ISOLATION AND ESTABLISHMENT OF A NEW EMBRYONIC-LIKE STEM CELL LINE FROM ZEBRAFISH

LEE YOUN SING

UNIVERSITI SAINS MALAYSIA
2014

ISOLATION AND ESTABLISHMENT OF A NEW EMBRYONIC-LIKE STEM CELL LINE FROM ZEBRAFISH

by

LEE YOUN SING

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

September 2014

ACKNOWLEDGEMENT

I would like to express my special appreciation and thanks to my supervisor Professor Alexander Chong Shu Chien, who has given me an opportunity in this project. I would like to thank you for leading my research and for allowing me to grow as a research scientist. Your advice on research has led to the success of this project. I would especially like to thank my lab members Crystal Goh, Jen Yang, Beng Siang, Boon Khai, Shu Shen, Karthi, Adelina and Wai Kwan for giving me support and help when I was collecting data for my Master thesis. I would like to express my appreciation to Malaysian Institute of Pharmaceuticals and Nutraceuticals (IPharm) and Universiti Sains Malaysia (USM) in providing facilities to complete the research. I would also like to appreciate Malaysian Ministry of Higher Education for providing MyBrain Scholarship so that I can complete my studies.

A special thanks to my family. Words cannot express how grateful I am for all of the sacrifices that you have made on my behalf for the whole duration of this project. I would also like to thank all of my friends who assured me to strive towards my goal. At the end, I would like to express my appreciation to Sing Yee who shared invaluable teamwork and opinions. She was always my support in the moments when there was no one to answer my queries.

TABLE OF CONTENTS

CHAPTER 3: MATERIALS AND METHODS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percentage
±	plus-minus
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
ADME	absorption, distribution, metabolism and excretion
B27	A mixture of proteins, hormones and vitamins as medium supplement.
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic proteins
BRL	buffalo rat liver
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CHIR99021	6-[[2-[[4-(2,4-dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2 pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile, a highly selective inhibitor of glycogen synthase kinase 3 (GSK-3)
cm	centimeter

CM	conditioned medium
CMV	cytomegalovirus
c-Myc	*v-myc myelocytomatosis viral oncogene homolog*
CRISPR-Cas	clustered regularly interspaced short palindromic repeats – Cas
DAPI	2-(4-amidinophenyl)-1H -indole-6-carboxamidine
dGH	degrees of general hardness
dH_2O	distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
ERK	extracellular signal-regulated kinase
ES	embryonic stem
ESM2	ES medium 2
EST	expressed sequence tag
et al.	*et alii* (and other people)
FACS	fluorescence-activated cell sorting

FBS	fetal bovine serum
FEE	fish embryo extract
FITC	fluorescein isothiocyanate
g	gram
g	*relative centrifugal force*
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GPI	glucose-6-phosphate isomerase
GSK3	glycogen synthase kinase 3
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
hpf	hours post-fertilization
HSC	hematopoietic stem cells
ICM	inner cell mass
IgG	immunoglobulin G
IgM	immunoglobulin M
ISCI	International Stem Cell Initiative
K18	keratin 18
K19	keratin 19
K8	keratin 8

KCl	potassium chloride
Klf4	*kruppel-like factor 4*
L	liter
L15	Leibowitz's L15
LB	Luria-Bertani
LDF	Leibowitz's L15, Dulbecco's Modified Eagle's Medium DMEM and Ham's F12
LIF	leukemia inhibitory factor
loxP	locus of crossover in P1
M	molar
MAP2	microtubule associated protein 2
MBE	medaka blastula embryonic
MEF	murine embryonic fibroblasts
MEK-1	mitogen-activated protein kinase 1
mg	milligram
$MgSO_4$	magnesium sulphate
mL	milliliter
mM	millimolar
MPSS	massively parallel signature sequencing

mRNA	messenger ribonucleic acid
miRNA	microRNA
N2	A mixture of insulin, transferrin, progesterone, selenium and putrescine as medium supplement.
NaCl	sodium chloride
NaClO	sodium hypochlorite
NaOH	sodium hydroxide
NEAA	non-essential amino acids
ng	nanogram
^{o}C	degree Celsius
Oct4	*octamer-binding transcription factor 4*
Opti-MEM	improved Minimal Essential Medium
PBS	phosphate-buffered saline
PD184352	2-(2-chloro-4-iodophenylamino)-N-(cyclopropylmethoxy)-3,4-difluorobenzamide, a highly potent and selective inhibitor of mitogen-activated protein kinase 1 (MEK-1)
PFA	paraformaldehyde
pH	potentiometric hydrogen ion concentration
RA	retinoic acid
Rex1	reduced expression 1

RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
RTS34st	rainbow trout spleen stromal cell line
SAGE	serial analysis of gene expression
SOX17	SRY (sex determining region Y)-box 17
Sox2	*SRY (sex determining region Y)-box 2*
SSEA1	stage-specific embryonic antigen-1
SSEA3	stage-specific embryonic antigen-3
SSEA4	stage-specific embryonic antigen-4
SSEA5	stage-specific embryonic antigen-5
Stat3	signal transducer and activator of transcription 3
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
U	units
v/v	volume per volume
w/v	weight per volume
ZEB2J	zebrafish blastula cell line
ZEE	zebrafish embryo extract
ZES1	zebrafish embryonic stem cell-like line 1

ZES2	zebrafish embryonic stem cell-like line 2
ZES3	zebrafish embryonic stem cell-like line 3
ZES4	zebrafish embryonic stem cell-like line 4
ZES5	zebrafish embryonic stem cell-like line 5
ZFEM1	zebrafish embryonic medium 1
ZFIN	Zebrafish Information Network
ZFNs	zinc finger nucleases

LIST OF PUBLICATION

Ho, S.Y., Goh, C.W., Gan, J.Y., Lee, Y.S., Lam, M.K., Hong, N., Hong, Y., Chan, W.K. and Shu-Chien, A.C. (2014). Derivation and long-term culture of an embryonic stem cell-like line from zebrafish blastomeres under feeder-free condition. *Zebrafish*, 11: 407–420.

ISOLASI DAN PENGHASILAN TITISAN SEL SERUPA SEL TUNJANG EMBRIONIK YANG BARU DARIPADA IKAN ZEBRA

ABSTRAK

Sel tunjang embrionik (ES) yang dihasilkan daripada pelbagai spesies ikan dengan menggunakan kaedah tanpa sel penyuap telah dilaporkan dalam banyak kes. Walau bagaimanapun, titisan sel tunjang embrionik ikan zebra yang dihasilkan masih terhad dan memerlukan penambahan ekstrak embrio ikan (FEE) dalam formulasi media. FEE tidak mudah didapati oleh makmal-makmal yang kekurangan sistem perumahan akuatik. Tambahan pula, ia boleh menimbulkan isu-isu etika kerana ia melibatkan kemusnahan sebilangan besar embrio ikan untuk menyokong pertumbuhan sel dalam *in vitro*. Oleh itu, matlamat kajian ini adalah untuk mendapatkan satu titisan sel serupa ES daripada blastomer ikan zebra tanpa menggunakan sel penyuap dan FEE. Dalam kajian ini, titisan sel baru, ZES4 telah berjaya diasingkan dan dikekalkan dalam keadaan sel serupa ES dan menunjukkan potensi pembezaan *in vitro* apabila keadaan kultur berubah. Sel yang berbeza telah dikenal pasti melalui morfologi dan immunokimia dengan menggunakan penanda spesifik sel keturunan. Walau bagaimanapun, penanda spesifik sel keturunan ini juga didapati dalam sel ZES4 yang belum berbeza. Stage specific embryonic antigen 3 (SSEA3) dan SSEA4 dikesan dalam embrio pada peringkat blastula dan sel ZES4 yang belum berbeza. SSEA1 dikesan dalam minoriti sel ZES4 yang telah dikultur dalam kepadatan tinggi untuk pembezaan selama 40 hari. Sel ZES4 yang dilabel dengan GFP dapat menyumbang kepada pembentukan somit, bahagian berhampiran gonad dan jantung yang berfungsi dalam perumah. Kesimpulannya, ZES4 mempunyai keupayaan untuk membeza *in vitro* dan *in vivo* yang setanding dengan sel tunjang embrionik ikan lain-lain.

ISOLATION AND ESTABLISHMENT OF A NEW EMBRYONIC-LIKE STEM CELL LINE FROM ZEBRAFISH

ABSTRACT

Embryonic stem (ES) cells established from various fish species using feeder-free method had been reported in many cases. However, zebrafish ES-like cell lines were currently limited and required the addition of fish embryo extract (FEE) in medium formulation. FEE was not easily available to laboratories that lack of aquatic housing system. Furthermore, it may raise ethical issues as it involved destruction of large numbers of developing fish embryos to support the growth of *in vitro* cells. Therefore, the aim of this study was to derive an ES-like cell line from zebrafish blastomeres without the use of feeder cells and FEE. In this study, a new cell line, ZES4 was successfully isolated and maintained in ES-like cell state and showed *in vitro* differentiation potentials upon changing the culture conditions. Differentiated cells were identified through morphological and immunocytochemistry using cell lineage specific markers. However, these cell lineage specific markers were also found in undifferentiated ZES4 cells. Stage specific embryonic antigen 3 (SSEA3) and SSEA4 was detected in both blastula stage embryos and undifferentiated ZES4 cells. SSEA1 was detected in minority of ZES4 cells in high density differentiation culture conditions after 40 days. GFP-labeled ZES4 cells were able to contribute to chimera formation in somite, near gonad region and functional heart of the hosts. In conclusion, ZES4 has the ability to differentiate *in vitro* and *in vivo* which is comparable to other fish ES cells.

CHAPTER 1

INTRODUCTION

1.1 Research background

Embryonic stem (ES) cells are cells isolated from blastocyst and cultured in a specific culture environment which manage to maintain pluripotency (Rippon & Bishop, 2004). These cells are able to differentiate into various cell types found in an organism upon induction by certain stimulants or incorporated into a host (Amit *et* al., 2000; Reubinoff *et al*., 2000; Thomson *et al*., 1998). It holds a great promise in regenerative medicine to treat various diseases as well as the possibility to regenerate damaged organs or body parts (Hong *et al*., 2011; Rippon & Bishop, 2004).

Studies on human and mouse ES cells have been leading the way compared to other species. Pluripotency-associated markers in both human and mouse ES cells had been identified from time to time. Various differentiation protocols had also been gradually developed to direct differentiate human and mouse ES cells to more differentiated derivatives (D'Amour *et al*., 2005; Kattman *et al*., 2011; Ng *et al*., 2008; Tan *et al*., 2013). The differentiated cells were identified based on cell lineage specific markers as well as the downregulation of pluripotency-associated markers or even transcription profiles.

Fish ES cells derivation has been started about 20 years ago. However, the achievements of fish ES cells are far behind compared to human and mouse ES cells. Medaka fish has been the current leading model organism in fish ES cells studies with the achievement of feeder-free culture system (Hong *et al*., 1996). Attempts on

derivation of other fish ES cells have also been reported in red sea bream (Chen *et al*., 2003a), Indian major carp (Dash *et al*., 2010), Asian sea bass (Parameswaran *et al*., 2007) and also zebrafish (Fan & Collodi, 2004).

The exhaustive works to derive zebrafish ES cells for the past few years have not yielded much progress and FEE was used in medium formulations (Fan & Collodi, 2004). However, the requirement of FEE limited the culture of zebrafish ES cells in laboratories that lack of aquatic housing system. Destruction of large numbers of developing fish embryos to culture the cells might also raise ethical issues. The works on formulating a defined medium without FEE had been attempted on derivation of zebrafish ES cells and gave rise to a transient zebrafish embryonic culture (Robles *et al.*, 2011). Hence, a new long-term zebrafish embryonic-like stem cell line will be established in feeder-free culture system with culture medium formulated without FEE. The differentiation potentials of this cell line will be examined by using both *in vitro* and *in vivo* systems in this study.

1.2 Objectives

Establishment of new zebrafish ES-like cell lines with feeder-free method and FEE-excluded culture medium could provide a better culture system for future studies. Hence, the objectives of this study are:

I. to establish a new feeder-free zebrafish embryonic-like stem cell line with FEE-excluded culture medium.

II. to examine the *in vitro* and *in vivo* differentiation potentials of the established cell line.

CHAPTER 2

LITERATURE REVIEW

2.1 Embryonic stem cells

ES cells were cells that originated from the inner cell mass (ICM) of pre-implantation blastocyst (Rippon & Bishop, 2004). The ICM have the ability to form all of the tissues in the body, except extraembryonic structures like the placenta (Rippon & Bishop, 2004). These short-lived pluripotent cells in the embryo can be propagated and remained undifferentiated in culture with the presence of several growth factors with or without feeder layers (Smith *et al*., 1988; Williams *et al*., 1988). The ES cell lines derived from early embryos have self-renewal ability and capability to become more specialized cells or differentiated cell types. This unique characteristic of ES cells has offered an *in vitro* system to study the underlying mechanisms involved in embryogenesis (Evans & Kaufman, 1981; Martin, 1981).

Stable ES cell lines were initially restricted to mouse (Evans & Kaufman, 1981; Martin, 1981), while attempts to derived ES cell lines from other mammalian species (Campbell *et al*., 1996; Iannaccone *et al*., 1994; Notarianni *et al*., 1991; Sims & First, 1993; Sukoyan *et al*., 1992; Thomson *et al*., 1995) and non-mammalian species (Pain *et al*., 1996) had also been carried out. However, the cultivation of these early embryonic cells was limited for only a period of time (Sukoyan *et al*., 1992; Pain *et al*., 1996), while others were only able to partially maintain the pluripotency or differentiation ability of the cells in extended cultures (Campbell *et al*., 1996; Sims & First, 1993). Over the last 30 years, murine ES cells have had a

huge impact on research fields especially in modifying the early mouse embryos (Bradley *et al*., 1984). This has formed the foundation of genome manipulation technology in producing transgenic animals through knock-out or knock-in for *in vivo* gene expression and regulation (Thomas & Capecchi, 1987). ES cells also allow *in vitro* studies of fundamental mechanisms on pluripotency and cell lineage specification of initial stages of mammalian development to be carried out (Rippon & Bishop, 2004).

As ES cell technology showed attractive values and potentials, human ES cells were successfully established in 1998 (Thomson *et al*., 1998; Prelle *et al*., 2002). Among the various characteristics shared between human and mouse ES cells were diploid karyotype nature, expression of high telomerase activity that governs the immortality of cells as well as specific cell surface markers (Thomson *et al*., 1998). However, there are some important differences between human ES cells and murine ES cells in culture. Human ES cells grow slower, tend to form flat instead of spherical colonies and easier to be dissociated into single cells compared to mouse ES cells (Laslett *et al*., 2003). Human ES cells are also unresponsive to leukemia inhibitory factor (LIF) and need to be cultured on murine embryonic fibroblasts (MEF) feeder layers with the presence of basic fibroblast growth factor (bFGF) (Amit *et al*., 2000; Laslett *et al*., 2003; Reubinoff *et al*., 2000; Thomson *et al*., 1998), or on matrigel or laminin in MEF-conditioned medium (Xu *et al*., 2001). There are also differences in certain antigenic phenotypes in murine ES cells where the embryonic antigen, SSEA1, is expressed in undifferentiated state but not SSEA3 and SSEA4, while undifferentiated human ES cells showed the opposing phenotype (Reubinoff *et al*., 2000; Thomson *et al*., 1998).

The generation of chimeric mice is used as a method to illustrate the pluripotency of murine ES cells but this is not applicable to human cells for obvious reasons (Rippon & Bishop, 2004). The gold standard test for pluripotency of human ES cells involved the implantation of human ES cells into immunodeficient mice for teratomas formation in order to demonstrate *in vivo* differentiation potential to all three germ layers (Amit *et al*., 2000; Reubinoff *et al*., 2000; Thomson *et al*., 1998). There are increasing numbers of human ES cell lines from numerous research groups and commercials companies (Annas *et al*., 1999). It is unlikely to be acceptable for therapeutic purposes if human ES cells were grown on murine feeder layers or cultured in medium containing animal-derived products as it poses the risk to transmit animal pathogens to human population (Rippon & Bishop, 2004). As a result, the use of human feeder cells or feeder-free conditions to culture human ES cells under entirely animal-free conditions is becoming more preferable (Amit *et al*., 2003; Hovatta *et al*., 2003; Richards *et al*., 2002; Richards *et al*., 2003). The pluripotency of human ES cells also enable the supply or production of different cell types in large amount for *in vitro* analysis and regenerative medicine (Hong *et al*., 2011).

2.2 Fish embryonic stem cells

In fish ES cells, especially in small aquarium fish like zebrafish and medaka have raised attention in research to become a model system for vertebrate development studies (Howe *et al*., 2013; Ozato & Wakamatsu, 1994; Powers, 1989; Shima & Shimada, 1991). Fish embryos usually developed externally which is an advantage over mouse for easier initiation of embryonic cultures from fish (Alvarez

et al., 2007). Blastocysts remained undifferentiated, easier to handle and more capable for germ-line chimera formation compared to younger embryos (Lin *et al*., 1992; Wakamatsu *et al*., 1993). Fish blastocysts were easily dechorionated by using pronase in zebrafish (Sun *et al*., 1995a), hatching enzyme in medaka (Hong & Schartl, 1996), or mechanically tear off the chorions with fine forceps in red seabream (Chen *et al*., 2003a), gilthead seabream (Béjar *et al*., 1999) and sea perch (Chen *et al*., 2003b).

Establishment of specific conditions to prevent spontaneous differentiations in fish ES cells has become the main objective to maintain the embryonic cells population (Alvarez *et al*., 2007). The achievement of feeder-free and long-term culture of undifferentiated medaka fish embryonic cells has marked the major contribution to fish ES cell cultures (Hong & Schartl, 1996). The feeder-free conditions established in medaka were applicable to several other fish species from different taxons in feeder-free culture such as flounder, *Paralichthys olivaceus* (Chen *et al*., 2004); gilthead seabream, *Sparus aurata* (Béjar *et al*., 2002); red seabream, *Pagrosomus major* (Chen *et al*., 2003a); and sea perch, *Lateolabrax japonicus* (Chen *et al*., 2003b).

A different approach has been attempted by Barnes' group (Collodi *et al*., 1992) using conditioned medium (CM), a spent medium collected from feeder layer cultures, to establish embryonic cell culture in zebrafish. They formulated a medium consisted of a mixture of L15, DMEM and F12 media (LDF) containing embryo extract and serum from trout (*Oncorhynchus mykiss*), while the CM used was collected from Buffalo rat liver (BRL) cell line which had been demonstrated to have the capability in maintaining mouse blastocyst-derived cell cultures in ES phenotype. Their medium formulation could apparently support and maintain the

undifferentiated state of midblastula embryo cells for more than 40 population passages. The medium formulation used for zebrafish ES cells derivation in feeder layer culture conditions by Fan and Collodi, 2004 comprises of LDF as basal medium, supplemented with fetal bovine serum (FBS), trout serum, zebrafish embryo extract (ZEE), bovine insulin, bFGF, epidermal growth factor (EGF) and RTS34st cell-conditioned medium. The cells have been reported to maintain germ-line competent state for at least 6 passages or 6 weeks using these culture conditions (Fan & Collodi, 2004).

Establishment of zebrafish ES cells in feeder-free conditions to overcome the downsides of feeder layer system have also been done by Xing *et al*., 2008. The zebrafish blastula cells were initially seeded on RTS34st feeder layer and then switched to feeder-free culture conditions with a simpler culture medium consisting of only L15 and FBS. This cell line with limited differentiation potential was named as ZEB2J.

Another attempt has been made by Robles *et al*., 2011 to initiate zebrafish embryonic stem cell culture using feeder-free system. The resulting transient zebrafish embryonic cells was cultured in LDF basal medium added with B27 and N2 serum-free supplements with two small molecule inhibitors, CHIR99021 for glycogen synthase kinase 3 (GSK3) inhibition and PD184352 for mitogen-activated protein kinase 1 (MEK-1) inhibition. MEK-1 inhibitor was found to affect the growth and viability of cells but able to preclude the differentiation of ES cells. In order to support the self-renewal and growth of ES cells in a better condition, GSK3 inhibitor was also included in the medium formulation (Robles *et al.,* 2011). Status of fish stem cells in cell-mediated gene targeting, semi-cloning and transplantation have been mentioned in some reviews (Alvarez *et al*., 2007; Barnes *et al*., 2008; Fan &

Collodi., 2006; Hong *et al*., 2011). Haploid ES cells have also been developed in medaka fish and made it possible to study recessive genes in fish ES cells (Yi *et al*., 2010).

2.3 Components in embryonic stem cell culture media

Human ES cells were originally established in medium containing serum and cultured on fibroblast feeder layers (Reubinoff *et al*., 2000; Thomson *et al*., 1998) which is similar to the conditions initially used to isolate mouse ES cells (Evans & Kaufman, 1981; Martin, 1981). However, the factors involved in mediating self-renewal of mouse and human ES cells seem to be different (Xu *et al*., 2005). Human ES cells failed to be maintained with the supplementation of LIF in culture medium (Thomson *et al*., 1998) or activation of signal transducer and activator of transcription 3 (Stat3) (Humphrey *et al*., 2004) which is the culture conditions normally used to sustain mouse ES cells. The growth factor that has the greatest effect in supporting self-renewal in human ES cells among all the tested growth factors is bFGF (Xu *et al*., 2005). The role of bFGF in sustaining pluripotency of human ES cells become more apparent with the reports from numerous researchers who successfully used high concentrations of bFGF to culture human ES cells in feeder-free conditions (Genbacev *et al*., 2005; Klimanskaya *et al*., 2005; Levenstein *et al*., 2005; Li *et al*., 2005; Wang *et al*., 2005; Xu *et al*., 2005). Nevertheless, it is still remained unclear on the exact mechanism involved (Ludwig & Thomson, 2007).

Numerous supplements like fish serum, 2-mercaptoethanol, bFGF and FEE were found to be important for stable growth other than fetal calf serum as shown in the study of growth response assays in feeder-free fish ES cells (Hong *et al*., 2003).

Intriguingly, no effect has been shown with the addition of recombinant human LIF on the propagation and differentiation of medaka or zebrafish MBE cells (Hong *et al*., 1996) and also other marine species, the gilthead seabream (*Sparus aurata*) (Béjar *et al*., 2002). It is possible that MBE cell cultures do not require LIF or the LIF activity is already mimicked by the heterogeneous embryo extract (Hong *et al*., 2003). On the other hand, fish cells are not affected by LIF as mammalian LIF sequence is too specialized (Hong *et al*., 2003). This notion is further being supported as no LIF has been discovered in fish (Hong *et al*., 2003).

The culture medium formulation of zebrafish embryonic cell and other fish ES cell lines contained FEE from rainbow trout (*Oncorhynchus mykiss*) as mitogens to promote cell growth (Collodi & Barnes, 1990; Collodi *et al*., 1992). Mitogens for medaka and zebrafish early embryonic cells using FEE from seawater fish, sea bream (*Sparus aurata*) had been claimed not as effective as FEE from medaka (Hong & Schartl, 1996). Mitogenic activity could be present in teleostean embryos, but Hong & Schartl, 1996 showed that species-specific FEE seemed to perform better than FEE from other species. In order to be effectively mitogenic to the fish ES cell lines, FEE is no doubt must be obtained from the same species even though the conserved factors might be present among the teleostean fish embryos. Hence, it has become a norm that FEE is added into the culture medium in order to successfully maintain other fish ES cell cultures (Béjar *et al*., 2002; Chen *et al*., 2003a; Chen *et al*., 2003b; Dash *et al*., 2010; Ghosh *et al*., 1997; Holen *et al*., 2010; Yi *et al*., 2010).

Besides using FEE as mitogen, bFGF and trout serum have also contributed in mitogenic activity in fish ES cell lines. It was reported that bFGF was effective mitogens for medaka MBE cells (Hong & Schartl, 1996). Besides, bFGF has also been known for its mitogenic activity in culture for many other mammalian cell types

(Matsui *et al*., 1992) and zebrafish embryos (Bradford *et al*., 1994). Nevertheless, Bradford *et al*., 1994 found that bFGF can irreversibly inhibit spontaneous melanogenesis in zebrafish embryonic cells culture. This could affect the evaluation of transplanted cells contribution in host embryos using pigmentation markers. However, medaka MBE cells pigmentation were not affected by bFGF (Hong & Schartl, 1996).

FBS at high concentration which is around 10 % has been shown to inhibit cell growth from early embryonic cells and some adult tissue cells of zebrafish (Collodi *et al*., 1992). On the other hand, FBS is crucial for MBE cells to support optimum cell growth and viability (Hong & Schartl, 1996). This only occurred in early zebrafish embryonic cells (Collodi *et al*., 1992). In contrast, FBS at high concentration which was around 20 % had been shown to increase cell growth by approximately five times in MBE cells (Hong & Schartl, 1996). With the addition of 1 % fish serum in the formulation, the proliferation of cells was found to increase by three times (Hong & Schartl, 1996). The absence of any of the serum rendered low cell viability after seven days of culture (Hong & Schartl, 1996).

2.4 Background on zebrafish

Zebrafish (*Danio rerio*) is first described by Francis Hamilton in 1822 (Quigley & Parichy, 2002). It has been classified under the family of cyprinids (Cyprinidae), Teleostei or teleost bony fishes which is in the ray-finned fish class, Actinopterygii (Carpio & Estrada, 2006). Zebrafish maintained in laboratory usually grow until 3 to 5 cm in length (Kishi *et al*., 2003). Males and females are distinguished by their general appearances, the anal fins of adult males are more

yellowish and larger (Laale, 1977; Schilling, 2002); adult females have more rounded bodies and have a small genital papilla in front of the anal fin (Laale, 1977).

Zebrafish is maintained at temperature of 28.5 ^{o}C (Westerfield, 2007). pH of water in the range of 6.0 to 8.0 and water hardness of 5.0 to 19.0 degrees of general hardness (dGH) are tolerable for zebrafish (Kishi *et al*., 2003). 14-hour light and 10-hour dark cycle are easily controlled by using artificial light in laboratory (Brand *et al*., 2002). The diets formulated for other fish species are able to be used on zebrafish (Matthews *et al*., 2002). There are reports showing that zebrafish is omnivorous, but usually feed on insects and microcrustacea (McClure *et al*., 2006; Spence *et al*., 2007). *Artemia* nauplii (Carvalho *et al*., 2006) and bloodworm are probably more appropriate food for zebrafish (Spence *et al*., 2008).

A single zebrafish female can produce up to over 700 eggs per breeding (Spence & Smith, 2006). The zebrafish embryos are transparent, develop rapidly and externally which enable the observation of embryo development (Kimmel *et al*., 1995; Wixon, 2000) from a single cell until they become multicellular organisms. The generation time of zebrafish from larvae to adult is only 3 to 5 months (Bopp *et al*., 2006) and their lifespan are usually about 2 to 5 years (Gerhard *et al*., 2002).

2.5 The rise of zebrafish as model organism

The pioneer work using zebrafish as model organism for vertebrate embryology studies were started by Streisinger *et al*., 1981. Besides, dedicated online databases have been established such as Zebrafish Information Network (ZFIN) that funded by the National Human Genome Research Institute of the National Institutes

of Health (Bradford *et al*., 2011). Zebrafish genome has also been fully sequenced by Wellcome Trust Sanger Institute through a project which started in 2001 (Howe *et al*., 2013). Mutants screen has resulted in identification of genes in zebrafish regulating vertebrate development (Haffter *et al*., 1996). Zebrafish genome is about the half of the size of human genome which consisted of approximately 1700 million base pairs in length. The human genome has a lot of similarity with zebrafish in numerous developmental and disease associated genes (Howe *et al*., 2013).

The ease of observations, large number of embryos availability within a short period of time, rapid development and the externally developed transparent embryos make zebrafish comparable to other mammalian models in vertebrate developmental and functional studies (Carpio & Estrada, 2006). It has gained importance in the field of biomedical research (Dooley & Zon, 2000), a model of human disease (Berghmans *et al*., 2005; Guyon *et al*., 2006) as well as drugs screening (Rubinstein, 2003).

Other than being a powerful model organism for genetic studies, zebrafish is also known for its regeneration abilities (Poss *et al*., 2003; Poss, 2007). It has been reported that zebrafish is able to regenerate its organs and tissues such as skin, fins, pigment cells, barbels, muscle, heart, liver, pancreas and central nervous system (Antos & Brand, 2010; Antos & Tanaka, 2010; Becker & Becker, 2008; Brignull *et al*., 2009; Curado & Stainier, 2010; Hata *et al*., 2007; Huang & Zon, 2008; Keating, 2004; LeClair & Topczewski, 2010; Nakatani *et al*., 2007; O' Reilly-Pol & Jonhson, 2009; Poss *et al*., 2003; Raya *et al*., 2004; Stoick-Cooper *et al*., 2007; Tal *et al*., 2010; White & Zon, 2008). Heart regeneration studies have shown that cellular tracing on source of regeneration can be done in zebrafish (Lepilina *et al*., 2006).

2.6 Immunofluorescence labeling techniques

The techniques of immunofluorescence labeling were initiated about 60 years ago (Coons *et al*., 1941; Coons *et al*., 1942) had since then, rendered incomparable influence to life sciences in various fields. The exploitation of this technology enables the utilization of antibodies in order to recognize and detect individual or multiple antigens *in situ* (Brandtzaeg, 1998; Coleman, 2000). Most of the laboratories are not using covalent labeling of antibodies as their standard practice as a huge amount of purified antibodies are needed (Mao, 1999) and to maintain the plasticity of this multi-labeling techniques, choices for various kind of fluorophores conjugation with every primary and control antibody are also required. Moreover, direct immunofluorescence is also thought by many to be rather insensitive (Mao, 1999) which has resulted in the establishment of the indirect immunofluorescence method (Coons *et al*., 1955) to achieve multi-labeling (Ferri *et al*., 1997; Staines *et al*., 1988).

Two-step or indirect method in immunofluorescence labeling has been developed by Coons *et al*., 1955 following the demand on more sensitive antigen detection. Only secondary antibodies that are raised against the primary antibodies are labeled (Polak & Van Noorden, 2003). The intensity of the reaction has also been increased as labels per molecule of primary antibody are also greater (Ramos-Vara, 2005). At least two labeled immunoglobulins can attach to individual primary antibody molecule renders the ability of the usage of more diluted primary antibody or to detect minor quantities of antigens (Ramos-Vara, 2005). Different primary antibodies that are raised in the same species can therefore share the same secondary antibody, making this approach more useful (Polak & Van Noorden, 2003).

2.7 Generation of chimeras from embryonic stem cells

ES cells provided a connection between *in vitro* genetic alteration and *in vivo* phenotypic analysis through chimera formation from cultured ES cells (Hong *et al*., 1998). In 1960s, Kristoph Tarkowski and Beatrice Mintz initiated the production of chimeras through combining two eight-cell mouse embryos that developed into one normal-sized mouse consisted of the mixture of cells from two embryos (Mintz, 1964; Tam & Rossant, 2003; Tarkowski, 1961). Introduction of foreign cells into embryos for chimera formation has become possible with the idea from Richard Gardner and Ralph Brinster (Brinster *et al*., 1980; Gardner, 1968). The ES cells derived from ICM of blastocyst and cultured *in vitro* retained the ability to differentiate into all cell types in chimeras as well as germ-line (Robertson, 1986). Homologous recombination using mutated genes in ES cells has shown the possibility to perform directed mutagenesis in mouse (Capecchi, 1989; Doetschman *et al*., 1988; Smithies *et al*., 1985; Smithies & Koller, 1989; Thomas & Capecchi, 1987). Generation of mouse mutants is not the only use of ES cells but they are also important in studying how genetic changes affect cells (Tam & Rossant, 2003).

In transgenic experiment, chimera formation from the injected ES cells is a prerequisite in order for the ES cells to successfully differentiate into germ cells (Alvarez *et al*., 2007). It has been known as the ultimate test to confirm pluripotency of cells. Microinjection technique in fish was developed based on the injection protocols using blastomeres (Ando & Wakamatsu, 1995; Wakamatsu *et al*., 1993). Non-cultivated embryonic cells transplantation for chimera formation has also been done on trout (Nilsson & Cloud, 1992) and zebrafish (Lin *et* al., 1992). Dechorionated mid-blastula embryos were arranged on agarose ramps and injected with approximately 100 suspended cells using micro-transplantator equipped with

borosilicate glass needle (Alvarez *et al*., 2007). Inverse correlation had been claimed to exist and chimera success rate was affected by the duration of cells in cultivation (Alvarez *et al*., 2007). As high as 90 % of chimeric success rate had been obtained from injected cells which had been cultivated for 27 to 66 passages and the cells were still able to contribute in all three embryonic layers (Hong *et al*., 1998). Success rate of 37 % chimera formation injected with 2-day-old MBE cells in zebrafish had been reported by Collodi's group (Bradford *et al*., 1994) and success rate of 15 % with 14-day-old MBE cell cultures (Sun *et al*., 1995a). Furthermore, by using MBE cells up to 6 weeks in culture for injection, it had been reported that the cells were able to form germ-line chimeras in zebrafish (Fan *et al*., 2004a).

2.8 Green fluorescent protein: A biomarker for *in vitro* and *in vivo* imaging

The ability to trace host embryos or chimeras by using lineage markers on injected cells development is important for analysis (Tam & Rossant, 2003). The most ideal marker is one that can be present abundantly, self-governed by the cells or individual cell and yet does not affect the normal activities of cells (Tam & Rossant, 2003). An electrophoretic variant of housekeeping enzymes, glucose-6-phosphate isomerase (GPI) had been used as marker in chimeric experiments (Peterson, 1979). Nevertheless, the use of this variant failed to achieve the detection at spatial resolution observation histologically as in order to detect the GPI, the tissues have to be destroyed (Tam & Rossant, 2003). Strain-specific DNA satellite markers (Rossant *et al*., 1983) or a large globin transgene insert (Lo *et al*., 1987) were used as the genetic markers in chimera analysis and both allowed the observation of cells from different origins. However, a challenging process is involved in histological

preparation of DNA-DNA *in situ* hybridization of embryos. β-galactosidase enzyme had also been used as a marker with simple histochemical staining on whole embryos and segmented tissues (Tam & Rossant, 2003).

A greater marker used for chimera analysis was green fluorescent protein (GFP) of jellyfish (Hadjantonakis *et al*., 2002) which enabled the expression of living cells for chimera studies (Tam & Rossant, 2003). Nevertheless, the basic histological processing may affect the GFP activity in cellular examination purposes and immunostaining on the samples was needed for better detection (Tam & Rossant, 2003). The cloning of GFP gene by Prasher *et al*., 1992 and the work by Chalfie *et al*., 1994 as well as Inouye and Tsuji, 1994 had shown that fluorescence was able to be produced in other organism following the expression of GFP gene. Their work was a major discovery in GFP technology. GFP had started to play a major role in biological research by the successful cloning of GFP (Labas *et al*., 2002; Lukyanov *et al*., 2000) and the derivations of enhanced GFP (EGFP) mutants (Cubitt *et al*., 1995; Labas *et al*., 2002; Lukyanov *et al*., 2000; Ward & Cormier, 1979; Zacharias *et al*., 2000). These proteins that were able to form internal chromophore without the need of additional cofactors, enzymes or substrates except molecular oxygen made them more favorable compared to other fluorescent markers (Campbell *et al*., 2002; Labas *et al*., 2002; Zacharias *et al*., 2000). Therefore, chromophore formation in live cells, tissues and even live organisms has become a possible task (Cubitt *et al*., 1995). These fluorescent proteins are mostly being utilized as quantitative genetically encoded markers which include protein to protein interaction, protein and cell tracking studies (Zacharias *et al*., 2000).

2.9 Future prospects

2.9.1 Regenerative medicine

Zebrafish provides a primary model system for the studies on regeneration of vertebrate tissue (Gemberling *et al*., 2013). The studies are mainly focused on their regeneration of heart, fins and central nervous system structures but they also exhibited the ability to regenerate liver, kidney, pancreas and jaw (Anderson *et al*., 2009; Andersson *et al*., 2012; Brignull *et al*., 2009; Chitnis *et al*., 2012; Diep *et al*., 2011; Hata *et al*., 2007; Li & Wingert, 2013; Moss *et al*., 2009; Pisharath *et al*., 2007; Shin *et al*., 2012; Wang *et al*., 2012). Several advantages over other model organism in regenerative studies in zebrafish include the diversity and the ease of manipulations (Gemberling *et al*., 2013). The establishment of regeneration studies in zebrafish could be strengthened with the proper use of various developing technologies.

One of the challenges in zebrafish model system is the failure in creation of conditional lost-of-function alleles. Directed mutagenesis has been done by using transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats - Cas (CRISPR-Cas) system and zinc finger nucleases (ZFNs) (Doyon *et al*., 2008; Huang *et al*., 2011; Hwang *et al*., 2013; Meng *et al*., 2008; Sander *et al*., 2011). Site-specific homologous recombination induced by using TALENs in zebrafish embryos has been reported lately (Bedell *et al*., 2012). Co-injected short single-stranded DNA oligos sequences could be incorporated by breaking double-stranded DNA even at low occurrences in both somatic and germ-line cells (Bedell *et al*., 2012). The creation of conditional knockout alleles using introduction of two corresponding loxP sites targeting a gene of interest can be

predicted using this technology (Nagy, 2000). Furthermore, individual gene products studies in a tissue-specific manner in regeneration can also be done and provide the promise for pharmacologic, antisense morpholino-based and dominant-negative methodologies for loss-of-function studies (Bradley *et al*., 1992; Gemberling *et al*., 2013; Joyner, 1991; Wobus & Boheler, 2005). However, the varieties of treatment conditions or phenotypic penetrance can render it hard to link the numerous pathways and produce a coherent blueprint for regeneration. Standardization of conditional loss-of-function alleles will be able to eradicate an essential factor of speed and provide simplicity over weaknesses of other techniques (Gemberling *et al*., 2013).

Identification of small molecules with the possibility to boost regeneration capacity using zebrafish embryos in high-throughput screening is a robust technique (Gemberling *et al*., 2013). There was an example of transgenic zebrafish which had been used to discover numerous small molecules that were able to block or enhance cardiomyocytes proliferation in growing embryonic or injured adult heart following the expression of cell cycle indicators exclusively found in cardiomyocytes (Choi *et al*., 2013). Potential small molecules could possibly be applied straight in the mammalian systems to assess their effects on regeneration (Gemberling *et al*., 2013; Guyon *et al*., 2007). For instance, prostaglandin E2 synthesis and prostaglandin E2 is able to increase the number of hematopoietic stem cells (HSC) in zebrafish and similar effects were discovered in adult HSCs of non-human primates and mice (North *et al*., 2007). Besides, identification of cellular sources activated for regeneration in zebrafish lead to the understanding of complex events on organ regeneration (Fleisch *et al*., 2011; Gemberling *et al*., 2013). Hence, stem cell and

regenerative biology studies in zebrafish may someday shed light for development of new therapies in humans.

2.9.2 Expression profiling

There has been incredible advancement lately towards the understanding of molecular basis of pluripotency. Different pluripotent cell lines have been widely analysed using transcriptomes so as to recognize molecular factors and processes that govern self-renewal and differentiation (Grskovic & Ramalho-Santos, 2008). Genome-wide expression profiling utilizing serial analysis of gene expression (SAGE; Richards *et al*., 2004), DNA microarrays (Abeyta *et al*., 2004; Bhattacharya *et al*., 2004; Ivanova *et al*., 2002; Ramalho-Santos *et al*., 2002; Sato *et al*., 2003; Sperger *et al*., 2003), expressed sequence tag (EST) study (Brandenberger *et al*., 2004a) and massively parallel signature sequencing (MPSS; Brandenberger *et al*., 2004b; Wei *et al*., 2005) have uncovered gene networks and signalling pathways considered to be vital for regulation of the pluripotent state.

In order to identify the molecular signature of pluripotent stem cells, expression profiles of ES cells have been evaluated by numerous groups through comparison with differentiated cells (Bhattacharya *et al*., 2004; Brandenberger *et al*., 2004b; Liu *et al*., 2006; Miura *et al*., 2004; Zhou *et al*., 2007), other stages of the developing embryo (Falco *et al*., 2007; Sharov *et al*., 2003), other types of pluripotent and adult stem cells (Abeyta et al., 2004; Ivanova et al., 2002; Ramalho-Santos *et al*., 2002; Sharova *et al*., 2007; Zeng *et al*., 2004), or ES cells of other species (Sato *et al*., 2003; Sun *et al*., 2007; Wei *et al*., 2005). Gene expression profiling of ES cells where expression of an important transcription factor that has

been perturbed can offer the understanding of pluripotency transcription regulatory networks (Ivanova *et al*., 2006; Matoba *et al*., 2006; Walker *et al*., 2007).

Populations of ES cells are used for genome-wide transcriptional profiling as it normally involves a great number of cells. Nonetheless, it has been proposed that ES cells are not a homogeneous population as recent reports have shown that cell-to-cell variances exist in the expression patterns of numerous genes between undifferentiated ES cells (Carter *et al*., 2008; Chambers *et al*., 2007; Toyooka *et al*., 2008). In fact, two populations of cells have been recognized within ES cells that vary in Rex1 expression level and differentiation potentials (Toyooka *et al*., 2008). Likewise, Nanog is not found in a subpopulation of undifferentiated ES cells (Chambers *et al*., 2007). The subpopulation is inclined to differentiate but may remain undifferentiated and re-express Nanog afterwards. These demonstrate that ES cells are heterogeneous and comprised of dissimilar types or states of cells (Grskovic & Ramalho-Santos, 2008). The existence of metastable state with the oscillation among Nanog-Oct4-Sox2 stabilized state has been suggested in ES cells where differentiation-related transcription is sustained below threshold levels, and a Nanog-deficient state, where an increase in specific signals, for example phosphorylated-Erk, may activate differentiation-related transcriptional networks (Silva & Smith, 2008).

There are still more works to be done in order to reveal whether heterogeneity of undifferentiated cells is restricted to the cells tested or if it is an inherent property of all ES cells. Transcriptional regulatory network oscillations may affect ES cells to fluctuate between dissimilar states with different inclinations for self-renewal or differentiation (Grskovic & Ramalho-Santos, 2008). It will be noteworthy to examine whether probabilistic activity is the central of ES cell pluripotency as well as the likelihoods of self-renewal or differentiation to be adjusted (Ramalho-Santos,

2004). The possible role played by post-transcriptional regulation in pluripotent cells could be elucidated through the complementation of transcriptome analysis with proteomic studies (Grskovic & Ramalho-Santos, 2008).

Analysis showed that microRNAs (miRNAs) play important roles in ES cell self-renewal and differentiation as numerous miRNAs were discovered to be mainly expressed in ES cells (Blakaj & Lin, 2008). More studies on miRNAs in the future could help to reveal their functions in regulating the transcriptional regulatory network of pluripotency (Marson *et al*., 2008). Basic understanding on early embryogenesis and germ-line development may be strengthened by the revelation of processes involved in controlling pluripotency. Moreover, it will be useful for the generation of disease or patient specific pluripotent stem cells and to direct differentiation to cell types of therapeutic importance. New cancer markers and therapeutic targets may be discovered with the help of the understanding on regulation of pluripotency. Therefore, the pluripotent transcriptome is going to have substantial influence in a range of fields in the near future (Grskovic & Ramalho-Santos, 2008).

2.9.3 Models for drug discovery

The understanding of absorption, distribution, metabolism and excretion (ADME) properties and identification of potential therapeutic compounds using *in vitro* models for drug testing have shown a major improvement (Bahadduri *et al*., 2010). The pathway on revolutionizing and accelerating of drug discovery as well as development has been influenced by the development of numerous *in vitro* ADME models (Ishizaki, 1996; Roden, 1993). The most popular *in vitro* models utilized by

the biotechnology and pharmaceutical industries are tumor-derived or engineered immortalized cells acquired from human or animal sources (Bradlaw, 1986; Gomez-Lechon *et al*., 2010). However, these cell lines exhibit great inconsistency in their growth, abnormal genotype and physiological response to drugs despite having the advantages of suitability and scalability of the screening procedure. The confidence value and amount of lead molecules for drug development are confined by the irregularities related with these immortalized cells (Kitambi & Chandrasekar, 2011). Restricted expandability of specialized primary culture models such as keratinocytes, hepatocytes and human umbilical endothelial cells has constrained their usage. (Donato *et al*., 2008; Pol *et al*., 2002; Tremblay *et al*., 2005).

The utilization of stem cells in drug discovery has been made for the demand of an improved and uniform physiological response, normal growth pattern and genotype. The ability of stem cells to differentiate into numerous specialized cell types (Nirmalanandhan & Sittampalam, 2009) as well as the likelihood of isolating stem cells from a broad range of tissues (Brittan & Wright, 2002; Bryder *et al*., 2006; Bussolati *et al*., 2005; Dhawan & Rando, 2005; Kim *et al*., 2005; Peault *et al*., 2007; Schaffler & Buchler, 2007; Van Vliet *et al*., 2007) and growing them *in vitro* have offered a precious tool for drug discovery and validation. The utilization of stem cells for drug discovery will improve the likelihoods of identifying lead with a target or pathway relevant to the disease process as well as greater potential for translation to clinical settings (Kitambi & Chandrasekar, 2011).

The capabilities of stem cells are special as they are able to self-renew and differentiate into mature somatic cell types in both *in* vitro and *in vivo*. There are several types of stem cells with different life span as well as the ability to differentiate into various mature cell types (Hook, 2012). The most compelling stem

cells are embryonic or induced pluripotent stem cells (Hanna *et al*., 2010). The cells are able to differentiate into all somatic cell types and also have unlimited self-renewal *in vitro,* whereas the differentiation potential of adult stem cells is limited (Alison & Islam, 2009). The unlimited supply of physiologically relevant cells generated from stem cells rendered them appealing for numerous biopharmaceutical applications like cell replacement therapies, drug discovery, disease modelling and toxicology studies (Hook, 2012). The effects and side effects of drugs on cardiac, neuronal and hepatic toxicity would offer straight evaluation in lead compounds test (Laustriat *et al*., 2010).

Stem cells from normal individuals and diseased patients can be acquired and differentiated into related cell types for toxicity testing (Rubin, 2008). The somatic cells from normal individuals or diseased patients can also be reprogrammed into induced pluripotent stem cells for further differentiation and toxicity testing if the stem cells are not available (Kitambi & Chandrasekar, 2011). Nonetheless, the evaluation of molecular nature in induced pluripotent stem cells is still incomplete; hence their usage in clinical translation will still be limited (Jozefczuk *et al*., 2011). Cell-based *in vitro* assays are not able to show the complicated *in vivo* scenario but they can offer a platform to carry out high content or high throughput screens. The *in vivo* cross-talk between organs or different cell types as well as the general metabolism and side effects are not reflected in cell-based screens. Therefore, different animal models are needed to complement the assays between *in vitro* and *in vivo* (Kitambi & Chandrasekar, 2011).

There are numerous of vertebrate and invertebrate models available for toxicity and efficacy of lead molecules screens. The recent common models for examining many features of genetics and developmental biology from teleost

vertebrate are zebrafish (*Danio rerio*) and medaka (*Oriyzas latipes*) (Kitambi & Chandrasekar, 2011). These models offer several benefits such as husbandary, large sample size, rapid external development and transparency of embryos (Furutani-Seiki & Wittbrodt, 2004; Mitani *et al*., 2006; Scholz & Mayer, 2008). These models are chosen for toxicological or pharmacological screens as they hold the chance of performing toxicity and efficacy screening of pharmaceuticals, chemicals and pesticides which can be linked to human health risks (Kitambi & Chandrasekar, 2011).

Identification and characterization of molecules through small molecule screening which give certain effects against numerous disease processes in humans has been successfully developed (Kitambi *et al*., 2009; Sukardi *et al*., 2010). Several characteristics such as cellular structure, cognitive behaviour and signalling processes of both zebrafish and medaka are very similar to other higher order vertebrates and they show a high degree of anatomical and physiological homology as well (Kitambi *et al*., 2009; Sukardi *et al*., 2010). The availability of numerous molecular, biochemical and genetic techniques together with the collection of several mutant lines with developmental and functional defects of the various metabolic processes has enabled the expansion of a number of *in vivo* drug screens on metabolism, development and physiological conditions of several human diseases (Currie *et al*., 2008; Haffter & Nusslein-Volhard, 1996).