mutations generated result in silent rather than missense or nonsense mutation, thus producing a null phenotype in F1 generations that cannot be recovered. Nonetheless, to date, more than 200 mutant lines with developmental phenotypes from ENU mutagenesis have been identified (Amsterdam and Hopkins, 2006).

- **Insertional mutagenesis by pseudotyped retrovirus**

Retroviral-mediated insertional mutagenesis has been proposed and successfully launched by the Hopkins Lab (Lin et al., 1994). This approach utilizes the nature of retrovirus infection to assist mutagenesis, meaning that the RNA virus can convert itself to DNA (provirus) and randomly integrate its genome to an infected hosts' DNA. The retrovirus is tropism, requiring compatibility of its envelop-proteins and the host receptor for infection. The pseudotyped retrovirus is genetically engineered to be replication-defective and to accommodate high infectivity in zebrafish cells. It utilizes viral genome and core proteins of Moloney murine leukemia virus, and the envelope

glyco-protein of a pantropic vesicular stomatitis virus. The pseudotyped retrovirus can be delivered to zebrafish embryos at the blastula stage, which then generates mosaic mutations in germ cells of each founder animal. The progeny of each founder animal can inherit many different insertional mutations. However, only 1/3 of the viral inserts result in gene disruption (Golling et al., 2002). In addition to its remarkable transgenesis rate, the provirus also provides a landmark for screening mutated genes in the host genome since the inserted genes can be recognized by a 5'- or 3'- random amplification of complementary DNA ends (RACE). To date, there are more than 300 mutants recovered from this mutagenesis approach (Amsterdam and Hopkins, 2006; Amsterdam et al., 2004).

- **Transposable element: gene trapping and gene breaking**

Recently, two transposon systems have been successfully introduced for mutagenesis in zebrafish. The two most popular are the medaka *Tol2* transposable element (Kawakami and Shima, 1999), and the Sleeping beauty (Davidson et al., 2003). Both systems vary in the origin of the transposable elements, yet their mechanism of action is based on the same principle. The transposable elements, characterized by flanked DNA with inverted repeats, are capable of moving from one locus to another in the presence of transposase. The insertional mutagenesis can then be introduced by delivery of transposase mRNA together with the transposable element through direct injection to embryos. The trapped gene can also be identified by 5'- or 3'-RACE. Similar to most insertional mutagenesis, the random integration usually causes a null phenotype, as the inserts land mostly in the intronic sequences. Many attempts have

been made to increase the disruption of the gene, among those included 3' or 5' gene trapping and gene breaking system (Balciunas et al., 2004; Kawakami et al., 2004; Nagayoshi et al., 2008).

When gene trapping is used as an aid, the 5' gene trapping system (or promoter trap) is simply done by including the splice acceptor (SA) sequences at the 5'end of an insert cassette, a reporter gene such as green fluorescence protein (GFP) in the middle, and the polyadenylation (pA) signal at the 3'end of an insert cassette. This results in expression of the reporter gene under the endogenous promoter of the trapped gene. Hence the reporter gene only expresses if the transposable element lands in the region downstream of an active promoter. The trapped gene is commonly disrupted its gene expression as a reporter gene is followed by the pA signal. Mutants can be easily sorted by the expression of the reporter gene. However, only 1/3 of reporter loci are in the correct reading frame and able to express functional reporters.

The 3' gene trapping system is done in a similar manner, by including an exogenous promoter that drives the reporter gene expression, and following with splice donor (SD) sequences. This system utilizes the pA signal of a trapped host-gene for transcriptional termination. The exogenous promoter provided upstream of a reporter gene ensures a correct reading frame and proper expression of the gene. However, targeting the 3' end of a gene usually does not disrupt the function of trapped genes, and commonly causes a null phenotype. In addition, the transcripts are known to be unstable, with a highly compromised reporter gene expression.

Gene breaking system combines a gene finding cassette and a mutagenic cassette (Sivasubbu et al., 2006). The gene breaking cassette contains an SA, pA signal, exogenous promoter, reporter gene, and SD from 5' to 3', respectively. The 5'end of the cassette, containing the SA and pA signal, is used to trap the promoter and to terminate the endogenous transcript. The 3' end, containing independent expression of a reporter gene and SD, can ensure gene trapping. The mutants can be selected by expression of a reporter gene. Sivasubbu, *et al.* (2006) reported 53% insertional mutagenesis into functional genes using this system. As gene trapping and gene breaking technology contain a reporter gene, they do not require phenotype-driven insertional mutagenesis screening.

iii. Gene-targeting mutagenesis

The completion of the human genome project has accelerated study of functional genomics in humans, as well as other vertebrate systems. As the zebrafish genome sequencing project is ending, many zebrafish orthologs of human genes have been identified, increasing the demand for studies of functional genomics in this vertebrate model system. Reverse genetics approaches are possible in zebrafish, however, much improvement is necessary. The technologies reviewed below have been widely used in the zebrafish research community and are an attempt to efficiently implement the forward genetics approaches described.

- **TILLING**

Targeting Induced Local Lesions In Genomes (TILLING) utilizes the existing mutant library of ENU-induced mutagenesis, as described above, to perform reverse

genetic studies (Wienholds et al., 2003b). This approach is called target-selected gene inactivation instead of gene-targeting. The study begins with the identification of desired mutated-genes, then searches the mutants from the ENU-mutant library, and later utilizes the desired mutant in the study. The point mutation in a desired gene can simply be identified by hybridization of DNA of the mutant with that of a wild-type animal. The heteroduplex DNA, if mismatched, can be recognized and digested by endonuclease, *Cel* I. Later, the mismatch can be diagnosed using high-resolution denaturing polyacrylamide gel electrophoresis. Alternatively, DNA sequencing of the region of interest can be used to identify the mutation. It is possible that the point mutation in desired genes may give a null phenotype. However, since the gene-targeting approach is not yet available in zebrafish, TILLING is known as the most utilized knock-out approach to date. A good example of a mutant identified by this approach is the *dicer1* mutant (Giraldez et al., 2005; Wienholds et al., 2003a).

- **RNA interference**

RNA interference (RNAi) technology has been utilized widely for *in vitro* cultured cells. The RNAi is designed to have its short RNA sequences complement the targeted transcript (antisense). Upon delivery to the cells, it binds specifically to targets, makes double stranded RNA, and subsequently causes destruction of transcripts or inhibition of translation. As a result, the specific expression of a given gene is temporarily knocked-down. This approach has been successfully implemented in studies of functional genomics in mammalian cells. Despite the successful application of RNAi-based technology to *in vivo* nematode model systems (Fire et al., 1998), in zebrafish, the

RNAi seems to cause non-specific destruction of RNA *in vivo* (Gruber et al., 2005; Li et al., 2000; Zhao et al., 2001).

- **Morpholinos**

An alternative antisense technology has been successfully implemented to knock-down gene expression in zebrafish (Nasevicius and Ekker, 2000). Morpholinos (MO) are oligonucleotides that contain morpholine instead of riboside moiety, and phosphorodiamidate instead of phosphodiester linkages. This replacement stabilizes MOs and its function for up to 120 hours post-fertilization *in vivo* (Smart et al., 2004). The targeted gene knock-down by MO is mediated by a steric blocking of translation followed by destruction of transcripts, hence it has less non-specific effect than small interference RNA. MO can be delivered to embryos or adult fish in a tissue specific manner. Since the knockout fish is not readily available, most of the functional genomic studies in zebrafish have utilized MO. The advanced technology, adopted from caged RNA, allows for a conditional knockdown of the desired gene (Shestopalov et al., 2007).

- **Target gene inactivation in ES cells and chimeras**

The study of reverse genetics using embryonic stem (ES) cells – chimera system has been extensively conducted in mice. Knockout mice are routinely generated using gene targeting homologous recombination in ES cells, followed by the production of germline chimeric founder animals. The ES cell-chimera technology has allowed for the disruption of thousands of genes in mice and provides a valuable tool for studying gene function. By definition, ES cells have three important characteristics: they are capable of renewing themselves indefinitely, differentiation into derivatives of the three germ

layers, and generating germ-line competent chimeras. In zebrafish, the derivation of ES cells has been successful (Ma et al., 2001). The reported cells meet the defined characteristics and are capable of contribution to the germ line of chimeric fish, albeit a decline in the rate of germ line contribution over ES cell passages *in vitro* was reported. Gene-targeting by homologous recombination in zebrafish ES cells was also achieved (Fan et al., 2006), and the mutant ES cells can be preserved as frozen cells for a long period of time. Despite substantial efforts to date, however, there are no reports of a knockout zebrafish produced by this approach. This is possibly due to (i) the rate of germ-line contribution of the ES cells being initially low, (ii) the ES cells have undergone too many passages *in vitro* for the positive/ negative selection, or (iii) the homologous recombination event is rare in the zebrafish ES cells.

- **Gene knock-out/-in followed by fish cloning using SCNT**

In those species in which ES cells are not a feasible approach to make knock-out/-in animals, somatic cell nuclear transfer (SCNT) can become a method of choice for germ line genetic modifications (Kuroiwa et al., 2002; Lai et al., 2002; McCreath et al., 2000; Richt et al., 2007). This approach calls for gene-targeting by homologous recombination in cultured cells, and subsequently, transferring the mutant nuclei into recipient eggs. As a result, cloned animals will be mutants, just as their donor cell. One drawback of this technique in vertebrates is its inefficiency that in turn negatively impacts the overall cost. The first cloned zebrafish produced by SCNT using long-term-cultured cells was reported in 2002 (Lee et al., 2002), opening the possibility to use cultured cells to perform gene targeting followed by nuclear transfer. Unfortunately, the

technique as described by Lee and collaborators did not work in our hands and those of others. More work was required to optimize SCNT in zebrafish.

- **Zinc-finger nucleases**

Zinc-finger nuclease (ZFN) technology has been introduced to increase the efficiency of homologous recombination in cultured cells (Perez et al., 2008; Santiago et al., 2008; Urnov et al., 2005). ZFN proteins are genetically engineered to have zinc-finger subunits for recognition of specific DNA sequences of the target locus, and an associated restriction endonuclease for generating double strand breaks (usually referred to as lesions) at the targeted DNA site (Kim et al., 1996; Smith et al., 2000). The activity of ZFN depends upon a formation of a heteroduplex of two endonuclease subunits. Zinc-finger proteins of each subunit are designed to bind to sequences flanking a target locus which accommodates interaction of its associated endonuclease subunits. Note that the number of zinc-finger proteins constructed correlates to the specificity of binding to the targeted locus. Only when two units of zinc-finger proteins bind to a correct locus, and are in close proximity to each other, will the endonuclease subunits form an active heteroduplex, and subsequently introduce a lesion to the target locus. The mutations introduced by ZFNs is target specific, and since ,in most cases, DNA repair following ZFN-induced double strand breaks is by non-homologous ends joining, a mutation is produced. The lesions can be either deletion or insertion, ultimately causing disruption of the target gene.

Recently, the ZFN technology has been utilized in germ-line gene targeting in zebrafish (Doyon et al., 2008; Meng et al., 2008). It is done by mRNA transfer of two ZFN

subunits into one-cell embryos. Although, the mutant animal normally harbors mosaic lesions, its germ cells are usually affected with a single lesion. Indeed, its mutant progeny bears the same lesion. As of yet, the combination of ZFNs and DNA repairing by homologous recombination has not been reported in zebrafish.

iv. Conclusion remarks for transgenesis and mutagenesis

In summary, substantial effort has been put towards implementation of methods for transgenesis and mutagenesis in zebrafish, broadening the appeal of this animal as a reliable model of human disease and development. However, each one of the techniques described above has its own limitations and numerous factors should be taken into consideration when implementing them (Table 1). To date, random mutagenesis seems to be the best approach to produce mutant fish lines. Nonetheless, large-scale screening is very laborious and, more importantly, gene targeting is not possible.

In order to truly understand gene function, gene targeting, i.e. knock-in or knock-out, is the method of choice. Its advantages have been clearly demonstrated in mouse studies. In zebrafish, morpholinos and ZFN are the only two techniques available that can be used to generate mutants using reverse genetics. Morpholinos can effectively down-regulate gene expression of the desired gene. However, it is transient and if the inactivation of gene expression is not complete, non-specific phenotypes can be observed. ZFNs have been shown to produce germ line competent mutants at reasonable mutagenesis rates, but it is still not amenable to knock-in experiments.

ES cells-chimera and SCNT are probably the most promising techniques for gene targeting in zebrafish. Of the two, SCNT is the best since it would require the least amount of time to generate a founder animal. At the moment though, the low rate of homologous recombination in cultured cells in both ES cells and somatic cells, hinders the broad applications of the technique. Problems associated with the low efficiency of germline transmission of ES cells and poor developmental rates in SCNT embryos are an additional drawback.

It is possible that by combining some of the techniques reviewed, the ultimate goal of creating a knock-in/ -out zebrafish can be achieved. As previously reported, ZFNs can increase the rate of DNA repair mechanisms, and may be used to generate cultured cells with gene targeting homologous recombination. Subsequently, the cultured cells, either ES cells or somatic cells, can be exploited in either ES cells-chimeras or the SCNT system.

Table 1 Summary of the techniques for transgenesis and mutagenesis studies

Technique	Mutagenic rate	Target gene	Type of mutation	Gene trapping	Gene targeting	Conditional	Identification of mutation	Detail identifications	Advantages	Disadvantages
ENU	high	random	point mutation	No	No	No	mutant phenotype	positional cloning, candidate gene approach, TILLING, etc.	high rate of mutation	require large scale screening for mutants
Pseudotyped retrovirus	moderate	random	insertion	possible	No	No	mutant phenotype	trace viral remnant by 3'- or 5'-RACE or blotting to cDNA	easily identify the mutated genes	most insertional mutagenesis cause null phenotype
Transposable elements	Low	random	insertion	Yes	No	Yes	analysis of a reporter gene	3'- or 5'-RACE	gene trapping/ breaking	low mutagenic rate
TILLING	same as ENU	target selected	point mutation	No	Yes	No	large scale screening from ENU mutant	hybridization based with *Cel* I endonuclease	mutant library	laborious, timely, costly
Morpholino	None-only knockdown	site specific	none	No	Yes	Yes with caged morpholino	rescue phenotype by mRNA transfer	analysis of gene expression	transient effect is available	cannot ensure complete inactivation and possibly non-specifc phenotypes
ESCs-Chimera	Low in cultured cells	site specific	insertion/ deletion	Yes	Yes	Yes with Cre-Lox or FLP-FRT system	positive/ negative selection in ESCs	using molecular markers	mutant ESCs can be selected and confirmed prior to use	the technology is still unavailable
SCNT	Low in cultured cells	site specific	insertion/ deletion	Yes	Yes	Yes with Cre-Lox or FLP-FRT system	positive/ negative selection in cultured cells	using molecular markers	mutant cells can be selected/ confirmed prior to SCNT	the technology is still unavailable
ZFNs	Moderate	site specific	insertion/ deletion	Yes	Yes	No	PCR-RFLP or sequencing	using molecular markers	germline mutation is not mosaic	target lesion cannot be selected, still need screening of mutant progeny
ZFN with HR	Moderate	site specific	insertion/ deletion	Yes	Yes	Yes	PCR-RFLP or sequencing	using molecular markers	possibly increase rate of HR	the technology is still unavailable

*conditional knock –in/-out; having a control over time and site of gene targeting, usually done by Cre-LoxP and FLP-FRT recombination

C. The recipient cell for nuclear transfer

In addition to the source of donor cells and the techniques for nuclear transfer, the recipient cell – the egg – is one of the most important parameters in the success of nuclear transfer across species. The cytoplasm of oocytes, not the nucleus, contains maternal factors that potentially implicate the development of cloned embryos. While SCNT protocols may vary among species, the stage in the cell cycle in which the egg is best suited for cloning remains mostly constant, i.e., arrested metaphase II (MII). This review focuses on the physiology of zebrafish eggs from the time of maturation to activation.

i. The oocytes/eggs for nuclear transfer

The first successful nuclear transfer experiments were conducted with amphibian eggs of *Rana pipiens* and *Xenopus laevis* (Briggs and King, 1952; Gurdon et al., 1958; Gurdon and Uehlinger, 1966). In part, this was due to their size since frog eggs (approximately 1 mm in diameter) are substantially larger when compared to mammals (approximately 100 μm in diameter), permitting micro-manipulation by nuclear transfer without much demand for sophisticated laboratory equipment. Furthermore, matured frog eggs can be obtained in the thousands following hormonal injection. The Xenopus eggs are collected in high salt medium, Holtfreter's solution, and remain arrested at MII until nuclear transfer. In *Rana pipiens*, prior to enucleation, the recipient eggs are subjected to activation to enter metaphase II enabling the visualization of the egg's nucleus. Removal of the chromosomes from the egg can be done using the now 'conventional' mechanical method using a glass needle (Briggs and King, 1952) or by UV

irradiation (Gurdon and Uehlinger, 1966). Donor cells are prepared by gently disrupting the cell membrane with an injection needle or pre-treated with membrane permeabilizing agents (lysolecithin or streptolysin O) prior to nuclear transfer (Chan and Gurdon, 1996). In Xenopus, simple pricking of the egg with an injection needle while transferring a nucleus can cause the egg to activate and start embryogenesis.

In mammalian cloning, the recipient oocytes are mostly arrested at MII, obtained from either *in vitro* or *in vivo* maturation. For *in vivo* maturation, MII oocytes are collected from animals that were previously hormonally stimulated to produce large quantities of eggs, a process called superovulation. In farm animals in which ovaries can be easily accessible from the abattoir, oocyte *in vitro* maturation is the method of choice. In this case, oocytes at germinal vesicle stage are collected, and cultured under conditions that are optimal for obtaining the MII oocytes. MII oocytes are usually enucleated using a glass pipette to remove the metaphase plate. Delivery of the donor nucleus can be done by simple injection (Wakayama et al., 1998) or by cell fusion (Willadsen, 1986). During mammalian cloning, oocyte activation is done by various techniques including an electrical pulse, ethanol, strontium, calcium ionophore as the first step, and in some instances, with the addition of an inhibitor of protein synthesis or protein kinase as the second step (Cibelli et al., 1998; Kato et al., 1998; Wakayama et al., 1998; Wilmut et al., 1997).

The first successful nuclear transfer in Medaka fish, used cells from the blastula stage (Wakamatsu et al., 2001). The technique involved the collection of MII eggs from the abdominal cavity of a female fish, and holding them at MII stage in a balanced salt

solution. MII eggs were then enucleated by X-ray irradiation prior to nuclear transfer. The cell membrane of the donor cell was then gently disrupted and transferred to the animal pole of the eggs, a process that also triggers egg activation. The description of this technique, i.e., irradiation of the egg first followed by nuclear transfer does not explain the subsequent work that the same group later did. We speculate that irradiation of the recipient egg was not as efficient as first probably thought (Wakamatsu et al., 2001). In a follow-up manuscript, medaka fish was cloned using non-enucleated egg as recipient and cells of embryonic or adult origin as donors (Bubenshchikova et al., 2005; Bubenshchikova et al., 2008; Bubenshchikova et al., 2007; Kaftanovskaya et al., 2007; Niwa et al., 1999). Note that using such technique, the cloned fish usually bears triploid chromosomes since the donor cell is diploid and the egg still contain its haploid-maternal chromosomes. Occasionally, diploid-fertile fish can be obtained by this technique as well (Wakamatsu, 2008). In addition, haploid ES cells of Medaka fish were recently generated and have produced cloned fish following nuclear transfer (Yi et al., 2009), albeit at low efficiency. In the future, knock-in / out Medaka fish could be generated if the non-enucleated unfertilized eggs are used together with haploid ES cells, since they can be amenable to genetic manipulations in culture.

Nuclear transfer in zebrafish was successfully reported in 2002 (Lee et al., 2002). MII eggs were collected using the stripping technique, by gently applying pressure to the abdominal cavity, and kept in Holfreter's solution at the time of egg collection. Egg enucleation was done by removal of the portion of the egg's cytoplasm underneath the

polar body using a Hank's balance salt solution with bovine serum albumin. Nuclear donor cells were gently disrupted in the injection needle prior to transfer to the egg.

Although the authors described the use of MII stage eggs with the technique as described, it is possible that eggs had exited MII stage. This is a conclusion we arrived at our laboratory after attempting to replicate the results. We have speculated that the collection media or the pronase used for dechorination (removal of a zona pellucida-like shell called chorion) triggered premature activation and therefore SCNT was not successful in our hands.

In summary, the recipient cells for nuclear transfer in frog and fish can be either activated eggs or mature arrested eggs (Table 2). In the latter case, the reconstructed eggs are activated right at the time of nuclear transfer. The success rate to produce clones, however, is different across species (Di Berardino, 2006). In mammalian cloning, especially in mice, the recipient cells at the metaphase stage are thought to be the most efficient cells to support the development of cloned animals (Egli et al., 2007; Wakayama et al., 2000). We have found that the quality of the eggs, as well as the cell cycle stage in which the eggs are found at the time of nuclear transfer, is a determining factor governing the success of the procedure. A thorough understanding of the physiology of the zebrafish egg at the time of activation and/or fertilization is necessary.

Table 2 Summary of nuclear transfer in frogs and fish

Species	Oocyte at time of NT	Enucleation Stainning	Enucleation by	Nuclear transfer by	Activation	Cloned animals from cells of Blastula	Embryos	Adult	Ref
Rana pipiens	MI and MII	no	glass needle	direct injection	pricking	Yes	n/a	Tadpole	1
Xenopus laevis	MII	no	UV irradiation	direct injection	pricking	Yes	Yes	Tadpole	2
Medaka	MII	no	X-rays irradiation	direct injection	pricking	Yes	n/a	n/a	3
Medaka	diploidize egg (n/a)	no	not remove	direct injection	pricking	Yes	Yes	Yes	4
Zebrafish	telo-prophase	no	blind removal	direct injection	Spontaneous before NT	n/a	Yes	n/a	5
Zebrafish	MII	H33342	laser firing	inject through a micropyle	Spontaneous after NT (15m)	n/a	Yes	abnormal embryos	6

- n/a is not applicable

- References (REF)

[1] (Briggs and King, 1952; DiBerardino and Hoffner, 1983)

[2] (Gurdon et al., 1958; Gurdon et al., 1975; Gurdon and Uehlinger, 1966)

[3] (Wakamatsu et al., 2001)

[4] (Bubenshchikova et al., 2005; Bubenshchikova et al., 2008; Bubenshchikova et al., 2007; Kaftanovskaya et al., 2007; Niwa et al., 1999)

[5] (Lee et al., 2002)

[6] (Siripattarapravat et al., 2009b)

ii. The zebrafish egg

After stimulation by gonadotropic hormone (GTH), the oogonium enters meiosis and starts its development. Oocyte growth in fish at pre-vitellogenesis is mediated by GTH-I (follicle stimulating hormone). GTH-II (lutinizing hormone) is responsible for maturation of fully-grown oocyte: germinal vesicle break down, stimulating follicle cells to synthesize maturation inducing hormone (MIH), and inducing ovulation. MIH stimulates synthesis of the maturation promoting factor (MPF), and maintains it at a high level in the mature MII-arrested oocytes.

In zebrafish, the ovulation is completed after the female is exposed to a male fish at dawn. It is not clear what really induces the ovulation in zebrafish upon mating, but it is thought to be pheromones. In Medaka, hydrolytic enzymes are responsible for follicular rupture prior to ovulation (Ogiwara et al., 2005). The mature egg detaches from the ovaries as denuded eggs, and is held in the ovarian cavity until the female is ready for mating. The eggs are released upon the natural mating behavior, at the same time that the milt is released from the male fish. The gametes are then fertilized externally within a few minutes. The mating zebrafish release eggs and milt many times over the course of a single mating.

In zebrafish, eggs at the mature arrested stage seem to be the most accessible samples. The female fish usually holds hundreds of mature eggs in the ovarian cavity. These eggs can be released from the abdominal cavity of the female following the natural courtship behavior of the mating pair (Westerfield, 1993). Alternatively, germinal vesicle oocytes from the fish ovary can be obtained using a combination of the

enzymes trypsin, collagenase and hyalurodinase (Guan et al., 2008). A reliable *in vitro* maturation technique has been reported by using Leibovitz L-15 medium supplemented with 17alpha, 20 beta-dihydroxy-4-pregnen-3-one (DHP) and bovine serum albumin at high pH (Seki et al., 2008). The quality of the *in vitro* matured eggs is comparable to one of the *in vivo* counterpart. However, since the mature arrested eggs are easily prepared, the *in vitro* maturation system is not yet implemented for the preparation of recipient cells for nuclear transfer.

Scanning electron microscopy studies revealed that the sperm of zebrafish must enter the egg through a micropyle, a single cone shaped entrance on the chorion (Wolenski and Hart, 1987). At the time of exposure to water, the fish egg undergoes egg activation independent of the contact with sperm. The sperm must find the micropyle within seconds of ovulation or fertilization fails. The process is facilitated by the abundance of sperm surrounding the egg; the micropylar grooves surrounding the micropylar pit (Amanze and Iyengar, 1990); and the chorionic glycoproteins that can promote binding affinity of the sperm to the micropyle area as well (Iwamatsu et al., 1997). Only one sperm per egg can go though the micropyle (Wolenski and Hart, 1987), and when one does, a filamentous actin network in the egg helps the fusion of the sperm to the egg at the fertilization cone (Hart et al., 1992; Wolenski and Hart, 1988).

The major mechanism of egg activation is similar across all species. The activated egg increases intracellular calcium concentration in waves that vary in frequency and magnitude these are known as calcium oscillations (Ducibella and Fissore, 2008). In zebrafish, the egg undergoes parthenogenetic activation as soon as it comes into

contact with water, and a single calcium wave is recorded (Lee et al., 1999). The activated eggs can also show other signs including exocytosis of cortical granules, metaphase exit to complete meiosis, extrusion of the second polar body, expansion of the chorion, and the formation of blastodisc after the ooplasmic segregation toward the animal pole of the egg.

The artificial oocyte activation methods used in nuclear transfer of mammals is not as efficient as fertilization, because only the single-exponential calcium wave is achieved after treatment. In bovine and murine, phospholipase C-zeta cRNA mimicks the calcium oscillation pattern of fertilized oocytes, and can promote nuclear reprogramming (Ross et al., 2009). In zebrafish, it is thought that egg activation by fertilization is not much different from the one of parthenogenesis since both produce a monotonic wave following activation (Lee et al., 1999). New line of evidence suggests that fertilization, not parthenogenesis, activates a Src-family protein kinase, the Fyn kinase (Sharma and Kinsey, 2006; Wu and Kinsey, 2000). The same group also showed that calcium oscillation are separated in two compartments, one that occurs at the center of the cytoplasm, and another one that starts from the micropyle and diffuses cortically throughout the cortex of the egg (Sharma and Kinsey, 2008). The latter is shown to be a sperm-specific wave and is different from the one triggered by parthenogenetic activation.

CHAPTER 2

THE DONOR CELLS FOR ZEBRAFISH SOMATIC CELL NUCLEAR TRANSFER: OPTIMIZATION OF *IN VITRO* CULTURE CONDITIONS, DNA TRANSFECTION, AND CHEMICALLY-ASSITED EPIGENETIC MODIFICATIONS.

Kannika Siripattarapravat, Chia-Cheng Chang, and Jose B Cibelli

Abstract

We characterized the essential parameters of donor cells for somatic cell nuclear transfer including culture conditions, growth and characteristic of cultured cells, and transfection efficiency of cultured cells. We also investigated the possibility of using histone deacetylase inhibitors, Trichostatin A (TSA) and Valproic acid (VPA), to treat the donor cells prior to somatic cell nuclear transfer.

Introduction

In somatic cell nuclear transfer (SCNT), there are at least three main factors that play a role in the success or failure of the procedure: the SCNT technique, the oocytes, and the donor cells. In simple terms, the reprogramming unit – referred to as the oocyte's cytosol, must be exposed to the appropriate substrate – referred to the donor cells' nuclei – and given substantial amount of time to completely remodel the somatic cell's epigenetic state to a state of pluripotency.

It has been suggested that modifying the chromatin structure of the somatic cells prior to their exposure to the egg's cytosol, may enhance the ability of these cells to be more readily reprogrammed following transfer into recipient eggs (Loi et al., 2003; Simonsson and Gurdon, 2004) creating a greater capacity for normal embryonic

development. The comparative study between *in vitro* fertilized embryos and cloned embryos suggested that defects of gene expression are due to incomplete epigenetic resetting (Wee et al., 2006). Furthermore, an improvement of cloning efficiency was reported in mice using trichostatin A (Kishigami et al., 2007; Kishigami et al., 2006). Trichostatin A is a histone deacetylase inhibitor that indirectly induces acetylation of histones by suppression of the activity of multiple histone deacetylases. In turn, multiple genes can be reactivated, and subsequently trigger an array of both, morphological and physiological changes in cells.

Enzymes that modulate the epigenetic status of cells were found to be conserved among plants, invertebrates, lower vertebrates and mammals. Xenopus egg extracts can reprogram both mouse and human somatic cells to express a pluripotent marker, the *OCT4* gene, which is regulated by DNA methylation (Byrne et al., 2003; Simonsson and Gurdon, 2004). The inter-species cross reactivity implied that functional properties of these enzymes are also conserved. There is evidence of epigenetic modification enzymes in zebrafish which suggest their roles as gene expression regulators as well (Mhanni and McGowan, 2004; Yokomine et al., 2006).

Multiple enzymes work in concert to equilibrate levels of DNA methylation and histone modifications in cells (Lachner et al., 2003; Shi and Whetstine, 2007). These enzymes are known to interact with different associated-regulatory proteins and are thought to recruit and work at preferential sites on the chromatin. Each enzyme also has its counter-partners that balance and maintain their substrates for regulation of gene expression (Freitag and Selker, 2005). Many reagents were investigated to globally alter

the epigenetic status of the cells, including histone deacetylase and DNA methyltransferase inhibitors (Bourc'his et al., 2001; Kishigami et al., 2006; Rybouchkin et al., 2006; Sullivan et al., 2004). However, the effects of these reagents are known to be non-specific, inconsistent from cell to cell, dependent upon the epigenetic status of each cell at the time of treatment.

In all cases, the inhibitors are thought to modify global epigenetic status of the cells by loosening the chromatin structure and favoring accessibility of transcription factors to the promoters. Histone deacetylase inhibitors, i.e., TSA, ultimately induce histone hyperacetylation as previously stated. Histone methyltransferase inhibitors decrease overall histone methylation, and DNA methyltransferase inhibitors, i.e., 5-Aza-2'-deoxycytidine , help open/ loosen the heterocentromeric regions in the chromosomes making the chromatin easily accessible for the transcription factors to promote gene expression. Valproic acid, a drug widely used therapeutically in humans, has been found to trigger replication-dependent and -independent DNA demethylation through its effects on histone acetylation (Detich et al., 2003).

In zebrafish, embryogenesis happens very rapidly following fertilization (Kane and Kimmel, 1993; Kane et al., 1992). This would require, in the case of SCNT, that the donor cell must be fully reprogrammed within 3 hours to reach the time of zygotic gene activation with the proper epigenetic modifications. We hypothesized that epigenetic alteration of donor nuclei prior to nuclear transfer, can potentially implicate the success of nuclear transfer and cloning in zebrafish. Therefore, the epigenetic state of donor

cells must be characterized. This information should be beneficial to increase the reprogramming efficiency through epigenetic modifications of the donor cells.

Established primary zebrafish cell lines are in short supply. The *in vitro* system for culturing embryonic stem cells in zebrafish (Fan et al., 2004a) and primary cells derived from embryos and adult tissues (Collodi et al., 1992; Driever and Rangini, 1993; Ghosh and Collodi, 1994) was previously described. However, there is limited information on the specific nature of the cultured cells and their optimal *in vitro* culture requirements, especially while using 5% CO_2 with atmospheric air. The types of cells and ability to genetically manipulate these cells are also left to be explored.

Our objectives were then to first optimize cell culture conditions and to characterize cultured zebrafish somatic cells from embryonic source. The ability of these cells to uptake the foreign DNA, using several transfection mediators, was also evaluated. As we obtained the optimal culture condition, we further aimed to characterize the donor cells following global epigenetic modifications by chemical means and render them more susceptible for reprogramming in the egg's cytosol under the window of time of 3 hours or less. We expect that the use of modified donor nuclei will then increase the SCNT efficiency in zebrafish.

Results

i. The culture media

The cell culture conditions were optimized by comparing two types of medium, Dulbecco's Modified Eagle Medium (DMEM) and Keratinocyte SFM (K), with different combinations of supplements including, fetal bovine serum (FBS), trout serum (TS),

zebrafish embryonic extracts (EE), N-Acetyl-L-Cysteine (NAC) and L-Ascorbic acid 2-Phosphate sesquimagnesium salt (A2P). In assorted culture conditions, cell growth in different cell seeding densities were also determined.

- **Effect of TS and EE supplemented in DMEM on cell growth**

Embryo-derived cells were plated in 5% or 10% FBS in DMEM, either supplemented with TS or/and EE, or without supplement (Figure 1). There were no significant differences in growth rate between 5% and 10% FBS supplemented DMEM. The effect of 0.1% EE was shown to be minimal. Only the presence of 1% TS in culture medium significantly increased the growth rate of zebrafish cells ($p<0.05$).

- **Growth of zebrafish cells depends upon cell seeding density**

Primary cultured cells were derived from zebrafish embryos, 15-25 hours post-fertilization (hpf). Two different cultured media were compared; 1) DMEMs; DMEM with 15% FBS, 1% TS, 0.1% EE, and 10 ng/ml Bovine insulin and 2) K-NACs; Keratinocyte SFM (comes with epidermal growth factor and bovine pituitary extracts) supplemented with 5%FBS, 1%TS, 2mM NAC and 1 μM A2P (Lin et al., 2005). During the first 2 weeks, 25 ng/ml of basic fibroblast growth factor (Invitrogen) was added to the medium to inhibit melanocyte formation (Bradford et al., 1994). Different cell-lines and passages were then compared.

Cells in low seeding concentration; 5×10^4 ($p=0.003$) and 1×10^5 ($p=0.007$) grew significantly better in K-NACs than DMEMs (Figure 2). No difference was found at $2\text{-}3 \times 10^5$ cell seeding density ($p<0.05$). However, at $4\text{-}5 \times 10^5$ cell seeding density, cells grew slightly better in DMEMs than K-NACs ($p<0.1$). We observed that cells plated more

efficiently in K-NACs than in DMEMs. The results suggest that cells propagated better at a higher cell seeding density and that K-NACs may improve plating efficiency, likely due to the presence of NAC and A2P in the media.

- **The growth of zebrafish cells at different %FBS and cell seeding density**

We determined the effect of NAC and A2P on cell growth in DMEMs (D-NACs) at different FBS concentration (Figure 3). Independent of the cell seeding density on day 1 of culturing, no difference was found for cell growth in either media supplemented with 5%, 10% or 15% FBS ($p<0.05$). Cells cultured in D-NACs grew significantly better than those cultured in DMEMs ($p<0.05$). Our results clearly show that the use of NAC and A2P in the culture medium has a positive effect on cell growth regardless of the amount of FBS added. This result further supports that the presence of NAC and A2P can increase cell plating efficiency, and possibly enhance propagation of cells.

- **Growth of cells in D-NACs and K-NACs**

Experiments were carried out to compare the growth of cells at different cell seeding density in two media; D-NACs (DMEM based) and K-NACs (Keratinocyte SFM based), supplemented with 5% FBS, 1% TS, NAC and A2P. Only in D-NACs 10 ng/ml bovine insulin and 0.1%EE were added. There was no significant difference in the growth rate observed between these two culture media (Figure 4). These results also indicate that cell growth in two different media is density- dependent.

The doubling time and cumulative population doubling level were calculated as described previously (Lin et al., 2005). Doubling time of embryo-derived cells was recorded over multiple passages *in vitro*, using embryo-derived cells from embryos at 15

or 25 hpf primarily cultured in either D-NACs or K-NACs media. The doubling time of early passage (p1-5) was at 5-6 days in D-NACs, and 6-7 days in K-NACs. In both types of media, cells were at passage 5-10 and cultured for more than 10 passages. Doubling time was at 5-6 days for cells at passage 5-10 and 2-3 days for cells over passage 10. Cumulative population doubling levels exceeded 50 in some cell lines cultured in either medium. The activity of telomerase was also evaluated in some of these lines at passages 2 and 20 (Figure 5), using TRAP assay (Chemicon, MA). The results showed that zebrafish cells, long-term cultured in either D-NACs or K-NACs, still have high telomerase activity, at the same level as earlier passaged cells. It remains to be determined if the high cumulative population doublings and telomerase activity detected in our Zebrafish primary cell cultures is cell type or cell culture dependent, i.e., if we have randomly selected tissue specific stem cells or our culture conditions were responsible for the seemingly robust *in vitro* cultures.

- **Characterization of embryo-derived cells**

The types of cells that present in the culture were characterized by immunocytochemistry. We investigated for the expression of Vimentin, Sox17, Nestin and Oct-4, these are markers for fibroblasts, endoderm, neuronal progenitors, and pluripotent cells, respectively. When the analysis was done at early passages, the population of cells was heterogeneous (data not shown). In late passages however, the majority of cells in both D-NACs and K-NACs media were positive for Vimentin (Figure 6). None of them were positive for Sox17, Nestin or Oct-4 markers (data not shown), indicating that our cells were most likely fibroblasts.

ii. Transfection efficiency

We aimed to define the parameters necessary for an efficient introduction of foreign genes into cultured cells prior to SCNT. We focused on somatic cells derived from zebrafish embryos and transfected them with foreign DNA. The expression plasmid vector containing Medaka elongation factor 1-alpha promoter driving green fluorescence protein (pEF1α-A-GFP) expression (Kinoshita et al., 2000) was used. These cells were transfected using four different liposome-mediated transfection reagents: Lipofectin (Invitrogen, CA), Lipofectamine2000 (Invitrogen, CA), Fugene6 (Roche, IN) and Exgen500 (Fermentus, MP). DNA uptake rate, evaluated by transient transfection, was quantified by counting the percentage of green cells out of the total number of cells in culture (Figure 7a). Transfection efficiency was invariably low across all reagents tested, except Exgen500 which yielded the highest transient transfection efficiency in its group ($p<0.05$), although large variations were found within replicates.

Electroporation was introduced as an alternative to liposome-mediated transfection reagents. We tested several combinations of Amaxa Nucleofactor® programs and solution kits as per the manufacturer recommendation (Lonza, Switzerland). The combination of program T-020 with solution kit V was used, as it gave highest transfection rate and best cell recovery. We used a pCS2-GFP plasmid containing cytomegalovirus promoter driven GFP expression and found that more than 50% of the cells were found to transiently express GFP (Figure 7b). The transfection efficiency of electroporation is by far better than one of liposome-mediated agents.

iii. Epigenetic modifications

We first determined the toxicity of TSA and VPA, which are both small molecules known to modify histone residues in the cells. We aimed to define an effective concentration for these inhibitors that is non-toxic to zebrafish embryos as well as capable of producing the desired modifications in the global histone acetylation status. Dose ranges were selected based on previous reports (Collas et al., 1999; Gurvich et al., 2005; Phiel et al., 2001).

- **Toxicity of TSA and VPA in zebrafish embryos**

Embryos were *in vitro* fertilized and place in a solution of either TSA or VPA within 5 minutes post-fertilization. Embryos were treated for 3.5 hours, then washed and moved to new egg water. We tested the TSA at concentrations of 5 nM, 50 nM, 100 nM, 500 nM, 5 μM, and 10 μM, and the VPA at concentrations of 0.1 mM, 0.5 mM, 1 mM, and 5 mM. Signs of embryonic toxicity for TSA were found at the 500 nM concentration. Treated embryos stopped their development at gastrulation, although no detectable abnormality prior to this stage was observed. A dose higher than 500 nM of TSA was lethal for 100% of the embryos. No detectable abnormality in embryos at day 4 post fertilization was observed when 100 nM TSA was used.

The VPA caused abnormality of embryos after segmentation period when a concentration of 500 μM or more was used (Figure 8). The embryos showed signs of retarded growth, blunt tail, and pericardial edema. All treated embryos died at day 4 post fertilization. There was no detrimental effect when a concentration of 100 μM was used. Therefore, the concentration of TSA and VPA that were used in the following experiment was based on the upper limit of toxicity recorded in zebrafish embryos.

- **Effect of TSA and VPA on histone acetylation**

Both, TSA and VPA are known to be histone deacetylase (HDAC) inhibitors. So, the levels of acetylation of histone H4 at lysine 5 residue (H4K5) were analyzed in culture cells following the treatment. Cells were incubated in vehicle controls, TSA at 100 nM and 200 nM, or VPA at 100 μM, 200 μM and 500 μM, for 4 hours. Then cells were fixed and analyzed for acetylation at H4K5 by immunocytochemistry.

The treatment with 100 nM TSA was sufficient to inhibit HDAC and cells displayed a marked increased in the levels of histone acetylation after treatment (Figure 9). In contrast, the treatment of VPA at 100 μM had no effect on histone acetylation (data not shown). We then increased the concentration of VPA to either 200 μM or 500 μM, however, only a minimal effect on the level of acetylation at H4K5 was observed after 4 hours incubation (Figure 9). When incubation time was prolonged to 12 hours using 500 μM of VPA, we observed more cells with an increased level of acetylation at H4K5 (data not shown). We did not find any sign of cell dead following both treatments in cultured cells.

Discussion

We have established the optimal *in vitro* culture system for zebrafish cells, including the media and cell seeding density for better cell propagation. The approach to yield high transfection efficiency in the cultured cells was also described. We reported the toxicity of TSA and VPA in the embryos and the efficacy of both reagents to increase the levels of histone acetylation at H4K5.

Our results showed that trout serum stimulated growth of zebrafish cells when heat inactivation was applied prior to its use. Embryonic extracts also have a positive effect on the growth of cells as well, however special care must be taken to avoid contamination and the quality fluctuations between embryo batches prompted us to exclude it from the final media preparations.

Cultured cells had better plating efficiency when NAC and A2P were added to the media. Both NAC and A2P are known for their capacity to reduce oxidative stress of the cells, and possibly help maintain the culture conditions similar to those observed under a low oxygen environment (Lin et al., 2005). Zebrafish cells seem sensitive to trypsin-EDTA treatments used to dissociate the cells. The culture system we chose calls for the use of low calcium medium (LHC) instead of phosphate buffer saline to wash cells, as well as a low concentration of trypsin-EDTA for dissociation of the cells. It is possible that NAC and A2P may facilitate a quick recovery of the cells after enzymatic treatment or mechanical injury at the time of dissociation. As a result, more cells survived and propagated in the next passages.

We clearly observed that zebrafish cells grow better at a higher cell plating density. In our experience, the cells rather go to quiescence when low numbers of cells are plated in the cultured vessel. We found that the doubling time of zebrafish cells is longer in early passages than later passages. We also found, that many cells died off at early passages, possibly because the culture was a mixed cell population and some of the cells were not capable of re-plating. It is possible that cells at early passages grew approximately at the same rate as those during later passages, however, from our

calculations; the doubling time was extended as it was adjusted for the dead of those cells.

Regarding cell survival and growth, there is no difference found between DMEM and K-SFM based media. In both cases, the culture conditions favored the growth and expansion of vimentin positive cells. The cells in either media, are capable of long-term *in vitro* propagation, as the cumulative population doubling level (50) is quite high, and the telomerase activity also remains high.

In some of the embryo-derived lines, we observed chromosomal aneuploidy at their late passages (P15-20). This is consistent with other reports in embryonic cells of zebrafish, i.e., cells have genomic instability during prolonged culture (Driever and Rangini, 1993). Although we did not characterize all the lines established, we found that some of the embryo-derived cells indeed had a normal karyotype.

We had used D-NACs medium to culture adult fin explants as well. The fin explants propagated well in D-NACs medium but their doubling time was long (3-4 days). Adult cells displayed normal karyotypes in D-NACs media, suggesting that the aneuploidy observed may be more related to the cell type rather than the culture conditions.

The efficiency of DNA transfection using liposome-mediated reagents was low in all reagents tested, making it impractical for routine use. Alternatively, electroporation showed high DNA uptake in the cultured fibroblast cells and should be considered the method of choice when genetic modifications must be introduced in cultured cells.

Considering the concentration of TSA and VPA used, TSA was more toxic than VPA. However, it seems to be a more powerful HDAC inhibitor since 100 nM concentrations are sufficient to increase histone acetylation. In the case of VPA, although well tolerated at higher concentration, its efficacy as an inhibitor of HDAC remains low, at least in the cells tested. By increasing the length of time the cells are exposed to VPA, the level of acetylation at H4K5 in some cells increased, suggesting either slow activity of the reagent or replication-dependent efficacy. It is also possible that VPA induces changes at other histone residues besides H4K5. Early developing embryos treated with VPA showed multiple signs of abnormalities after the exposure to the drug was discontinued. This indicates that treatment of VPA may affect gene expression of embryos in early development, triggering abnormal phenotypes later in their development. Much work remains to be done in order to elucidate the specific effect VPA has on zebrafish cells.

Culture conditions and techniques have been established to optimize the *in vitro* culture system of zebrafish cells and to genetically manipulate the cultured cells. All of which can be beneficial to handlings of donor cells for somatic cell nuclear transfer. The study of chemical treatments of cultured cells has set the foundation for future experiments that will demonstrate whether these chemicals can increase the success rate of SCNT in zebrafish.

Methods

Primary cultures of embryo-derived cells. Embryos were obtained from natural breeding of Tuebingen Zebrafish line, incubated in egg water at 28°C until 15-25 hour

post-fertilization. A pool of 50 embryos was utilized in each line. Embryos were de-chorinated in 3 mg/ml pronase (Sigma, MA) for 5 minutes, and moved to LHC basal media (Invitrogen, CA) with 100 μg/ml gentamicin. Embryos were disinfected in 0.04% sodium hypochlorite (bleach) for 3 minutes, washed extensively in LHC and followed by mechanical disassociation in LHC by pipetting. Cells were resuspended in specific culture media depending on the experiment, and cultured at 28°C with 5% CO_2 in atmospheric air. More than 3 different cell lines, obtained from different batches of embryos, were utilized for each analysis.

To subculture the cells, they were washed twice with LHC and subjected to trypsinization using 0.025% trypsin-EDTA (0.05% trypsin-EDTA and LHC in a ratio of 1:1) at room temperature. As soon as the cells started to dislodge from the culture dish, 5% FBS in LHC was added to inhibit the activity of trypsin. Cells were counted, and plated at designated numbers in culture dishes. Culture media was replaced every 3-4 days and subculture was performed when cells reached 80-90% confluency.

***In vitro* fertilization (IVF) and toxicity test.** IVF was done as described in the standard protocol (Westerfield, 1993), with slight modifications. The eggs were briefly kept in Chinook salmon ovarian fluid at room temperature prior to IVF. The milts were collected from at least 3 males and kept in Hank's balance salt solution on ice until used. The eggs and milt were mixed and activated using egg water then incubated undisturbed for 5 minutes. Subsequently, fertilized embryos were moved to new egg water with VPA or TSA at designated concentrations. Embryos were incubated with each treatment for 3.5

hours, then washed extensively with egg water and raised at 28°C. The development of embryos was recorded until 4 days post-fertilization.

Transfection. DNA transfection was performed according to the manufacturer's recommendation. Plasmid DNA was prepared using a midi-prep as described in a standard protocol (Sambrook and Russell, 2001). Two μg of DNA was used in each transfection.

Immunocytochemistry. Cells were washed in LHC twice and fixed with freshly prepared, cold, 4% paraformaldehyde for 5-7 minutes. The fixative reagent was then removed and phosphate buffered saline (PBS) was added. Cells were treated with 0.5% triton X100 in PBS and allowed 15 minutes for permeabilization before they were washed twice in PBS with 0.1% triton X100 (PBSTx). Five percent bovine serum albumin (BSA) in PBSTx was then used as a blocking reagent for 90 minutes. Primary antibodies were diluted using 3% BSA in PBSTx. Cells were incubated in designated primary antibody overnight at 4°C with gentle rocking. Cells were then washed with PBSTx, and incubated with the couterpartners – AlexaFluor® labeled secondary antibody (Invitrogen, CA) for 90 minutes. Cells were washed extensively with PBSTx and their DNA stained with Hoechst33342. Images were taken with CoolSNAP™ Pro camera using Image-Pro Express (Media Cybernetics, MD).

We used the following antibodies in the experiments; primary mouse anti-vimentin antibody (dilution 1:200, Sigma-Aldrich), primary goat anti-Sox17 antibody (dilution 1:200 , Santa Cruz Biotechnology), primary goat anti-Oct4 antibody (dilution 1:200, Santa Cruz Biotechnology), primary rabbit anti-Nestin antibody (dilution 1:200,

Abcam), primary rabbit anti-H4K5 acetylation antibody (dilution 1:200, Upstate), secondary donkey anti-mouse AlexaFlour 594 (dilution 1:500, Invitrogen), secondary donkey anti-goat AlexaFlour 594 (dilution 1:500, Invitrogen) and secondary donkey anti-rabbit AlexaFlour 488 (dilution 1:500, Invitrogen).

Statistical analysis. Each experiment was repeated at least 3 times. Data was analyzed with SigmaStat version 3.1 (Jandel Scientific, San Rafael, CA), using analysis of variance (ANOVA). The level of significance was set to a p-value of < 0.05.

Figure 1 Effect of FBS, TS and EE on cell growth in DMEM. DMEM was supplemented with 10 ng/ml bovine insulin, 5% (solid) or 10% (blank) of FBS in combination with either 1% TS, 0.1% EE or both TS and EE. A total of $1X10^5$ cells/$3.8cm^2$ were seeded. Total number of cells were counted at day 7 after plating. (Error bar = standard error)

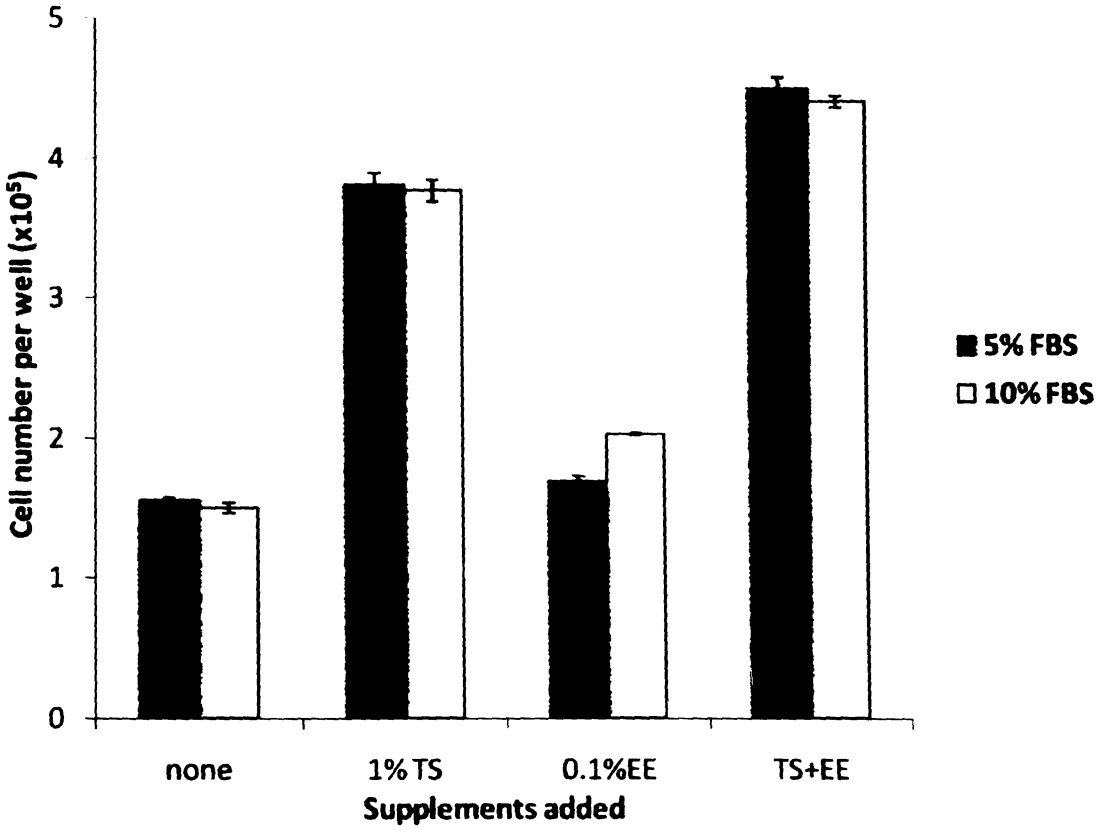


Figure 2 Comparison of cell growth in DMEMs and K-NACs. Cells seeded at 0.5, 1, 2, 3, 4 and 5 X 10^5 cells/ 3.8 cm^2 were propagated in both media. Numbers of cells were counted at day 4 after plating. (Error bar = standard error)

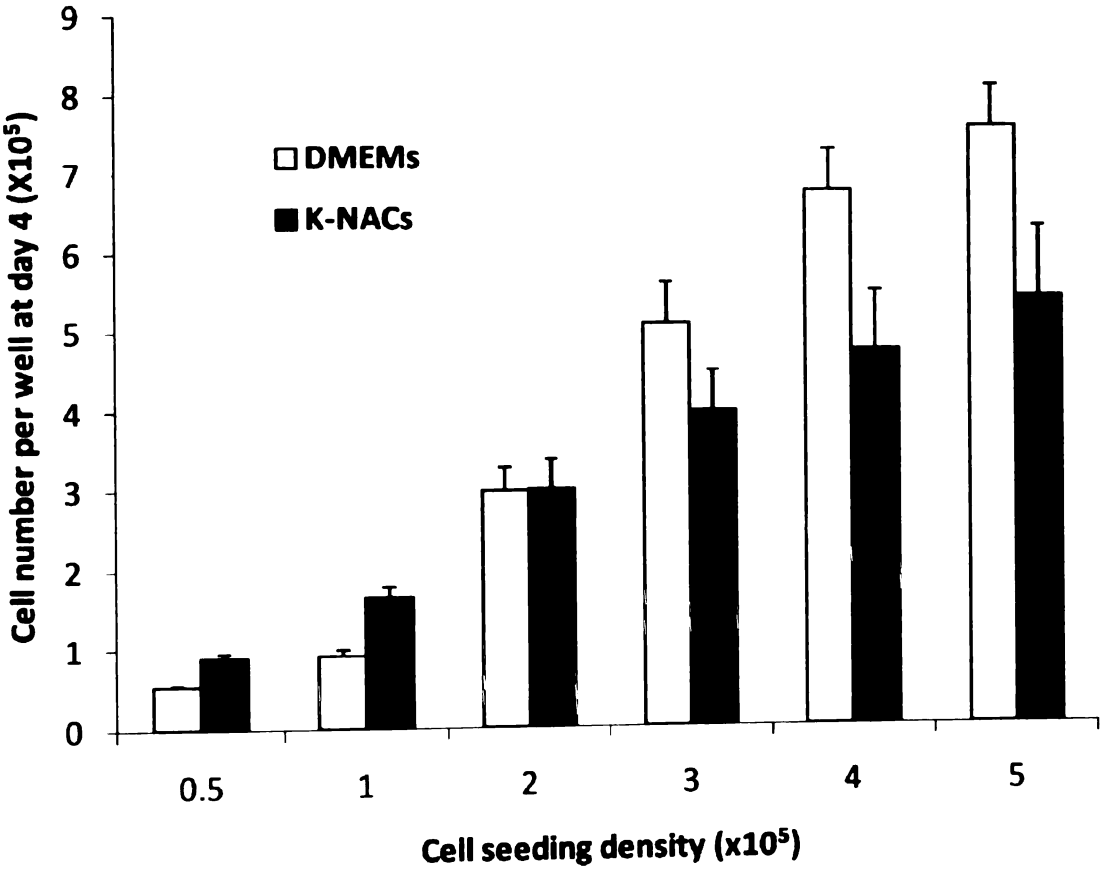


Figure 3 Comparison of cell growth in DMEMs and D-NACs using different FBS concentrations. Cells were seeded at 1, 2 and 3 X10^5 cells/3.8 cm^2 and propagated in both media. Numbers of cells were counted at day 4 after plating.

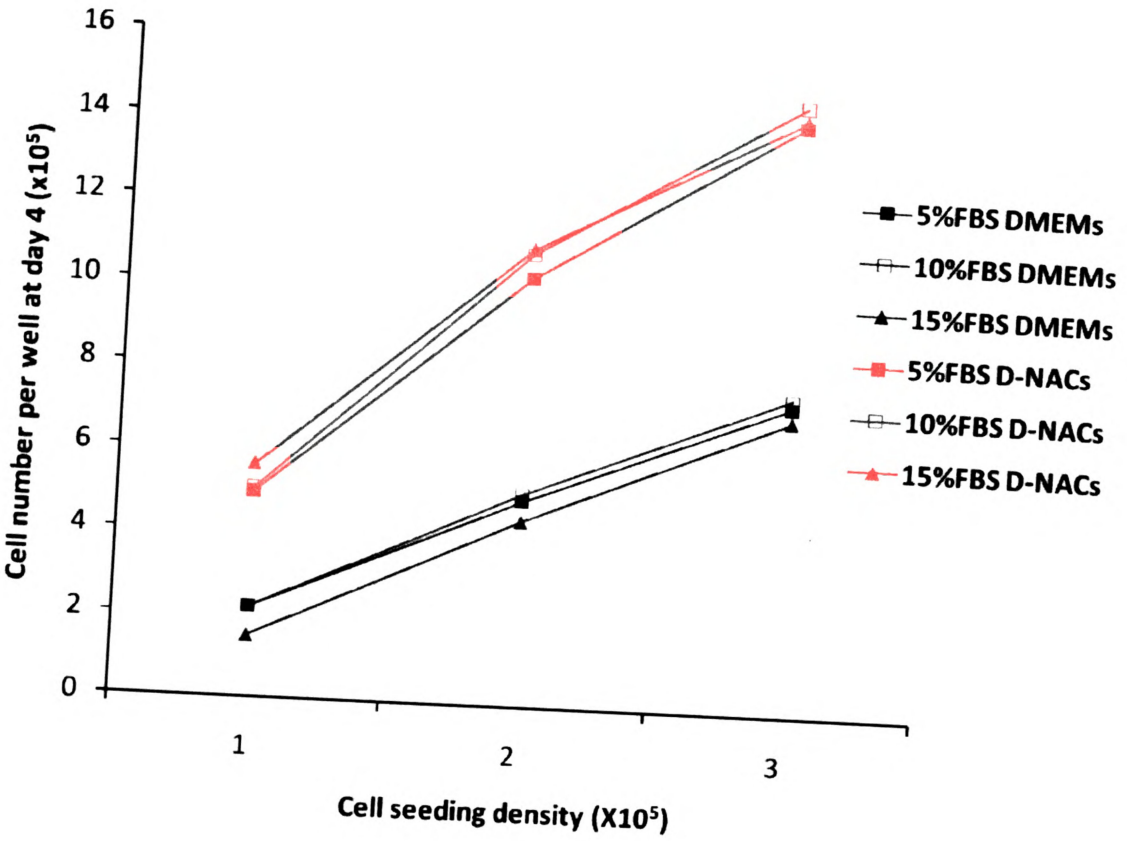


Figure 4 Comparison of cell growth in D-NACs and K-NACs media. Cells were seeded at at 0.5, 1, 2, 3, 4 and 5 X10^5 cells/ 3.8cm^2 and propagated in both media. Numbers of cells were counted at day 5 after plating. (Error bar = standard error)

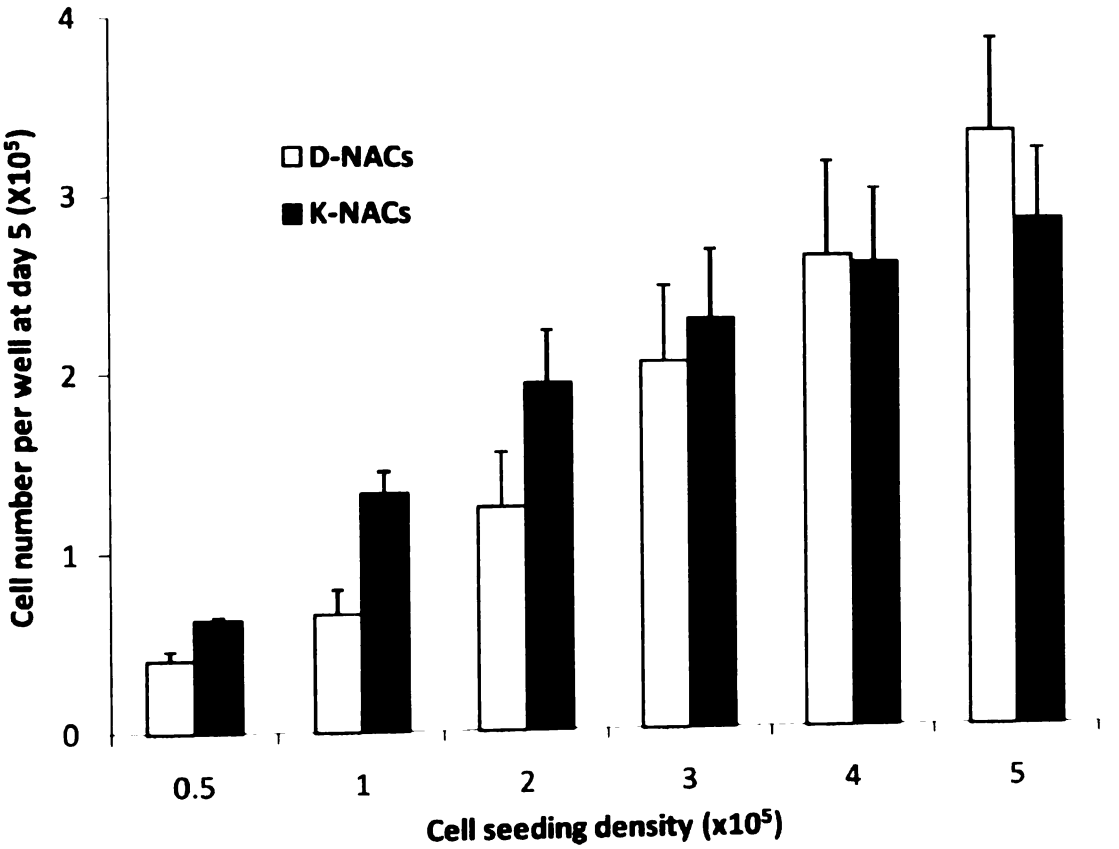


Figure 5 Telomerase activity by TRAP assay. The telomerase activity of cultured cells at passage 2 (a), passage 20 (c), and positive control cells (e). Control ladder (g) is from a positive control DNA of the TRAP assay. Lane b, d, and f are heat inactivated cells of a, c and e, respectively, showing negative results for TRAP assay.

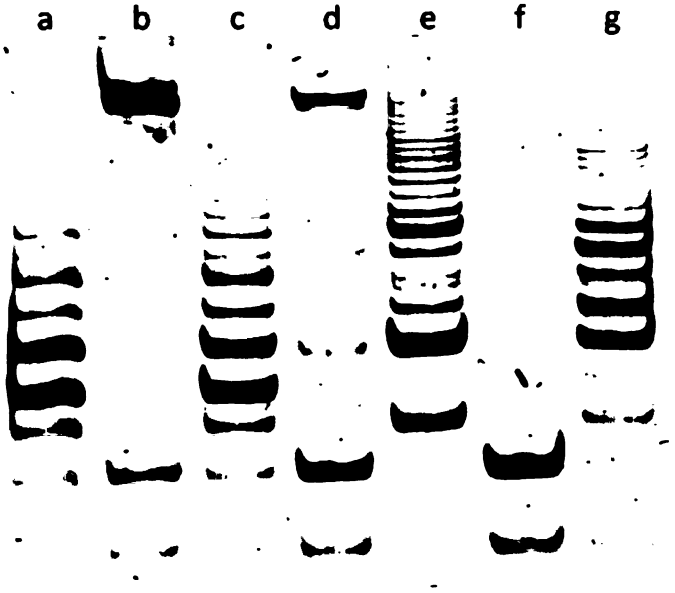


Figure 6 Expression of Vimentin in cultured cells. Cells were grown in either D-NACs (a,b) or K-NACs (c,d) and stained for Vimentin (green) at passage 1 (a,c) and passage 15 (b,d). Nuclear staining is shown in blue. Scale bar is 50 µm.

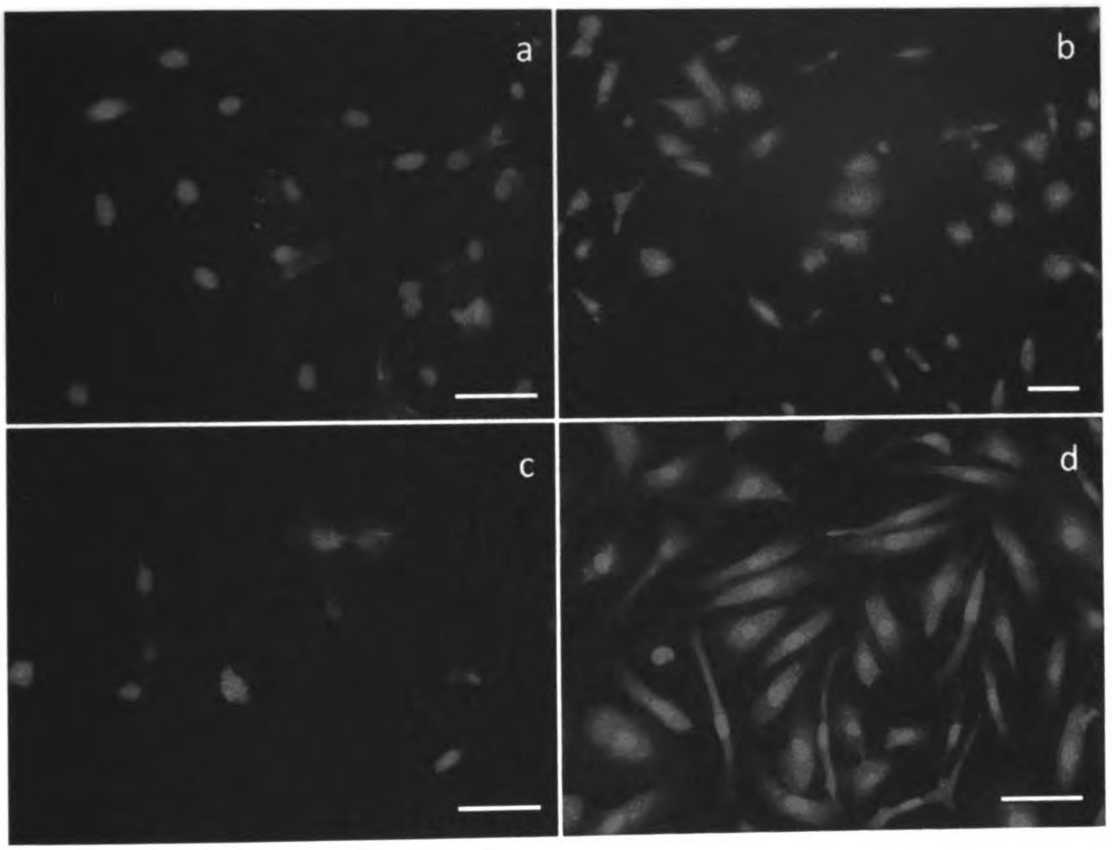
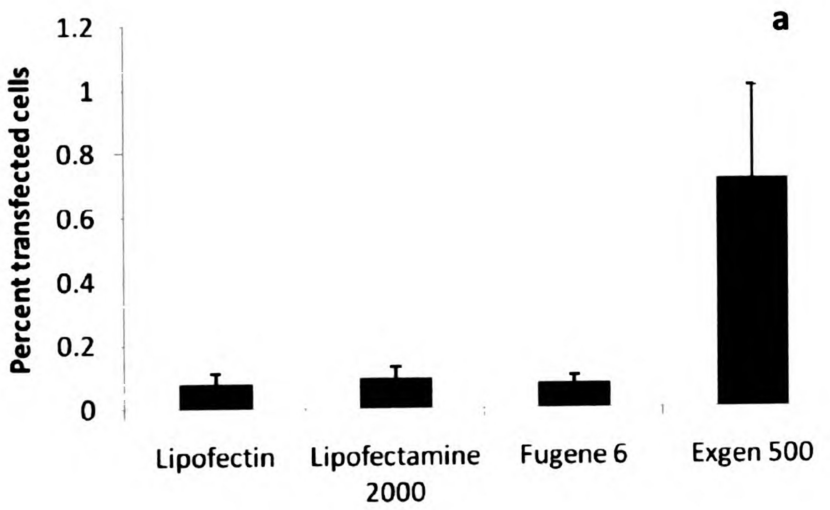


Figure 7 DNA uptake rates of zebrafish cells using liposome-mediated transfection reagents (a), or electroporation (b). The image is merged from phase contrast and green fluorescence channel, as green color depicted expression of green fluorescence protein under pCS2-GFP (b). Scale bar is 100 μm.



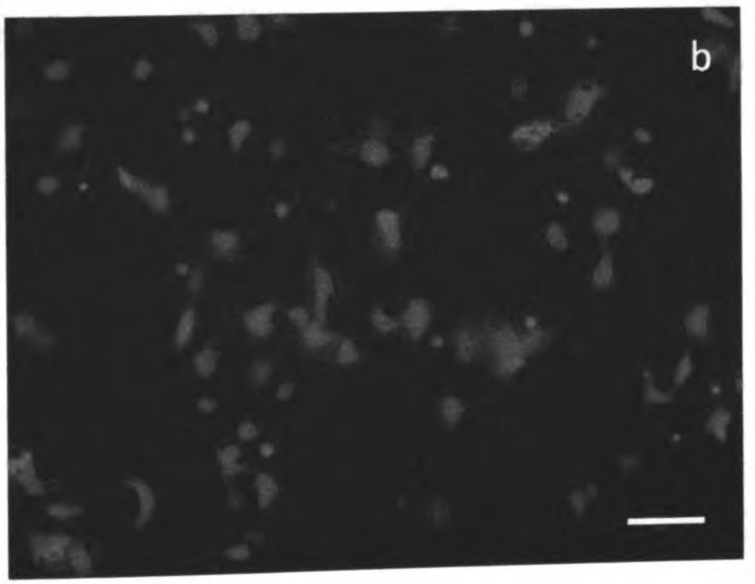


Figure 8 Toxicity of VPA in zebrafish embryos. Embryos at day 1 post fertilization, following the treatment of VPA at indicated concentrations. Treated embryos showed abnormalities at their segmentation period. Except control IVF embryos, all treated embryos died at day 4 post fertilization. Scale bar is 1 mm.

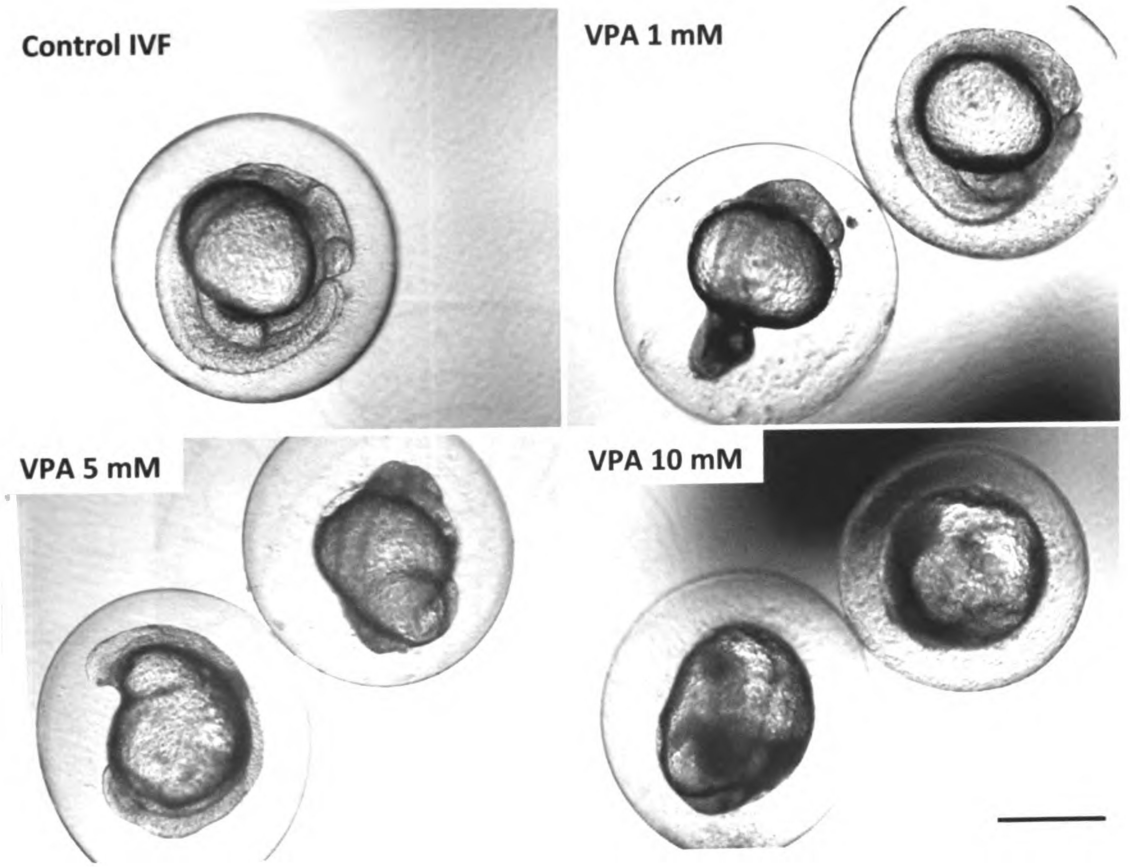
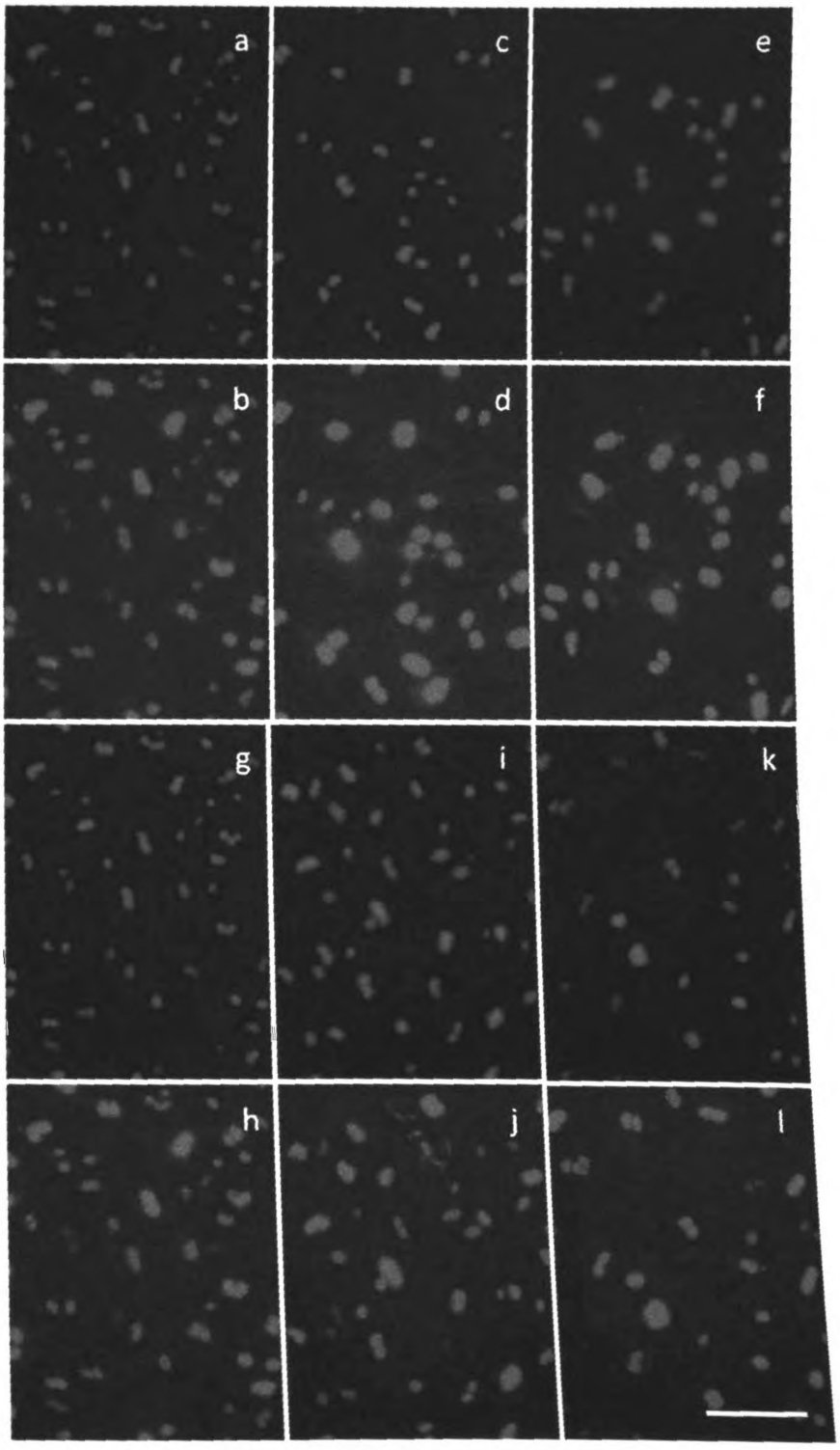


Figure 9 Level of histone acetylation in cultured cells. Acetylation of histone H4K5 in control cells (a-b, g-h), and treated cells with 100 nM TSA (c-d), 200 nM TSA (e-f), 200 µM VPA (i-j), and 500 µM VPA (k-l). Blue color depicts nucleus of the cells, and green color is levels of acetylation at histone H4K5. Scale bar is 100 µm.



CHAPTER 3

CHARACTERIZATION AND *IN VITRO* CONTROL OF MPF ACTIVITY IN ZEBRAFISH EGGS

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Abstract

We describe the characterization of maturation-promoting factor (MPF) in zebrafish eggs and used different defined conditions to maintain its activity *in vitro.* MPF activity levels are high in freshly ovulated mature eggs and decline rapidly within 5 min after either fertilization or parthenogenetic activation. The MPF activity of eggs matured *in vitro* declines faster when the eggs are incubated in Hank's culture medium supplemented with 0.5% BSA (H-BSA) than when incubated in Chinook salmon ovarian fluid (CSOF). MPF activity in non-activated, aged eggs remains high in H-BSA supplemented with 75 μM MG132 or 10 mM caffeine, but neither MG132 nor caffeine can sustain high MPF activity in activated eggs. MG132-treated eggs showed delayed completion of metaphase and extrusion of the second polar body. Nuclear staining of the activated eggs confirmed the correlation between their cell cycle stage and MPF activity at each time point. An embryo toxic effect was found when matured eggs were held in 100 μM of MG132 or 20 mM caffeine for 1 h. Calcium-depleted medium and 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid also showed detrimental effects on the embryos. Conversely, nonactivated, aged matured eggs maintained high MPF activity and developmental potential when CSOF was used as a holding medium.

Introduction

Changes in maturation promoting factor (MPF) activity in matured zebrafish eggs through postfertilization zygotes have not been reported. This study primarily focuses on the characterization and *in vitro* modulation of MPF activity in zebrafish, with the long-term aim of defining the best recipient eggs to use in somatic cell nuclear transfer.

In general, it is widely accepted that a more efficient protocol for nuclear transfer can be obtained when oocytes are at metaphase arrest at the moment of fusion with the somatic cell. A recent publication has shown that enucleated zygotes can be used as recipient cytosol, as long as they are arrested at metaphase (Egli et al., 2007). The data suggest that reprogramming factors are not depleted after oocyte activation but can be retained in the zygote (Egli et al., 2007). The first cloned zebrafish were also reportedly produced by transferring nuclei into unfertilized eggs (Lee et al., 2002). More work needs to be done, however, to determine exactly what stage of the zebrafish egg's cell cycle is most suitable for nuclear transfer. Importantly, the efficiency of zebrafish cloning is very low. It could be possible to increase it by exerting better control of the egg's cell cycle. To accomplish this goal, it is necessary to first characterize and later attempt to modulate MPF activity.

Mature zebrafish eggs arrest at the second metaphase of meiosis before spawning (Nagahama, 1994). Metaphase arrest is regulated by cytosolic factor, which inhibits the anaphase-promoting complex/cyclosome. Several pathways have been shown to play roles in regulating activity of cytosolic factor and have been reviewed elsewhere (Schmidt et al., 2006). Briefly, regulation of activity of cytosolic factor is

mediated by (i) activity of MPF, (ii) the Mos-MAPK pathway, and (iii) the Erp1/Emi2 pathway. In Xenopus, these pathways have been recently described to be linked by p90rsk (Inoue et al., 2007; Nishiyama et al., 2007).

Zebrafish MPF consists of a catalytic subunit, Cdc2, and a regulatory subunit, cyclin B (Yamashita, 1998). While the level of cyclin B changes in relation to the different cell cycle stages, Cdc2 in eggs is constitutively expressed and maintained at a constant level (Kondo et al., 1997). Therefore, the activity levels of MPF depend upon the levels of cyclin B. Both, cyclin B levels and MPF activity are low in immature eggs, are high in arrested MII eggs, and decline again once the egg is activated (Figure 10). Pretranscribed cyclin B mRNA aggregates in growing zebrafish eggs (Kondo et al., 2001). This stored/masked mRNA disperses in response to maturation-inducing hormone and is subsequently translated after additional polyadenylation (Kondo et al., 1997). Phosphorylation of Cdc2 at threonine 161 (T161) by Cdk7 forms active MPF and that promotes metaphase arrest. Cyclin B in fish is degraded by the 26S proteasome. The truncated proteins are initially cut at lysine 57 and subjected to ubiquitination for further destruction (Tokumoto et al., 1997). Degradation of cyclin B results in reduction of MPF activity and promotes meiotic exit. Persistent cyclin B levels can be maintained by introduction of a nondegradable protein mutated at lysine 57 (Tokumoto et al., 1997), which prevents eggs from meiotic exit.

Calcium signaling is important for egg activation at fertilization and for initiation of embryogenesis (Ducibella and Fissore, 2008; Webb and Miller, 2000; Webb and Miller, 2003; Whitaker, 2006). There is evidence of a role for calcium in promoting

meiotic exit. In Xenopus, degradation of Erp1/Emi2 is regulated by calcium/ calmodulin-dependent kinase II and Plx1 (Schmidt et al., 2005). During spontaneous activation of matured rat oocytes, cyclin B1 and Mos are degraded by a calcium-dependent proteasome pathway (Ito et al., 2007). No direct evidence demonstrates the role of calcium signaling in the meiotic exit of zebrafish eggs. However, intracellular calcium concentrations increase exponentially at the time of egg activation (Webb and Miller, 2003), and, as in other species, it is thought to trigger many downstream developmental pathways.

Many reagents have been used to modulate MPF activity, including MG132, caffeine, and 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA). MG132 is a potent proteasome inhibitor known to inhibit degradation of cyclin B. A broad range of MG132 concentrations have been used to block cell cycle progression from metaphase. In Xenopus eggs, 100 μM of MG132 inhibits the degradation of cyclin B for up to 1 h (Chesnel et al., 2006). The effective, as well as reversible, dose of MG132 needed to block pig oocytes at metaphase is 10 mM for a period of 30 to 48 h (Chmelikova et al., 2004). In rats, 5 μM of MG132 maintains MPF activity for up to 105 min after oocyte collection. Rat oocytes treated with MG132 were reported to display higher MPF activity than controls, promoting premature chromatin condensation after nuclear transfer (Ito et al., 2005). Later reports showed that cyclin B and Mos levels were maintained in rat oocytes in the presence of 10 to 25 μM of MG132 (Ito et al., 2007). MG132 (5 μM) was shown to reversibly hold rat mature oocytes in metaphase up

to 3 h (Zhou et al., 2003). These data suggested broad efficacy of MG132 across species; therefore, it should also maintain MPF activity in zebrafish egg as well.

Caffeine is another potent reagent known to maintain MPF activity. It has been proposed that caffeine acts through stabilization of the MPF complex (Kikuchi et al., 2000). In the pig oocyte, 5 mM of caffeine elevated MPF activity and promoted metaphase arrest (Kikuchi et al., 2000). Caffeine, at a dose of 2.5 mM, maintained high levels of MPF during pig nuclear transfer; it subsequently promoted premature chromosome condensation and increased the number of reconstructed embryos (Kawahara et al., 2005). Caffeine increased MPF activity and MAPK activity in sheep oocytes at concentrations greater than 10 mM (Lee and Campbell, 2006). Further, incubation of loach fish embryos in 2.6 mM caffeine for 1 h was not found to be toxic (Kopeika et al., 2003). To date, there have been no reports describing the use of caffeine in zebrafish eggs.

BAPTA is a potent calcium chelator. It chelates both intra-and extracellular calcium and is reversible. BAPTA could potentially be used to prevent calcium signaling and to block oocyte activation. At a concentration of 10 μM, BAPTA has been shown to prevent murine oocyte activation (Zernicka-Goetz et al., 1995). It produced a transient effect without affecting embryo viability. Porcine oocytes loaded with 10 μM BAPTA were also blocked from activation (Ruddock et al., 2001). Cyclin B and Mos levels were maintained in rat oocytes in the presence of 10 μM BAPTA with a significant decrease in polar body extrusion, a sign of release from metaphase arrest (Ito et al., 2007). The use of BAPTA in calcium-free medium could potentially inhibit calcium oscillation in

zebrafish eggs and, therefore, prolong the metaphase. All three reagents, MG132, caffeine, and BAPTA, can potentially be of great help in modulating MPF activity in zebrafish.

The present study characterized MPF in zebrafish eggs before and after activation. In addition, the efficacy of reagents that potentially maintain MPF in the eggs was investigated, as well as their toxicity. The results will be applied to direct optimal methodology toward increasing success of cloned zebrafish production.

Results

- **MPF activity, cyclin B, and phosphorylated Cdc2 in unfertilized and fertilized matured zebrafish eggs**

While MPF has been well characterized in some fish species (Iwamatsu et al., 1999; Katsu et al., 1993; Yamashita, 1998; Yamashita et al., 1995), little information is available for zebrafish (Kondo et al., 2001; Kondo et al., 1997; Tokumoto et al., 1997). MPF activity was measured in matured zebrafish eggs upon activation either by fertilization or spontaneous parthenogenetic activation. The differential effect of these two different egg activation protocols on changes of MPF activity was also investigated.

Similar to Medaka (Iwamatsu et al., 1999), zebrafish MPF activity declined within a few minutes after egg activation (Figure 11A). No significant difference was found between fertilization and parthenogenetic activation (Figure 11A). Protein levels of cyclin B and phosphorylated Cdc2 (Cdc2p) declined in a similar fashion (Figure 11B). In addition, the declining pattern of MPF was correlated with mitotic changes in the nucleus of fertilized eggs (Figure 12). Nuclear staining revealed that anaphase II of

meiosis occurred within 5 min postfertilization. Twenty-five minutes later, the zygote nucleus was condensed again and the first cleavage of embryo began at around 35 min postfertilization followed by the second cleavage 25 min later.

- ***In vitro* fertilization rate of arrested matured eggs aged in Chinook salmon ovarian fluid**

Zebrafish eggs undergo spontaneous activation at spawning, within seconds of contact with water. Morphologically, this can be observed as a detachment of the chorion and formation of the fertilization cone (Wolenski and Hart, 1987). Two major holding media are known to maintain the nonactivated stage of zebrafish eggs: Hank's balanced salt solution with 0.5% bovine serum albumin (H-BSA; osmolarity 290 mosmol/L) and Chinook salmon ovarian fluid (CSOF; osmolarity 298 [+ or -] 6 mosmol/L). H-BSA is reported to maintain the fertilization capacity of eggs for up to 1 h (Sakai et al., 1997), whereas CSOF has done so for up to 6 h (Corley-Smith et al., 1999). An experiment was conducted to reevaluate the capacity of CSOF to maintain eggs *in vitro* and the subsequent developmental potential of these fertilized embryos. As previously described (Corley-Smith et al., 1999) an egg's capacity to be fertilized is compromised when aged *in vitro*; the longer the period between collection and fertilization, the lower the developmental rate (Figure 13). Sakai, *et al.* observed that the fertilization rate of eggs aged in H-BSA declined rapidly in 2 h, and no eggs were fertilized at 5 h (Sakai et al., 1997). The current study showed that, in contrast to H-BSA, CSOF could hold egg fertilization capacity for up to 6 h (Figure 13), although a significant decline of developmental rate was observed ($p < 0.0001$). A significant proportion of eggs held in

CSOF for as long as 6 h maintained their fertilization capacity and developmental potential to blastula stage (51%, $p < 0.0001$) and hatch stage (20%; $p < 0.01$).

- **MPF activity, cyclin B, and Cdc2p in arrested matured eggs aged *in vitro***

To determine whether the decrease in fertilization capacity was due to a decline in MPF levels during *in vitro* incubation of arrested matured eggs, MPF activity was measured in eggs that were held in either H-BSA or CSOF. MPF levels declined in both holding media, although at significantly different rates. MPF level decreased faster in H-BSA than in CSOF (Figure 14). Within 1 h, the MPF level of eggs incubated in H-BSA dropped to 60% of its initial value, whereas the MPF level of eggs incubated in CSOF continued to maintain its constant value. The decline in the fertilization rates accompanied the decline in MPF activity.

Eggs incubated in CSOF maintained the appearance of freshly isolated eggs (Figure 15). In contrast, eggs in H-BSA showed various degenerative changes, such as spontaneous activation, detachment of the chorion, and disappearance of yolk granules (Figure 15). The spontaneous activation observed in some eggs in the H-BSA group was accompanied by the degraded form of cyclin B protein found in a Western blot analysis (data not shown). The overall findings support the hypothesis that MPF activity directly correlates with fertilization rates in eggs incubated in H-BSA.

- **Toxicity of MG 132, caffeine, and BAPTA-acefoxymefhyl (AM) esfer in zebrafish embryos**

As described previously, MPF activity in mammalian oocytes can be maintained by MG132, caffeine, and BAPTA. To further evaluate the efficacy of these agents in

maintaining MPF levels in zebrafish eggs, a toxicity test was first conducted to determine what dosage and incubation time were not detrimental to embryonic development. Eggs were collected in CSOF and assigned to control and treatment groups. Either H-BSA or CSOF was designated as a control. The treatment groups included 1-100 μM of MG132 in either HBSA or CSOF for 1 h, 1-50 μM of caffeine in either H-BSA or CSOF for 1 h, and 5-10 μM of BAPTA-AM in calcium-depleted H-BSA for 0.5 to 1 h. After each treatment, eggs were extensively washed in H-BSA and immediately fertilized. Embryonic development at the blastula stage, 1 day and 4 days postfertilization, was recorded (data not shown).

The first signs of MG132 toxicity, observed at a concentration of 100 μM, began on day 1 and continued until day 4 after fertilization. They manifested as an increase in the percentage of abnormal embryos. No toxicity was detected when lower concentrations (less than or equal to 75 μM) were used for 1 h. Eggs treated with 75 μM of MG132 showed a normal appearance and were not different from eggs in H-BSA (Figure 15). While 100 μM of MG132 showed embryonic toxicity, nuclear staining of parthenogenetically activated eggs demonstrated that those eggs were capable of completing metaphase (Figure 16); however, the second polar body was not extruded as rapidly as in nontreated eggs.

Caffeine produced significant detrimental effects on eggs at 50 mM. In embryos previously incubated for 1 h in 20 mM of caffeine or less, a slightly negative effect on development was detected. However, when the treatment time was extended to more than 1 h, an increase in toxicity was observed, suggesting a cumulative detrimental

effect. Caffeine-treated eggs showed signs of degeneration and disappearance of yolk granules (Figure 15). Nuclear staining revealed condensed nuclei in eggs treated with 20 mM of caffeine. After parthenogenetic activation, no extrusion of the second polar body was observed in such eggs (Figure 16).

Calcium-depleted H-BSA was used as a control for BAPTA-AM treatment. A detrimental effect on eggs was observed in the control group when incubated for 1 h. The addition of BAPTA-AM to the holding media produced a more detrimental effect than the controls. Concentrations as low as 5 µM of BAPTA-AM were toxic to the eggs after 30 min of incubation. BAPTA-AM was, therefore, excluded from further studies.

- **Effect of MG 132 and caffeine on MPF levels in matured eggs aged *in vitro* and in activated eggs**

The levels of MPF decline in zebrafish eggs aged *in vitro* and immediately after egg activation. This suggests that treatment of eggs with either MG132 or caffeine could be used to modulate MPF levels in these eggs. To minimize the spontaneous activation after egg collection, eggs were maintained in CSOF for approximately 1 h before starting the treatments. As in the toxicity tests, concentrations of 75 and 100 µM of MG132, and 10 and 20 mM of caffeine in H-BSA were used. In each drug treatment, including controls, eggs were aged *in vitro* by incubation at room temperature. MPF activity, that is, total cyclin B and Cdc2p proteins measured by a Western blot analysis, of *in vitro*-aged eggs remained high during the incubation period in CSOF, and in treatments with 75 µM MG132 in H-BSA, with 10mM caffeine in H-BSA, and in HBSA, respectively (Figure 17).

To investigate the effects of MG132 and caffeine on activated eggs, eggs were treated with either one of these two reagents for 1 h before activation. Egg activation was triggered by dechorination, treating them with 3 mg/mL pronase in the presence of either reagent, to simulate the protocol for somatic cell nuclear transfer. Note that eggs were dechorionated; otherwise, they could not be manipulated using a conventional nuclear transfer protocol (Lee et al., 2002). MPF activity declined within a few minutes in activated eggs, regardless of which tested compound was supplementing the holding media (data not shown). Nuclear staining revealed metaphase exit in activated eggs treated with 100 μM MG132 (Figure 16).

Discussion

This study characterized zebrafish MPF activity in both (i) activated eggs and (ii) mature arrested eggs aged *in vitro*. We found that while CSOF can maintain MPF activity in arrested mature eggs, MG132, caffeine, and BAPTA can be toxic for arrested mature eggs before fertilization. Attempts to modulate MPF activity in activated eggs using these reagents were unsuccessful. Only CSOF maintained high MPF activity in nonactivated, aged matured eggs. After *in vitro* fertilization, CSOF aged eggs were capable of sustaining normal development as well.

These results also confirmed what others have observed in mammalian and in teleost systems (Iwamatsu et al., 1999), that is, MPF levels in zebrafish also increase at metaphase and decline rapidly after the completion of metaphase in fertilized and in parthenogenetically activated eggs. The failure to observe a rapid oscillation of MPF activity during the first and second cleavages may have been due to technical limitations

(Figures 11 and 12), because the samples were analyzed in pools, and it is possible that eggs in the same pool were asynchronous with each other. Besides the rapid decline of MPF activity, cyclin B protein was shown to degrade when H-BSA was used as a holding medium. The decline in the rate of fertilization can be partially attributed to spontaneous activation, as activated eggs showed a detachment of the chorion and the disappearance of the micropyle, the region in the chorion where the sperm enters (Wolenski and Hart, 1987). Another explanation for this decline in the fertilization rate could be a continuous loss of egg viability and overall protein degradation in the holding medium used (Figure 15). Considering that a higher fertilization rate was achieved in nonactivated eggs than in activated eggs when intracytoplasmic sperm injection was performed (Poleo et al., 2001) it is reasonable to conclude that the egg activation process triggers not only morphological changes that hinder the fertilization capacity, but also irreversible physiological changes that underlie embryonic development.

CSOF, H-BSA, MG132, and caffeine were tested in an effort to find a compound or set thereof that could maintain MPF activity and delay egg activation. The results showed that both MG132 and caffeine partly help sustain MPF activity in eggs aged *in vitro*. MG132 may have partly mimicked the effect of CSOF, because the level of cyclin B protein remained relatively high in treated eggs (Figure 17). However, there was no advantage in using MG132 rather than CSOF in terms of improving the developmental rate of *in vitro* fertilized-embryos and maintaining nonactivated egg quality (Figure 15). Even though caffeine-treated eggs might maintain high MPF, caffeine's toxic effect, demonstrated by degenerative changes in appearance (Figure 15), chromatin

condensation (Figure 16), and embryo toxicity, makes it an unsuitable candidate for such use.

While these agents seem to help maintain MPF activity in nonactivated eggs, none of them can alter MPF activity in previously activated eggs. Only delayed resumption of meiosis II was observed using MG132; however, an increase in their concentration only exacerbated its embryonic toxicity.

The current results demonstrate that to maintain high MPF activity as well as fertilization capacity, none of the reagents tested can replace the fluid obtained from the ovaries of Chinook salmon. The Chinook salmon is a Pacific salmon that reproduces only once, at the end of its life cycle. Although the composition of CSOF has not been reported, several studies on the ovarian fluids of closely related salmonid species have been conducted (Coffman and Goetz, 1998; Lahnsteiner et al., 1995; Olsen et al., 2001; Rime et al., 2004). Some of the components of this fluid include heat-/acid-stable serine protease inhibitor, antibacterial substances (lectins), apolipoprotein A-I-1, and hormones. However, no direct evidence links any of these compounds with the ability to maintain the nonactivated stage of zebrafish eggs *in vitro*. There is speculation that the osmolarity of ovarian fluid may prevent egg activation *in vivo*, though it is not the sole reason, as demonstrated by Sakai, *et al* using HBSA. Perhaps antiproteases act to prevent degradation of several proteins, including MPF, extending the egg viability period *in vitro*.

The activity of MPF declines slowly over time in nonactivated eggs aged *in vitro* in CSOF, MG132, caffeine, and H-BSA, respectively. Toxic effects were found when 100

μM MG132, 20 mM caffeine, and calcium-depleted medium were used as holding media before fertilization. The current study argues in favor of using CSOF to maintain nonactivated eggs for extended periods before performing somatic cell nuclear transfer or intracytoplasmic sperm injection experiments. Considering that activated eggs show irregular levels of MPF, the overall success of somatic cell nuclear transfer may fluctuate depending on whether the time of nuclear transfer matches the high point of MPF activity or not. Only nonactivated eggs with high MPF activity would be the best recipients for somatic cell nuclear transfer.

Methods

Egg/milt collections and in vitro fertilization. Eggs and milt from zebrafish (Tubingen strain) were obtained using the stripping technique described previously (Westerfield, 1993). Briefly, breeding pairs were set in individual tanks the day before the eggs and milt were collected. The male was introduced to a female tank in the morning after the light came on. Once breeding activity began, and before spawning, the female fish was immediately isolated from the male. Females were anesthetized using MS-222 (Sigma, St. Louis, MO), and the eggs were collected by gentle stripping and placed in either CSOF or H-BSA. CSOF was obtained from Chinook salmon (*Oncorhynchus tshawytscha*), kindly donated by the Michigan Department of Natural Resources. Eggs were selected under a stereomicroscope, and only those of good quality (yellowish and granular) were selected for subsequent experiments. At least three females were utilized for each experiment.

For milt collection, male fish were anesthetized in MS-222 and subsequently placed in the slit of a sponge with their tails hanging over and their genital openings at the edge. Excess water was dried out, and the milt was collected in a capillary tube under a stereomicroscope by gently stripping. Milt was immediately transferred to ice-cold Hank's solution. Before its use, a portion of milt was evaluated for sperm density and motility under a microscope. Good-quality sperm was of high density and nonmotile in Hank's solution, but became active swimming when egg water was added. Milt was collected from at least three males for each experiment, and each batch was used within 30 min of collection.

Eggs were activated by either fertilization or parthenogenesis. We performed in vitro fertilization according to a core protocol from The Zebrafish Book (Westerfield, 1993). For delayed *in vitro* fertilization, either H-BSA (Sakai et al., 1997) or CSOF (Corley-Smith et al., 1999) was used as a holding medium. Parthenogenetically activated eggs were moved from the holding medium to egg water. To measure MPF activity, pools of 10 eggs or embryos were collected at designated times before and after fertilization or parthenogenetic activation. All samples were snap frozen in liquid nitrogen and stored at -80°C.

Cell lysates. Before use, samples were thawed and lysed in sample buffer (50 mM Tris, 0.5 M NaCl, 5 mM EDTA, 2 mM EGTA, 0.01% Brij35, 50 mM 2-mercaptoethanol, 25 mM [beta]-glycerophosphate, 1 mM Na-orthovanadate, and protease inhibitors), with a ratio of one egg per 2 μL of sample buffer.

MPF activity by ELISA. MPF activity ($p34^{cdc2}$ kinase) was quantitatively measured using a Mesacup Cdc2 Kinase Assay Kit (MBL International, Woburn, MA). The activity of $p34^{cdc2}$ kinase measured by this kit showed high correlation coefficients (as high as 0.9961) to histone H1 kinase activity measured by a radioactive method (Ito et al., 2001). Results were read as the optical density (OD) at 492 nm. The OD_{492} values of each sample were quantified as a percentage of sample MPF activity to control MPF activity at time zero. At least three biological replicates were performed for each experiment.

Western blotting for cyclin B and Cdc2p. To confirm the presence of cyclin B and Cdc2p, the samples were analyzed by Western blotting using standard protocols (Sambrook and Russell, 2001). Briefly, lysates were prepared in SDS sample buffer, and according to its different molecular weight, cyclin B was analyzed in 10% SDS-polyacrylamide gel electrophoresis, while Cdc2p was analyzed in 12% SDS-polyacrylamide gel electrophoresis. Approximately 1 egg from the total pool lysates of 10 eggs was loaded to each lane. Proteins were separated by electrophoresis at 50 V for 4-6 h in Tris-glycine buffer and transferred onto the polyvinylidene fluoride (PVDF) membrane at 4°C, 100mA for 4h in transfer buffer. Blots were blocked in 5% skim milk in Tris-buffered saline and 0.1% Tween-20 (TBST), with agitation for 90min at room temperature. Mixed mouse monoclonal IgGs primary anti-cyclin B1 antibody (Upstate Biotechnology, Lake Placid, NY) was diluted to 1 μg/mL in TBS. Goat primary anti-Cdc2p (T161) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted to 1 μg/mL in TBST with 3% skim milk. Blots were incubated overnight at 4°C with each primary antibody and then washed extensively with TBST. Either horseradish peroxidase-conjugated goat anti-

mouse antibody or bovine anti-goat antibody (Santa Cruz Biotechnology) was diluted to 0.4 μg/mL in TBST with 3% skim milk. Blots were incubated with each counterpart secondary antibody at room temperature for 90 min and then washed extensively with TBST. Blots were immersed with SuperSignal West Chemiluminescent Substrate (Thermo Scientific Pierce, Rockford) for 5 min and subsequently exposed to X-ray films.

Nuclear staining. Eggs were dechorionated in 3 mg/mL pronase and washed extensively in Holtfreter's solution. At the time of collection, samples were immersed in 4% cold paraformaldehyde for 12 to 24h. Fixed samples were washed in 0.1% Triton-X100 in PBS (PBSTx) for 20 min and then stained with 1 μg/mL Hoechst 33342 in PBSTx for 15 min. Samples were triple-washed with gentle agitation in PBSTx. Images were taken under a fluorescence microscope with Image-Pro Express (Media Cybernetics, Bethesda, MD).

Statistical analysis. A two-way linear mixed model analysis of variance (Littell et al., 1996) was performed on the percentage of abnormal embryos. The fixed effects included in the model were treatment (16 levels), development stage (3 levels), and their interaction. The random effects of female and random interaction of female by treatment were included to account for repeated measures on the same individual. Model fit was assessed by analysis of the residuals. Our study revealed no evidence of association between treatment and development stage ($p = 0.66$). Significant main effects of treatment ($p = 0.015$) and developmental stage ($p < 0.0001$) were observed. Differences between the treatment levels were further assessed through pair-wise comparisons. Tukey's test was used to account for multiple tests, using an overall significance level of $p = 0.05$.

Figure 10 Morphological and molecular changes during parthenogenetic activation of zebrafish eggs. (A) Cdk7 phosphorylates Cdc2 at threonine 161 and when combined with cyclin B (ClnB) – present at high levels in mature eggs – form active MPF. (B) Mature eggs with high MPF activity are arrested at metaphase II of meiosis. The micropyle is closely associated with the egg's chromosomes and the first polar body. (C) Egg activation triggers degradation of ClnB by ubiquitin-dependent proteasome pathway, decreasing the activity of MPF. (D) Activated eggs exit metaphase, extrude the second polar body, and form the female pronucleus. MPF = maturation promoting factor, Cdc2P = phosphorylated Cdc2, UB = ubiquitination, MII = metaphase II of meiosis, 1st PB = first polar body, 2nd PB = second polar body, FPN = female pronucleus.

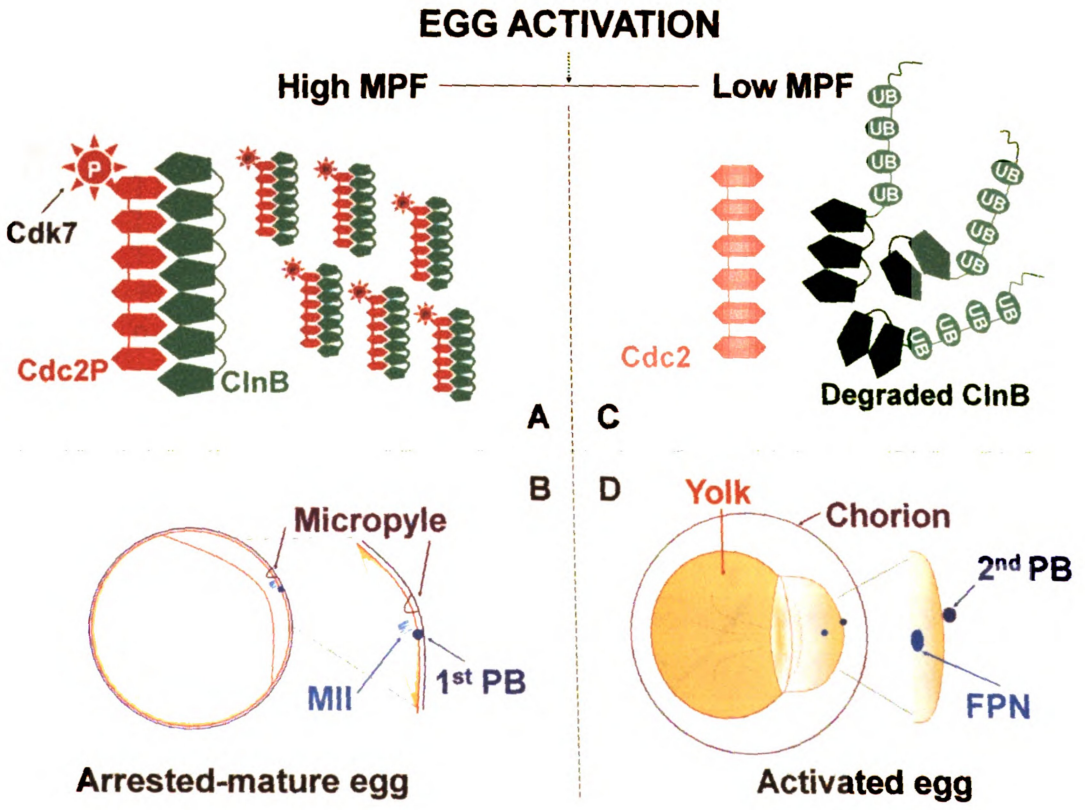
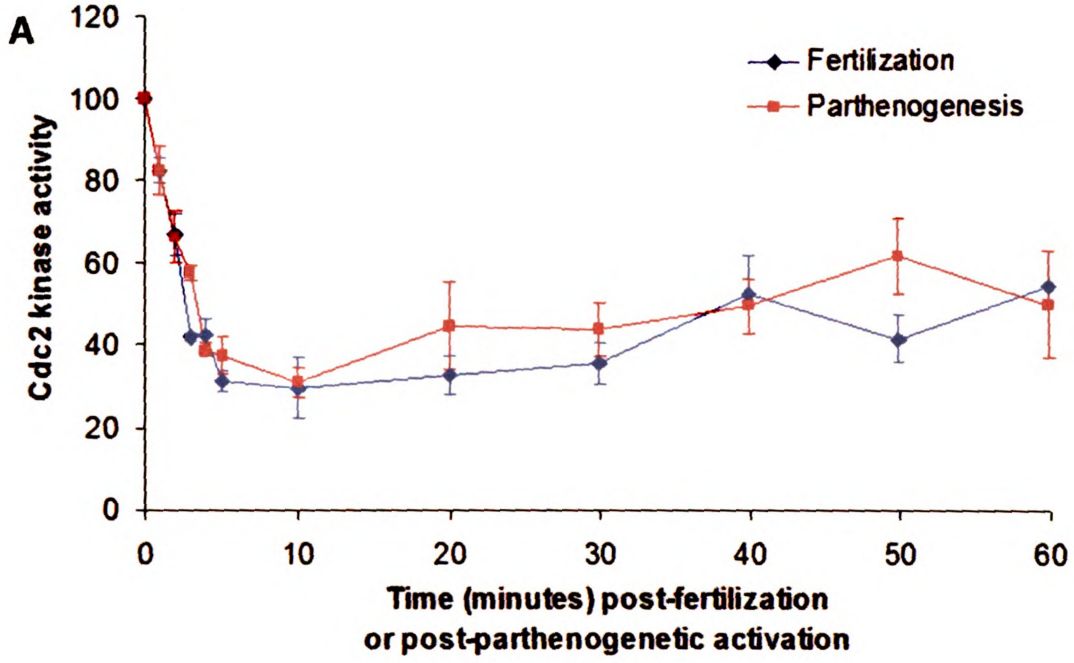


Figure 11 MPF activity in eggs activated either by fertilization or parthenogenesis. (A) Cdc2 kinase activity of activated eggs. (B) Western blot of cyclin B1 and Cdc2p of activated eggs. Time indicates minutes post-fertilization or minutes post-parthenogenetic activation. Error bars are standard error of means.



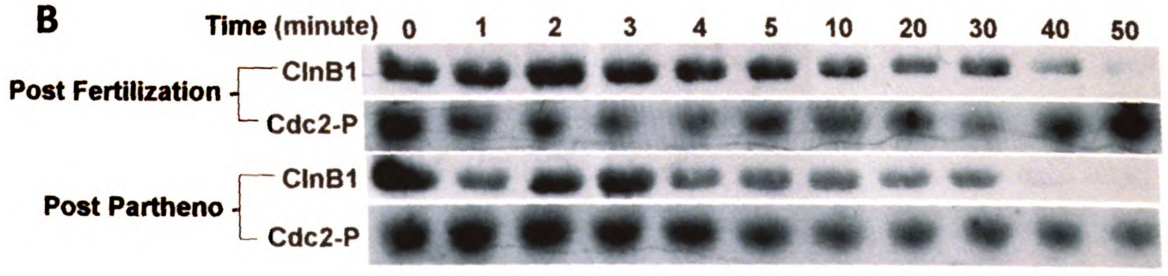


Figure 12 Nuclear staining of fertilized eggs. Numbers indicate the time (min) after fertilization; metaphase (0 min/3 min), completion of metaphase (5 min), extrusion of second polar body (10 min), decondensed male and female pronuclei (25 min), beginning of the first cleavage (30 min), anaphase (35 min), 1 of 2 cells (40 min), decondensed nucleus (45 min), and beginning of second cleavage (55 min). Scale bar = 10 μm.

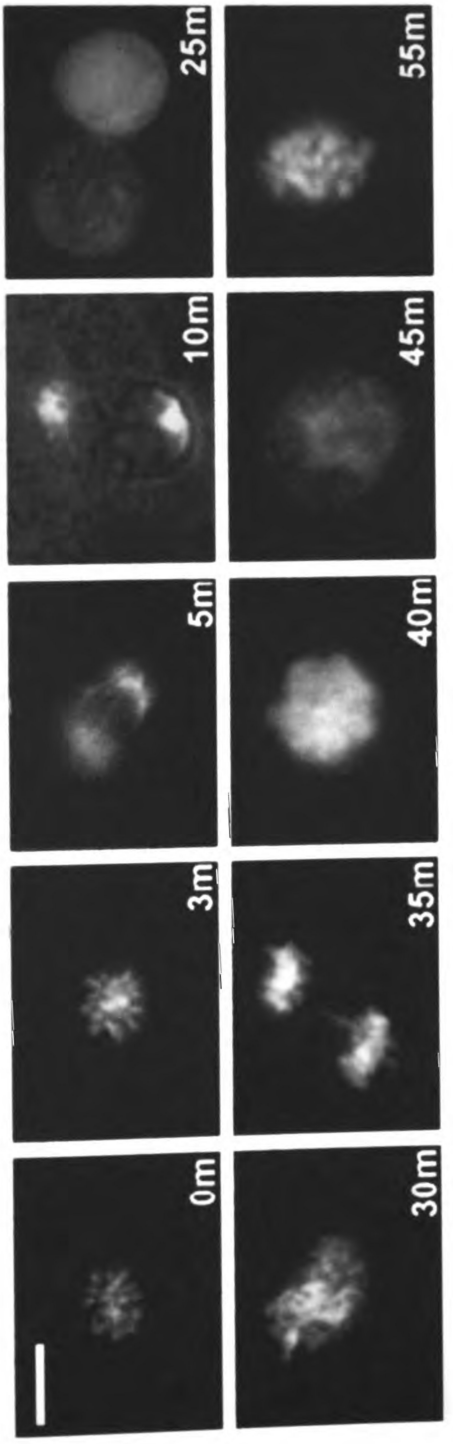


Figure 13 *In vitro* fertilization rates of eggs aged in CSOF. Graph shows percentage of fertilized embryos obtained from eggs aged in CSOF for 1, 2, 3, 4, 5, and 6 hr post-collection. Natural breeding (NB) indicates rate of fertilization at time 0. Solid bars indicate blastula development, and open bars indicate hatched embryos. Error bars show standard error of means of three biological replicates.

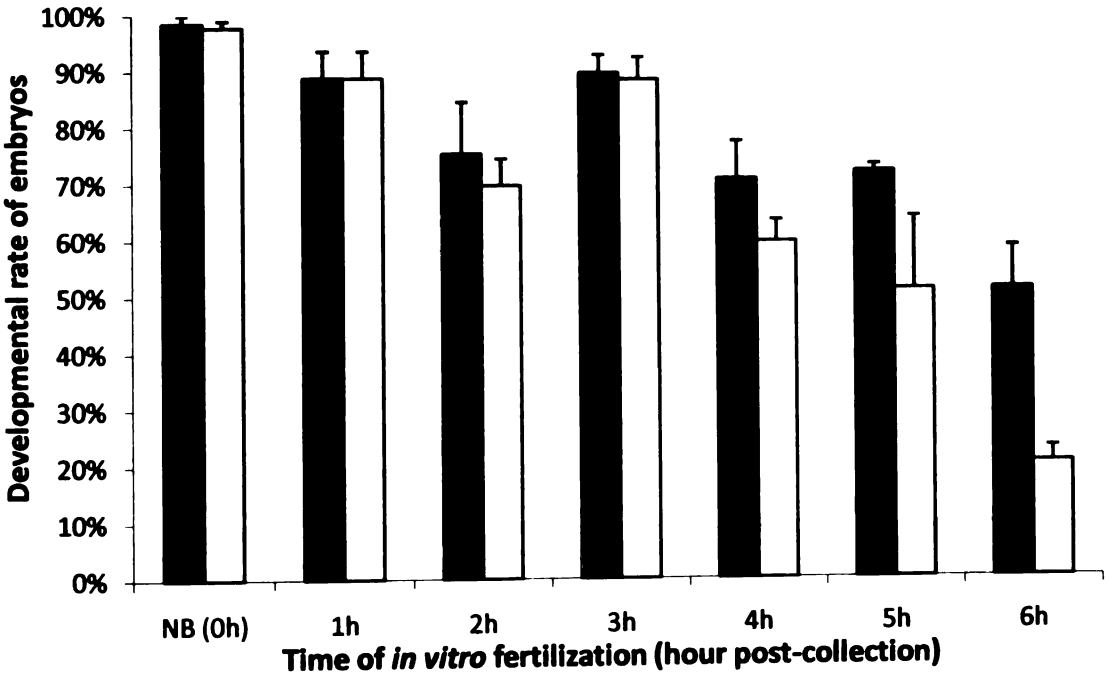
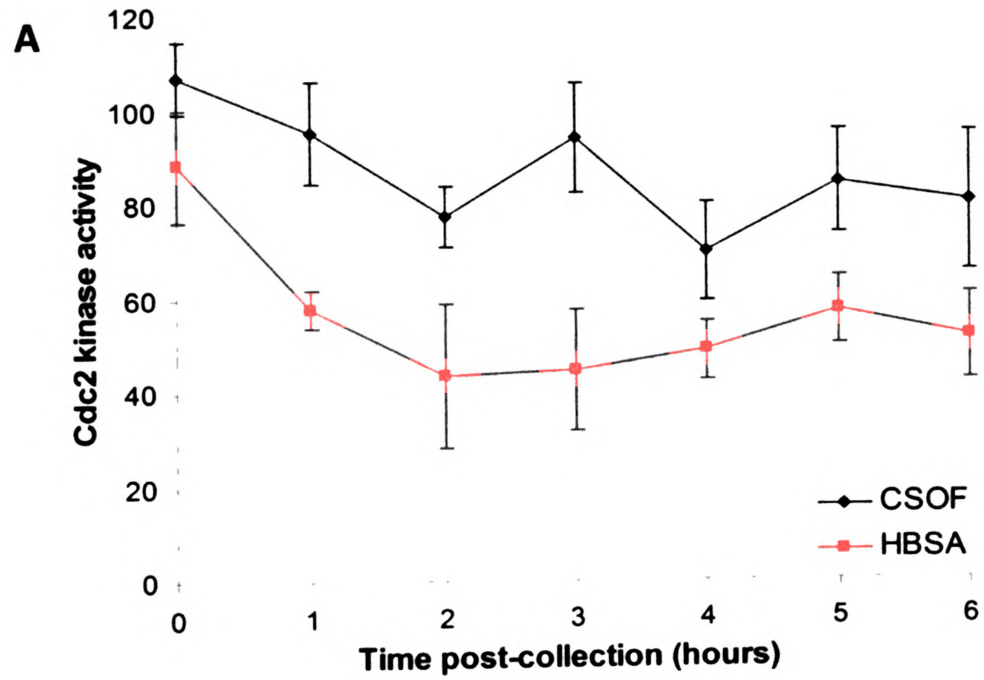


Figure 14 MPF activity of *in vitro* aged eggs in either CSOF or H-BSA. (A) Cdc2 kinase activity. (B) Western blot analysis of Cdc2p. Time indicates hr post-collection. Error bars are standard error of means.



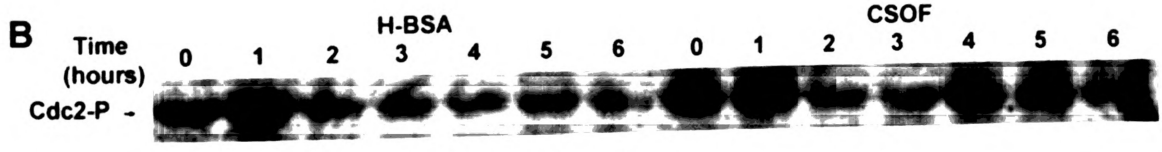


Figure 15 Pictures of arrested matured aged eggs in CSOF, H-BSA, 75 µM MG132 in H-BSA, and 10 mM caffeine in H-BSA. Images were captured at the indicated hr post-collection (hpC). Images were taken from random fields. Identical results were observed from the three biological replicates performed. Only one of the three is shown here. Scale bar = 500 µm. Arrows indicate eggs with degenerative appearances at 3 hpC.

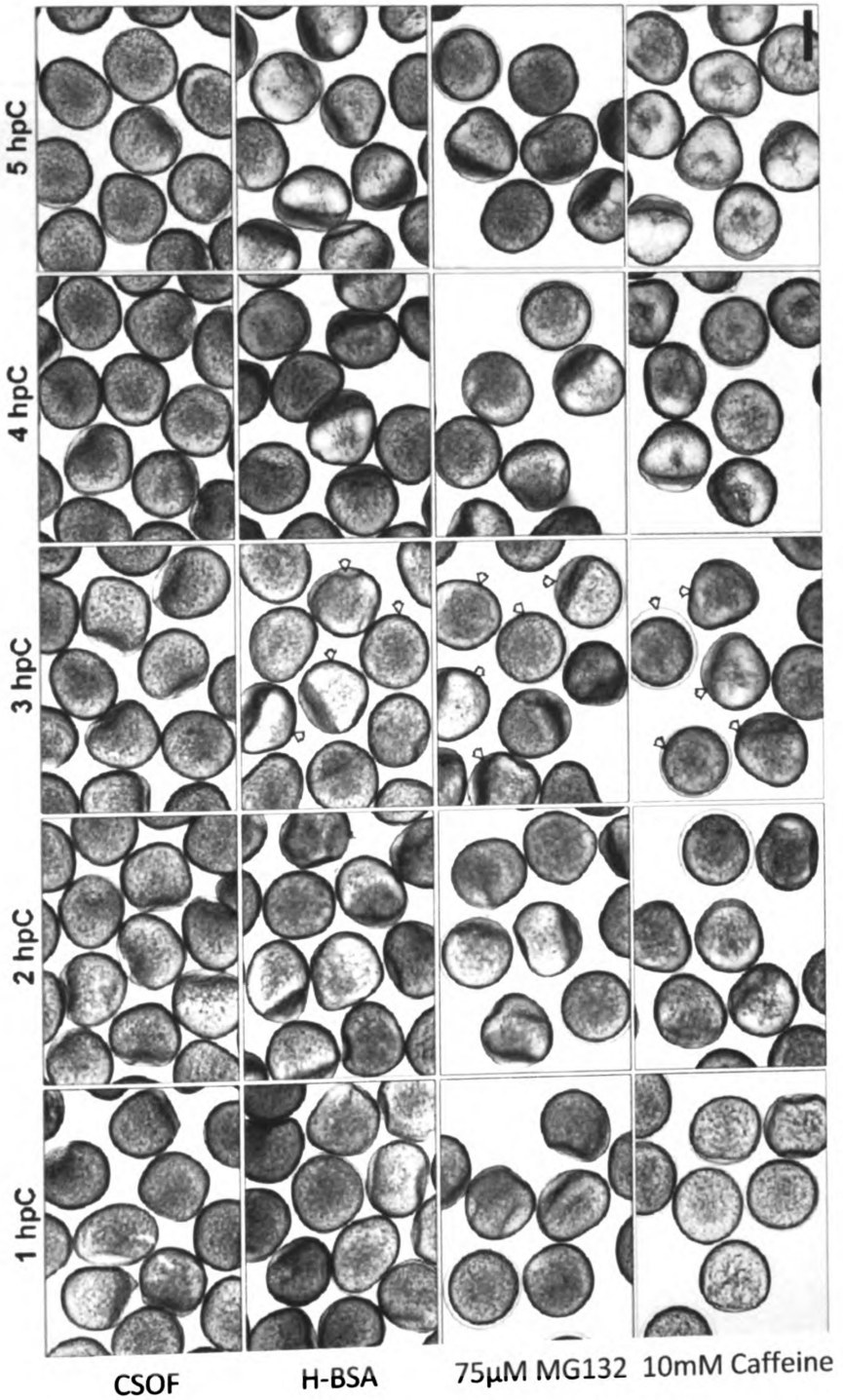


Figure 16 Nuclear staining of parthenogenetic embryos. Eggs were incubated in 100μM MG132, 20mM Caffeine and control medium (H-BSA) and then fixed at the indicated time of post-parthenogenetic activation. Following parthernogenetic activation, eggs underwent metaphase exit when either H-BSA or MG132 supplemented H-BSA were used, however, caffeine treated eggs showed chromatin condensation and no extrusion of second polar body. Scale bar = 10 μm.

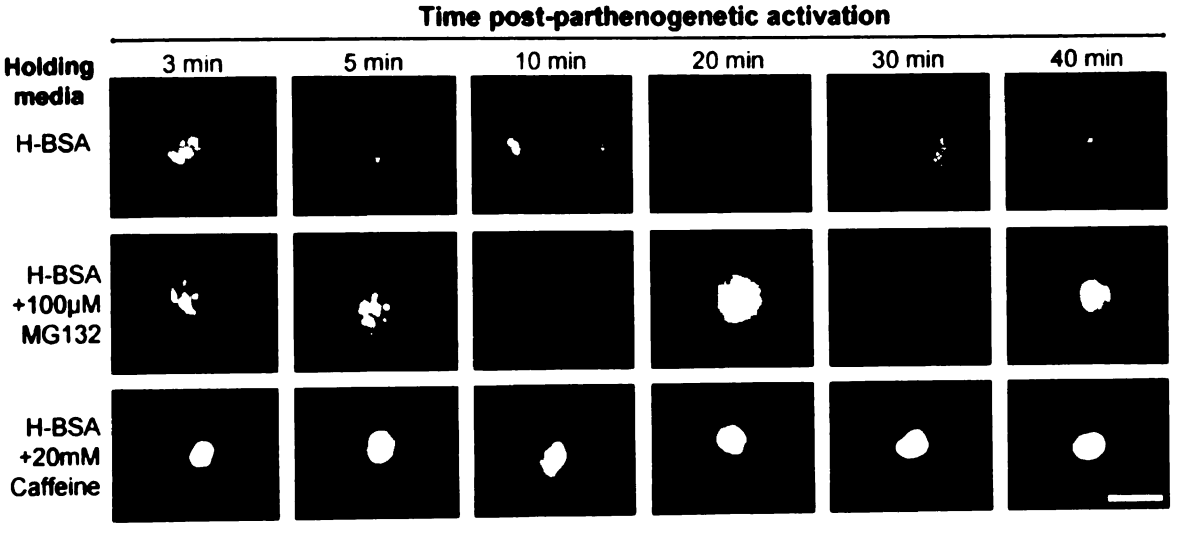
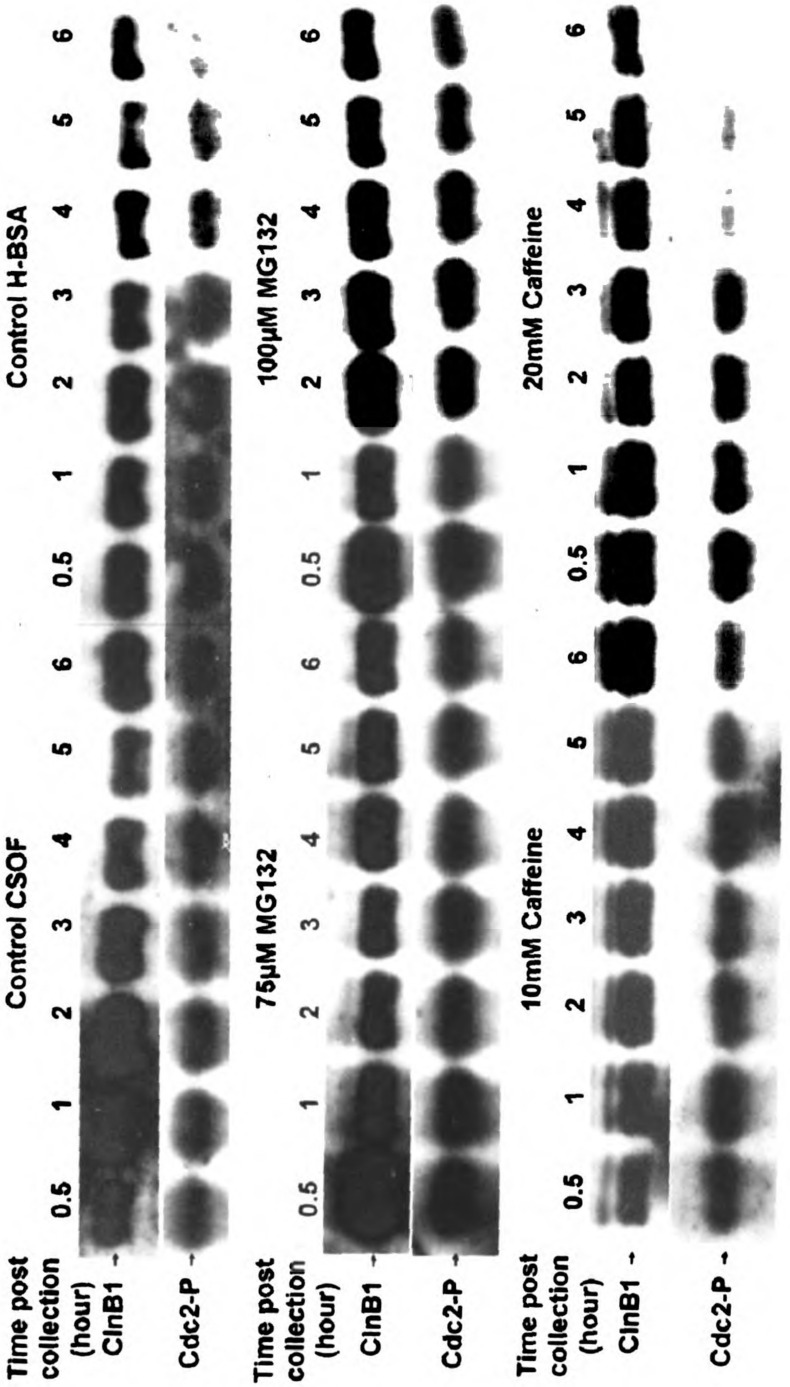


Figure 17 MPF activity in matured eggs aging *in vitro*. Western blot analysis for cyclin B1 and Cdc2p of mature eggs in H-BSA, CSOF, H-BSA with MG132 (75 or 100 μM), and H-BSA with caffeine (10 or 20 mM). Line numbers indicated hr post-collection.



CHAPTER 4

SOMATIC CELL NUCLEAR TRANSFER IN ZEBRAFISH

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Abstract

We developed a method for somatic cell nuclear transfer in zebrafish using laser-ablated metaphase II eggs as recipients, the micropyle for transfer of the nucleus and an egg activation protocol after nuclear reconstruction. We produced clones from cells of both embryonic and adult origins, although the latter did not give rise to live adult clones.

Introduction

Zebrafish is a convenient, relatively inexpensive and useful vertebrate animal model for the study of normal and pathological development, physiology, aging and disease. Large-scale mutagenesis and screening have proven to work efficiently in this organism. However, these 'forward genetic' approaches are highly laborious and time-consuming (Anderson and Ingham, 2003). A simple 'reverse genetics' method is necessary to bring the zebrafish model system into parity with rodent model systems. Mutant knockout or knockin mice are routinely generated using gene targeting in embryonic stem cells. Despite substantial effort (Fan et al., 2006; Ma et al., 2001), there

are no reports of transgenic zebrafish with germline transmission generated using this approach.

Somatic cell nuclear transfer (SCNT) has the potential to become the method of choice for germline genetic modification in fish. A previous report of cloned zebrafish demonstrated that nuclear transfer with cultured cells is possible, with an efficiency of cloned fish production at 2% or less (Lee et al., 2002). However, the reproducibility of the protocol is poor. Multiple factors may be responsible for this, among them are the use of activated eggs as recipients, which limits manipulation time to less than 1 h after egg collection; the technical challenge of removing the egg's chromosomes by removing a portion of egg's cytoplasm underneath the second polar body without staining the DNA; and the manipulation of dechorionated eggs and handling of the fragile reconstructed embryos. We describe here a simplified methodology that addresses each of these problems.

Our method relies on (i) the use of mature, arrested eggs at metaphase II of meiosis as recipients, making use of the observation that mature eggs can be maintained in an inactivated state in Chinook salmon ovarian fluid (CSOF) for up to 6 h with negligible detrimental effects (Siripattarapravat et al., 2009a); (ii) complete laser inactivation of the Hoechst-stained DNA in the metaphase plate of the egg, leaving the egg's cytosol intact; (iii) delivery of donor cells through the micropyle, the route that the fish sperm uses to enter the egg, using a human intracytoplasmic sperm injection needle to transfer the nucleus into the animal pole of the egg; (iv) egg activation after nuclear transfer; and (v) manipulation of cloned embryos with intact chorion (Figure 18). Using

this approach, the egg remains at metaphase II after reconstruction until activation in egg water (Figure 19a). Notably, in most of the species cloned to date, metaphase II oocytes have shown to be the most suitable recipient cytosol for SCNT (Egli et al., 2007). In comparison to control parthenogenetically activated embryos (Figure 19b), embryos of the laser-ablated group showed an absence of female pronuclei and second polar body extrusion (Figure 19c).

To facilitate phenotypic screening of clones, we used donor cells from two types of zebrafish: (i) *golden* fish (Lamason et al., 2005) in an AB strain background, in which homozygotes have *golden* phenotypes and heterozygotes appear as wild-type zebrafish, and (ii) zebrafish expressing GFP in the Tubingen long fin (TuLF) background (Nagayoshi et al., 2008). We isolated donor cells from the 15–20 somite-stage embryonic tailbud or cultured cells from adult caudal fin. We obtained recipient eggs from either wild-type zebrafish, transgenic zebrafish homozygous for histone H2A tagged with GFP (*H2AzGFP*) (Pauls et al., 2001) or a line derived by outcrossing Tubingen and AB lines (TAB).

We monitored the *golden* phenotype or the green fluorescence of transgenic TuLF in clones produced by this technique. The use of *golden* donor cells in combination with wild-type recipient eggs produced *golden* fish (Figures 20a and 20b). The use of transgenic TuLF donor cells in combination with TAB recipient eggs produced GFP^{+} fish that showed a long-fin phenotype upon reaching adulthood. SCNT offspring using recipient eggs from transgenic $H2AzGFP^{+/+}$ fish provided evidence of complete inactivation of the egg genome, as they showed a loss of nuclear localized green fluorescence (Figure 20c). In addition, to confirm that no genetic material of the

recipient cell was carried over to clones, we performed single-nucleotide polymorphism (SNP) genotyping analysis (Table 3). The DNA fingerprint of cloned fish showed a complete match to that of a donor cell (Table 4).

Approximately 40% of reconstructed embryos completed the blastula stage of development (Table 1). Of these, more than half paused between high (3 h) and sphere (4 h) stages, and later did not enter gastrulation. Clones that did not complete gastrulation (90% epiboly), usually completed the germ ring stage but did not form the embryonic shield, an involution of the hypoblast. Most of the clones from embryonic tailbud donor cells that completed 90% epiboly developed to 1 d (completed segmentation). For adult caudal fin donor cells, less than half of clones that completed gastrulation also completed segmentation.

Using *golden* donor cells, approximately 2–15% of reconstructed embryos developed to 1-d-old fry (Table 5). Up to 2.2% of reconstructed embryos, using embryonic tailbud cells and wild-type eggs, grew to fertile adult fish (Table 5). Clones from *golden* fish reached reproductive maturity and when crossbred with a *golden* counterpart produced 100% *golden* offspring (Figure 20d). Offspring (F_1 generation) of both *golden* clones were healthy and produced *golden* fish in the F_2 generation. One of the adult *golden* fish died at 21 months (Figure 20b) and we killed the other at 16 months because of signs of emaciation.

Using TuLF donor cells, 3.3–10.7% of reconstructed embryos developed to 1 d (Table 5). All of the clones expressed GFP. Six percent of reconstructed embryos using embryonic tailbud cells developed to eating fry and five clones survived to adulthood. At

the time of this writing, 3 clones were at 4 months of age, healthy and produced offspring carrying their genetic traits.

We observed various extents of abnormality in 1-d-old clones (Figure 21). Most abnormal clones showed more posterior rather than anterior developmental defects, that is, all abnormal embryos showed primitive development of the head and eyes but defective tail formation. Most of the cloned embryos that did not develop to 4 d displayed severe abnormalities such as growth retardation, a bent tail, a small head, a lack of hematopoiesis and a short trunk. Clones that developed to 4 d but did not eat showed minimal abnormalities, including no swim bladder formation and enlarged pericardium. Despite any detectable abnormality, some clones died after 7–10 d, and some clones could eat but died at 12–20 d. In the latter cases, it is uncertain whether the death was caused by cloning or could be explained by common loss of fish embryos at an early age. In all cases, abnormal clones of adult caudal fin donor cells showed more progressive abnormalities than those of embryonic tailbud donor cells. We did not observe the 'no head' phenotype as reported by others (Lee et al., 2002).

All clones examined had a normal karyotype ($2n = 25$) as shown by replication banding (Amores and Postlethwait, 1999)(Figure 22a). In addition, we used 11 SNPs to confirm the identity of cloned embryos, donor cells and female egg donors (Figure 22b). All cloned fish showed complete matched genotypes to one of the donor cells, but not to those of the female egg donors (Table 4).

The use of metaphase II eggs in CSOF allowed for longer manipulations sessions, approximately 50 eggs per person per day. For cell transfer, the use of intracytoplasmic

sperm injection needles in combination with injection through the micropyle avoided premature egg activation from dechorionation by pronase (Siripattarapravat et al., 2009a). Additionally, eggs with an intact chorion were more tolerant to micromanipulation and injection, as naked eggs are easily broken by the suction of the egg holder or sharp-point injection needles. Our technique overcomes these difficulties, allowing us to manipulate eggs more practically and monitor developing cloned embryos independently.

The combined use of SCNT and donor cells that can be grown *in vitro* would allow for the use of knockout and knockin methodologies in which the integration site and disposition of the transgene can be confirmed before generating cloned zebrafish. Furthermore, a permanent reservoir of cells with the desired genotype can be maintained in the form of cultured somatic cells and/or cryopreserved samples. Above all, the timeline to produce a founder fish carrying the targeted gene could be shortened by 6–7 months. A zebrafish SCNT procedure like the one described here could enhance the advantages of this model for studies of vertebrate developmental biology and human disease.

Methods

Zebrafish resources. For donor cells, we used homozygous *golden* (*slc24a5*$^{b1/b1}$) in the AB background (Lamason et al., 2005) and transgenic fish lines expressing GFP (HGn62A, HGn28A and HGn8E) in the TuLF background (Nagayoshi et al., 2008). Recipient eggs were obtained from wild-type, transgenic homozygous histone H2A–tagged with GFP

(*H2AzGFP*) in the AB background (Pauls et al., 2001) or outcrossed fish of Tubingen and AB line (TAB).

Preparation of recipient eggs. Eggs were obtained from sound females using the stripping technique (Westerfield, 1993). Each female fish was sedated with MS222 and gently squeezed in the abdomen. Eggs were collected directly in CSOF and sorted for quality under a stereoscope. Eggs were immersed in 50 μg/ml of Hoechst 33342 in CSOF for 20 min and held in CSOF in a moist chamber at room temperature (23 °C) until used. Note that eggs exposed to both Hoechst DNA staining and UV-light irradiation protocols showed no detrimental effect of this treatment to embryonic development after *in vitro* fertilization (K.S., unpublished data). Just before manipulation, eggs were washed in 5% polyvinyl pyrrolidone (PVP) in CSOF and transferred to a manipulation drop. The caudal fin of the egg donor was cut for genotyping analysis. PVP has been extensively used in human *in vitro* fertilization clinics and caused no detriment to zebrafish embryonic development (K.S., unpublished data).

Preparation of donor cells. We prepared primary culture of adult cells from caudal fin. The fin was washed with LHC medium (Biosource, Inc.), disinfected in 0.04% bleach and rinsed using LHC. The fin was minced and transferred to a culture dish in D-NACs medium (Lin et al., 2005), containing DMEM supplemented with 2 mM *N*-acetyl-L-cysteine, 1 μM ascorbate-2-phosphate, 1% SeaGrow (East Coast Biolab, Inc.), 5% FBS and antibiotics. The explants were grown in 5% CO_2 with atmospheric air at 28 °C for over a month. Cells were freshly prepared before SCNT by trypsinization. A portion of cells were also kept for genotyping analysis. Embryo-derived cells were freshly isolated

from the tailbud of embryos at the 15–20 somite stage before SCNT. The tailbud was mechanically dissociated in D-NACs medium. The remaining part of the donor embryo was collected for genotyping analysis. All donor cells were aliquoted into original medium and kept at 4 °C until used for SCNT. Fresh donor cells were prepared in a drop of 2% PVP in serum-depleted medium every 2 h along the SCNT manipulation.

Nuclear transfer. Nuclear transfer was performed using one pipette for holding the recipient egg and double injection needles for supporting and nuclear transfer. The egg holding pipette was cut straight and fire-polished to 200–300 μm inner diameter. As the micropyle is very small and allows access of only a single sperm, a human intracytoplasmic sperm injection (ICSI) needle (Humangen) was used for nuclear transfer. For embryonic cells, needles with inner diameter of 5–6 μm were used. For cultured adult cells, needles with inner diameters of 8–9 μm were used. The supporting needle (inner diameter 20 μm) was set up in parallel with the injection needle to help rotate the egg. Drops of 5% PVP in CSOF under mineral oil were used as manipulation medium. The x40 laser objective lens and controller (Hamilton Throne Bioscience, Inc.) were used to inactivate the egg genome. The egg was positioned with the micropyle facing the bottom of a manipulation dish, allowing the metaphase plate to be visualized best under UV light. The metaphase plate was burned twice using a laser beam (setting of 500 μs with 100% power). Donor cells, placed in 2% PVP in serum-depleted D-NACs, were loaded into the ICSI needle, a process whereby the cell membrane was broken. The egg was repositioned with its micropyle now facing the injection needle, so that the donor nucleus and its remaining cytosol could then be transferred to the animal pole of

the egg via the micropyle. Approximately 5 eggs at a time were manipulated. The reconstructed embryos were washed in CSOF for 15 min and subsequently activated in egg water (60 µg/ml sea salt). The developmental potential of cloned embryos was monitored and recorded at blastula (3 h), germ ring (6 h), 90% epiboly (9–10 h) and 1-day to adult fish stages.

To verify our manipulation technique, we produced zebrafish ICSI embryos by injecting sperm nuclei into 'off-target' laser-treated eggs (ablated location adjacent to the metaphase plate, sparing the egg's DNA). Approximately 5% fertile adult fish per total eggs manipulated were obtained using the ICSI technique (K.S., unpublished data).

DNA fingerprinting. We selected SNP markers from the dbSNP database in Genbank based on chromosomal regions and a presence of restriction enzyme cutting site(s) both at the polymorphic nucleotide (diagnostic site) and, if possible, at the adjacent nucleotide (internal control site). We analyzed the genomic region of interest using UCSC genome browser (Kent, 2002) and designed primers using primer3 (Rozen and Skaletsky, 2000). SNP genotyping was analyzed by restriction fragment length polymorphism (RFLP) analysis after PCR. The SNP markers were tested and those found to be highly polymorphic among individuals were selected (Table 3). Genomic DNA of cloned embryos, donor cells and donor eggs was isolated using DNAeasy kit (Qiagen). Information regarding PCR, primers and restriction enzymes is available in Supplementary Table 1. PCR was done in 20 µL reaction mixtures containing 0.2 U Platinum Taq DNA polymerase (Invitrogen), 0.5 µM each primer and 20–50 ng genomic DNA. The thermocycler program was 5 min at 94 °C, followed by 35–40 cycles of 94 °C

for 30 s, 55 °C for 30 s and 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR products were checked by 1% agarose gel electrophoresis and then digested with the restriction enzyme (NEB) at 37 °C overnight. RFLP was analyzed by 3% agarose gel electrophoresis using Ultrapure1000 (Invitrogen), except that SNP9 was analyzed by 6% PAGE (Table 3).

Karyotyping and offspring production. Cultured cells derived from caudal fin of cloned fish were expanded and prepared for karyotyping as described above. Replication banding was chosen because it provides substantial resolution to identify different chromosomes of zebrafish (Amores and Postlethwait, 1999). Karyotyping of such cells was performed by Cell Line Genetics, LLC. Cloned fish at reproductive maturity were allowed to breed naturally with either homozygous *golden* or transgenic Tubingen long fin counterparts. The phenotype of the offspring was recorded and their reproductive soundness was evaluated.

Figure 18 Protocol for SCNT. Eggs were collected in CSOF and stained with Hoechst 33342. Laser-assisted XYClone module (Hamilton Throne Bioscience, Inc.) was used for targeted ablation of the metaphase plate of the recipient egg. The donor cell is gently broken and transferred through the micropyle. The reconstructed embryo is washed in CSOF, activated in egg water and raised at 28 °C.

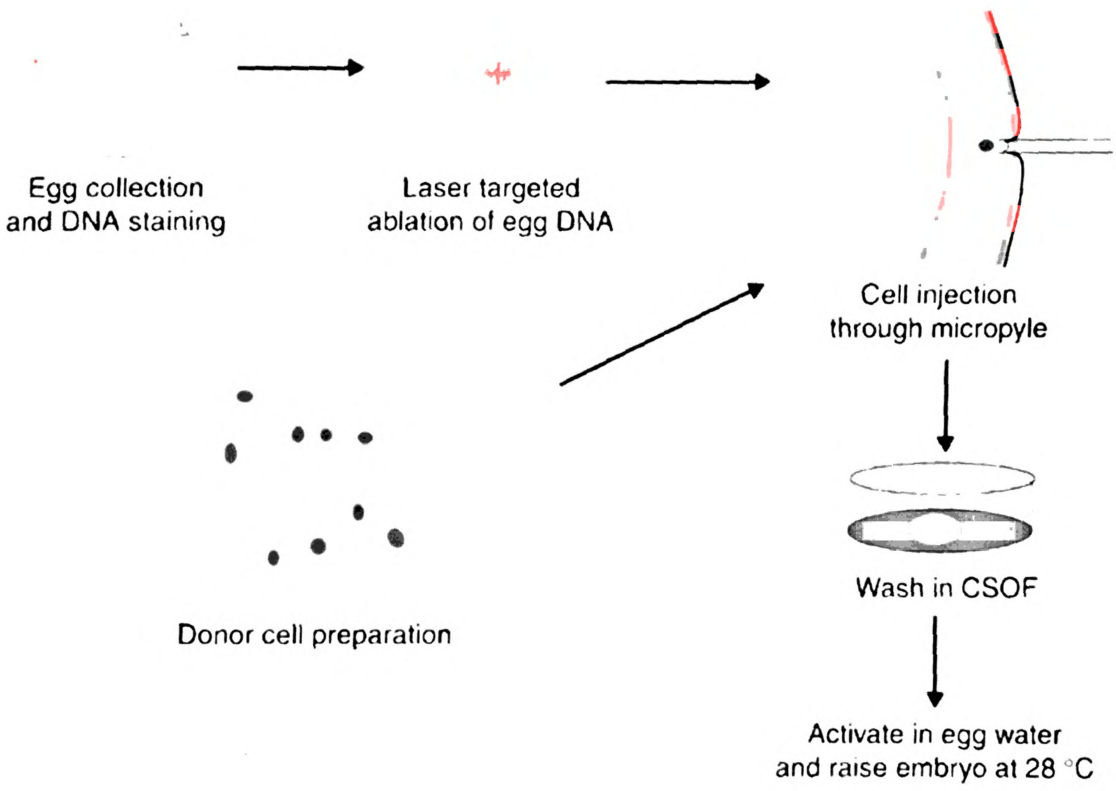


Figure 19 Recipient eggs. Matured-arrest eggs were held in CSOF, and image was captured at 1 hour post-collection (a). Inset in (a) depicts DNA stained with HOECHST in a fixed egg which remained at metaphase II of meiosis. (b) Nuclear staining of parthenogenetically activated egg at 20 min after egg-activation indicates female pronuclear formation and complete extrusion of second polar body – arrow (outside of the plane of focus). (c) In contrast to (b), nuclear staining of parthenogenetically activated egg at 20 min after laser-ablation shows complete inactivation of egg DNA and no extrusion of second polar body. Scale bar is 10 μm, otherwise indicated.

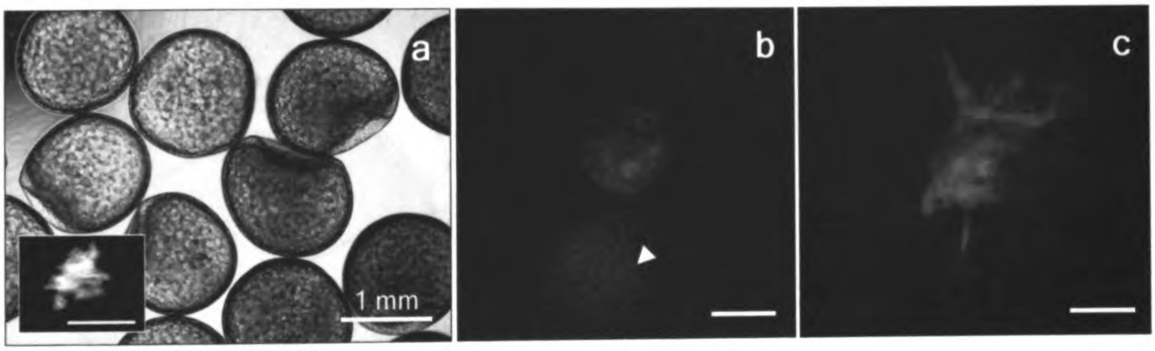


Figure 20 Phenotype of cloned zebrafish and its offspring. (a) Brightfield image of a cloned embryo at 2 d of age showing *golden* pigmented pattern. Inset, an image of a wild-type fertilized embryo of same age. (b) Images of adult *golden* cloned fish showing lack of heavy pigments (bottom) and of wild-type pigmented female egg donor (top). (c) Fluorescence image of cloned 3-d *golden* embryo (bottom; no green fluorescence) and an *in vitro*–fertilized embryo of the *H2AzGFP* egg donor (top). (d) Brightfield image of the offspring of female cloned zebrafish and *golden* male that inherited the *golden* phenotype. Scale bars, 0.5 mm (a,c,d) and 0.5 cm (b).

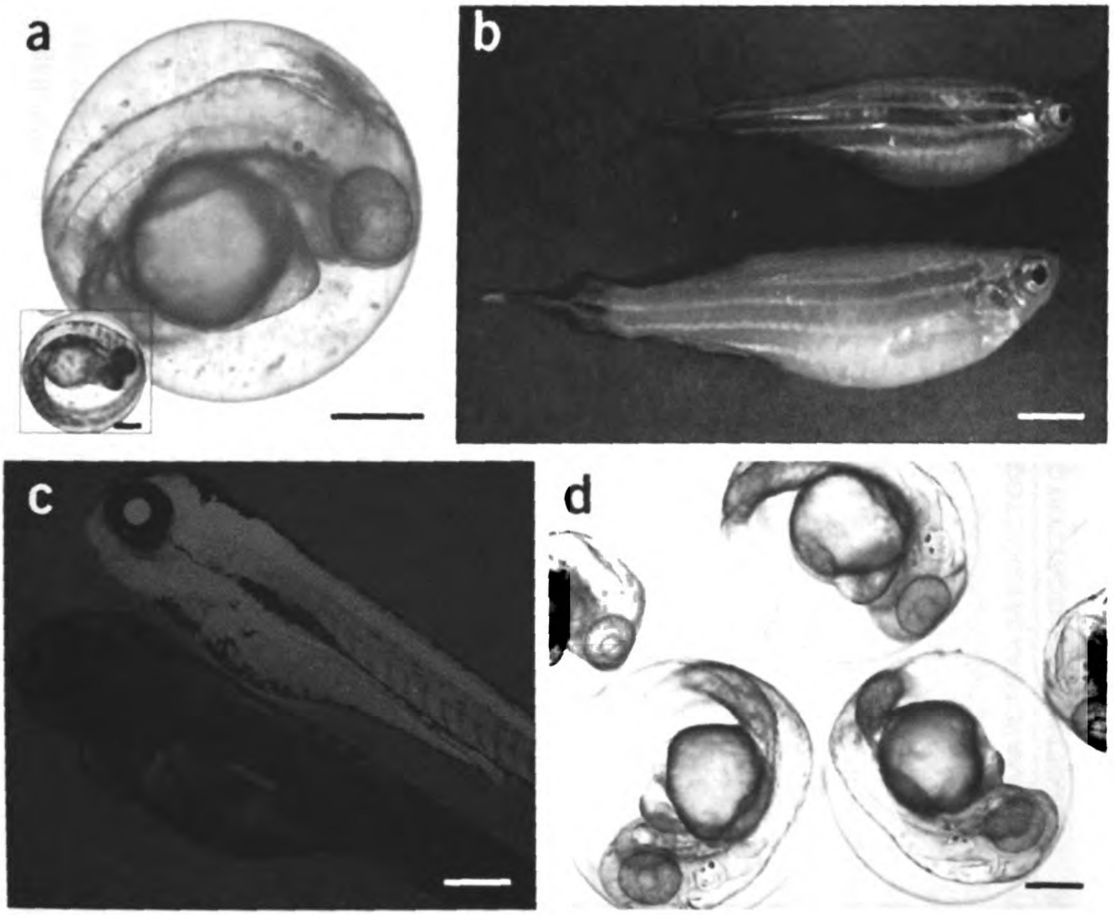


Table 3 Genotyping by SNPs. Primer sets, PCR condition, restriction enzymes, and diagnostic product sizes. All information obtained from GenBank, UCSC genome browser and primer3. Chr. is chromosome region of SNP marker. Lower cases indicate mutagenic sequence of primers.

Marker ID	Genbank no.		Primer (5'-3')	Enzyme	Chr.	Diagnostic bands (bp)		Remarks
zfSNP3	rs3729032	[F]	TGCAACTGACCGATGTATTTG	NlaIII	20	GG	172, 100, 28	1.5mM $MgCl_2$
		[R]	CCTTTCCTCCCTCTGATGTG			AA	272, 28	3% agarose
zfSNP5	rs3729483	[F]	TTGGCTGGCTTGAGATTGAT	HpyCH4IV	10	GG	130, 108	3mM $MgCl_2$
		[R]	TCATCAGAGTCAGTGAATAATTTTTGT			AA	238	3% agarose
zfSNP8	rs3728219	[F]	GACCGTCTAGAGGTTTCTGTGG	MspI	9	GG	122, 112	1.5mM $MgCl_2$
		[R]	CATTCTCCACGACCACTGC			CC	239	3% agarose
zfSNP9	rs3728151	[F]	TGCGCTTTTACAGACTGTGC	HpyCH4IV	5	CC	196, 188, 30	1.5mM $MgCl_2$
		[R]	AGCAATGCTTCACTCCATCC			TT	226, 188	6% polyacrylamide
zfSNP10	rs3729324	[F]	AATCCTGGTTGGCTTGTAGC	MspI	24	CC	240, 129	1.5mM $MgCl_2$
		[R]	AGCAGTACTTTTAGCCCTTGG			TT	369	3% agarose
zfSNP13	rs3729001	[F]	TGCCGTTTCTCCATAACGAC	HhaI	19	GG	214, 72, 45	1.5mM $MgCl_2$
		[R]	GTTGAGCGTTCCTCTTCAGG			AA	286, 45	3% agarose
zfSNP15	rs3728634	[F]	CAGCCCAGCACTATGACCTC	MspI	14	CC	170, 142, 50	1.5mM $MgCl_2$
		[R]	TTGGAGCCGAATGGATACTC			AA	312, 50	3% agarose
zfSNP16	rs3728553	[F]	GATGCCCTAAAGAAGGACCAC	TaqI	13	CC	118, 116, 80	1.5mM $MgCl_2$
		[R]	GCAGTGCATATTTCCTGCTG			GG	234, 80	3% agarose
zfSNP17	rs3728548	[F]	TGAAACGTAATCTTCGGACAAC	NlaIII	13	TT	217, 83, 63	1.5mM $MgCl_2$
		[R]	AGACGATCTTGGTCCCACTC			CC	280, 83	3% agarose
zfSNP19	rs3728348	[F]	ATTTTTGACTGGCCCAACAG	HpyCH4V	11	AA	113, 101, 37	3mM $MgCl_2$
		[R]	TTTCAAATCTGAAGATGATCTGG			TT	214, 37	3% agarose
zfSNP20	rs3729434	[F]	TTGGCTTACAGTGaGCATTTATC	HpyCH4V	9	TT	103, 89, 42	3mM $MgCl_2$
		[R]	CAGTTTGCtCCTTAAGGAAGTG			CC	131, 103	3% agarose

Table 4 Genotyping results. Genotypes of recipient egg's donor (R), donor cell (D), and cloned embryo (C).

		SNP identification number (S#)										
ID	Fish ID	S3	S5	S8	S9	S10	S13	S15	S16	S17	S19	S20
R	Fin♀, WT	GG	GA	GC	CC	TT	GA	AA	GG	TC	TA	CC
D	Donor, Gol$^{-/-}$ ET 20s	GA	AA	CC	CT	CT	GA	CC	GC	TT	TT	CC
C	clone NT2E1, 10dpNT	GA	AA	CC	CT	CT	GA	CC	GC	TT	TT	CC
C	clone 9-7 adult fish ♀, 3m	GA	AA	CC	CT	CT	GA	CC	GC	TT	TT	CC
R	Fin♀, WT	GG	GA	GC	CC	CT	GG	AC	CC	TT	TT	CT
D	Donor, Gol$^{-/-}$ ET 15s	GG	AA	CC	CT	CT	AA	AC	GC	TT	TT	CC
C	clone NT5E1, 7dpNT	GG	AA	CC	CT	CT	AA	AC	GC	TT	TT	CC
C	clone 9-12 adult fish ♀, 3m	GG	AA	CC	CT	CT	AA	AC	GC	TT	TT	CC
R	Fin♀, WT	GG	GG	GG	CC	CT	AA	AA	CC	TT	AA	TT
D	Donor, Gol$^{-/-}$ ET 18s	GG	GA	GC	CC	TT	GG	AC	CC	TT	TT	CT
C	clone NT8E1, 5dpNT	GG	GA	GC	CC	TT	GG	AC	CC	TT	TT	CT
C	clone NT2E1, 6dpNT	GG	GA	GC	CC	TT	GG	AC	CC	TT	TT	CT
C	clone NT5E1, 6dpNT	GG	GA	GC	CC	TT	GG	AC	CC	TT	TT	CT
R	Fin♀, WT	GG	GA	GG	CT	TT	GA	AA	GC	TT	TA	CT
D	Donor, Gol$^{-/-}$ P1♂, AF	GG	GG	GG	CT	CT	GA	CC	CC	TT	TT	TT
C	clone NT7E1, 6dpNT	GG	GG	GG	CT	CT	GA	CC	CC	TT	TT	TT
R	Fin♀, WT	GG	GA	GG	CC	TT	AA	AC	CC	TT	AA	CC
D	Donor, Gol$^{-/-}$ P2♀, AF	GA	AA	CC	CC	TT	GA	CC	GC	TT	TT	CC
C	clone NT1E1, 3dpNT	GA	AA	CC	CC	TT	GA	CC	GC	TT	TT	CC
R	Fin♀, H2AGFP	GG	AA	GG	CC	CT	GG	AA	GG	TC	TT	CT
D	Donor, Gol$^{-/-}$ ET 15s	GA	GA	GC	CT	CC	GA	AC	GC	TT	TT	CT
C	clone NT3E1, 14dpNT	GA	GA	GC	CT	CC	GA	AC	GC	TT	TT	CT
C	clone NT8E1ab, 7dpNT	GA	GA	GC	CT	CC	GA	AC	GC	TT	TT	CT
R	Fin♀ H2AGFP	GG	GG	GG	CC	TT	GA	CA	GG	TT	TT	CC
D	Donor, Gol$^{-/-}$ ET 20s	GG	AA	GG	TC	CT	GA	CA	GC	TT	TT	CC
C	Clone NT1E1, 4dpNT	GG	AA	GG	TC	CT	GA	CA	GC	TT	TT	CC
C	Clone NT2E1, 4dpNT	GG	AA	GG	TC	CT	GA	CA	GC	TT	TT	CC
C	Clone NT4E1, 4dpNT	GG	AA	GG	TC	CT	GA	CA	GC	TT	TT	CC
R	Fin♀ H2AGFP	GG	AA	GG	CC	CC	GG	AA	GC	TT	TT	TC
D	Donor, Gol$^{-/-}$ ET 20s	GG	GA	CG	TT	CT	GA	CC	GC	TT	TT	TC
C	Clone NT2E1	GG	GA	CG	TT	CT	GA	CC	GC	TT	TT	TC
C	Clone NT4E1, 10dpNT	GG	GA	CG	TT	CT	GA	CC	GC	TT	TT	TC
C	Clone NT5E1	GG	GA	CG	TT	CT	GA	CC	GC	TT	TT	TC
R	Fin♀ H2AGFP	GG	AA	CG	TC	CT	GG	AA	GC	TC	TT	TT
D	Donor, Gol$^{-/-}$ ET 20s	GG	AA	CG	TC	CT	AA	CC	GC	TT	TT	TC
C	Clone NT2E1, 10dpNT	GG	AA	CG	TC	CT	AA	CC	GC	TT	TT	TC
C	Clone NT2E2, 10dpNT	GG	AA	CG	TC	CT	AA	CC	GC	TT	TT	TC
C	Clone NT2E3, 12dpNT	GG	AA	CG	TC	CT	AA	CC	GC	TT	TT	TC
C	Clone NT3E1, 10dpNT	GG	AA	CG	TC	CT	AA	CC	GC	TT	TT	TC
C	Clone NT6E1, 8dpNT	GG	AA	CG	TC	CT	AA	CC	GC	TT	TT	TC
C	Clone NT6E2, 8dpNT	GG	AA	CG	TC	CT	AA	CC	GC	TT	TT	TC
C	Clone NT6E3, 8dpNT	GG	AA	CG	TC	CT	AA	CC	GC	TT	TT	TC
C	Clone NT7E1, 8dpNT	GG	AA	CG	TC	CT	AA	CC	GC	TT	TT	TC
R	Fin♀ H2AGFP	GG	GA	CG	CC	CC	GG	CA	GG	TC	TT	CC
D	Donor, Gol$^{-/-}$ ET 20s	GG	AA	GG	TT	CC	GA	CC	GC	TT	TT	CC
C	Clone NT5E1, 4dpNT	GG	AA	GG	TT	CC	GA	CC	GC	TT	TT	CC
C	Clone NT7E2, 4dpNT	GG	AA	GG	TT	CC	GA	CC	GC	TT	TT	CC
R	Fin♀ H2AGFP	GG	GA	CG	CC	CC	GG	AA	GC	TT	TT	TT
D	Donor, Gol$^{-/-}$ ♂ AF	GG	AA	GG	TC	CT	GA	CA	GG	TT	TT	CC
C	Clone NT5E1, 1dpNT	GG	AA	GG	TC	CT	GA	CA	GG	TT	TT	CC

Table 4 (continued)

		SNP identification number (S#)										
ID	Fish ID	S3	S5	S8	S9	S10	S13	S15	S16	S17	S19	S20
R	Fin♀ H2AGFP	GG	GA	CG	TT	CT	GG	CC	GG	TC	TT	TC
D	Donor, Gol$^{-/-}$ ♂AF	GG	AA	GG	TC	CT	GA	CC	GG	TT	TT	CC
C	Clone NT5E1, 2dpNT	GG	AA	GG	TC	CT	GA	CC	GG	TT	TT	CC
R	Fin♀ H2AGFP - HG1	GA	AA	GG	TC	CC	GA	AA	GG	TT	TT	TC
D	Donor, Gol$^{-/-}$ ♂AF	GA	AA	GG	TC	CC	GA	CC	CC	TT	TT	CC
C	Clone NT3E1, 2dpNT	GA	AA	GG	TC	CC	GA	CC	CC	TT	TT	CC
C	Clone NT3E2, 2dpNT	GA	AA	GG	TC	CC	GA	CC	CC	TT	TT	CC
R	Fin♀ H2AGFP	GG	AA	CG	CC	CC	GG	CA	CC	TC	TT	TC
D	Donor, Gol$^{-/-}$ ♂AF	GA	AA	GG	TC	CC	GA	CC	CC	TT	TT	CC
C	Clone NT2E1, 1dpNT	GA	AA	GG	TC	CC	GA	CC	CC	TT	TT	CC
R	Fin♀ TAB2	GA	AA	CG	CC	TT	GA	CA	GC	TT	TT	TC
D	Donor, HGn62A-Leo ET 15s	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
C	Clone NT1, 1 month	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
C	Clone NT2, 1 month	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
C	Clone NT3, 1 month	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
C	Clone NT1E1, 4dpNT	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
C	Clone NT1E2, 19dpNT	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
C	Clone NT3E1, 10dpNT	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
C	Clone NT3E2, 4dpNT	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
C	Clone NT3E3, 4dpNT	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
C	Clone NT3E4, 4dpNT	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
C	Clone NT4E1, 4dpNT	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
R	Fin♀ TAB2	GA	AA	CG	CC	TT	GA	CA	GC	TT	TT	TC
D	Donor, HGn62A ET 18s	GG	GA	GG	CC	CT	GA	CC	GG	TT	TT	CC
C	Clone NT1, 1 month	GG	GA	GG	CC	CT	GA	CC	GG	TT	TT	CC
C	Clone NT2, 1 month	GG	GA	GG	CC	CT	GA	CC	GG	TT	TT	CC
C	Clone NT1E2, 16dpNT	GG	GA	GG	CC	CT	GA	CC	GG	TT	TT	CC
C	Clone NT2E3, 3dpNT	GG	GA	GG	CC	CT	GA	CC	GG	TT	TT	CC
R	Fin♀ TAB2	GA	AA	CG	CC	TT	GA	CA	GC	TT	TT	TC
D	Donor HGn8E ET 18s	GG	GG	GG	CC	CT	GG	AA	GG	TT	TT	CC
C	Clone NT2E1, 14dpNT	GG	GG	GG	CC	CT	GG	AA	GG	TT	TT	CC
C	Clone NT1E1, 4dpNT	GG	GG	GG	CC	CT	GG	AA	GG	TT	TT	CC
R	Fin♀ TAB2	GA	AA	CG	CC	TT	GG	CA	GC	TT	TT	TC
D	Donor ♂HGn8E leo AF	GG	GG	GG	CC	CT	GA	AA	GG	TT	TT	CC
C	Clone NT1E1, 4dpNT	GG	GG	GG	CC	CT	GA	AA	GG	TT	TT	CC
C	Clone NT2E1, 4dpNT	GG	GG	GG	CC	CT	GA	AA	GG	TT	TT	CC
R	Fin♀ TAB2	GA	AA	CG	CC	TT	GA	CA	GC	TT	TT	TC
D	Donor, ♂HGn28A leo AF	GG	GG	GG	CC	CT	GA	AA	GG	TT	TT	CC
C	Clone NT4E1, 4dpNT	GG	GG	GG	CC	CT	GA	AA	GG	TT	TT	CC
C	Clone NT3E1, 4dpNT	GG	GG	GG	CC	CT	GG	AA	GG	TT	TT	CC
R	Fin♀ TAB2	GA	AA	CG	CC	TT	GG	CA	GC	TT	TT	TC
D	Donor, ♀HGn62A leo AF	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
C	Clone NT4E1, 7dpNT	GA	GA	GG	CC	CT	GG	CC	GG	TT	TT	CC
R	Fin♀ TAB2	GA	AA	CG	CC	TT	GA	CA	GC	TT	TT	TC
D	Donor,♂HGn8E leo AF	GG	GG	GG	CC	CT	GA	AA	GG	TT	TT	CC
C	Clone NT2E1, 2dpNT	GG	GG	GG	CC	CT	GA	AA	GG	TT	TT	CC
C	Clone NT5E1, 2dpNT	GG	GG	GG	CC	CT	GA	AA	GG	TT	TT	CC

Table 5 Development of cloned embryos

Strain of egg donor (donor cells)	Number of SCNT operations[a]	Number of eggs in all SCNT operations	Total number of embryos reaching indicated stage (mean percentage of embryos + s.e.m.; n = number of SCNT operations)						
			Blastula	Germ Ring	90%Epiboly	1 day	4 days	Live (Eat)	Live (Adult)
WT (*golden* ET)	3	109	44 (38.7±4.8)	11 (10.0±2.5)	7 (6.7±1.0)	7 (6.7±1.0)	7 (6.7±1.0)	3 (3.6±2.5)	2 (2.2±1.2)
WT (*golden* AF)	3	125	49 (39.7±6.5)	17 (13.4±2.2)	9 (7.3±2.6)	2 (1.7±0.9)	1 (1.0±1.0)	0 (0)	0 (0)
H2A (*golden* ET)	5	149	78 (51.8±6.0)	33 (21.4±3.9)	28 (18.1±4.0)	23 (15.0±4.2)	20 (13.0±3.4)	2 (1.0±0.6)	0 (0)
H2A (*golden* AF)	7	193	66 (33.7±2.7)	19 (9.8±2.2)	13 (6.7±1.4)	10 (5.3±1.1)	0 (0)	0 (0)	0 (0)
TAB (TuLF ET)	3	143	73 (50.1±10.5)	36 (24.4±7.9)	22 (14.7±6.0)	16 (10.7±4.2)	12(7.9±3.7)	9 (6.0±2.1)	5 (3.3±1.7)
TAB (TuLF AF)	4	207	86 (37.9±8.9)	25 (10.9±3.6)	15 (7.2±3.2)	7 (3.3±0.7)	1 (1.8±0.4)	0	0

SCNT operations were performed using wild-type eggs (WT), transgenic *H2AzGFP* eggs (H2A) or TAB outcrossed eggs (TAB). Donor cells were isolated from either freshly dissociated tailbuds of embryos at the 15–20 somite stage embryonic tailbud (ET) or cultured adult fin fibroblasts (AF), from homozygous *golden* or TuLF fish.

[a]Each SCNT operation was done with eggs and donor cell from separate individuals.

Figure 21 Abnormalities observed in cloned embryos. (a-b) Abnormal cloned embryos derived from embryonic cells at 1 day and (c) 3 days of age, (d-f) and cultured adult fibroblasts at 1 day of development. Scale bar is 0.5 mm. Most of the abnormal cloned embryos showed severe abnormalities of posterior development, and mild to moderate changes of anterior parts.

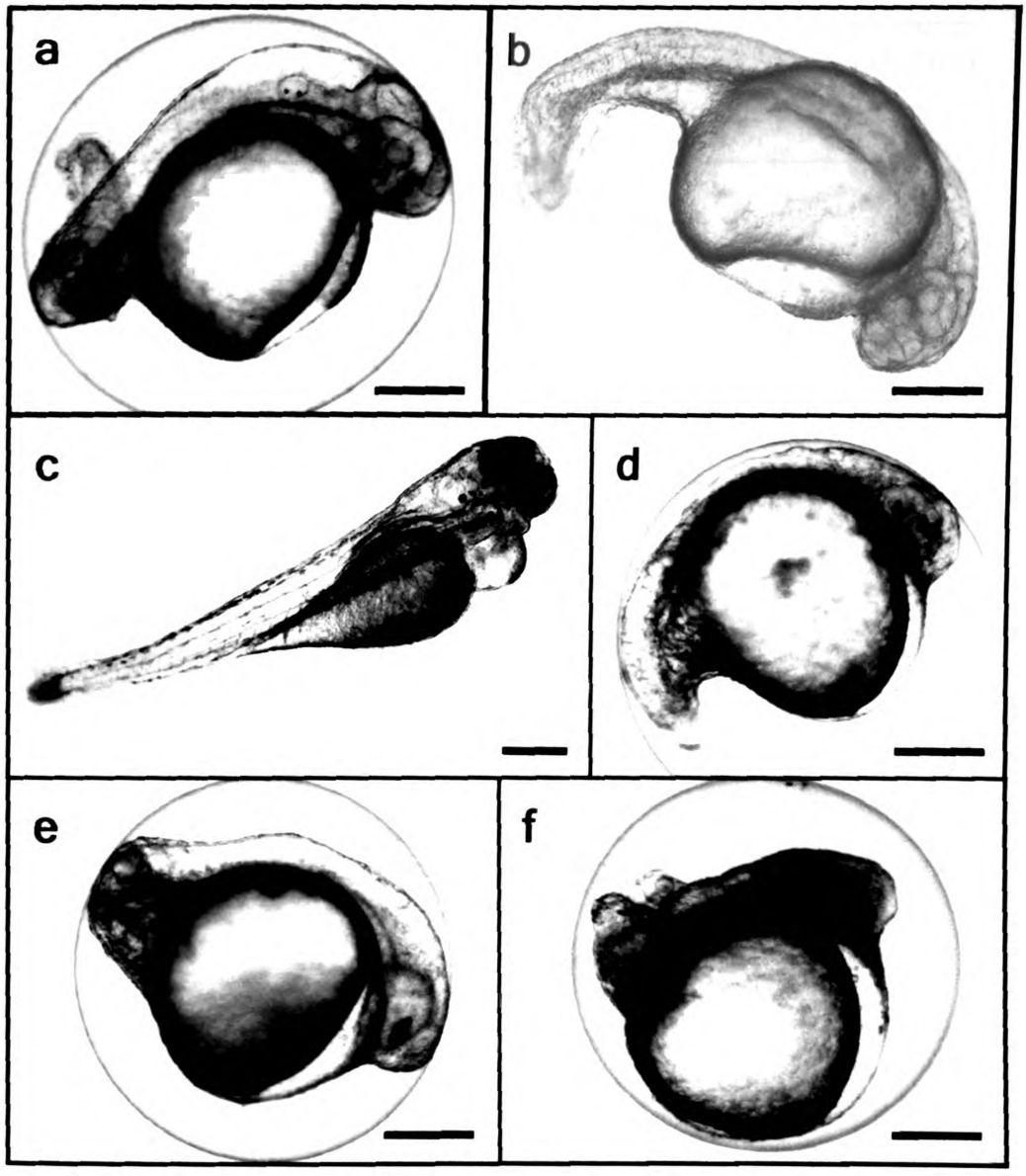
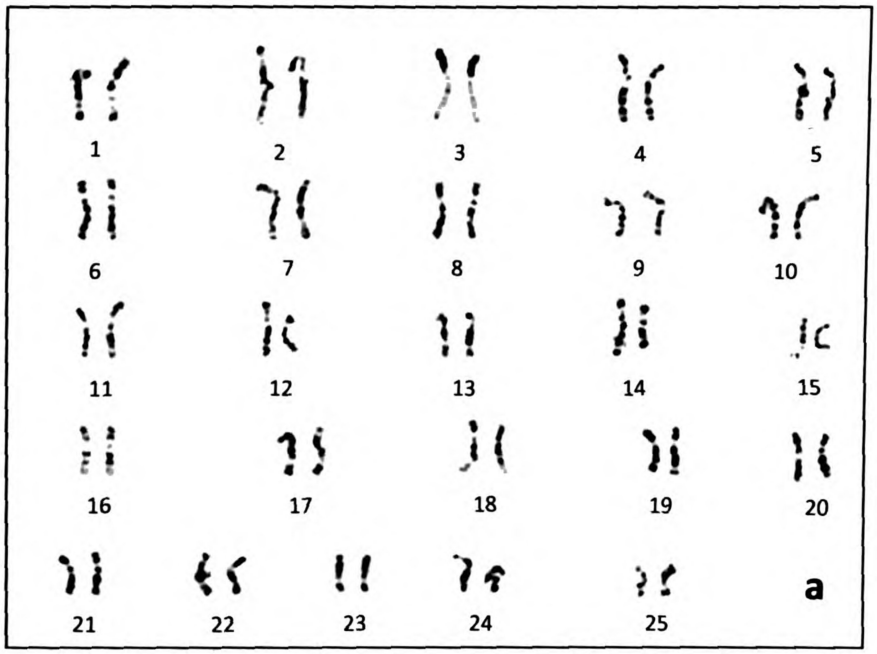
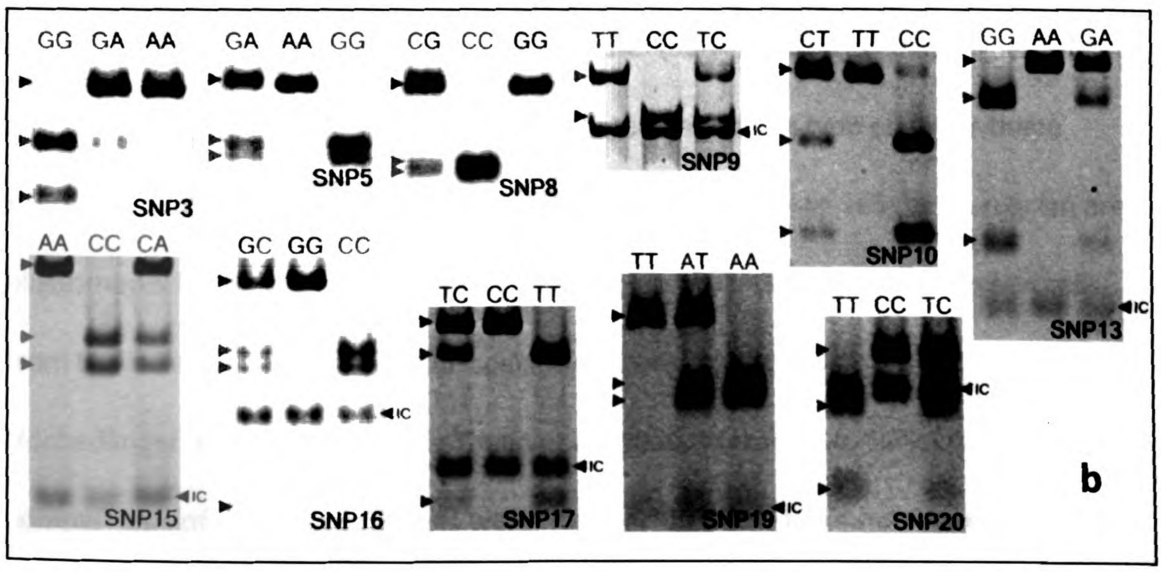


Figure 22 Karyotyping and genotyping of cloned fish. (a) Karyotype analysis of cloned zebrafish by replication banding. Cloned zebrafish possessed normal diploid karyotype (2n=25). (b) Single nucleotide polymorphism (SNP) markers for genotyping analysis of cloned zebrafish by Restriction Fragment Length Polymorphism following polymerase chain reaction. A total of eleven informative markers are shown. Blue and red letters indicate genotypes. Arrows point to diagnostic bands of each genotype. IC is internal control for restriction enzyme activity. Of eleven SNP markers tested, we found a complete matched genetic identity between the donor cell and the cloned fish, no matching with the recipient egg donor was observed (Table 4).





CHAPTER 5

INFLUENCE OF DONOR NUCLEUS SOURCE IN THE OUTCOME OF ZEBRAFISH CLONING PROCEDURES

Kannika Siripattarapravat, Boonya Pinmee, EunAh Chang, Juan David Munoz, Koichi Kawakami, and Jose B Cibelli

Abstract

The donor cells from five different tissues of transgenic zebrafish were compared for their capacity to be reprogrammed following somatic cell nuclear transfer. Donor cells of the HG21C line, cells of fin and notochord origin, gave the best rate of cloned fish production. While cells from other lineages were tested and, indeed produced cloned fish, the efficiency of cloning was significantly lower than the ones selected from the HG21C fish line.

Introduction

It is known that as a cell differentiates, its developmental potential gets more restrictive. Following somatic cell nuclear transfer (SCNT), cells can be reprogrammed to an embryonic state at different efficiencies depending on the type of cell or tissue origin. We have learnt from mouse experiments that the easiest cells to reprogram are blastomeres from a morula, however the efficiency progressively declines when cells from the inner cell mass and tissue specific cells are used (Gurdon and Melton, 2008; Hochedlinger and Jaenisch, 2006; Thuan et al., 2010; Wakamatsu, 2008). Side by side comparisons of different cell types were made by the same laboratory and found that ESCs are more amenable to cloning than somatic fibroblasts (Rideout et al., 2000) and

even among different somatic cell populations, the variations of cloning efficiency are significant (Oback, 2009; Oback and Wells, 2007). Adult frogs were obtained when donor nuclei was isolated from blastomeres (Gurdon et al., 1958) and embryonic-intestinal cells (Gurdon and Uehlinger, 1966). But only tadpoles were produced using adult cells as donor nuclei (Gurdon et al., 1975; Laskey and Gurdon, 1970). These evidences point toward the existence of a cell to cell variability that can be attributed to the epigenetic state that defines the phenotype of a given cell.

Effective nuclear reprogramming requires shutting down somatic-cell specific gene expression and turning on embryonic-specific genes in a carefully choreographed manner. In SCNT, errors of the nuclear reprogramming were observed in almost all species cloned (Cibelli et al., 2002). In mice, when muscle cells were used as donor nuclei, the GLUT4 glucose transporter gene continued to be active in the early cloned mouse embryos (Gao et al., 2003). Furthermore the pluripotentcy-related gene, *Oct 4*, is expressed incorrectly in the majority of the cloned embryos produced using nuclei from cumulus cells (Boiani et al., 2002). In frogs, tissue-specific gene expression of a donor cell was found to persist in cloned embryos, a phenomenon known as epigenetic memory (Ng and Gurdon, 2005; Ng and Gurdon, 2008). Despite these abnormal patterns of gene expression, a small population of cloned animals can develop into seemingly healthy adults.

It has been suggested that one of the most important steps towards successful SCNT is the selection of a donor population of nuclei that are intrinsically more reprogrammable by the recipient oocyte (Santos and Dean, 2004). There are evidences

indicating that the cell donor is responsible for variations in the efficiency of SCNT (Kato et al., 2000; Wakayama and Yanagimachi, 1999), yet it is inconclusive (Oback, 2009). Work done in our laboratory has shown that in zebrafish, the efficiency to produce cloned hatched-fry can be 6-13% when donor cells are freshly isolated from embryonic tail-bud (Siripattarapravat et al., 2009b). Based on the evidence, we hypothesized that nuclear reprogramming efficiency in zebrafish varies from one tissue-specialized cell to another. In other words, cells from different lineages could have different developmental potential when used as donor cells for SCNT.

To test this hypothesis, we proposed to evaluate the efficiency of cloning zebrafish (measured by reconstructed embryos that develop to normal hatched-fry stage) when donor cells derived from three different sources, ectoderm, mesoderm, and endoderm, were used. We used transgenic zebrafish expressing green fluorescence protein (GFP) under a very 'tight' endogenous tissue specific promoter (Figure 23) (Nagayoshi et al., 2008). All transgenic lines were generated with the *tol* 2 vector system developed by the Kawakami lab (Nagayoshi et al., 2008). These fish express GFP in tissue specific manner, approximately at 24 hpf, allowing for a rapid-live cell type identification.

We report here the cloning efficiency of 5 different cell types in Zebrafish. We used 5 different transgenic lines: 1) HGn62A–skin (ectoderm), 2) HGn28A–skin (ectoderm), 3) HGn8E–heart (mesoderm), 4) HG21c–fin/ notochord (mesoderm), and 5) HGn30A–hatch gland (endoderm) (Nagayoshi et al., 2008). The result showed that GFP^{+}

cells from lines HGn21C are much more amenable to nuclear reprogramming than the others.

Results

SCNT was performed as previously described (Siripattarapravat et al., 2009b), with the exception that the donor cells were from embryos 24 hours post-fertilization (hpf) and were selected for GFP^+ prior to nuclear transfer (Figure 23). We observed developmental capacity of cloned embryos derived from donor cells of different sources (Table 6 and Figure 24). All cell types used yielded cloned embryos albeit at different rates of developmental capacity and degree of normality. We were able to clone adult zebrafish from donor cells of HGn28A and HGn8E. Following back-crossed with the wild type strain, the offspring of these clones were normal and carried lineage specific GFP^+ as their cloned parental lines (Figures 23F and 23G).

All cell types from the different transgenic fish line had different sizes, morphology, and appearances. GFP^+ cells of HGn28A and HG21C lines were the smallest, and just fit the needle with a diameter of 7-8 μm. The cytoplasmic membrane of both cells was easily broken in the injection needle. GFP^+ cells of the HGn62A line, whose cytoplasmic membrane is not easily broken, were the largest and only fit in the injection needle with diameter of 9-10 μm. All of the GFP^+ cells from HGn28A, HG21C, and HGn62A lines were abundant in the embryos and easily identified in the manipulation drop, likely shortening the amount of time that the cells were exposed to the UV light when compared to HGn30A and HGn8E lines. GFP^+ cells of the HGn30A line were large and contained multiple cytoplasmic vesicles. Though cells were big in size, they were

very fragile as they broke at suction and could be squeezed easily through an injection needle with diameter of 8 μm. GFP^+ cells of the HGn8E line were of medium size with a cell membrane which was very elastic and had to be forced through the needle a few times prior to disruption of the cytoplasmic membrane. It remains to be determined if all these physical characteristics of the cells have any impact on the overall efficiency of SCNT, however for the purpose of this study, we assumed that the most important determining factor is the epigenetic status of the cell nucleus.

The developmental rates of cloned embryos were recorded at the blastula stage, germ ring (entering gastrulation), 90% epiboly (complete gastrulation), day 1 (complete segmentation), day 4 – hatched fry, eating fry, and adult (Kimmel et al., 1995). Cloned embryos were classified according to their morphology and recorded as either normal embryos or total embryos (including abnormal counts). Results are shown in table 6 and figure 24. At the blastula stage, cell division is the major event with cells undergoing approximately 10-11 cell divisions before entering mid-blastula transition, when the cell cycle is no longer homogeneous and lengthens; at this point zygotic gene transcription starts (Kimmel et al., 1995). Except 18% in HGn8E, approximately 40% of cloned embryos completed development to normal blastula. The abnormality of cloned embryos recorded at this stage was partial blastula embryos. At gastrulation stage, the cells start differentiation, migration, and form the germ ring (GR). Only 5% of cloned embryos from HGn62A, HGn30A and HGn8E developed to normal GR, while observing 12% of HGn28A and 20% of HG21C. The abnormal cloned embryos at GR showed unequal migration of the cells toward vegetal pole of the egg. Upon finishing

gastrulation, at 90% epiboly (EB), cells have completely migrated from the animal pole to vegetal pole, and the three germ layers are formed. Except 16% of HG21C, only 3-5% of cloned embryos made to normal EB. Most of abnormal cloned embryos at EB stage showed lower cell density than the normal embryos at this stage. The segmentation period is followed, and as cells progress in differentiation they form somites, and start organogenesis. By day 3, the embryos finish organogenesis, hatch from the chorion, and develop swim bladder. At day 1 to 4, the number of cloned embryos dropped dramatically to 0.4% in HGn62A and HGn30A. For HGn28A and HG21C, the number of cloned embryos remained at 2-3% in day 1, and dropped by half at day 4. The cloned embryos of HGn8E remained at 1.6% until day 4. As previously reported (Siripattarapravat et al., 2009b), we observed various degrees of abnormalities in cloned embryos at 1-4 days. All of the normal cloned embryos at day 4 started to eat. Only two fry from GFP^+ donor cells of HGn28A and HGn8E lines were made to adult fish

A two-way analysis of variance was used to analyze the data. The model was set for binomial distribution of counting dataset under PROC GRIMMIX (SAS system). Since the variances are discrete in 2 developmental stages from others, i.e., the number of eating fry and adult, they were excluded from the statistical analysis.

Statistical analysis has shown no significant interaction found between sources of GFP^+ donor cells and numbers of cloned embryos at all developmental stages recorded. When contemplating donor cell individuals and accounting for numbers of total embryos counted from all developmental stages (from blastula to 4 days), cloning efficiency from HG21C donor cells was significantly higher than any other donor cells (p

< 0.05). When comparing numbers of normal embryos, no difference was found among donor cells of all lineages.

Discussion

We have found that the developmental capacity between cell lines was different when analyzed at blastula to hatched fry stage. All cell lines were capable of generating cloned fish with the HG21C line being the most efficient.

A significantly large number of cloned fish failed to develop normally most likely due to failures of reprogramming. We speculate that no altered phenotypes were due to the transgene present in the fish lines used since four out of the five lines harbor the inserted gene in the intronic region of the genome, and while the HG21C line has its transgene inserted into the *tcf* 7 gene (transcription factor 7) sequences, only the homozygous mutants show abnormal development of fins.

A potential confounding factor that could have negatively impacted the rate of normal fish generated, could be the use of UV light to locate the DNA in the egg nucleus and to select transgenic cells. However, three pieces of evidence argue against such speculation. First, we ran control experiments in which eggs were exposed to UV light and their cytoplasm laser irradiated followed by *in vitro* fertilization, and healthy fish were generated after this manipulation. Second, our method explicitly minimized UV exposure to less than five seconds; and third, fresh donor cells were freshly loaded every 30 minutes. Regardless, if there was a potential for increasing the rate of abnormalities due to UV irradiation, all experiments would be equally affected since all five transgenic fish lines were subjected to the same treatment. Taken together we can

conclude that UV exposure should not be a confounding factor in the overall efficiency comparison between treatments.

It has been demonstrated in several mammalian species that SCNT is possible with donor cells are at either G1/G0 or G2-M, but not S phase (Campbell et al., 1996; Cibelli et al., 1998; Egli et al., 2007; Wakayama et al., 1999). We did not test the cells for their stage in the cell cycle prior to SCNT, and it is possible that the cells used as donors were at different stages in the cell cycle. However, in an attempt to standardize our protocol, we purposely selected cells that were the smallest in the pool, likely selecting only cells in the G0 to G1 stage. Nonetheless, more work is needed to determine whether cells at different stage of cell cycle will have different cloning efficiency in zebrafish.

GFP^+ cells in the HGn30A and HGn8E lines are distinctly specialized cells. Cells from the HGn30A line contain multiple cytoplasmic vesicles, possibly storing proteolytic enzymes produced from the cells of the hatch gland. The injection technique was done by delivery of all cytoplasmic components together with the nucleus at the time of nuclear transfer. It is possible that the components in those vesicles have negative impact onto the reconstructed embryos and the capacity for nuclear reprogramming. GFP^+ cells of HGn8E line are heart muscle cells, some of them are multinucleated cells. It is possible that more than one nucleus was transferred and that may have caused ploidy abnormalities in the cloned embryos. In addition, muscle cells are known to continue to express muscle specific genes, possible making them more resilient to epigenetic modifications (Gao et al., 2003).

In mouse cloning, the abnormality observed in cloned mice was not only due to epigenetic reprogramming, but also the karyotypic abnormalities from manipulation of donor nucleus (Wakayama and Perry, 2002). We have not analyzed for karyotypes in the abnormal clones embryos therefore we cannot rule out all possible causes of abnormalities in the clones.

In summary, we found that GFP^+ cells isolated from the HG21C zebrafish line yield the highest capacity for nuclear reprogramming following SCNT. A thorough analysis of the epigenetic signatures of these cells may help us elucidate specific factors that are responsible for their enhanced reprogramming capacity. In a more practical application, we expect to use these cells for gene targeting experiments in vitro followed by SCNT. In turn, this work could facilitate the generation of generation of knock-in/ -out zebrafish founder animals providing the zebrafish research community with an unparalleled tool for studies of gene loss/gain of function.

Methods

Zebrafish strain. The outcrossed between Tubingen and AB line, named TAB, was used as female egg donors. The transgenic zebrafish (Nagayoshi et al., 2008) with Tubingen long-fin background, expressing tissue specific green fluorescence protein (GFP), HGn30A-hatch gland, HGn28A and HGn62A-skin, HG21c-fin/notochord, and HGn8E-heart were used to isolate donor cells.

Preparation of recipient eggs and donor cells. The recipient eggs were obtained by stripping technique (Westerfield, 1993). The eggs were immediately placed in Chinook salmon ovarian fluid (CSOF)(Siripattarapravat et al., 2009a). The eggs were stained with

50mg/ml Hoechst33342 for 20 minutes as described previously (Siripattarapravat et al., 2009b), and kept in CSOF until used for nuclear transfer. The donor cells were freshly prepared from embryos at 24 hour post-fertilization. The embryos were sorted for GFP positive under fluorescence microscope. For HGn30A, HG21c and HGn8E, the embryos were dissected and selected for the GFP positive tissues. For HGn28A and HGn62A, the whole embryos were extracted from the yolk prior to use. Subsequently, embryos were briefly minced in LHC basal media, and trypsinized (with 0.025% trypsin) at room temperature for 10-15 minutes. The activity of trypsin was then inhibited by using 5% fetal bovine serum in LHC. The cells were washed twice with LHC, and kept in DNACs medium (Siripattarapravat et al., 2009b) until used for nuclear transfer. In case of HGn30A and HGn8E, more than 20 embryos were utilized in each manipulation as there were limited numbers of GFP expressing cells in each embryo. For other strains, only 5-10 embryos were used. The cell suspension was added to a new drop in manipulation dish every 30 minutes to minimize repeated UV exposure of donor cells.

Somatic cell nuclear transfer. The nuclear transfer was performed as described previously (Siripattarapravat et al., 2009b), with minimal modifications. For enucleation, the DNA-stained egg's metaphase plate was ablated within the chorion using laser firing. The injection needle 8 μm in diameter was used for all cell types, except HGn62A in which the injection needle with 9 μm in diameter was used. Prior to injection, the cells were selected for GFP expression and individually picked for nuclear transfer. The individual donor nucleus was then transferred to the egg through the micropyle (the sperm entry site). Reconstructed embryos were activated in 'embryo medium'

(Westerfield, 1993) and allowed to develop. The developmental potential of cloned embryos were monitored and recorded every 3 hours after egg activation to 1 day, and continued every day until adulthood.

Statistical analysis. Developmental potential of cloned embryos was analyzed statistically by using two-way ANOVA and testing for the effects of two factors: the types of donor cells and the number of live embryos at 5 developmental stages. Since the response variable was the number of living embryos from the initial total reconstructed embryos, we considered modeling the count nature of the data assuming a binomial distribution under PROC GLIMMIX (SAS Institute, 2008). Repeated measures analysis was considered for developmental stages which required the modeling of a covariance structure across time points. Autoregressive model for covariance structure was preferred based on Akaike's information criterion values. Least squares means were estimated after back transforming from the binomial distribution using a logistic link function. Means for percent of embryos obtained from each type of cells were compared for significant difference within each developmental stage using Fisher's protected LSD and alpha<0.05.

Figure 23 GFP$^+$ donor embryos and offspring of cloned zebrafish. Tissue specific GFP$^+$ embryos at 24 hours post-fertilization of HGn30A (A), HGn62A (B), HG21C (C), HGn28A (D), and HGn8E (E) that are sources of GFP$^+$ donor cells (Nagayoshi et al., 2008). Back-crossed with wild type strain, offspring of cloned zebrafish from HGn28A GFP$^+$ donor cells (F) and HGn8E GFP$^+$ donor cells (G) of the same age. Scale bar is 250 μm.

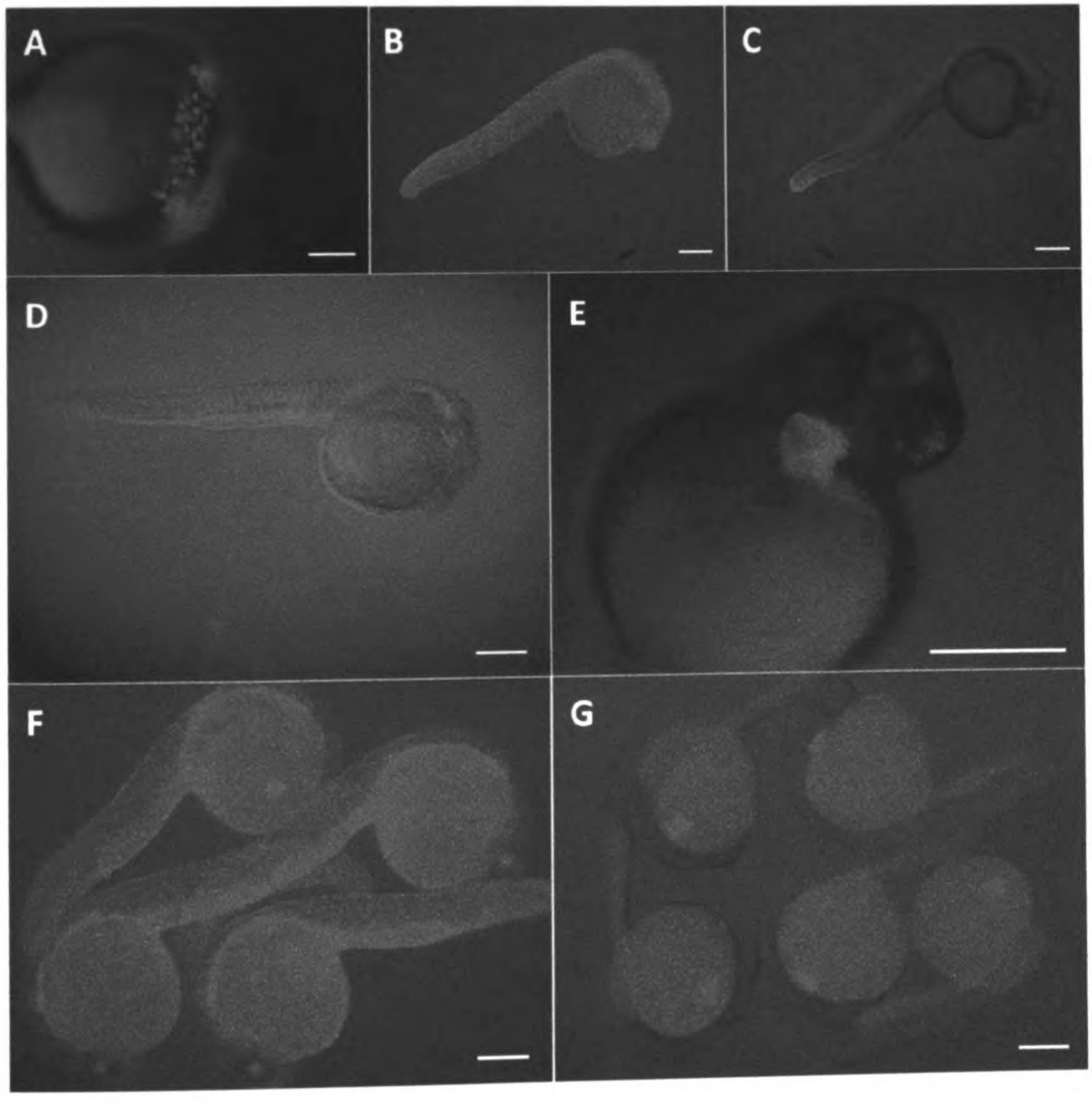
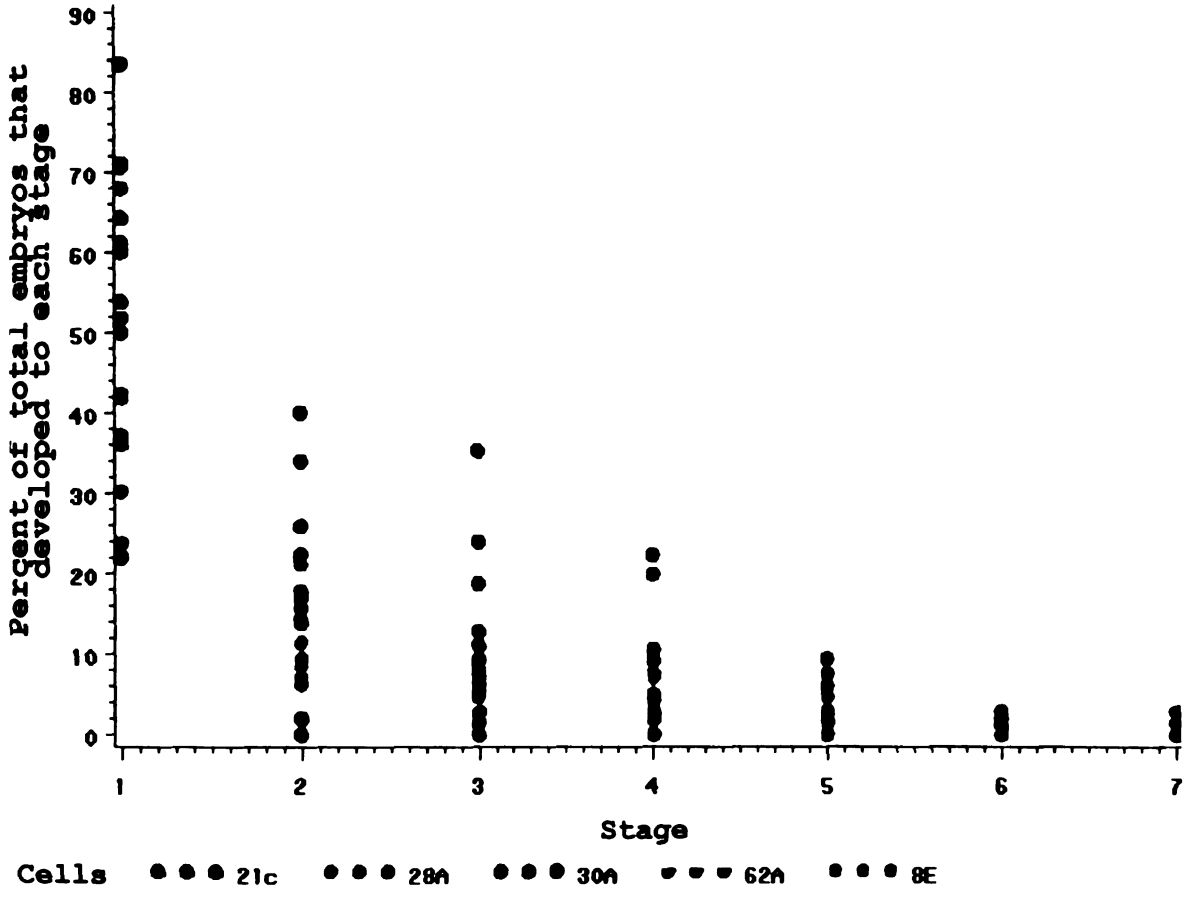


Table 6 Efficiency of zebrafish SCNT from GFP$^+$ donor cells of different lineages.

			Developmental stages - Number of embryos (%mean+SE)						
Donor G+	**NT**	**eggs**	**Blastula**	**Germ Ring**	**90%Epiboly**	**1 day**	**4 days**	**Live (Eat)**	**Live (Adult)**
HGn62A-N	4	290	107 (37.33+6.26)	18 (6.60+3.64)	9 (3.23+1.31)	1 (0.38+0.38)	1 (0.38+0.38)	1 (0.38+0.38)	0.00
HGn62A-T			180 (61.85+3.00)	26 (9.36+3.86)	15 (5.30+1.93)	12 (4.27+1.94)	2 (0.74+0.43)	1 (0.38+0.38)	0.00
HGn30A-N	3	225	95 (37.04+18.57)	11 (4.48+1.24)	7 (2.68+1.40)	1 (0.39+0.39)	1 (0.39+0.39)	0.00	0.00
HGn30A-T			146 (58.88+18.79)	39 (15.22+7.01)	18 (6.84+3.46)	8 (3.05+1.53)	2 (0.78+0.78)	0.00	0.00
HGn28A-N	3	225	79 (35.10+1.24)	29 (12.38+3.90)	13 (5.60+2.80)	5 (2.13+1.08)	2 (0.95+0.95)	2 (0.95+0.95)	1 (0.48+0.48)
HGn28A-T			116 (52.21+6.43)	35 (15.15+3.65)	17 (7.51+3.32)	9 (3.95+2.10)	4 (1.74+0.88)	2 (0.95+0.95)	1 (0.48+0.48)
HG21c-N	4	256	115 (41.12+7.47)	61 (20.81+7.47)	49 (16.67+6.28)	6 (2.78+1.23)	4 (1.58+0.92)	1(0.29+0.29)	0.00
HG21c-T			147 (54.62+9.11)	75 (27.56+8.20)	59 (20.22+6.76)	39 (13.93+4.50)	12 (4.55+2.03)	1(0.29+0.29)	0.00
HGn8E-N	4	185	32 (17.88+2.68)	12 (6.72+1.07)	8 (3.95+1.51)	3 (1.64+0.60)	3 (1.64+0.60)	3 (1.71+0.61)	1 (0.71+0.71)
HGn8E-T			62 (33.24+3.95)	16 (8.82+1.02)	12 (6.32+0.66)	8 (4.25+1.26)	8 (4.25+1.26)	3 (1.71+0.61)	1 (0.71+0.71)

The letter following the strain of donor cell sources, 'N' refers to only normal embryos – as their appearances looked normal, and 'T' refers to total embryos. Each SCNT operation was done with eggs from a separate individual and GFP$^+$ donor cells from a pool of embryos.

Figure 24 Developmental rate of GFP^+ cells from different zebrafish lines, included all normal and abnormal embryos. Statistical analysis was done by using proc GRIMMIX under SAS. Stages are blastula (1), germ ring (2), 90% epiboly (3), 1 day (4), 4 days (5), eating fry (6), and adult (7). The GFP^+ donor cells were isolated from transgenic lines; HG21C (21C), HGn28A (28A), HGn30A (30A), HGn62A (62A), and HGn8E (8E).



CONCLUSION AND FUTURE DIRECTIONS

In this dissertation, we successfully demonstrated nuclear reprogramming in zebrafish by introducing a somatic cell nucleus in to an enucleated egg. We optimized three major parameters: the donor cells, the recipient eggs, and the nuclear transfer technique. We described new cell culture protocols and DNA transfection for cultured cells and explored the possibility of modifying the donor nuclei using histone deacetylase inhibitors. The ideal stage of recipient cells (eggs) and their physiology were also investigated. Lastly, we compared different cell types for their ability to be reprogrammed following SCNT. Our work will facilitate gene knock-in / -out experiments in zebrafish (Figure 25), enhancing its prominent role as animal model for human disease.

Novel *in vitro* culture techniques for embryonic and adult zebrafish cells were described in chapter 2. We found that the cells in culture, possibly fibroblasts, were amenable to high rate DNA transfection using electroporation. Considering that homologous recombination events have been shown to work in embryos (Wu et al., 2006) and cultured cells of zebrafish (Fan et al., 2006), it is possible that the same event could happen in our cultured fibroblasts as well. Cultured fibroblasts, however, grow much slower than the ES cells, possibly hindering homologous recombination events. Zinc finger nucleases have been shown to increase the rate of the DNA repair mechanism by homologous recombination in cultured human cells (Porteus and Baltimore, 2003; Urnov et al., 2005). In addition to the successful implementation of ZFNs in zebrafish (Doyon et al., 2008; Meng et al., 2008), the combination of ZFNs and

the high rate of DNA transfection described could possibly enhance the success rate of gene targeting events in cultured fibroblasts (Figure 25). Since telomerase activity in the cultured embryo-derived fibroblasts remains high in the late passages, the clonal selection of gene-targeted cells might be possible.

In the future, some of the strategies used to increase the efficiency of SCNT in *Xenopus* should be tested in Zebrafish. Though *Xenopus* SCNT was described more than 50 years ago, its efficiency has always remained low. It has been speculated that this is due to a failure of DNA replication in the first round of cell division (Lemaitre et al., 2005). The origins of replication in red blood cells, following the treatment with egg-mitotic extracts for 45 minutes, decreased the inter-origin spacing from approximately 90 kb to the length of 15 kb, similar to one of embryonic cells (Lemaitre et al., 2005). Such a finding could explain why cloning efficiency increased as cells were subjected to serial transplantation (DiBerardino and Hoffner, 1983; Gurdon et al., 1975). Thus it suggests that, unlike one of the embryonic cells, the genome of fully differentiated cells possesses a chromosomal architecture that does not accommodate for the rapid DNA replication in early embryos and that the mitotic-cytoplasmic environment somehow promotes the shortening of the inter-origin spacing. We also observed in zebrafish SCNT, that the efficiency to produce cloned fish declined dramatically when cultured adult fin cells were used. The pre-treatment of donor cells with egg mitotic extract might promote the success of cloning in zebrafish by altering the chromosomal structure of differentiated cells to those of early embryos prior to entering rapid DNA replication.

Throughout all species cloned, once the somatic cell is fused to, or injected into the egg, its nucleus will relinquish control of the cell cycle in favor of that of the egg. In mice, a longer incubation of the donor nucleus in the MII oocyte induced a formation of the metaphase, and upon activation, a pseudo-polar body was extruded (Wakayama and Perry, 2002). Subsequently, diploid cloned-embryos could be obtained by either treatment with inhibitors of cytokinesis or by using of G2 (4n) donor cells, to inhibit the extrusion of the pseudo-polar body or to correct the diploidy, respectively. Unlike frog and Medaka SCNTs, where egg activation is triggered by nuclear transfer itself, the protocol for zebrafish SCNT calls for an incubation of donor cells in the MII eggs for 15 minutes prior to activation. While we did not investigate the role of the cell cycle and the changes to the donor nuclei following SCNT, this is an area that deserves more investigation. At the moment, there are transgenic zebrafish available which can express dual fluorescence proteins, degradable (red and green), upon entering each cell cycle stage (Sugiyama et al., 2009). The use of donor cells derived from these transgenic fish, together with the study of changes in cloned embryos upon egg activation, may reveal that indeed the impact of the cell cycle stage in the donor cell is as important in zebrafish as it is in all other cloned species.

The MII eggs of zebrafish are easily obtained, however, the quality of the eggs declines within an hour after collection in the synthetic medium (Sakai et al., 1997). The use of Chinook salmon ovarian fluid as a collection medium can extend the quality of the eggs for up to 4-6 hours (Corley-Smith et al., 1999; Siripattarapravat et al., 2009a). However, the ovarian fluid of Chinook salmon is not commercially available, and more

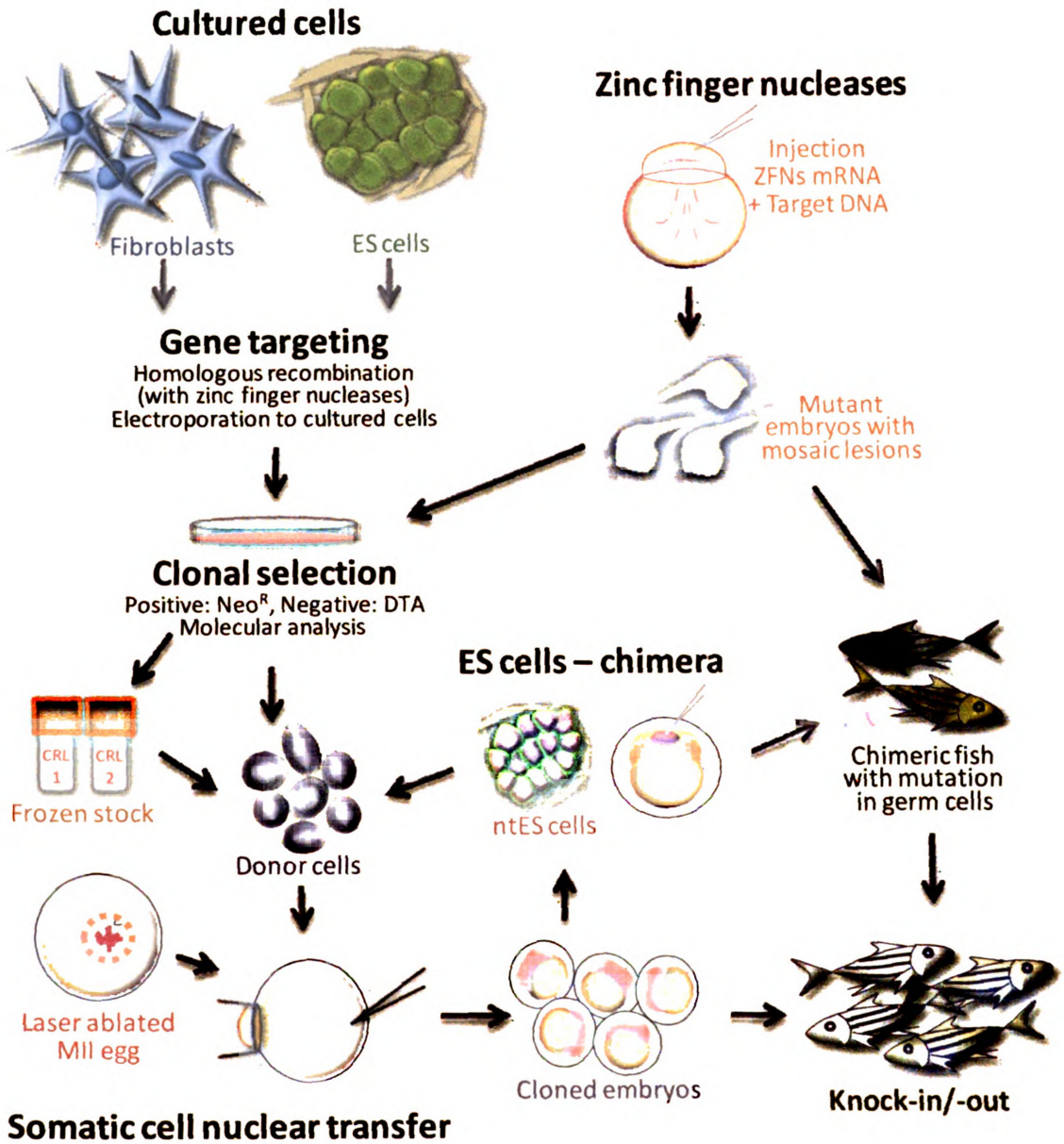
importantly, the quality of the fluid might fluctuate with the collection technique and the health of the Chinook salmon. While the ionic composition of Chinook salmon ovarian fluid has been reported (Rosengrave et al., 2009), until now, there is a lack of understanding on how the fluid works to maintain the zebrafish egg at the MII stage. Synthetic ovarian fluid, if at all possible to manufacture, will be a great tool to better control the cell cycle in the unfertilized eggs.

The pattern of calcium oscillation following fertilization is important for subsequent embryonic development (Ducibella and Fissore, 2008) and nuclear reprogramming (Ross et al., 2009). Recent publications demonstrated that the physiology of eggs activated by fertilization differs from eggs activated by parthenogenesis, in which eggs usually lack calcium oscillation at the cortical compartment (Sharma and Kinsey, 2008). The eggs injected with zebrafish sperm extracts elicit calcium oscillation at the cortex, similar to that of fertilized eggs (Sharma and Kinsey, 2008). It is possible that the protocol for egg activation following SCNT in zebrafish, i.e., exposure to egg water, is not sufficient to trigger all the downstream pathways essential to embryonic development. The use of sperm extracts following SCNT to mimic the calcium oscillation pattern of fertilized eggs might improve the nuclear reprogramming efficiency in cloned embryos.

It has been speculated that incomplete nuclear reprogramming is due to an insufficient gap period between the time of nuclear transfer and the zygotic genome activation, leading to aberrant gene expression in the cloned embryos. The first zygotic transcripts appear as early as the first cell division – approximately 20 hours in mice, and

at the sixth cell division – approximately 2-4 hours in frogs and fish (Tadros and Lipshitz, 2009). Cloned zebrafish at the dome stage, were reported to have differential expressed genes from those of fertilized counterparts (Luo et al., 2009). It suggests that, within a few hours following nuclear transfer, the donor nucleus of successful frogs and fish SCNT will need to turn off the genes of differentiated cells and switch on the embryonic genes. In parallel to the transition to rapid cell division of early embryos, cloned embryos have to initiate the embryonic gene expression as well, thereby increasing the probability of failure. Cloned frogs were reported to fail the first round of cell division, referred to as partial blastula (Gurdon et al., 1975), as well as having aberrant expression of donor cell type-specific genes in other embryonic cells (Ng and Gurdon, 2005). It is not surprising that with the time constraints imposed by rapid cell divisions in the early embryos, frogs and zebrafish from somatic nuclei in adult donor cells cannot be reprogrammed into an adult individual (DiBerardino and Hoffner, 1983; Gurdon et al., 1975; Siripattarapravat et al., 2009b). In mouse cloning, the efficiency of nuclear reprogramming can be increased using histone deacetylase inhibitors (Thuan et al., 2010). Therefore, the treatment of either donor cells or cloned embryos prior to zygotic genome activation with chemical reagents that potentially modify the global epigenetic status of the donor cells, i.e., inhibitors of histone deacetylase, might facilitate the subsequent nuclear reprogramming following SCNT in zebrafish.

Figure 25 Possible approaches to generate Knock-in/ -out zebrafish.



The culture cells, either fibroblasts or ES cells, can be subjected to gene targeting by homologous recombination (HR) *in vitro*, with the help of ZFNs to increase the rate of DNA repairing by HR. The cells, harboring, targeted mutagenesis, can be selected as clonal lines, by using positive/ negative selection and molecular approaches. These cells can be kept as frozen stocks, and some used as donor cells for SCNT. Using SCNT, the knock-in/-out zebrafish can be directly generated. As the rate of adult cloned fish production is low, one can use cloned embryos to generate ntES cells. The ntES cells can be used for another round of SCNT or subsequently generate chimeric fish. The chimeric fish harboring mutation in germ cells can be bred to produce offspring that are mutants. Alternatively, the gene targeting by co-injections of ZFNs and targeted constructs for HR can be done in embryos. The cells of embryos, harboring mosaic mutation, can be cultured to isolate the clonal lines and later used as donor cells as well.

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