Regenerative medicine: *in vitro* and *in vivo* models and the role of small-molecule compounds

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Summary

Regenerative medicine, as a gospel for masses of incurable diseases, holds great promise for replacing damaged tissue and/or stimulating the organisms' own repair mechanisms to replenish the damaged ones. One of the challenges in developing regenerative medicines is the lack of reliable and cost effective *in vitro* and *in vivo* models for assessing cell self-renewal and tissue regeneration.

Zebrafish, human embryonic carcinoma cells (EC cells) and human embryonic stem cells (hES cells) were developed in this project to explore the possible function and mechanism of a series of novel small molecules. Multiple techniques including *in-situ* hybridization, quantitative real-time PCR, fluorescence-activated cell sorting, RNAi high–throughput screening and kinase profiling, etc. were used to investigate the biological activities of these compounds in relevant cellular or animal models. Based on the data gained so far, it is believed that compound 672 is able to increase stem cell proliferation *in vitro* and zebrafish fin repair *in vivo* in ERK1-dependent manner, acting through interacting with insulin receptor substrate-1 (IRS-1). The other compound 689 was shown to accelerate the growth of stem cells and fin repair through preventing cell death by upregulating superoxide dismutase 2 (SOD2).

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I. General Background:

1. Introduction

Regeneration in animals can be defined as a complex process for restoring a portion of the body lost through traumatic injury. At multiple levels within an animal body, the regeneration can replace the lost cell, tissue, organ or even the entire body (Figure 1.1; Bely A & Nyberg K., 2010). Regeneration at the cell or tissue level has been seen in a wide range of animals, while regeneration at organ or whole organism level is relatively rare and only occurs in a small number of animals. Animals such as cnidarians and flatworms can regenerate an entire body from a small body fragment; Species like *Drosophila* (imaginal disc), *Xenopus* (limb), *Zebrafish* (heart and fin), *Planarian* (body), etc. own an extensive ability to regenerate the missing portion of body lost to trauma (Martin and Parkhurst, 2004; Brockes, J. P. & Kumar, A., 2002; Reddien and S´anchez Alvarado, 2004; Tu and Chi, 2012), but most vertebrates including human beings have a very limited capacity to restore and replace even tissue or organs.

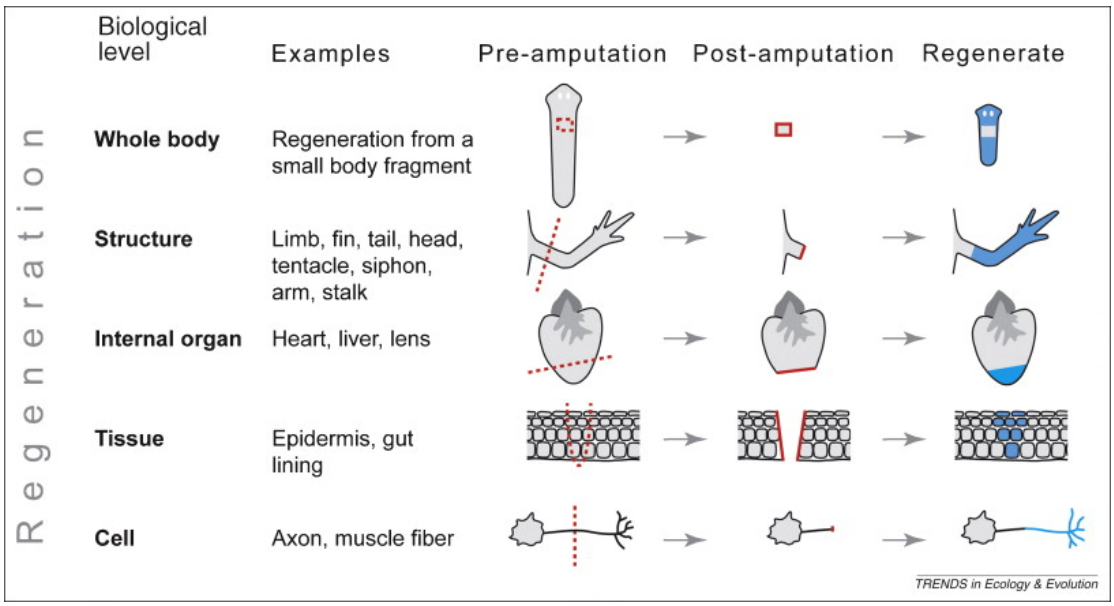


Figure 1.1 Regeneration in animals could replace part of the cells, tissue, organs, structures or even body (Bely A & Nyberg K., 2010).

The whole regeneration process in an animal after wounding could be classified into three steps: wound healing/tissue repair, tissue regeneration and cellular turnover. In wound healing/tissue repair, a scar is formed immediately after the injury, which protects the wound plane from the external environment. A blastema (a niche formed by dedifferentiated cells or stem cells) will proliferate and differentiate into different cells in order to replace the lost cells around the wound plane. Then morphology and functionality are both completely restored during tissue regeneration (Nguyen et al., 2009). Cellular turnover, also known as homeostatic regeneration, involves the changing of cell type and it is only occurred in few specific tissues and organs like mammalian epidermis, intestinal mucosa, blood cells, lung epithelium, bone marrow, thymus, testis, uterine lining (endometrium) and mammary glands (Belacortu et al., 2011). One of the mechanisms associated with tissue regeneration is dedifferentiation. It involves the turning back of a differentiated/mature cell to a less differentiated stage within its own lineage by losing of phenotypic specialization (Heinrich C et al., 2015). This reversing process allows the cell to proliferate again before re-differentiation, which will then replace lost cells.

Stem/progenitor cells based therapies have been introduced to clinics more than three decades ago with the hope to improve the limited regenerative capacity of human beings and devise new methods to cure patients suffering from serve injuries or chronic diseases such as cancers, heart failure, osteoporosis, Alzheimer's, Parkinson's diseases, severe burns, spinal cord injuries and diabetes mellitus, etc (Ferrari G et al., 1998; Jiang Y et al., 2002;

Prockop DJ, 1997; Reubinoff BE et al., 2000; Pagliuca FW et al., 2014) where the malfunctioned organs or tissues cannot be sufficiently restored by patients' own repair capacity in these diseases. However, due to the complex nature of human body, it is still difficult to establishing a method allowing expansion of progenitor cells, permitting cells responding to the environment and being differentiated before and after transplantation in a clinically relevant manner. This dilemma brings more research attention to the naturally occurred regeneration processes in order to understand the signaling network and discover molecules that can control the behavior of progenitor cells.

Studying the naturally regeneration process in different vertebrate animal models can enrich our knowledge of the mechanisms underlying the modulation of the cell fates alteration in tissues and the factors that can regulate de-differentiation, gene activation, cellular proliferation, also the molecules that stimulate the differentiation or stem cells towards a single type of cells. Research into small molecules that can influence the regeneration process will aid the development of chemical-based regenerative medicine.

In this chapter, the current understanding of regenerative processes, experimental models as well as the mechanism of small-molecule modulators impacting on cell fate will be discussed.

2. *In vivo* regeneration

2.1 Regeneration in invertebrate models

Unlike most vertebrates, invertebrate species such as echinoderms, planarians, hydras, and ascidians could replace the missing part of their body from a small fragment (Belacortu et al., 2011). Tissue regeneration recapitulating part embryonic developmental processes have been demonstrated using different animal models (Stocum, 2008) and the molecular mechanisms occurred in these processes are believed to be conserved between different species (Belacortu, 2011), which makes it is easier for exploring the process of natural regeneration in appropriate models.

2.1.1 Planarians

Planarians are flat, soft-bodied worms and are a classic animal model system for studying regeneration. The astonishing regenerative abilities and a large amount of adult stem cells that *Planarians* have make them one of the widely used models for regenerative medicine research. Working on fresh water species of *Planarians* is easier and cheaper comparing with the other animal models in the laboratory. Two lines of them, *Schmidtea mediterranea* (Smed) and *Dugesia japonica* (Dj) have been established as the models for studying basic regenerative mechanism (Sheiman IM et al., 2015). Both of these two species have excellent regenerative abilities (regenerate a full body from a single fragment) and numerous mutant strains (Ishizuka et al., 2007).

Organ systems in *Planarians* are similar to other triploblastic animals: a brain with diverse neurotransmitter systems, an arborized gastrovascular cavity

containing both the digesting and distributing function and a protonephridial excretory system (Rink, 2012). Though *planarians* share similarity with other species on morphology, they are still able to regenerate their entirety body even from a very small fragment (Tan et al. 2012). During *Planarian* regeneration, an outgrowth zone (blastema) near wound plan was shown to be established post trauma, and from which all missing tissues were replaced. Base on this observation that a brand new animal can be regenerated from random small fragments of *Planarians*, all cell types or even organ systems in *Planarians* are believed can be reproduced. It was suggested that in adult planarians, pluripotency could be seen at all tissue levels (Tanaka, 2011).

Recently, Wagner demonstrated transplanting a single Neoblast into an irradiatly damaged worm can rescue the recipient and generated a healthy one with the same phenotype of donor animal (Wagner et al. 2011. Fig.1.2). This experiment clearly showed the pluripotency of Neoblasts, which may even be identified as totipotent. Neoblasts are naturally generated pluripotent adult stem cells, which are different from mostly lineage-restricted adult stem cells in all other animal models (Rink, 2012). So, understanding the mechanistic basis of adult stem cells regeneration has been and believed will continue to be a major focus in *Planarian* research.

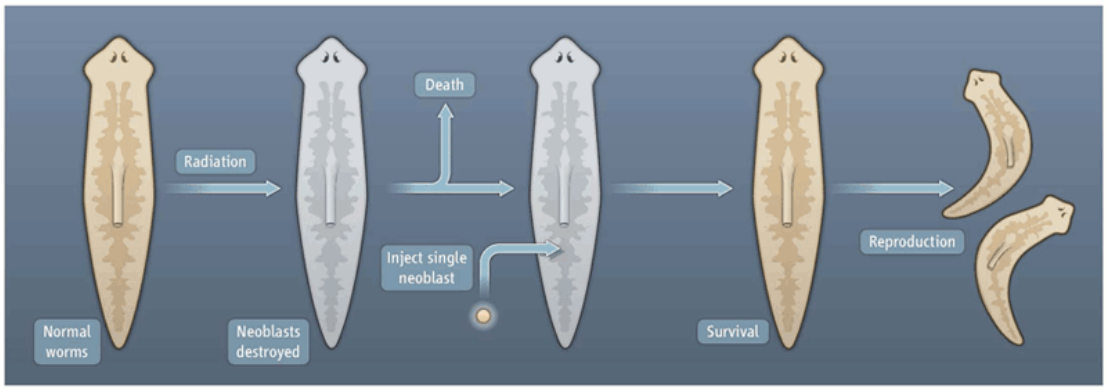


Figure 1.2 Pluripotent cells reside among the Neoblast population and can regenerate an entire worm. (Slack, 2011). As shown in picture, a single neoblast cell is able to replace the damaged tissues.

2.1.2 Drosophila

As a simple and less genetic redundancy model comparing to others, *Drosophila melanogaster* is the most commonly used insect model. Studies of this model have contributed greatly to the understanding of developmental genetic, cellular and molecular aspects. Initially, injured larval imaginal discs were able to regenerate after being implanted into an adult female fly (Hadorn and Buck, 1962), which provided an ideal model for revealing how genetic and cellular mechanism occurs underlying tissue repair as shown in Figure 1.3. The organs of fly like midgut and ovaries are also employed in the uncovering molecular mechanism involved in homeostatic regeneration and controlling the behavior of residential stem cell (Ohlstein and Sprading, 2006). Since most developmental genetic signaling and molecular mechanisms are conserved among animal kingdom, genetic screens in *Drosophila* has significantly contributed to current understanding of genome-wide genetic interactions of natural regeneration processes based on numerous mutant phenotypes modified by overexpression or inactivation certain genes during development (Brand and Perrimon, 1993).

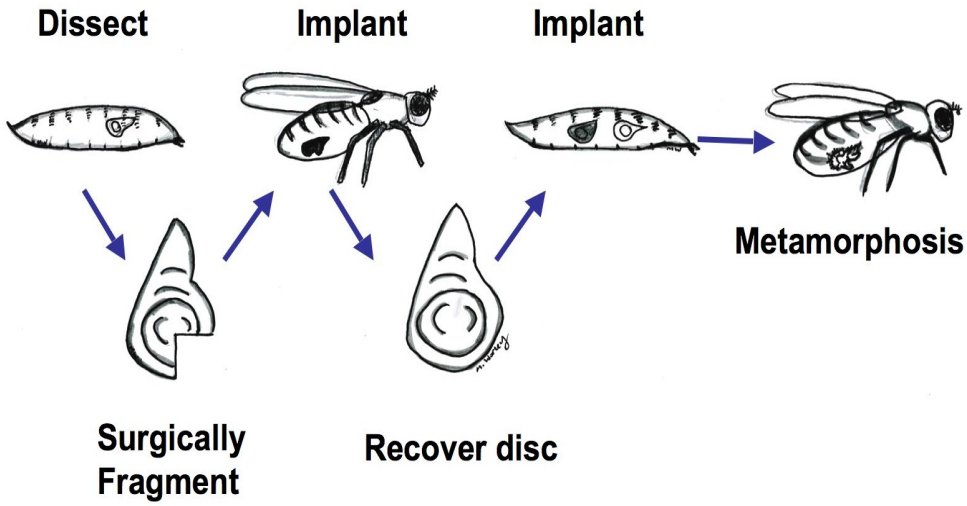


Figure 1.3 The process of imaginal disc regeneration study on Drosophila (https://mcb.berkeley.edu/labs/hariharan/regeneration.htm)

2.2 Vertebrate model

2.2.1 Urodele

Urodele amphibians are unique with their regenerative capacities among all vertebrates because their jaw, lens, limb, spinal cord, tail, and intestine could regrow easily (Brockes and Kumar, 2005). Limb regeneration in salamanders, axolotls and newts serve as the compelling evidence showing the tissue regeneration in vertebrates.

Dedifferentiation is believed to be the main mechanism in limb regeneration in salamanders. During this process, certain types of cells reprogrammed into

undifferentiated cells, which are then recruited from the wounded area to form a blastema in the wounded plane, and the blastema will proliferate and re-differentiate into cells forming the replacing limb. Moreover, there is evidence showing that retinoblastoma protein (RB), a member of pocket protein family, functions as the most important regulator modulating the differentiated cells turning back into cell cycle (Tanaka. et al., 1997). However, there is also data showing that lineage-restricted progenitors within the blastema and only supply the same type of tissues where they originated from. The most widely accepted on the main cell source of blastema formed after appendage amputation is skeletal muscle (Echeverri.et al., 2001; Gargiolo, et al., 2004; Morrison, et al., 2006). Elongated cells called muscle fibers or myofibers, which are developed by emerging of multiple myogenic progenitor cells called myoblasts, form skeletal muscle.

The most compelling evidence supporting that regrown tissues mainly originated from muscles during regeneration comes from *in vivo* cell tracking experiments. For example, using transgenic *Axolotls* expressing a GFP transgene as the model for tracing limb epidermis, muscle has been demonstrated (Tanaka E & Reddien P, 2011. Fig.1.4).

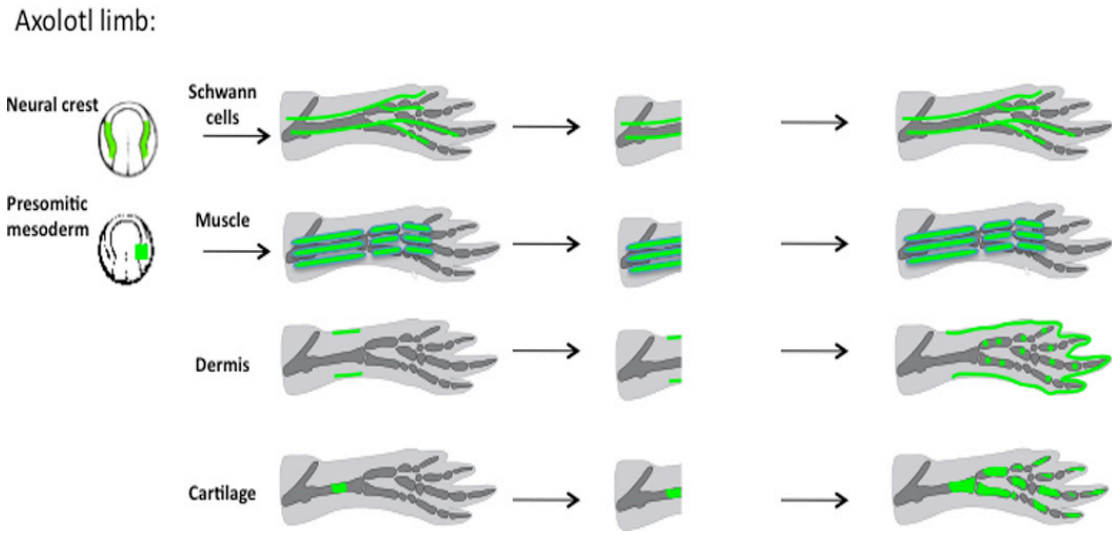


*Figure 1. 4 Cell tracking of GFP-labeled cells in amphibians shows the progenitors in Axolotl limb blastema are lineage-restricted. (*Tanaka E & Reddien P, 2011*)*

GFP labeling was used to detect the cell fate regulation in *Axolotls* tissues by labeling random cell types initially. During the regeneration experiment, limbs of *Axolotls* were amputated then different GFP-labeled cell types were determined following the whole process of regeneration. Results showed that labeled muscle cells did contribute to the regrown muscle but not the other tissues like cartilage or epidermis. Taken which, it is undeniable that limb regeneration in *Axolotls* is lineage restricted. However, because when the muscle was labeled by GFP before experiment, the grafts of mesodermal cells, blood vessels were not excluded. If mesodermal cells were also positive with GFP, it was not safe to eliminate the possibility that muscle progenitor cells potentially contribute to endothelial cells.

While the limb experiments resolved the *Axolotl* limb blastema cells are lineage restricted. Amputation of the tails with different color labeled cells also showed that each tissue layer regenerated independently and do not interact

with each other (kragl M et al., 2009; Le Grand and Rudnicki, 2007). Nonetheless, it is certain that natural regeneration in amphibian is lineage-specific, but there is still be possibility that the cellular mechanisms of recruiting blastema cells from the same type of tissues involves activation of residential progenitor cells, or the blastema cells are dedifferentiation from somatic cells.

2.2.2 Anurans

Xenopus tadpole tail regeneration is the first demonstration that tissues like muscle or nerve can be regenerated from progenitor cells of different phenotype in a vertebrate. Results from these species have been a milestone in the study of developmental biology. As a successfully established research model, *Anurans* have opened a window of how animals develop from a single fertilized egg into an entire body formed by complex tissues and organs, which are structured by a large amount of fate-organized cells.

Anuran amphibians can regrow limbs and tails such as tadpoles. However, what is interesting is this regenerative ability fails for a period during development and metamorphosis (Fig 1.5.1; Love et al., 2013), which also provides us an exploitable angle for analyzing the losing capacity of regeneration. It has been noted that tadpoles only can regrow complex structure like appendage or organs during the morphological change, such as limb development and tail regression during metamorphosis (Slack et al, 2004). It is possible that regeneration in anuran amphibians may be driven by resident progenitor cells. Once differentiation has elicited the capacity to

regenerate is limited. Furthermore, the widely studied examples of this animal model are the regeneration of lens and retina. In both adult and larvae of *Xenopus*, lens and retina were demonstrated to be able being fully regenerated from dorsal iris pigmented epithelium and retinal-pigmented epithelium respectively (Filoni, 2009). It was shown that in larvae, the ability to regrow a new lens was eventually diminished alongside with development and disappearing on metamorphosis. The early stage of eye development in vertebrates can be characterized into forebrain bilateral evagination (turn inside out) and formation of the optic vesicles. The optic vesicles were facing the surface ectoderm, main components of the lens placode (Henry, 1990; Grainger, 1992). During the process of formation of the lens vesicle by lens placode, the optic vesicle will cover the developing lens and give rise to the optic cup. The optic cup has two layers and the inner one then eventually generated the neural retina. This structure makes the blood vessels can reach the eye and gather the retinal axons into the optic nerve.

Therefore, the anuran amphibian serves as an ideal model to investigate the transition of regenerative capacity between the loss and gain in animals.

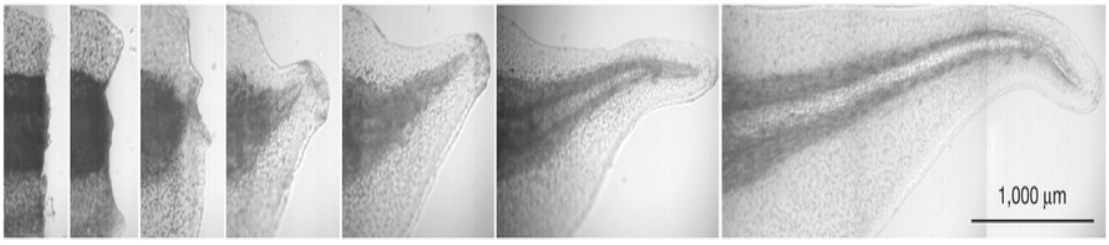


Figure 1.5.1 Images of a representative regenerating tadpole tail from 6 hours post amputation (hpa), 1dpa, 2dpa, 3dpa, 4dpa to 6dpa (Love, 2013).

2.2.3 Fish

Zebrafish, as the other widely used vertebrate model for studying the principle of regeneration, has become a very popular vertebrate animal system for studying developmental processes due to its external fertilization, early optical clarity and genetic tractability during development (Tu and Chi, 2012; Shi WC et al., 2015; Rennekamp AJ et al., 2105). Moreover, new technical breakthroughs in imaging techniques and the establishing of zebrafish transgenic lines make the *in vivo* analysis of the cellular mechanism possible. Hence, all these features could help to gain a deeper insight of how specific genes may influence the development or regeneration. In this section, how the zebrafish has been utilized to figure out not only the mechanisms of heart and fin regeneration, but also the potential signaling pathways that may be controlled in order to modulate the regeneration processes will be discussed.

2.2.3.1 Heart regeneration in zebrafish

The heart is known to be the first organ developed in all vertebrates (Tu and Chi, 2012). In zebrafish, at five hours post fertilization (hpf), atrial and ventricular cardiac progenitor populations could be seen in the lateral marginal zone on either side of the embryo and are restricted by retinoic acid signaling. That is, the ventricular pool appears to reside more dorsally and closer to the margin than the atrial pool.

Following amputation, nearly 20% of the ventricle of the heart in a zebrafish could be fully regenerated (Raya et al., 2003). It is believed that differentiated cardiomyocytes may dedifferentiate and proliferate to regenerate the missing

tissue (Jopling et al., 2011). While data shown by Poss group also suggested that the new myocardium is originated from a population of resident progenitor cells. For verifications, double transgenic animals with both red fluorescent protein (RFP) and enhanced green fluorescent protein (EGFP) tagged by cardiomyocytes-specific promoters were analyzed at multiple times. Since EGFP fluorescence will show up earlier but much shorter than RFP (Verkhusha et al., 2003), which will be able to show if dedifferentiation occurs during regeneration. Cells positive with RFP but not EGFP never detected throughout the whole regeneration process, which suggests that regenerated cells of the myocardium were from the undifferentiated cells, but not from dedifferentiation (Lepilina et al., 2006). It is still needed to be considered that since the mechanism involved in protein degradation is still unclear and the protein products involved in the regenerative process may rapidly degrade in a dedifferentiating cell. Thus, it is too early to conclude that dedifferentiation do not contribute to the formation of progenitor cells.

Additional investigation of cell proliferation using more markers is required to critically evaluate the origin of dedifferentiated or residential progenitor cells. However, there is still no direct evidence supporting this hypothesis, and little is known whether any specific signaling pathways mainly involved with this process, which leaves a large amount of following work to address these questions.

2.2.3.2 Fin regeneration of zebrafish

The fish caudal fin is another widely employed experimental model for studying appendage regeneration in vertebrate animals. The structure of the caudal fin in adult fish consists of fibroblasts coupled with segmented bony fin rays (Akimenko et al., 2003). Following amputation, blastema will be formed near the tip of fin rays and cells in the region will proliferate to replace the missing ones. Similarly, epithelial cells around the stump will contract and seal the wound in zebrafish larvae. From 3-48 hours post amputation, proliferating cells within the region adjacent to the stump will provide cells that needed for fulfilling the amputated part of fin fold (Fig. 1.5.2).

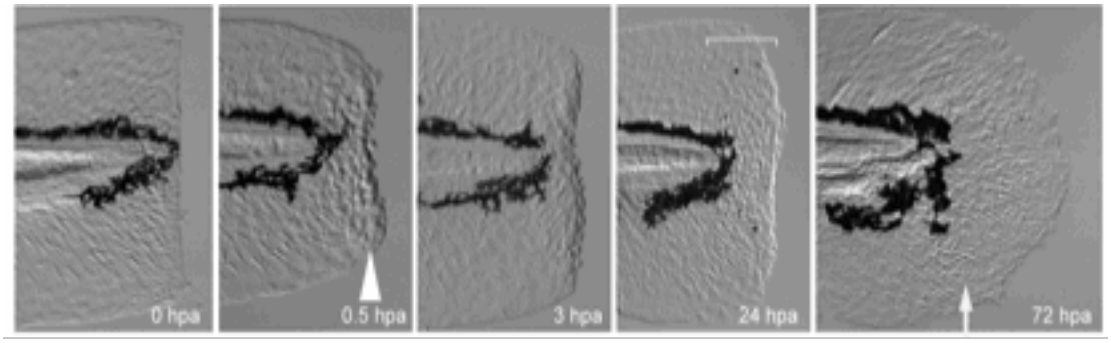


Figure 1.5.2 Fin fold regeneration in zebrafish larvae from 0 hpa to 72 hpa (from left to right): the epithelial cells around the stump start to contract after about 0.5 hour post amputation to seal the wound (arrowhead). Obvious larger portion regrown can be seen on 24 hpa. Following, the lost part was fully regenerated (arrow) on 72hpa (Yoshinari and Kawakami, 2011).

It is still unclear about the origin of cells that form the blastema, i.e. whether blastema cells consist of only pluripotent cells that had the potential to form all cell types of the fin or multiple progenitor cells at the point of injury. A sporadic Tol2 transgene insertion method was applied to track the fate changing of cells during the regeneration of caudal fin in zebrafish (Tu and Johnson, 2011;

Tornini V & Poss KD, 2014). In this study, the fin was shown to be regenerated by the cells from nine different cell lineages: vessel/artery, osteoblast, fibroblast, glial, xanthophore/melanophore, epidermis, iridiphore and lateral line. Following fin amputation, it was shown that the labeled cells regenerated only the same cell type and did not contribute to other cell types. Similar results were also reported by other groups from studying the development of other vertebrate appendages (Arques et al., 2007; Pearse et al., 2007). However, fin dermal bone in zebrafish may be originated from neural crest (Tanaka and Reddien 2011). This suggests that progenitor cells may potentially contribute to ossification directly hence results in no cartilage being detected during this process. Nevertheless, clonal cells analysis does not show whether the all types of cells in fin were labeled or not, so it is still safe to conclude that the process of regeneration is fully restricted.

3. *In vitro* cellular regeneration models

Cell-based regeneration commonly involves the use of stem cell due to its inherent capacity of proliferation and differentiation. Therefore, cell models derived from stem cells have been the base of many studies. An important aspect of stem cell based regenerative medicine is to elucidate the molecular mechanisms allowing progenitor stem cells to differentiate in desired manner or to maintain their pluripotency so that the lost cells or damaged tissues can be replenished.

Stem cells are undifferentiated cells that can self-renewal indefinitely and can be differentiated into specialized cells where and when it is needed. Stem cells from different sources have different differentiation capacities and can be divided into following categories according to their abilities in differentiation:

- Totipotent stem cells – such as fertilized eggs, which can give rise to all types of embryonic cells and placenta cells.
- Pluripotent stem cells – such as embryonic stem cells, which are able to give rise to nearly all cell types but not placenta cells.
- Multipotent stem cells – e.g. mesenchymal stem cells that can differentiate into a number of cell types, but usually restricted to a single lineage.
- Oligopotent stem cells - can give rise to only a few cell types, such as lymphoid or myeloid stem cells.
- Unipotent cells – such as certain progenitor cells, which can only produce only one cell type but can self-renewal in contrast to fully differentiated cells.

In mammals, there are two main types of stem cells commonly used in cell-based therapy: embryonic stem cells, which are isolated from the inner cell mass of blastocysts, and adult stem cells, which are found in various tissues. The formal is pluripotent while the later one can be either multipotent or oligopotent.

Embryonic stem cells are pluripotent, are able to both self-renewal (reproduce at least one daughter cell with stem cell character leading to a perpetuating population) and generate progenitor/precursor cells, which are able to differentiate into functional cells of any of the three germ layers (ectoderm, mesoderm or endoderm), which can then process independent differentiation to produce all cells in body (Yu and Thomson, 2008). This highly efficient symmetric-dividing character of pluripotent cells provides clue for cell-based therapies: a small amount pluripotent stem cells contribute to differentiated cells needed for residential repair, which has yet been achieved since it used to lack suitable method maintaining pluripotency of stem cells and modulating differentiation in controllable manner. Unlike in animal models, cell models maintained *in vitro* provide a cellular platform, which is easier and cheaper for investigating the behavior of stem cells under a less complicated circumstance by minimizing the interference brought by xeno-factors.

3.1 Embryonic stem (ES) cells

In the mid-1990s, ES cell were firstly isolated from the rhesus monkey and the common marmoset (Thomson et al. 1995, 1996). The experience of

maintaining these initial ES cell lines in culture conditions greatly improved the understanding on growing human embryo *in vitro* (Gardner et al. 1998), which leads to the first derivation of human ES cells from inner cell mass of the blastocyst in 1998 (Thomson et al. 1998).

Human ES cells are usually karyotypically normal. And the high telomerase activity and differentiation potential of hES cells permit the advanced derivatives of hES cells into all three germ layers even after relative long-term culturing in lab. To demonstrate these characters of human ES cells, Amit et al (2000) showed dissociated human ES cell colonies from different origin are capable to differentiate into all three lineages arose from cells borne, which convincingly proved the pluripotency of human ES cells.

Moreover, subsequent studies also revealed that various cell types can be differentiated from ES cells using novel methods such as high-density culture dosed with different growth factors and 3D culture methods (Baker BM & Chen, 2012). However, these observations are still not enough to solve the pluripotency issues related to spontaneous differentiations that is commonly observed during the routinely passage. Investigation into how to maintain undifferentiated human ES cells *in vitro* in order to produce large quantity of stem cells for cell based therapy in vivo is one of the crucial aspects of stem cell research that is important to regenerative medicine.

3.1.1 Characterization of human ES cells

Fluorescence-activated cell sorting is usually utilized for assessing the purity of ES cells based on distinct cell surface markers. It allows the cells being sorted into pluripotent cells and differentiated cells. Therefore pluripotent cells can be separated from differentiated cells and used for transplantation (Pera et al. 2003). The stage-specific embryonic antigens 1, 3, and 4 (SSEA-1, SSEA-3, and SSEA-4), antigens interacted with keratin sulfate/chondroitin sulfate proteoglycan like TRA (Tumor rejection antigen)-1-60, TRA-1-81, GCTM (embryonal carcinoma antigen)-2, TG-30, TG-343 and the tetraspanin molecule CD9 are most widely used stem cell surface markers (Andrews, 2002). SSEA-1, SSEA-3 and SSEA-4 are globoseries glycolipids and expressed by all human ES cells. However, SSEA-1 is not detected on the undifferentiated human ES cells but on differentiated ones (Pera et al. 2000). Specific monoclonal antibodies, which can recognize cell-surface carbohydrate epitopes, are able to detect SSEA-3 and SSEA-4 on undifferentiated human ES cells. Undifferentiated human ES cells are also shown to be positive for TRA-1-60, TRA-1-81, GCTM-2, TG-30, TG-343 and CD9 staining, all of which cannot be detected or present in a much decreased quantity on differentiated cells. Unfortunately, none of these are unique markers for the undifferentiated human ES cells because these makers have also been seen on somatic cells in later stage during development. Currently, many genetic studies are focusing on embryonic development, it is therefore hoped that more robust gene markers will be discovered and used as specific markers for identifying truly pluripotent stem cells.

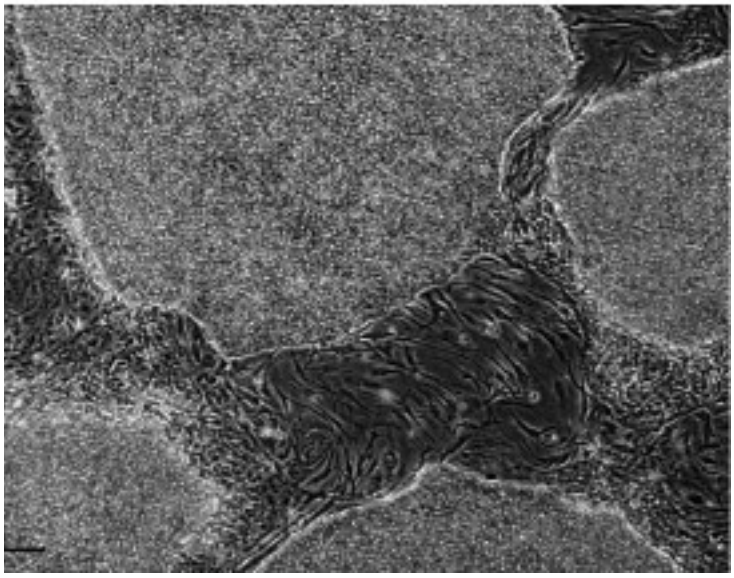


Figure 1.6 Classical morphology of human embryonic stem cell colonies (Amp KJ et al., 2010).

1.1.2 Maintaining of Human ES Cells

Human ES cells have unique phenotypes (small in size overall, high nucleus to cytoplasm ratio and prominent nucleoli). Colonies are usually tightly packed have a smooth surface under culture conditions (Fig 1.6). ES cells display more characteristic phenotype in serum-free condition than in medium with fetal bovine serum (FBS) (Laslett et al., 2003), which makes serum-free medium a more favorable choice to maintain the pluripotency of stem cells while FBS containing medium is preferred when more differentiation is required. The most commonly employed method, also the method firstly established, is to use murine embryonic fibroblasts (MEFs) in Dulbecco's modified Eagle's medium (DMEM) containing glutamine, β-mercaptoethanol, amino acids and 20% FBS (Thomson et al., 1998). However, this classic method is still under dispute since unexpected infection was reported to be caused by the xenogeneic components, which contains non-human pathogens. In 2000, Amit et al reported that hES cells can be successfully

passaged for many generations using MEFs in serum-free medium when FBS was replaced by knockout serum replacement. However, the medium still contained bovine serum albumin.

Richards et al demonstrated the use of animal-free condition for culturing hES cells on human feeder cells with human serum. The group reported that stem cells can maintain stem cell characters even after 70 subsequent passages on human foreskin fibroblasts or mitotically inactivated human adult marrow cells (Cheng et al., 2003). Though it is an ideal solution for culturing cells necessary for clinical therapy since all components are originate from human, the whole process is very time consuming and expensive in cost which makes this method unsuitable for drug screening work.

A xeno-free culturing system was initially suggested by Xu's group in 2001. However, after improvement over the past decade, this method has shown some unbeatable advantages when compared with above mentioned conditions (Prestwich GD et al., 2015). Firstly, culturing flask or plates were pre-coated with Materigel, a soluble matrix membrane similar to the extractor used originated from Engelbreth-Holm-Swarm mouse tumor tissues and supplemented with laminin, collagen IV, entactin, heparin sulfate proteoglycan and a serial of growth factors (FGF, epidermal growth factor, nerve growth factor, platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and TGF- β 1). Secondly, the serum-free medium consisting of only necessary components is used. Different ES cells lines were all shown to be able to maintain pluripotent characters using this system.

Maintaining the pluripotency of human ES cells after repeat passaging is always an issue because hES cells have a strong tendency to differentiate upon dissociating induction. This has imposed significant challenges for standardizing culture protocols for hES cells. Both cell-cell adhesion and cell-ECM (extracellular matrix) attachment are complex processes and important for maintaining colony integrity. Cell adhesion is mediated by a group of cell surface adhesion molecules (CAMs), which include the integrin, selectin, immunoglobulin, and cadherin super-families etc (Munro et al., 1996). However, what are the essential components in the cell matrix that allowing the binding with cell membrane is still not clear. Studies aiming to improve the knowledge of the structure and components of ECM are supposed to contribute greatly to the maintaining and modulation of hES cells.

Another most important factor for the low efficiency of ES cells passage is the physical damage caused by cell dissociation, because single human ES cell were shown to be able to survive when being seeded in high density (Amp KJ et al., 2010). Multiple drugs and compounds that can increase the efficiency of human ES cell passage have been reported, such as a specific Rock kinases inhibitor Y-27632 (Watanabe et al. 2007), an ATP-competitive inhibitor of p160Rock (Ki=140nM) and Rock-II in ROCK (Rho-associated protein kinase) signaling. Y27632 has been applied to hES cells for overcoming the sensitivity of these cells to dissociation during passaging. Short-time treatment (one hour) with high concentration (10μM) Y-27632 blocks apoptosis of dissociated hES cells.

3.2 Embryonic carcinoma (EC) cells

The initial hint igniting an explosion of research interest to investigate the mechanism involved in differentiation of human embryonic cells, as well as in drug screen and toxicity testing using pluripotent stem cells was from studying teratocarcinomas in the 1950s. Teratocarcinomas, a type of malignant germ tumor cells containing an undifferentiated embryonic carcinoma (EC) (Yu and Thomson, 2008). The first experiment demonstrated that EC cells are pluripotent stem cells was showing that EC cells were capable to self-renewal and differentiation into all germ layer, indicating that this cell type can serve as an substantial model for human ES cells research (Kleinsmith and Pierce, 1964). The first EC cell line was derived from teratocinomas of mice (Finch and Ephrussii, 1967). To prove the stem-cell character, over 1700 single EC cells were injected into syngeneic adult mice, formation of teratomas in injecting zone indicating that EC cells are able to differentiate into the same types of cell and tissue from where they are originated. Moreover, several subsequent studies also showed EC cells would participate in development when injected into blastocysts (Andrews 2002).

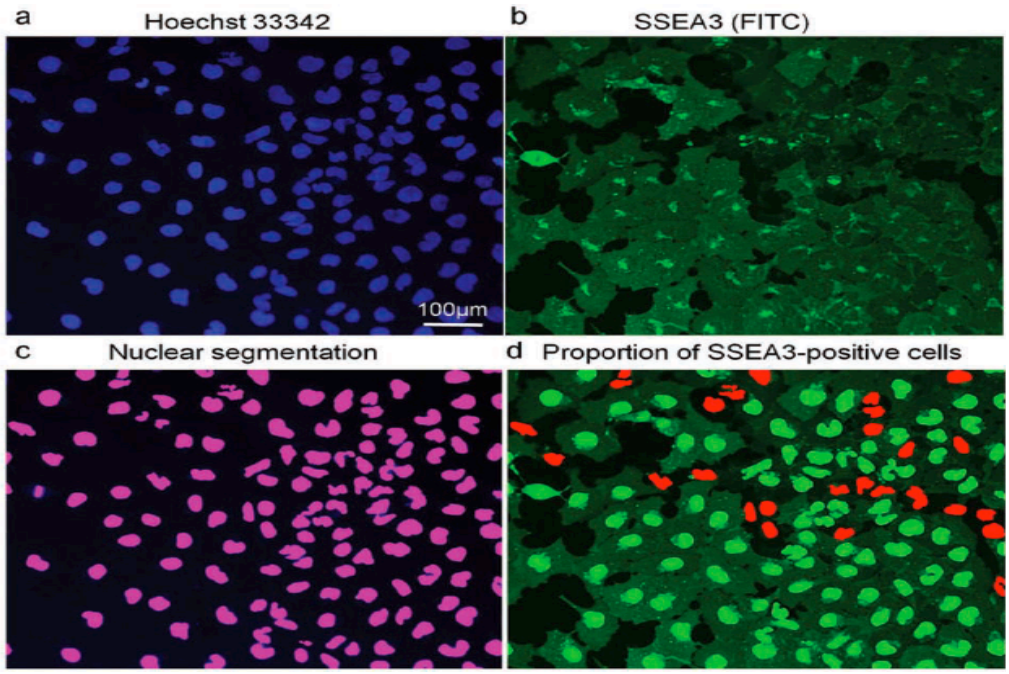


Figure 1.7 NTERA2 cells stained with (a) the Hoechst 33342 nuclear dye and (b) FITC-labeled SSEA3, a marker of undifferentiated cells. (c) Nuclei are identified by segmenting Hoechst 33342-labeled objects in the images (purple). (d) Presence of an FITC signal on an individual cell is assessed on the basis of FITC intensity above the nuclear region. Nuclei of SSEA3-positive cells are green and SSEA3-negative nuclei is red (Barbaric, 2011).

However, due to the chromosomal abnormality, the potential of EC cells utilization in clinics is limited because the cell types could be differentiated from EC cells are less than hES cells. But human EC cells are easier and cheaper to maintain in laboratories when comparing to human ES cells. Unlike hES cells requiring complex and expensive media formulations and feeder cells or extracellular matrices for growth (Barbaric, 2011), EC cells could be easily cultured in decent quantities in lab for experiments, which were impossible on intact mammalian embryos. And surface antigens as SSEA-3 (glycolipid antigens stage-specific antigen 3) and SSEA-4, proteoglycan antigens like TRA-1-60, and TRA-1-81 can be detected on these cells (Andrews et al. 1982, 1984; Barbaric et al, 2011; Kannagi et al. 1983).

All these features make EC cells an appropriate model for large-scale drug screening. And one of the best-characterized pluripotent human EC cell lines is ntera2 (Andrews, 1984). Similar with hES cells, almost all pluripotency genes (e.g. OCT4 and NANOG), surface antigens (e.g. SSEA-3; Fig. 1.7) and proteoglycan antigens (e.g. TRA-1-60) were detected on Ntera2 cells. (Huang G et al., 2015; Draper, 2002; Adewumi, 2007; Andrews, 1984).

3.3 Induced pluripotent stem cells (iPSCs).

Since Shinya Yamanaka reported the successful reprogramming somatic cells into induced pluripotent stem cells (Takahashi, K & Yamanaka, S, 2006), this technology has been widely applied into regenerative medicine studies. Because embryonic stem cells can only be derived from embryos, which means the destruction of a pre-implantation stage embryo, while iPSCs can be converted from any type of mature cells theoretically. The other important reason is iPSCs can be generated using the tissue from patients (Takahashi et al., 2007), which means patients could have the chance to be provided with the autologous cells for therapeutic transplantation without the risk of immune rejection. With the development of the understanding of this unique cell type, iPSCs have been revealed to be with all characters of pluripotent cells (both morphologically and immunologically; Maherali et al., 2008), and several certain cell types have been reported to be dervided from iPSCs, such as neurons, pancreatic, liver and heart (Pang et al., 2011; Pagliuca et al., 2014; Aoi et al, 2008; Cao N et al., 2013).

It is undeniable that there is limitation of the utilization of iPSCs in clinics because reprogramming of adult cells has the chance to cause the expression of oncogenes in the manner of genetically modification. Several reports have suggested different methods to overcome this issue: removing the oncogenes could be triggered during the induction of pluripotency (Ji et al., 2012); induction of pluripotency by treating cells with proteins channeled into the cells via poly-arginine anchors (Zhou et al, 2009) and using combination of small-molecule compounds (Hou, P et al., 2013).

4. General molecular functions in regenerative medicine

4.1 FGF signaling pathway

FGF pathway, as part of FGF superfamily, plays a major role in a number of extracellular and intracellular signal transduction processes (Fig 1.9). A series of downstream signaling can be stimulated in response of the FGF-activation. Phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and phospho-lipase Ck (PLCk) pathway are believed to be the key members of FGF pathway downstream networks (Smith et al., 1997). The activation of FGF pathway starts with the auto-phosphorylation of FGFRs (Tal, Franzosa et al. 2010), which then trans-phosphorylate its intracellular kinase domain. In general, after FGFR activating, FGFR homodimer sequester and phosphorylates FGFR substrate 2, which then promotes the landing of its adaptor protein and Son of seven- less (Sos, guanine nucleotide exchange factor) to the plasma membrane. Following the relocation, Sos is able to active Raf kinase on the cell membrane, which leads to a series phosphorylation of kinases like RAS, MEK and ERK. The MEK/ERK kinase cascade targets multiple genes including cyclin (cell cycle check point) families and sequentially regulates cell cycle progression.

Four types FGFR have been identified, and they share a general structure with a high homology similarity but are not identical. Full structure of FGFR is demonstrated being formed by two parts (Green et al., 1996), extracellular part containing three immunoglobulin-like domains (divided by a series acidic amino acids) and a transmembrane part conjugated with the cytoplasmin

domain with a tyrosine kinase and a short C-terminal. Functions of four FDFRs are also believed not to be the same. FGFR 1 is linked with phosphorylation of MAPK kinases while FGFR 4 is shown not to be involved in the process of kinase activation.

Due to the multiple downstream networks of FGF pathway in cell fate regulation and growth, it has been observed that numerous human diseases are involved with genetic lesions of this pathway (Wang et al., 1994). FGF 3 is shown being important in the development of the outer ear and teeth, congenital deafness and morphological change of outer ear were observed in FGF3-mutant animals. Moreover, Kallman syndrome (abnormal, reduced or lost sense of smell) is believed to be caused by losing normal function of FGFR1. Losing of FGF 8 is observed in animals with salivary and lacrimal gland defects.

FGF signaling is also shown to be important in the formation of mesoderm and posterior structures of the vertebrate embryo. During Xenopus embryo development, FGF is shown to be important in downstream of early mesoderm initial-activated signals, to contribute to maintain the expression of the Xenopus brachyury gene (Xbra) in mesoderm precursor cells (Smith et al., 1997 & Umbhauer et al., 2000). And activation of FGF4 is shown to participate in the auto-regulatory loop maintaining the expression of Xbra.

Furthermore, FGF signaling pathway is also revealed as one of the prominent signaling pathway in the process of initiation of epidermal/mesenchymal interactions in blastema formation in natural regeneration in zebrafish (Yokoyama et al., 2001; Yokoyama et al., 2000). FGF signaling is detected highly expressed in the first 6 hours in the blastema area around wound plane. FGFR1 can also be observed expressing during 18 to 24 hours post amputation in fibroblast-like mesenchymal cells, which are shown proximal and distal to the amputation region. SU5402/ Ri, a commonly used inhibitor of FGFR1, was shown with negative effects on FGF pathway, which results in a significant deficient in forming blastema and reduction of blastema gene expression like msx (muscle segment homeobox) home box domain genes, msxb and msxc (Poss et al., 2000. Fig 1.8.1), which are believed to be the markers of undifferentiated, proliferating cells in regenerating tissues (Akimenko et al., 1995).

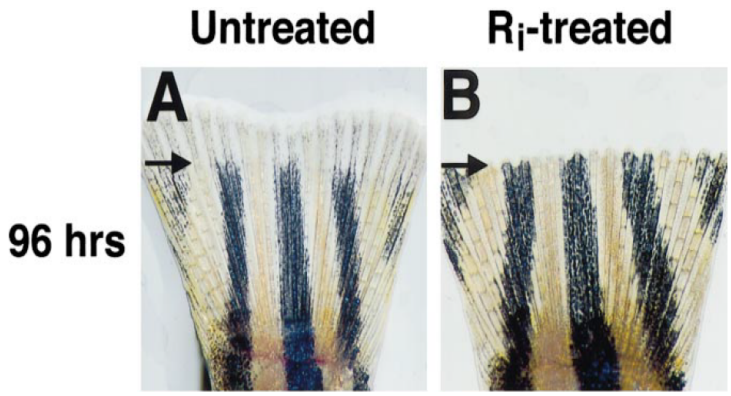


Figure 1.8.1Fgfr1 inhibition blocks regeneration: Fin from untreated (A) and treated (B) fish with R_i at 96 hpa, showing normal regrowth and new segmentation. Arrows demarcate amputation plane in each (Poss et al., 2000).

In summary, these data cement the role of FGF signaling in wound healing, either directly or indirectly.

4.2 Retinoic acid signaling

Retinoic acid (RA) is an active derivative origin from the liposoluble vitamin A (retinol), which is the vital molecule sensitising light and altering to retinaldehyde leading to the phototransduction in retina cells (Parker and Crouch, 2010). The action of vitamin A was elucidated years ago and its acidic metabolite, RA, acts as a biding site regulating the transcription of the retinoic acid receptor (RAR). RAR serves as a nuclear receptor stimulating the transcriptional activators (Rhinn, 2012).

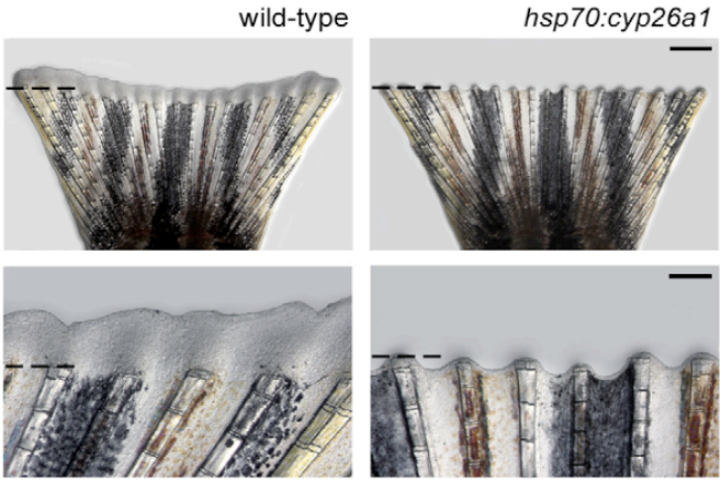


Figure 1.8.2 Inhibition of RA signaling in hsp70: cyp26a1 fish results in an early and complete block to fin regeneration (Mathew et al., 2009).

RAR was detected to be highly expressed in the blastema (White et al., 1994). Another gene in this pathway aldh1a2 (raldh2), which encodes a major enzyme catalyzing RA synthesis in embryos was also found to be highly expressed during regeneration (Mathew et al., 2009). During epimorphic regeneration on zebrafish fin, RA was shown to be associated with a specific cell type leading to the differentiation of blastema cells (Stoick-Cooper et al.,

2007). Retinoic acid receptor gamma (RAR gamma) was observed in the blastema cells and exposure to exogenous RA during fin regeneration resulted in morphological changes of the caudal fin (Geraudie et al., 1995). Furthermore, gene knockout studies confirmed the crucial functions of RARs in mouse development (Mark et al., 2009). Inhibiting RA signalling by knock-down RA gene shows that blastema formation requires normal RA signalling for cell proliferation (Fig 1.8.2, Blum, 2011) but whether RA is involved in corroborating with any major signalling is still not clear.

4.3 Sonic hedgehog and Wnt signaling pathway

Studies on fin regeneration in adult zebrafish revealed the stable expression of Sonic hedgehog (shh), the membrane-bound receptor patched1 (ptc1) of shh and bone morphogenic protein 2b (bmp2b), bmp4 and bmp6 in re-growing region (Laforest et al., 1998; Smith et al., 2006). Both shh and bmp signaling pathways have been demonstrated to be involved in the regeneration of dermal bone and the abnormal level of shh or bmp2 induced artificially in the blastema resulted in excess bone deposition and abnormal patterning in the regenerating fin (Quint et al., 2002).

Sonic Hedgehog (Shh) was named after Sega's famous video game hero, and Indian (Ihh) and Desert (Dhh) were named after existing hedgehog species. The Hedgehog signaling (Hh) pathway (shh in mammalian) is a developmental signaling pathway that regulates embryonic patterning and adult tissue maintenance by directing cell differentiation and proliferation (Ingham and McMahon, 2001). Shh was first identified in mutation lines of

Drosophila melanogaster in 1980 (Nüsslein-Volhard and Wieschaus, 1980). These mutant flies were observed with abnormal embryonic development, which suggests shh directly affected embryonic development. A study focused on cancer stem cells also suggests Hh pathway plays a role in regulating the proliferation of cells, as well as initiating metastasis (Clevers, 2011; Singh BN et al., 2015).

Hh pathway that controls cell-fate in concentration-dependent manner, and the stimulation of this pathway is conserved among animals (Yun et al., 2012). In response to the regulation from two transmembrane proteins Hh acyltransferase (Hhat) and Dispatched (Disp), Hh will activate the membrane receptor patched (Ptch1), which are shown linking to the regulation of activation of Hh pathway (Fig 1.9). When Hh protein binds to the receptor, the inhibition of membrane receptor on the G protein coupled receptor-like protein Smoothened (Smo) is stopped. Following the translocation of Smo, the degradation of Fused Glioma-associated oncogene homologue (Gli) complex (Gli1, Gli2 and Gli3) into from the nucleocytoplasmic factor Suppressor of Fused (Sufu) is reduced by disrupting its functional docking sites. Then with help of the stable complex of Gli2 and Gli3, the Gli proteins are able to enter the nucleus leading to the stimulation of targets of the downstream pathways.

Furthermore, injection of exogenous shh and the bmp inhibitor chordin remarkably reduced abnormal bone fusions caused by ectopic shh expression (Smith et al., 2006). Data from the same group demonstrated that if only injecting chordin did not inhibit activation of shh and Ptch1.

It has also been observed that the Hh signaling pathway undergoes cross-talk with several other key molecular signaling pathways, such as Wnt, p53, retinoic acid and growth factors, both during embryonic development and carcinogenesis in context dependent manners (Yun et al., 2012). It is also widely accepted that Hh signaling may work with the association of BMP signaling.

Similar to Hh pathway, Wnt signaling was revealed to be involved in modulating the self-renewal and differentiation of progenitor cells, also tumorigenesis (colorectal cancer cells and melanomas). Disruption of blastema formation and wound epidermis is observed when inhibiting or stimulating of Wnt signalling by overexertion its specific inhibitor Dickkopf (DKK) and up-regulation of Wnt5a respectively (Kawakami et al., 2006; Stoick-Cooper et al., 2007). A common pathway regulation step is also shared between Hh and Wnt. Two similar transmembrane proteins, acyltransferase Porcupine (Porc) and seven-pass transmembrane protein Wntless (Wls) are required for the stimulation of Wnt too, (Fig 1.9). Furthermore, Frizzled (Fzd) family, the receptors of Wnt, is even homologues of Smo. β-catenin was revealed as a key target in pathway activity regulation in Wnt, which suppresses ubiquitination and degradation of β-catenin through re-localising Axin1 (scaffolding proteins, promotes proteosomal degradation of β-catenin) and multifunctional protein adenomatosis polyposis coli (APC). Then in nuclei accumulated β-catenin will bind T-cell factor/lymphoid enhancer-binding factor family of transcription factors, which generally suppress Wnt genes leading to the up-regulation of signaling.

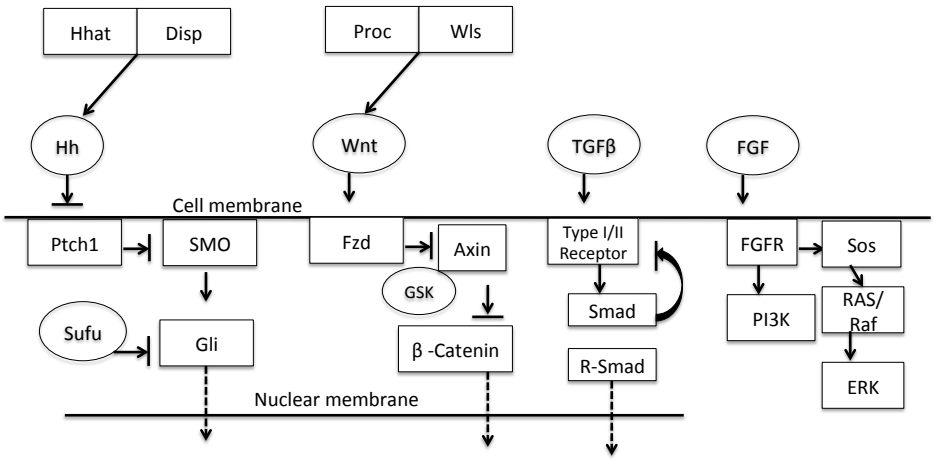


Figure 1.9 Signaling maps of Shh, Wnt, TGF-β and FGF pathways.

4.4 BMP and activin/TGFβ signaling pathway

A heterotetrameric receptor complex (Type I and Type II TGFβ receptors) coupled with two types of serine/threonine kinases were demonstrated to be in the responsibility to transfer the stimulating signal from all TGFβ family proteins (Kawakami et al., 2006). Seven and five isoforms of type I and II receptors of TGFβ have been observed in mammalian cells. Following protein binding with type I receptor, the type II receptors will be spontaneously activated and then the activated receptors stimulate intracellular receptor-dependent Smads family. Once the level of Smads protein increases in nucleus, they will be able to regulate the transcription activity of TGFβ genes.

It is commonly accepted that TGFβ pathway is involved in regulating cellular senescence, differentiation, and apoptosis. Data from microarray-based assay revealed that activin-βA (a member of the secreted TGFβ superfamily) is

detected as early as 1 hour after amputation in zebrafish (Jazwinska et al., 2007). Suppression of activin/TGFβ signalling pathway leads to significant altering of mesenchymal morphology, expression of msx, and cell proliferation (Jazwinska et al., 2007). While over-expression of TGFβ pathway was shown to suppress tumorigenesis.

BMPs, members of the TGFβ superfamily, were characterized as a family of proteins that could induce ectopic bone formation through an endochondral process (Ten et al., 2003). BMPs are extracellular ligands that transfer the signals by binding to complexes of BMP receptors (co-formed by type I and type II receptors conjugated with serine/threonine kinases) that are located on the cell membrane (Derynck, 2007).

Regulation of BMP pathways was revealed to be linked with four type I receptors (ACVR1 [ALK2], BMPR1A [ALK3], BMPR1B [ALK6], and ACVRL1 [ALK1]), which modulate the signal transduction of the pathway. In addition, three type II receptors involved have been identified: BMPR2 (BMPRII), ACVR2A (ActRII), and ACVR2B (ActR-IIB), which are associated with type I receptors by phosphorylating the GS domain, leading to the activation of downstream pathways. Type I receptors is commonly inhibited by FK506-binding protein 1A (FKBP1A protein), which is released by ligand binding. And in downstream signalling, BMP specific R-Smads have been demonstrated to play a vital role in pathway regulation (Shen et al., 2009). R-Smads are able to form a complex and enter nucleus leading to modulating the activity of transcription of specific genes.

As one of the most important sub-family in TGFβ family, BMP is also shown to be involved in vertebrate early development by regulating embryogenesis and cell patterning along dorsal-ventral axis (Derynck, 2007).

4.5 Notch Pathway

Notch pathway is the only one that has been shown to be with a prominent role in cell fate determination in the retina (Dorsky et al., 1997). It is believed that normal level of Notch signalling is vital to stem cells for maintaining the pluripotency. A mutant of HES1 gene in the Notch pathway has also been shown to be able to differentiate into neuronal cells when differentiation occurred prematurely (Ishibashi et al., 1995). A zebrafish study also suggests Notch promotes the development of glia and its differentiation to retina cells (Scheer, 2011).

Moreover, the knock-down of Notch signalling pathways inhibits normal tail regeneration while over-expression rescues regeneration during the refractory period on Xenopus tadpole (Beck et al., 2003). As a result, Notch regulating cell-cell interaction and modulating a balance of pluripotent cells in a region leads to the formation of a complex tissue during somitogenesis (Scheer, 2011).

Activation of Notch signalling in mammalian cells starts with the binding of mature Notch protein with heterodimeric trans-membrane protein receptors and Delta-like or Jagged families shown on adjacent cells (Scheer, 2011).

Following this, a tumor necrosis factor-alpha converting enzyme (TACE) is activated and proteolyses Notch protein undergoing conformational changes. The remaining fragments of Notch protein will be cleaved, which promotes Notch intercellular domain (NICD) entering nucleus leading to the activation of Notch gene expression, which is usually inhibited by CSL families.

Nonetheless, some human disorders and diseases have been shown associating with Notch pathway. Mis-patterning was observed in organs like liver, eye or bone when suppressing Notch2. Overexpression of Notch1 was linked with breast cancer (Scheer, 2011).

The Hh, Wnt, activin/TGFβ, FGF, and RA pathways therefore constitute a core set of cell fate regulators. However, these pathways alone cannot determine the cellular diversity, which is regulated by a complex network of genetic and epigenetic mechanisms. Moreover, cell metabolism modulation pathways like Insulin/IGF-1 can interact with multiple proteins or kinases involved in developmental signaling to achieve the regulation of cells. In response to Insulin/IGF-1 stimulation, the intracellular tyrosine kinase of IR/IGF-1R will stimulate IRS-1 (Vigneri et al., 2010; Skolnik et al., 1993), the phosphorylation of which on its tyrosine residues will associate and provide over 30 potential recognition sites for series of kinases, PI3K, Akt, GSK3 and MAPK .etc (Chardin et al., 1993; Pawson T, 1995). Heavily phosphorylated IRS-1 also provides these binding sites to adaptor proteins like Grb-2, Fyn, Nck, Crk (Gual et al., 2005). The recruitment of Grb-2 and Sos complex to IRS-1 onto the plasma membrane by Shc (a SH2– phosphotyrosine-binding

domain adaptor) will stimulate the guanine nucleotide exchange factor Sos, which in turn leads to the exchange of GDP to GTP in Ras. Then the activated Ras will trigger the MEK-MAPK kinase cascades (Lawrence et al., 2007; Skolnik et al., 1993).

4.6 p53

As a transcription factor, p53 mainly acts on tumor suppression through transcriptional regulation of its target genes (Levine, 2009; Vousden, 2009). When stimulated by various stress signals like DNA damage, hypoxia, and oncogenic signaling, p53 is activated primarily through post-translational modifications, which elicits p53 protein accumulation by increasing p53 protein half-life in cells. The activated p53 protein is able to bind a specific DNA sequence to regulate their expression to start seriously of cellular responses, such as cell cycle arrest and apoptosis, which in turn inhibits the proliferation of cells may potentially become cancerous. Furthermore, studies have revealed that p53 regulates cellular antioxidant defense (Budanov, 2004; Sablina, 2005), which also supports the tumor suppression role of p53. For instance, a recent study showed that while p53 deficiency results in the elevated intracellular reactive oxygen species (ROS) levels. P53-Null mice fed with antioxidant N-acetylcysteine showed to significantly improve karyotype stability, which in turn prevent the formation of tumor (Sablina, 2005).

Mitochondrial oxidative phosphorylation is also revealed being involved with regulation of several downstream target gene of p53 (Matoba, 2006; Vahsen,

2004; Stambolsky, 2006; Hu W, 2010; Suzuki S, 2010; Zhang C, 2011). SCO2 (synthesis of cytochrome c oxidase 2) is one of the most important modulators. P53 was shown stimulating the expression of SCO2, which in turn regulate the cytochrome C oxidative activity. Moreover, transcription of apoptosis-inducing factor (AIF) and Parkin (the vital molecule involved in Parkinson diseases) are also believed to be reduced by p53 leading to balanced integrity of mitochondrial complex. Furthermore, p53 was recently revealed important in regeneration too, which will be discussed in corresponding p53 regulation chapter.

4.7 Small-molecule modulators of cell fate

Although it will be decades before sophisticated restoration of organ function becomes routine in clinical practice, significant progress has been made in developing first generation therapies, the majority of which involve transplantation of a patient's own cells (autologous) to partially restore tissue function (Allsopp et al. 2010). With the contribution from both *in vitro* and *in vivo* research models, there is now a greater understanding of the signaling pathways regulating cell growth and expansion, the mobilization characteristics of resident progenitor cells in tissues, and the mechanisms promoting cell reprogramming. The manipulation of fundamental cell features by small molecules is an area, which receives increasing attention and has already had significant impacts on regenerative medicine.

Since both in vitro and in vivo models have benefited our understanding greatly on how nature regeneration occurs, it has become possible for human beings to modulate the fate of progenitor or even differentiated cells for trauma repair or disease treatment. Thus, the discovery and design of small molecule, which can modulate stem cells therapeutically, have attracted significant attentions. It is hoped that these potential small molecule leads can be further developed into clinical useful drugs for patients suffering from degenerative diseases like neurological disorders, cardiovascular dysfunction and macular degeneration (Davies SG et al., 2015). Small molecules that have been reported to modulate stem cells will be reviewed in this section (Fig. 1.10.1 for 1.7.1 and Fig. 1.10.2 for 1.7.2).

4.7.1 Small molecules that modulate cell fate by inducing cell multipotency

Murine myoblast C2C12, a type of myogenic lineage restricted cell, has been shown could be induced reprogramming to multipoint cells that are able to re-differentiate to osteogenic cells by Reversine (Chen et al., 2007). Further investigation reveals that this small molecule suppresses both myosin II and MEK1 allowing reprogramming of stem cells. Similarly, MLN-8054 and VX-680 (inhibitor of Aurora A kinase, Lens s et al., 2010) can also promote dedifferentiation.

Treatment with Sulfonyl Hydrazones (Sadek et al., 2008) is able to turnover mouse embryonic carcinomas into cardiovascular progenitor, which can further develop into spontaneously beating cardiomyocytes.

5-aminoimidazole-4-carboxamide-1-D-ribofuranoside (AICAR) (Adamo et al., 2009) has been demonstrated to be with the capacity of induction pluripotency in mouse cells by up-regulating Klf4, Klf2 and c-Myc.

E-616452 (Ichida et al., 2009), a specific TGF-β receptor pathway inhibitor, stimulates cell reprogramming by activating the transcription of Nanog leading to the increased pluripotency of stem cells. In combination with Oct4, KlF4 and c-Myc, this compound showed high efficiencies in deriving mouse fibroblasts into progenitor cells.

Klf4, as a cancer suppressor gene, was utilized as a reliable marker of pluripotent. And reprogramming mouse somatic cells through modulating these genes was shown could be replaced by a small-molecule Kenpaullone (Lyssiotis et al., 2009). Kenpaullone was also shown to inhibit GSK3 and cyclin-dependent kinases, which suggests the mechanism of kenpaullone action might be novel, but complex in nature. It might also be non-specific because it hits multiple targets.

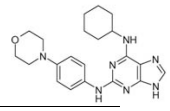
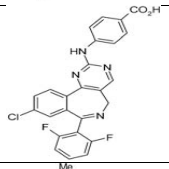
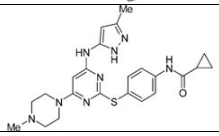
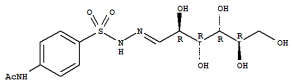
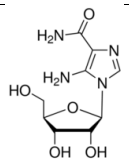
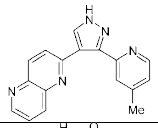
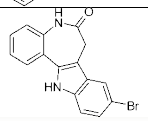
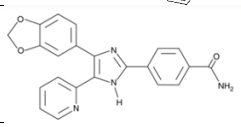
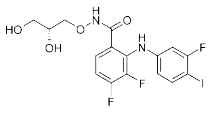
Furthermore, as one of the TGF-β pathway antagonist, SB-431542 (Maherali et al., 2009) can enhance the efficiency of not only reprogramming the four genes (c-Myc, Nanog, Sox2 and KlF4) on mouse fibroblast cells into pluripotent cells by 30-fold, but also deriving these cells into iPSCs by modulating three out of the above four genes. And the efficiency of reprogramming could be increased by nearly 100 fold if cells were dosed with SB-431542 together with an MEK inhibitor PD0325901 (Lin et al., 2009).

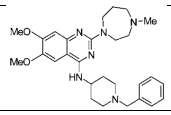
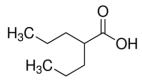
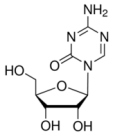
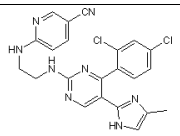
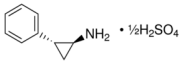
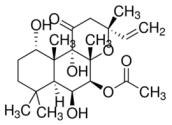
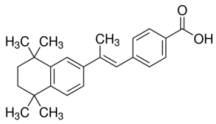
The efficiency of cell reprogramming in lab can be increased by another small-molecule BIX-01294 (Jakovcevski M et al., 2012) on neural progenitor cells by being transduced with Oct3/4 and Klf4. BIX-01294 is an inhibitor of a histone methyl-transferase (HMT, G9a), which demethylases the histone on its H3K9 residue to disrupt gene transcription. And data from the same lab also suggested that BIX-01294 was able to promote reprogramming even better when using together with a potassium channel blocker BayK8644 (Gao Z et al., 2013).

DNA methyltransferase inhibitors like Valproic acid (Ichida et al., 2009) or 5-azacytidine (Mikkelsen et al., 2008) are similar small-molecules improving cell-turnover efficiency by over 100 fold on c-Myc induced cells.

A large-scale screening has successfully revert mouse fibroblast cells to pluripotent cells by only dosing with a chemical cocktail (Hou, P et al., 2013) containing Valproic acid together with E-616452, 3-deazaneplanocin A (DZNep) CHIR99021 (GSK3 inhibitor; Sineva et al., 2010), tranylcypromine (Monoamine oxidases inhibitor; Aker J et al., 2013), forskolin (activatior of adenylyl cyclase; Bellomo et al., 2011) and TTNPB (agonist of Retinoic Acid Receptor; Mukhopadhyay et al., 2010).

Figure 1.10.1

Name	Structure	Reference	Target
Reversine		Chen et al., 2007	Myosin II and MEK1
MLN-8054		Anastasia et al., 2006	Aurora A kinase
VX-680			Aurora A kinase
Sulfonyl Hydrazones		Sadek et al., 2008	unclear
5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)		Adamo et al., 2009	unclear
E-616452		Ichida et al., 2009	TGF-β receptor
kenpaullone		Lyssiotis et al., 2009	GSK3
SB-431542		Maherali et al., 2009	TGF-β
PD0325901		Lin et al., 2009	MEK

BIX-01294		Jakovcevskim et al., 2012	histone methyl-transferase
Valproic acid		Ichida et al., 2009	DNA methyltransferase
5-azacytidine		Mikkelsen et al., 2008	DNA methyltransferase
CHIR90211		Sineva et al., 2010	GSK3
Tranylcypromine		Aker J et al., 2013	Monoamine oxidases
Forskolin		Bellomo et al., 2011	adenylyl cyclase
TTNPB		Mukhopadhyay et al., 2010	Retinoic Acid Receptor

4.7.2 Small-molecule modulators modulating proliferation or differentiation

Replacement cells derived from human stem cells is the key interest in the realm of regenerative medicine. Numerous age-related diseases could benefit greatly from adapting cell-transplantation such as retinal-pigmented epithelial (RPE) cells (Allsopp et al. 2010). Due to the unstable character of human embryonic stem cells, it will be crucial for the achievement of inducing stem cells into lineage-restricted cells for transplantation in lab. Thus the discovery of small molecules that are able to enable such cell differentiation is really attractive to scientists.

Stauprimide (Zhu et al., 2009) was identified from a large-scale screen performed in the presence of low amount of activin A. Data suggests this molecule significantly increased the efficiency of turnover of mouse ES cells into endoderm cells.

The *in vitro* differentiation of hES cells into retinal cells and iPSCs was reported in Japan when they were exposed to CKI-7 (Rachidi N et al., 2014), a casein kinase I inhibitor. It was revealed that Wnt pathways can be suppressed by this compound co-dosed with SB-431542. And this observation supports the hypothesis that modulating cell differentiation artificially can be achieved through inhibiting Wnt signalling.

Based on a high-throughput screening targeting Wnt signaling, IWP-1 (Chen et al., 2009) was shown to inhibit Wnt pathway by suppressing porcupine, which modulates the synthesis of Wnt proteins as an acyltransferase. This

small-molecule also showed an effect on stabilizing Axin, a scaffold protein of Wnt pathway mediating β-catenin.

Similarly, PKF115-584 (Huang et al., 2009; an antagonist of β-catenin) and XAV939 (Fancy S et al., 2011. promoting β-catenin degradation) have been shown to have negative effects on the proliferation in β-catenin-dependent tumor stem cells.

A Wnt signaling negative regulator kinase GSK3B was shown to be suppressed by a group of 5-thiophene pyrimidine derivatives (Wang, W et al., 2009), one of which enhances proliferation of mouse β-cell. Similarly, BayK8644 (Gao Z et al., 2013) also showed a similar effect via stimulating a negative regulator of GSK3B. By targeting GSK families, a series of small molecules have been revealed with the capacity in modulating stem cells' differentiation. BIO (Sato et al., 2004), an inhibitor of GSK3 was observed to promote self-renewal on both mouse and human embryonic stem cells. Bisindolylmaleimides (Bone et al., 2009) selectively suppress GSK-3 in mouse ES cells leading to high efficiency in self-renewal. And another GSK inhibitor, TWS119 (Ding et al., 2003) was demonstrated to stimulate neuronal differentiation.

BMP pathway, as the other crucial signaling pathway during development, has also been explored for specific small-molecule modulators. Phenamil (Morehouse K et al., 2011), a potential promoter of bone healing, promotes osteoblast generation through BMP pathway. Dorsomorphin (Hong and Yu,

2009) is another example of small molecule that has been reported to suppress BMP by inhibiting BMP type I receptors. BMP7, a key intermediate molecule in BMP pathway is shown down-regulated by a cocktail of small-molecule compounds LY294002, CCG1423 and a Janus-associated tyrosine kinase 1 inhibitor (Silva et al., 2009).

The other small-molecule compound showing enhancement of differentiation from human bone marrow progenitor cells into bone marrow cells is eltrombopag (SB-497115. Erickson-Miller et al., 2009), which is revealed as a non-peptide agonist of thrombopoietin receptor and utilized in clinic for treating thrombocytopenia.

Small-molecule inhibitors of a γ-secretase, such as MRK003 (Ramakrishnan V et al., 2012), is able to stimulate Notch pathway suppressing tumorigenesis by inhibiting the proliferation and promoting programmed cell death of its precursor cells.

Inhibitors of Hh signalling have also been reported to promote the differentiation of progenitor cells. One of them firstly being announced is cyclopamine (Von Hoff et al., 2009), a steroidal jerveratrum alkaloid, which was shown with reliable inhibition of Hh pathway by directly inhibiting one of Hh membrane receptors, Smoothened. A high-throughput screening following this report also discovered two antagonists: cyclopamine SAG (Stanton et al., 2009) and purmorphamine (Moon J et al., 2013).

Robotnikinin (Stanton et al., 2009), a 12-membered macrocycle, is reported in a microarray-based screening. Robotnikinin was shown to have a strong affinity to shh receptor proteins (especially Ptch1), which results in the formation of a protein complex that blocks the signal transduction of Hh pathway.

Kinases, as the executors of numbers of important bioactivity, are also a potential target for modulating cell pluripotency. Rho kinase inhibitors are well-known modulators of stem cells that have been widely used in research laboratory. H1152 (Hwang et al., 2008) is one of them and was shown to promote mouse ES cells differentiating into dopaminergic neurons. It is also reported that the other Rho kinase inhibitor Y27632 (Hotta et al., 2009) induces generation of multipotent neural crest cells from human neural stem cells.

Meanwhile, a well-known inhibitor of phosphatidyl inositol-3-K (PI3K. Gupta et al., 2009), wortmannin, a known negative regulator of apoptosis, was revealed to stimulate differentiation of hES and iPS cells into insulin-positive pancreatic cells. In contrast, Pluripotin (Chen, S et al., 2006) was found in an affinity chromatography based assay to enhance the proliferation of mouse ES cells through stimulating ERK1 and activate PI3K by binding with Ras, which can also directly phosphorylate ERK. In the same paper, differentiation of ES cells was shown to be blocked by Pluripotin. Moreover, a recent study also revealed that two compounds, miconazole and clobetasol (Najm et al., 2015; Barnes L et al., 2013) can promote oligodendrocytes differentiation on both

human and mouse progenitor cells through suppressing activity of MAPKs.

Histone deacetylases play important roles in regulating TGF signaling, and the inhibitors of histone deacetylases are discovered to robustly induce the differentiation of mouse stem cells into pancreatic progenitor cells (positive of pancreatic marker Pdx-1). A combination of IDE1 and IDE2 (Chen, S et al., 2009) was shown in this report with better efficiency on differentiation induction than Activin A (Borowiak et al., 2009), which was commonly used in previous studies. Furthermore, Indolactam V (Miron V et al., 2013), an activator of Protein kinase C was also found to increase the expression level of Pdx1. It was shown that when dosed with Indolactam V, ES cells are able to differentiate into pancreatic progenitor cells and generate to endocrine cells (secrete insulin) after being transplanted into animals.

Nonetheless, hepatocytes have also been demonstrated to be derived from human pluripotent stem cells by treating with small-molecule compound sodium butyrate (another inhibitor of histone deacetylase; Cuisset L et al., 1998) and Activin A (for activating TGF-β pathway; Siller R et al., 2015).

SGX523 (Guessous et al., 2010) is another kinase inhibitor on hepatocyte growth factor receptor and was shown have strong suppression effect on its target even in very low concentration. SGX523 was shown as a potent inhibitor on the proliferation of glioma xeno-grafts due to its lethal effects towards cancer stem cells (CSC). 3-Deazaneplanocin A (DZNep. Suvà et al., 2009), an inhibitor of S-adenosylhomocysteine hydrolase, was also shown to

enhance the self-renewal of CSC. And a potassium ionophore regulator, bidirectionalion Salinomycin (Gupta et al., 2009) was reported to selectively enhance differentiation of cancer stem cells into epithelial cells.

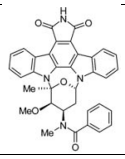
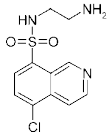
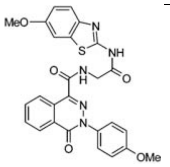
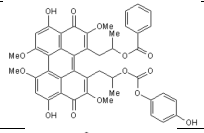
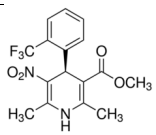
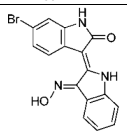
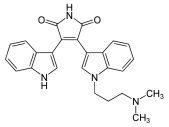
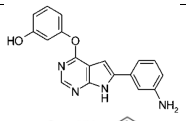
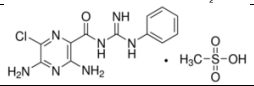
Targeting differentiation of neural stem cells has the potential to cure neurodegenerative such diseases such as Parkinson's and Alzheimer's diseases. Ding and Schultz have described the discovery of Neuropathiazol (Warashina et al., 2006), which can decrease the efficiency of adult hippocampal neuro-progenitor cells differentiating into neurons in rats, but the mechanism was not detailed. Moreover, Phenazopyridine (Soundararajan et al., 2006) was shown to promote differentiation of human ES cells into multipotent neural progenitors. And a screening of over 20000 compounds for discovering promoters on neuronal differentiation from mesenchymal stem cells has identified Quinoxaline (Kim et al., 2009). Quinoxaline induced neutron cells were found to have functional electrophysiological and cholinergic activities.

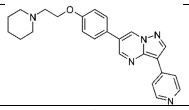
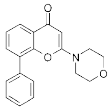
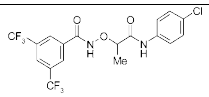
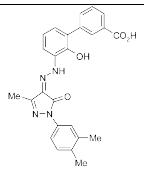
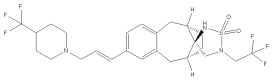
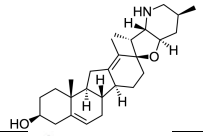
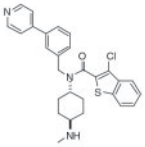
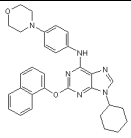
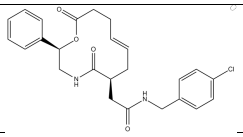
To achieve directly differentiating human pluripotent cells into lineage-specific progenitor cells for transplantation, large volume of research has been carried out to identify reliable culturing conditions for inducing differentiation. Insulin-expression pancreatic progenitor cells have been successfully derived from human embryonic stem cells by exposure to series combination of small molecules (Pagliuca et al., 2014). When dosed with a combination of Phorbol 12,13-dibutyrate (PdbU; activator of Protein kinase C; Hori T et al., 1999), LDN193189 (LDN; inhibitor of BMP receptor; Derwall M et al., 2012), SANT1

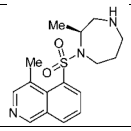
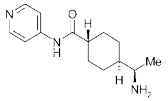
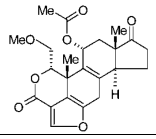
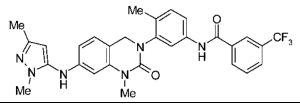
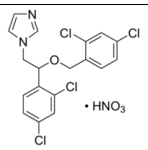
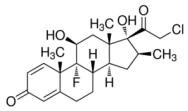
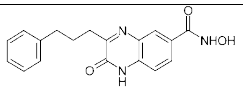
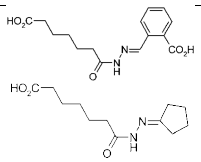
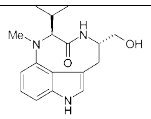
(inhibitor of Hh pathway; Dixit D et al., 2013), Alk5 Receptor Inhibitor (Alk5i; inhibitor of TGF-β signaling; Stähli A et al., 2014), Triiodothyronine (agonist of thyroid receptor; Munuganti et al., 2013) and supplied with Retinoic Acid, and recombinant protein agonist of Notch pathway, human pluripotent cells were shown to differentiate into pancreatic progenitors cells, which was also observed to secrete insulin in high glucose medium.

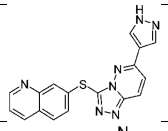
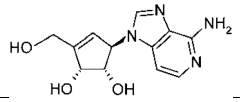
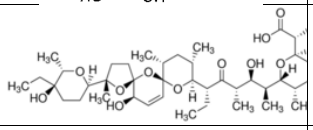
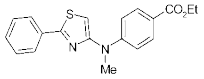
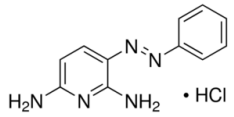
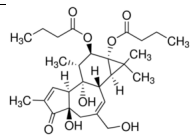
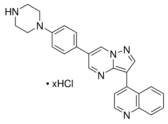
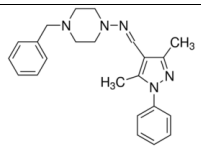
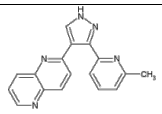
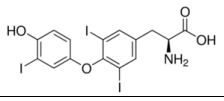
Similar assay using a combination of small-molecule compounds, CHIR99021, ascorbic acid (nature antioxidant; Bradshaw et al., 2011) and BMP4 have also been reported to induce human pluripotent stem cells giving rise to multipotent cardiovascular progenitor cells (Cao N et al., 2013).

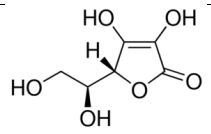
Figure 1.10.2

Name	Structure	Reference	Target
Stauprimide		Zhu et al., 2009	unclear
CKI-7		Rachidi N et al., 2014	casein kinase I
IWP-1		Chen et al., 2009	Wnt
PKF115- 584		Huang et al., 2009	β-catenin
BayK8644		Gao Z et al., 2013	GSK3B
BIO		Sato et al., 2004	GSK3B
Bisindolylmaleimide		Bone et al., 2009	GSK3B
TWS119		Ding et al., 2003	GSK
Phenamil		Morehouse K et al., 2011	BMP

Dorsomorphin		Hong and Yu, 2009	BMP
LY294002		Silva et al., 2009	BMP
CCG1423		Silva et al., 2009	BMP
Eltrombopag (SB-497115)		Erickson-Miller et al., 2009	thrombopoietin receptor
MRK-003		Ramakrisham an V et al., 2012	Notch
Cyclopamine		Chen, J.K et al., 2002	Hh
SAG		Stanton et al., 2009	Hh
Purmorphamine		Moon J et al., 2013	Hh
Robotnikinin		Stanton et al., 2009	Hh

H-1152		Hwang et al., 2008	Rho kinase
Y27632		Hotta et al., 2009	Rho kinase
Wortmannin		Zhang, D et al., 2009	phosphatidyl inositol-3-K
Pluripotin		Chen, S et al., 2006	ERK1
Miconazole		Najm et al., 2015	MAPKs
Clobetasol		Barnes L et al., 2013	Histone deacetylases
Quinoxaline		Kim et al., 2009	unclear
IDE1 and IDE2		Chen, S et al., 2009	TGF
Indolactam V		Miron V et al., 2013	TGF

SGX523		Guessous et al., 2010	kinase
3-deazaneplanocin A (DZNep)		Suvà et al., 2009	S-adenosylhomocysteine hydrolase
Salinomycin		Gupta et al., 2009	potassium ionophore
Neuropathiazol		Warashina et al., 2006	unclear
Phenazopyridine		Soundararajan et al., 2006	unclear
Phorbol 12,13-dibutyrate (PdbU)		Hori T et al., 1999	Protein kinase C
LDN193189 (LDN)		Derwall M et al., 2012	BMP receptor
SANT1		Dixit D et al., 2013	Hh
Alk5i		Stähli A et al., 2014	TGF-β
Triiodothyronine		Munuganti et al., 2013	thyroid receptor

ascorbic acid		Bradshaw et al., 2011	nature antioxidant

4.8 Cellular prion protein and stem cell

Despite the proteins or kinase directly involved within the process of development or regeneration, some membrane protein has also been revealed to be with a vital role in the modulation of pluripotent cells. Prion diseases, which include as bovine spongiform encephalopathy (BSE) and variant Creutzfeldt–Jakob Disease (vCJD) (Watts, 2007 & Murdock B et al., 2015) has been demonstrated to be caused by the transformation of a membrane protein cellular prion protein (PrP^C), which has also been demonstrated to play a role in enhancing proliferation and promote self-renewal of stem cells (Mouillet-Richard S, 2000; Zhang CC et al., 2006; Steele AD et al., 2006 and Miranda A et al., 2013). Engraftment of hematopoietic stem cells (HSCs) to bone marrow in multiple transplants using HSCs from PrP-null mice showed to be inefficient in contrast to normal. The defect was rescued when these PrP-null HSCs genetically modified to express prion protein (Zhang et al., 2006). Similar observation is also reported that only those cells expressing prion protein are able to regenerate the mammary gland when being transplanted into animals (Liao et al., 2007). In adult neurogenic regions, cell proliferation was increased in mice overexpressing prion protein compared to wild-type type and *prnp* (the gene

coding PrP^C) knockout mice (Steele et al., 2006). Therefore, prion protein may play a role in the signal transduction between intracellular and extracellular sides within the progenitor cells during the process of regeneration *in vivo*.

Moreover, PrP^C might also be able to block some of the internal or environmental factors initiating apoptosis and function as anti-apoptotic agent (Bounhar Y, 2001; Roucou X, 2005). Because PrP^C is capable of binding copper in vivo (Brown et al., 1997), it has also been proposed that PrP^C may function similarly like a superoxide dismutase (SOD). Cu^{2+}-bound SOD was shown to be associated with the detoxification of reactive superoxide radicals by converting them into oxygen and hydrogen peroxide, which allows further degradation. Decreased SOD1 activity was observed in brain homogenates derived from *prnp*-knockout mice (Brown et al., 1997) or brain homogenates with immuno-depleted PrP^C (Wong, 2000), and this phenomenon domonstrates that PrP^C is able to regulate the incorporation of Cu^{2+} into SOD (Brown, 1998). Furthermore, it was suggested that PrP^C might also possess some intrinsic SOD function (Brown, 1999), although another group has failed to find any SOD1 activity with recombinant PrP^C (Jones, 2005). The ability of PrP^C to regulate SOD activity remains contentious because few studies demonstrated the activity of neither SOD1 nor SOD2 was altered *in vivo* with varied *prnp* gene dosage (Hutter, 2003; Waggoner, 2000).

Recently, cloning and sequencing of *prnp* genes in zebrafish demonstrated that there are two PrP orthologs, PrP-1 and PrP-2, existing in fish. Data gained from Northern and Western blotting indicated that these two fish

proteins are expressed at particularly high levels in brains, muscle, skin, heart and gills in adult fish (Málaga-Trillo, Salta et al. 2011). Compared with mammalian counterparts, PrP-1 and PrP-2 are quite variable in size and amino acid sequence but display characteristic features of PrPs seen in mammals: an N-terminal signal peptide, conserved N-glycosylation sites, two cysteine residues that form a disulphide bond, a central hydrophobic stretch, and the C-terminal signal for the attachment of a GPI-anchor (Málaga-Trillo, Salta et al. 2011). Based on this, zebrafish might be utilized to serve as an alternative *in vivo* research model for investigation into the function of prion-aiming compounds in tissue regeneration.

5. Previous work leading to the project

The wider application of cell-based therapy using stem cells from other tissue sources has been hampered by their small number in tissues, rapid aging in vitro and limited differentiation potential (Colin, 2007). Both the number and potency of these stem cells declines with aging in vitro making the identification, isolation and purification a very challenging task. Methods that can overcome these problems would be most advantageous.

Small molecules modulators for stem cell functions have several advantages, as they are usually cell permeable, non-immunogenic and can be manufactured to the standards of good manufacturing procedures (GMP) for therapeutic applications. Small-molecule compounds, which can robustly benefit the growth or expansion of stem cells in vitro or in vivo, will significantly contribute to the development of regenerative medicine.

As the availability and accessibility of adult stem cells research materials are limited, the well-characterised teratocarcinoma cell line Ntera2 (NT2) was chosen as the cellular screening model in a project previously carried out by a group of research student in the group. In the initial screening using EC cells, 350 small molecules were dosed onto EC cells for 4 days. Ms Sandra Knight used a fluorescent plate-reader (BMG Flurostar Optima) and the InCell Analyzer 1000 (GE Healthcare) to assess the capabilities of the chemical library in enhancing the proliferation of EC cells by correlating the fluorescence emission of the dye–nucleic acid complex with cell number. The compounds were then ranked using a waterfall plot for their ability in

enhancing the proliferation of EC cells (Fig 1.11). 25 Hit compounds were then tested 10 times until consistent activity was observed (Fig 1.12) in the confirmation assay.

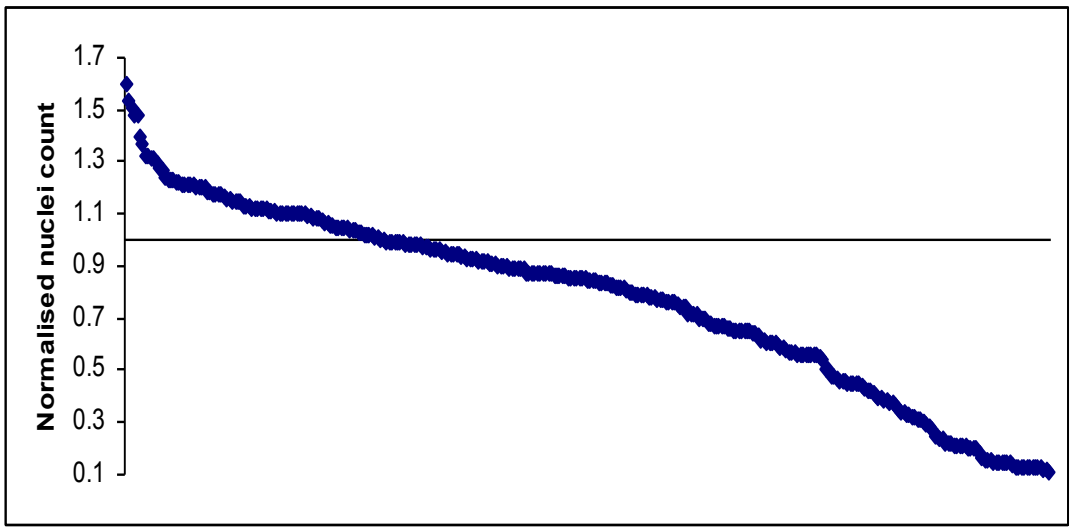


Figure 1.11. Graphical representation showing the spread of normalised nuclei counts obtained from the primary screening of 350 compounds. 5 day assay using Ntera 2 cells, 1500 cells/well in 96-well plate. Cells stained with Hoechst 33342, analysed using InCell Analyzer. Results are normalised against the 0.25% DMSO. n>5.

Four of them, 3000165, 3000432, 3000689 and 3000672 (Fig 1.13) were shown to consistently increase the cell number, mostly at the concentration of 1 μM.

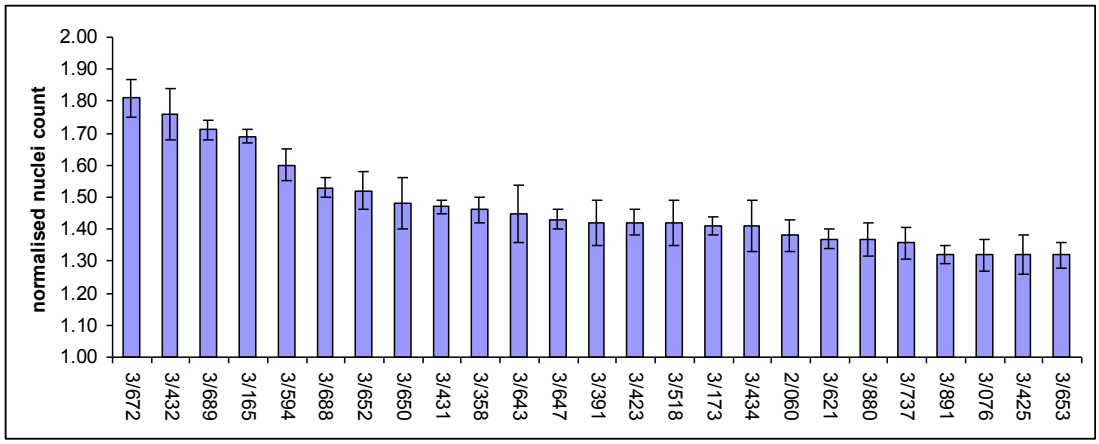


Figure 1.12. The normalised nuclei count for the twenty five compounds inducing the greatest increase in proliferation of Ntera 2. 5-day assay, 1500 cells/well, 1 µM compound, 96-well plate, stained with Hoechst 33342. Counts show the average value ±SD.

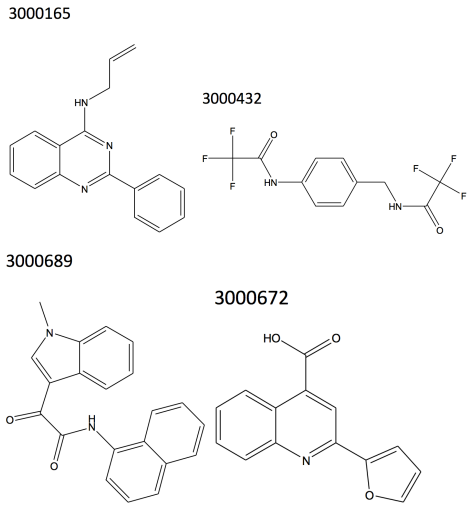


Figure 1.13 Structure of 3000165, 3000432, 3000689 and 3000672.

However, it is unclear how these small molecules were able to have effect on cell numbers and whether they would influence progenitor cells number in vivo. Thus, further evaluating these compounds in cellular models and developing an invertebrate model for exploring the role of these four small molecules might play during a whole process of natural occurring regeneration would help to gain some useful insights into novel mechanisms involved in tissue regeneration, hence the development of regenerative medicines.

6. Aims and Objectives

The initial in vitro screening has clearly demonstrated that 3000689, 3000165, 3000672 and 3000432 are able to increase the total cell number of EC cells. But how these compounds accelerate cell proliferation is still not clear. Moreover, it is still necessary to use different cell model to confirm the activities of these small molecules and investigate their mechanism of action. As these four compounds belong to different families and do not share similarities in structure, whether all of them are with positive influence on cell growth generally or just on EC cells is the question also needed to answer. Therefore, it is necessary to try different approaches for exploring the possible mechanism of how these compounds increase the cell population of EC cells.

With the hope to be developed as regeneration modulator, testing these compounds on in vivo model is also crucial for this program. An easy and cheap in vivo model will be required for studying the mechanism of these compounds. And how they act in tissues and the side effect they may bring to the animal model will also need to be evaluated.

Thus, the aim of this research program is to use the fin regeneration of zebrafish larvae as the invertebrate model, and both human EC and ES cells as the cell models for exploring the role of these four small molecules, especially the best hit compound 672 play during the whole process of natural occurring regeneration. Establishing a reliable in vivo model for evaluating the mode-of-action for these molecules with the hope to develop them as modulators for tissue regeneration will also be involved.

Multiple assays (Fig 1.15) were applied to both *in vitro* and *in vivo* models to address the questions below:

Question 1 (Q1) - How do these compounds increase the cell population of EC cells during the treatment period, through enhancing cell proliferation or preventing cell death?

To address this question, cell cycle and apoptosis analysis using FCM will be used to detect if treatment with the selected compounds can enhance cell proliferation or prevent apoptosis.

Question 2 (Q2) –Can these compounds also promote the growth of the other human pluripotent stem cell lines or not?

Different human embryonic stem cell lines will be employed to answer this question.

Question 3 (Q3) - Will any of these four compounds have any positive influence on tissue regeneration in an in vivo animal model? (Toxicity and validation test on an *in vivo* model, Cell apoptosis and mitosis analysis)

Fin regeneration of wildtype zebrafish larvae will be utilized as the *in vivo* model to test if the selected compounds can improve the fin regeneration through promoting cell proliferation or preventing cell death.

Question 4 (Q4) – What is the correlations between results from in vitro and the ones from in vivo studies?

Comparing the results from both *in vitro* and *in vivo* models will contribute to address this question.

Question 5 (Q5) - What is the possible mechanism of action for these compounds?

RNAi high-throughput screening, qPCR, Thermal shifting, *in situ* hybridization and Kinase profiling will be used to identify the mode of action the hitting compounds act as.

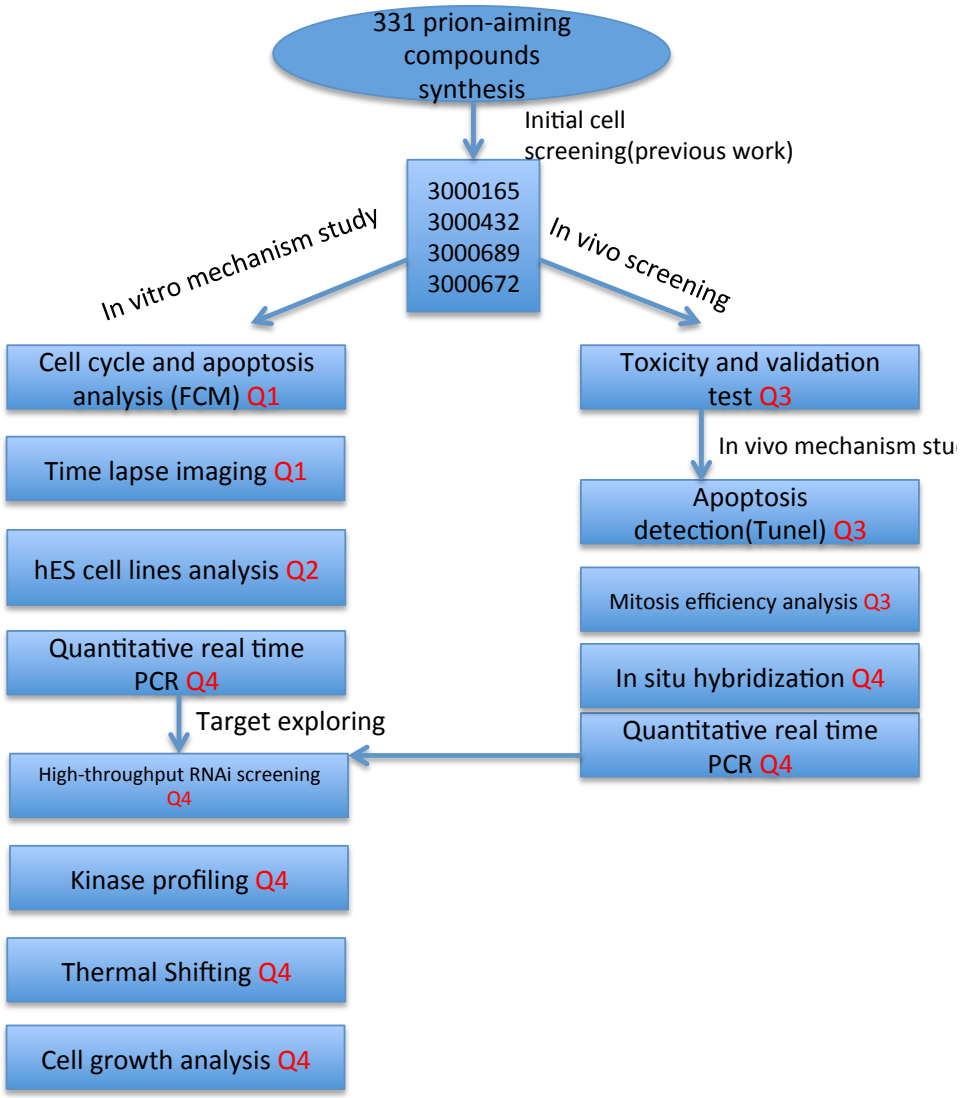


Figure 1.14 Flow chart of work Plan.

All detailed information of each assay will be documented in following three chapters. Chapter II will discuss the work based on stem cells and the *in vivo* model analysis will be talked about in Chapter III. In Chapter IV, the procedure of exploring the mode of action of the hitting compounds will be described. After the conclusion in Chapter V, the work analyzing the regulation of p53 during zebrafish fin regeneration (p53 is one of the hypothetical targets of anti-prion compounds) will be documented in Chapter VI.

II. *In vitro* study

1. Materials

EC cell: Ntera2/D1 cells were maintained and routinely cultured in Centre for Stem Cell Biology in University of Sheffield.

ES cell: Mshef 11, Mshef 13 and Mshef 14 were maintained and routinely cultured in Centre for Stem Cell Biology in University of Sheffield.

Small-molecule compounds 689 (analogue 1170, 1173 and 1178) and 672 were synthesized in-house. Purity of all compounds used for screening and analysis is over 95% by HPLC. Solid form of the tested compounds was kept at -20^{o}C before using. Absolute DMSO (Sigma, USA) was used to dissolve all tested chemicals and make them into 10 mM stock solution each, which was aliquoted into multiple portions and kept at -20^{o}C before using. 1 μM final concentration of compounds was used for the treatment.

Information of all the other reagents used is shown in Appendix.1.

2. Methods

2.1 Maintaining and passaging of stem cells

EC cells (Ntera2/D1) were routinely cultured in high glucose (4500mg/L) DMEM medium (Sigma, D5796) supplemented with 10% FBS (Gibco, 10270-106). They were incubated at 37°C and supplied with 10% CO_2. Cells were mechanically passaged at a ratio of 1:3 using sterile glass beads, when 80 - 90% confluent was reached. 0.25% trypsin with 2% EDTA is used for suspending cells enzymatically.

Mshef stem cells were routinely maintained in Nutristem medium (Biological Industries) on CELLstart™ xeno-free substrate (Life Technology) coated flask or plates or flasks. They were incubated at 37°C and supplied with 5% CO_2. Cells were mechanically passaged at a ratio of 1:3 using colony-cutting method, when 80- 90% confluent was reached. TrypLETM (Life Technology) was used to fully suspend cells.

Cell suspension was counted based on Countess Automated cell counter (EC, Invitrogen) and Scepter Automated cell counter (hES, Millipore) following the instruction provided by the manufacturer respectively.

2.2 Flow cytometry assays

Cell flow cytometry, as one of the most popular technologies in cell research, has been widely used in cell counting, cell sorting, biomarker detection and protein engineering. It is a laser-based, biophysical technique, which allows simultaneous multi-parametric analysis of the physical and chemical characteristics of up to thousands of particles per second (Eggeling L et al., 2015). Flow cytometry is routinely used in clinical settings including the diagnosis of health disorders such as blood cancers, but has many other applications in basic cell research (Eggeling L et al., 2015). In cell biology, this technique is commonly referred as fluorescence-activated cell sorting (FACs). The instrument built based on this principle can provide fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest. It is frequently used for sorting a heterogeneous mixture of biological cells into two or more

containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. Cell suspension is firstly prepared or incubated with a dye or a fluorescence-conjugated maker each has a specific excitation and emission wavelength. After the staining, cell suspension will flow in a stream of fluid, and then a beam of light of a particular excitation wavelength (wavelength adjustable depending the type of fluorescence used) is shone at each cell when it pass through. The coupled electronic sensor will firstly identify the size of each cell in the fluid based on the level of light reflected, at the same time detects the emitted fluorescence signal (Figure 2.2) and the specialist software is used to de-convolute the signal. Data shows as a histogram (single parameter, EdU) or dot-plot (two parameters or more, Annexin V & PI) graph, the percentage of each proportion will be automatically read out based on the graph. The methods of how to analyze the results based on the histogram or dot-plot graph will be described in the corresponding assays.

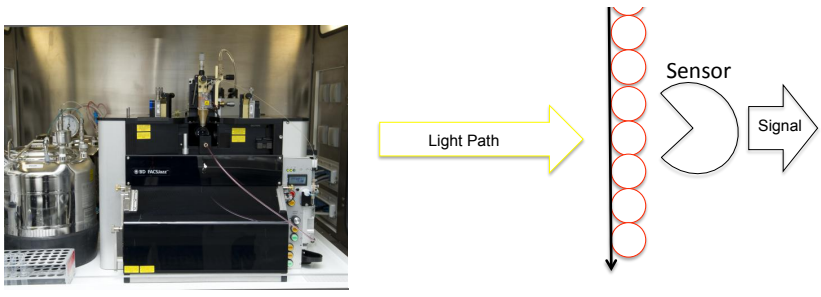


Figure 2.1 Picture of basic FCM instrument and the principle of how FCM works.

2.2.1 Cell cycle phases analysis by propidium iodide staining

Eukaryote cells normally undergo a full cell cycle, which can be divided into three phases (Fig 2.3): interphase (cells accumulate supplements preparing for mitosis and duplicate DNA), mitotic (M) phase (nuclear division where nuclei and cell content are divided into two through mitosis) and cytokinesis (cytoplasmic division where plasma membrane and cytoplasm are divided to form two identical daughter cells).

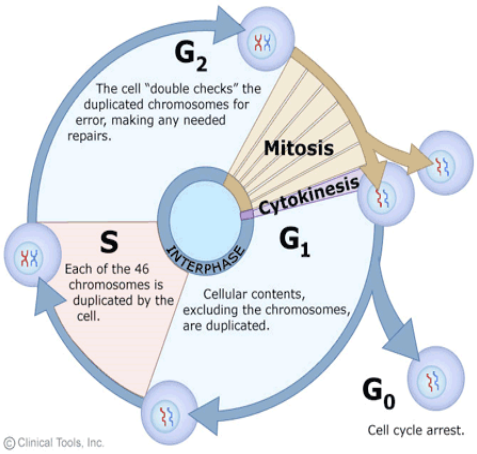
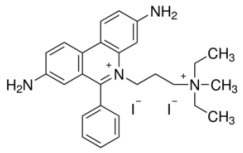


Figure 2.2 Demonstration of procedure of a full cell cycle (images from Clinical tools).

Interphase can also be characterized into three sub-phases including Gap 1(G1), S and G2. G1 phase is the preparation phase where, cells grow in size and all proteins and enzyme needed for DNA replication are recruited/synthesized. This makes the duration of G1 phase variable in length between different types of cells due to their different doubling time required (Ruiz et al., 2011). In S phase, DNA is synthesized and centrosome is duplicated. G2 is a checking phase. Unlike most somatic cells, stem cells proliferate rapidly with relatively short doubling time, which is an important character of the pluripotency (Becker et al., 2006). It is commonly understood that compared to other cell types, stem cells have a relatively shorter cell

cycle due to the reduction of the time when they stay in G1 phase (Li et al., 2012), which allows more efficient proliferation and also results in relatively small cell size. Therefore, analyzing the proportion of cells undergoing different phases of cells cycle especially the time they spend in G1 phase will help the understanding of the mechanism of action of our compounds.



Propidium iodide (PI), a widely used fluorescent agent (wavelength: 535-617nm), was used for cell cycle detection by FACs. PI, as shown on left, can bind nuclei acids by intercalating between bases of DNA (in chromatin) or RNA in fixed cells.

Based on the fact that once PI bond to nuclei acids in chromatin, the fluorescence of PI will be enhanced (Ruiz et al., 2011). Cells detected with more than one fluorescence will be recognized as G2 or M phase since it has two copies of DNA after the S phase, and positive cells showing only one fluoresce dot population is characterized as G1 phase because DNA replication does not occur in this phase.

Cells in 4 x12 wells (seeding density: 5× 10^5 cells/well) were kept in incubator for 16 hours, then compounds were added into the medium to the designated concentrations. The cells were harvested and washed twice with 4mL of cold phosphate buffer saline (250 × g, 4 min) at 6, 12, 18 and 24 hours post treating. For PI staining, the cells were fixed with 70% cold ethanol overnight. After washing with 4 mL PBS twice, cells were re-suspended in 500 µL cell-staining buffer with 20 mg/mL PI and 10mg/mL RNAse (Sigma, US) for 30 min

in dark. After being washed, the treated cells were analyzed by flow cytometry (FCM) (Beckman Coulter, Brea, CA).

2.2.2 EdU cell cycle analysis

EC cells were treated with DMSO or the selected compound for 24-72 hours. Cells were incubated with 10 μM EdU for 0.5 h, and then harvested using trypsin (Invitrogen). After being washed in PBS with 1% BSA, cells were fixed in 4% formaldehyde in PBS (PFA) for 20 min at room temperature. Following 15 min incubation in 1x Saponin-based permeabilization and washing reagent at room temperature, cells were stained with Click-iT reaction buffer for 0.5 hour. After being washed again with 1x Saponin-based permeabilization and washing reagent, cells were re-suspended in 500μl Saponin-based permeabilization and wash reagent with 2 μL Click-iT EdU Cellcycle 488-red for detecting cells not in S-phase based on the protocol provided by the supplier. CyAn ADP flow cytometer with the O2 optics (Beckman Coulter) was used to detect the fluorescence in cells.

2.2.3 Annexin V apoptosis assay

EC cells grown in six-well plates were incubated with DMSO (control) or the selected compounds in DMEM (10% FCS). Triplicate wells were used for each condition. After 24 hours incubation, old medium was replaced with DMEM containing 350 μM hydrogen peroxide (Sigma). All cells including detached and adherent cells were collected from each well by spinning (1100 rpm) after 2 hours. Cell pellets were washed with PBS and then binding buffer (10 mM hepes, 2.5 mM $CaCl_2$ and 140 mM NaCl then set pH as 7.4). One

million cells from each well were incubated in 100μl binding buffer with 10μl of annexin V-FITC (invitrogen) at room temperature in dark. After 10 minutes, PI (10 μg/mL; sigma-aldrich) was added into each tube right before the fluorescence measurement of cell was taken using a CyAn ADP flow cytometer with O2 optics (Beckman Coulter).

2.2.4 Statistical analysis

Percentage of cells in assays above in DMSO control and each compound treatment wells was collected and analyzed in Sync Wizard Model. Student t-test was applied to analyze the significance between results on Prism. P values of <0.05 being considered statistically significant. ** $P < 0.01$, *** $P < 0.001$.

2.3 Time-lapse cell cycle analysis

Human EC or ES cells were seeded into 6 well plates in fresh culturing medium, and live image of focused cells was taken every 15 min prior to the analysis using Luma time-lapse scope in incubator. 1 mM stock solution of tubulin tracker was prepared following the protocol suggests by the manufacturer. Cells were incubated in medium containing 100 nM final concentration of Tubulin Tracker in incubator for 30 min then rinsed with fresh medium and maintained in fresh medium for live imaging.

2.4 Clonogenic analysis on hES cells

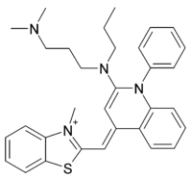
Human ES cells were fully dissociated using TrypLE (Life technology) and seeded into 6-well plates at the same density in Nutristem medium either with

or without the selected compounds. The cells were maintained at 37°C and supplied with 5% CO_2 and kept for 5 days prior to analyzing and staining (Tra-1-60, 1:200 Abcam and anti-mouse ALEX 488, 1:1000 Molecular Probes).

In pre-dosing assay, Mshef cells were harvested by colony cutting method using pipette tips and kept in medium with the selected compounds for 3 hours. After dissociation and being washed with PBS, cell suspension was seeded into 6-well plates in the same density in fresh medium as that without compounds. Image J (open source) was used to identify the boundary of cell colony and measure the acreage.

2.5 Quantitative real-time PCR

Polymerase chain reaction (PCR), as the most popular assay in molecular genetics, can amplify target DNA molecule for a relatively high amount by a simple reaction composed by DNA polymerase, DNA sample, primers of target DNA and NTPs. Real-time quantification of PCR products means quantifying the PCR products while the PCR reaction is processing. A nuclei acid dye like SYBR (as structure image shown below) can bind double-strand DNA and be stimulated to show fluorescence (green, 522nm).



During the amplification, amount of DNA products will increase as well as the intensity of fluorescence, which will be detected by sensor and quantified (Vandesompele J et al., 2002; Nolan T et al., 2006). After normalizing with the level of a housekeeping gene in the same batch of genomic cDNA sample, it will be possible to gain

the level of target DNA in the sample. Taken that cDNA is reversed from the RNA isolated from cell or tissue samples, the level of target cDNA can be taken as the relative expression level of this interest gene (Richardson A et al., 2013)

One day after cells were seeded in six-well plates with 3mL medium in each well, the compounds of desired concentration were added into wells. Then total RNA of $1.5x10^4$ EC cells that were dosed with selected compounds for 4 days was extracted using Trizol reagent (Sigma, USA). $CHCl_3$ was then added in to remove proteins, and the supernatant was transferred into new eppendorf after centrifuge (4^oC, 12000rpm, 10 min). After being pelleted using pure isopropanol, RNA was washed with 80% ethanol and air-dried. After being dissolved in nucleic acid-free H_2O, RNA samples were measured by NanoDrop for concentration (BioPhotometer, Eppendorf). The mean value of two tests was used as the amount of each RNA sample. Then all RNA was reverse transcribed using the High Capacity RNA-to-cDNA KIT (Applied Biosystems, USA) following the protocol recommended by the manufacturers.

PCR products were quantified using the SYBR-Green method based on the corresponding standard curve (SYBR Green JumpStart Taq ReadyMix, SIGMA, USA). The primer sequences are designed on Primer3 shown in table 2.1. Each pair of primers was picked based on: GC content (50-60%), size of PCR product (60-150bp) and melting temperature (60-65^oC). Random cDNA sample were tested with gradient descending concentrations of each primers (different ratio of two primers in a pair) on Mx3000 qPCR system (Agilent

Technologies, USA) using SYBR Green analysis program. Standard curve was generated using MxPro software (Agilent Technologies, USA) based on the C_T value gained from the reaction. If the slope of the standard curve from -3.1 to -3.6 in a reaction, this pair of primers and the concentration setting of each components involved will be taken as a reliable system for analyzing the relative expression level of the corresponding gene in different samples. Student t-test was applied to analyze the significance of results on Prism. P values of <0.05 being considered statistically significant. ** $P < 0.01$, *** $P < 0.001$.

3. Results and Discussion

3.1 Cell maintaining and passaging

Both EC and ES cells were maintained as described in methods. As shown in Figure 2.3, routinely maintained cells were observed with all classical morphology as described in Chapter I section 3 (cells were seen small in size overall, high nucleus to cytoplasm ratio and prominent nucleoli and cell colonies were observed as tightly compacted). Any cells with abnormal morphologies (usually spontaneously differentiated) would be abandoned when passaging.

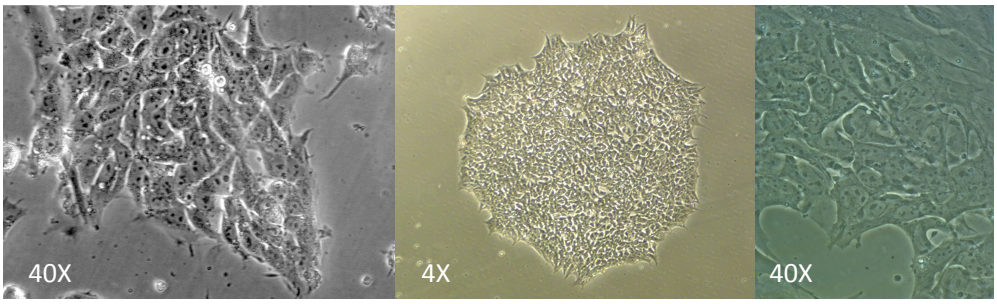


Figure 2.3 Pictures of classical human EC (Left) and ES (shef 11, Middle & Right)

3.2 Flow cytometry assays

3.2.1 Cell cycle phases analysis by Propidium iodide (PI) staining using FACs

In this assay, EC cells maintained in medium with or without compounds were fixed in ethanol then stained with PI for running through FACS machine. After analyzing three different passages of EC cells, it was found that in Y27632 (Ri) treated group, there are relatively smaller portion of cells undergoing G1 comparing to control group. Y27632, as the inhibitor of Rho, was also reported to accelerate the progress from G1 to S phase during cell cycle in human fibroblast cells (Roovers K et al., 2003). Similar results were also obtained from 689 and 672 treated cells (Fig 2.4). Based on this finding, it is possible that cells treated with 689, 672 and Y27632 all have a shorter duration of G1 phase. However, t-test does not suggest there is a significant difference in subpopulation of cells in G1 phase between treated or control group of cells.

Nonetheless, due to the naturally high efficient proliferation feature of EC cells, it is also difficult to say that cell cycle is promoted by the slight shifting of

cell cycle phases. Moreover, Y27632 is commonly understood to prevent apoptosis induced by dissociation during passaging of stem cells, which might permit a more rapid cell expansion. Because 689 or 672 does not share structural similarity with Y27632, it is unlikely that any of them will act via the same mechanism. Therefore, further investigation into the mode of action of these compounds is required.

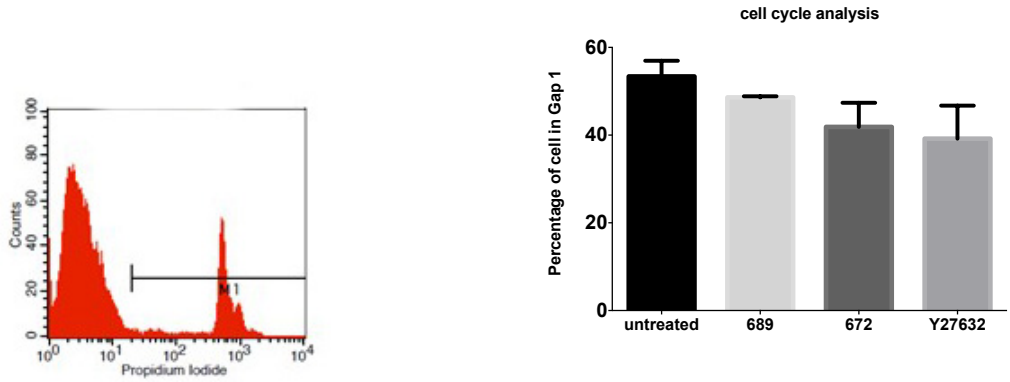


Figure 2.4 Left: Histogram figure shows the result of PI staining on FACs, the portion excluding M1 was taken as cells in G1. Right: EC cells were dosed with compounds 689, 672 or Y27632 for 24hours. Data shows the average percentage of cell proportion in G1 ±sd. Student t-test does not suggest a significant difference between untreated and treated group. p>0.05. n=3.

3.2.2 Cell proliferation analysis by EdU incorporation

Because it was difficult to tell the altering of cell cycle phases precisely by PI staining, a mitosis biomarker, EdU (5-ethynyl-2-deoxyuridine) was employed for labeling the newly synthesized DNA during S-phase of cell cycle, which allowing the tracking of reorganization of DNA in mitotic phase (Iovino et al., 2014). More adaptable than traditional BrdU (bromodeoxyuridine) staining, EdU incorporation is much less time consuming and can be detected by a binding reaction without disturbing cells or denaturing DNA during the procedure (Salic & Mitchison, 2008). As a nucleoside analog, EdU contains

an alkyne group that could stably coupled with the fluoresce-bound picolyl azide via 'Click' chemistry catalyzed by copper, which allows EdU to covalently bind to DNA and becomes the part of it during the reorganization of dividing cells (Figure 2.5; Buck SB et al., 2008). FACs can easily pick up the fluorescent signals and sort the cells according to the strength of the signal within a particular population.

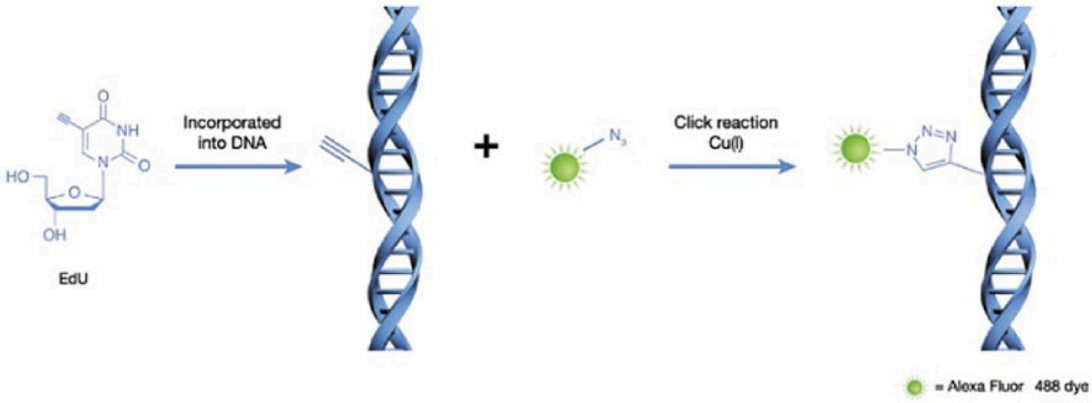


Figure 2.5 Principle of EdU labeling (Buck SB et al., 2008).

Here, to understand whether 672 influences cell proliferation or not, the same batch of EC cells were used. Following cell attachment, 672 was added into medium in treatment wells and same concentration of DMSO was used in the controlled wells. After being incubated with EdU for 30min, EC cells treated by 672 for different length of time (24-72 hours)as well as untreated controls were harvested and analyzed using the method described in 2.2.2. As data shown in Fig 2.6, P4 was taken as the portion of dividing cells that were undergoing S-phase for DNA synthesis during EdU incubation. It seems that after 24 hours dosing, the proportion of cells undergoing S-phase incorporated by EdU is about 50% in control wells but nearly 60% of the cells

was detected in 672 dosed wells (Fig 2.6 a top panel &b). And this enhancement can be also observed in cells treated with 672 for longer time (48h and 72 h; Fig 2.6 c), even when about 85% confluent was reached. Given that percentage of cells with newly synthesized DNA has been consistently higher in 672-dosing wells, it is likely that more cells are dividing when 672 exits. This strongly suggested that 672 is able to boost the proliferation of cells and can consistently improve the efficiency of cell division in a reasonable low concentration.

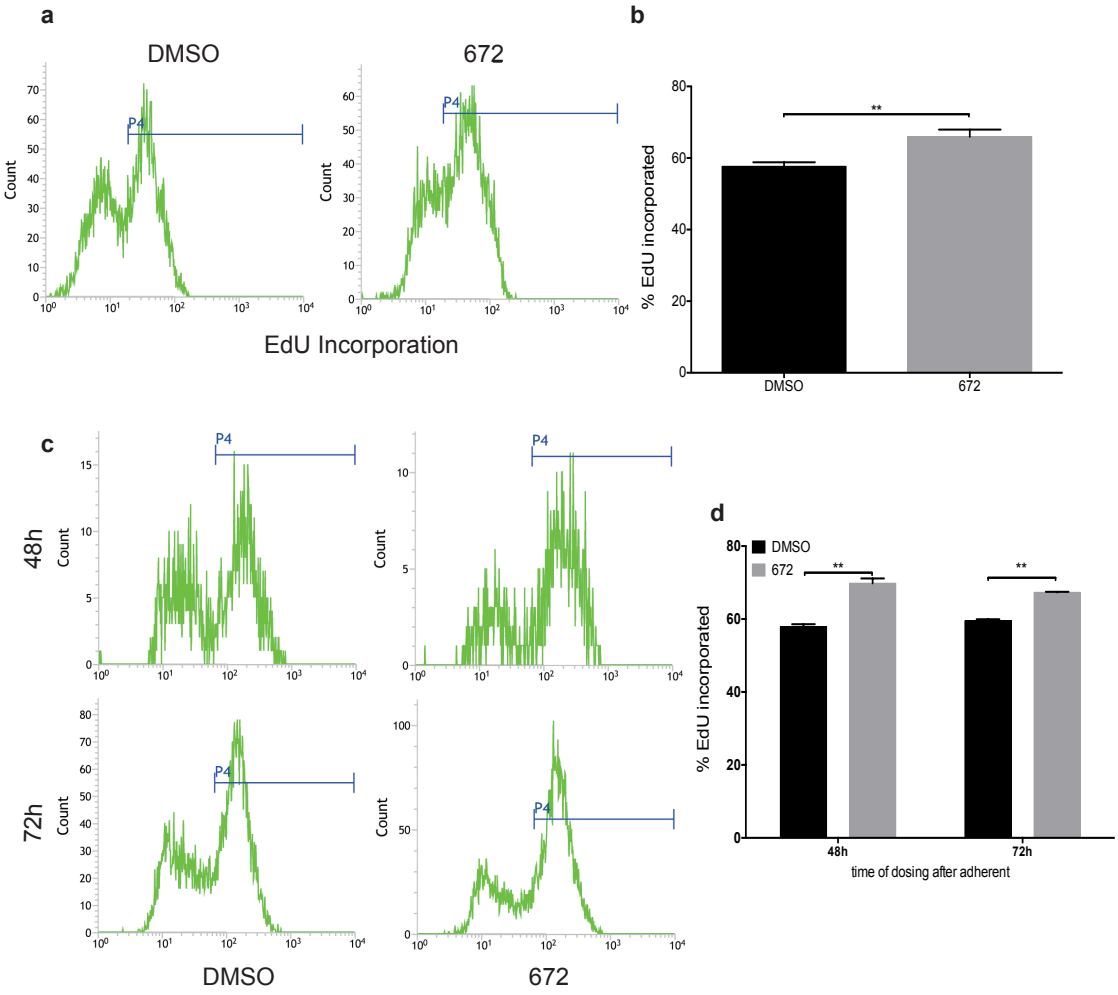


*Figure 2.5 Cell population incorporated with EdU is detected increased by 672 consistently after adherent: a & c) Histogram graphs showing the EdU incorporated cell sorting results of cells treated with DMSO (Left) and 672 (Right) for 24-72 hours; b & d) columsn represents the proportion of EdU-incorporated cells. ** p<0.01. n=3.*

3.2.3 Cell apoptosis analysis by a combination of Annexin V and PI staining using FACs

In FACs-based assays, cell fragments containing DNA are often interfering with the apoptosis analysis due to the fact that some cell fragments (generated during harvesting and washing) are fluorescent and produce false positive signal during the assay. To avoid this problem, the combined staining of both Annexin V and PI on live cells were used to assess if 672 can prevent programmed cell death. Annexin V (FITC conjugated, green) and PI (red) combined staining was firstly reported in 1995 (Vermes et al., 1995). As the surface of cell initiating apoptosis will reorganize, and phosphatidylserine (PS) will translocate from intracellular side to extracellular side on the plasma membrane, which allows Ca^{2+}-dependent binding of PS with Annexin V. Although this phenomenon not only occurs on apoptotic cells but also necrotic cells, the membrane of cells in early stage of apoptosis is still intact while necrotic cells will be leaky due to the loss of integrity. Thus, fluorescein isothiocyanate (FITC)-labeled Annexin V can label cells undergoing apoptosis ($FITC^+/PI^+$ and $FITC^+/PI^-$), and both PI (can pass through membrane and bind to DNA) and Annexin V label will distinguish these population of necrotic/ late-stage apoptosis cells ($FITC^+/PI^+$) from early stage apoptotic cells ($FITC^+/PI^-$).

To find out if 672 can prevent cell damage induced by exogenous hydrogen peroxide, which has been seen in a separate study on 689 using human mesenchymal stem cells. EC cells were dosed with 672 then harvested and incubated with Annexin V-FITC and PI for analysis using FACs. As shown in Figure 2.6 Left, the dots-plotting graph shows the distribution of different type

of cells analyzed in two parameters: Y axis represents the intensity of red fluorescence (PI) and X axis shows green fluorescence (FITC-Annexin V). To distinguish the different portions, all dots were divided into four areas: cells in Q4 are healthy cells ($FITC^-/PI^-$); Q3 are cells initiating apoptosis ($FITC^+/PI^-$); while Q2 represents necrotic cells ($FITC^+/PI^+$) and Q1 are cell fragments containing nuclei acids.

When cells were dosed with only 672 for 24 hours, no obvious alteration in the subpopulation of $FITC^+/PI^+$, $FITC^+/PI^-$ and $FITC^-/PI^-$ was detected when compared to the DMSO control (Figure 2.7 top). This indicates that 672 may not have an effect on preventing cells from apoptosis. It is interesting to note that when cells were exposed to 300 μM hydrogen peroxide for 2 hours, the population of apoptotic cells ($FITC^+$) increased significantly, which is consistent with previous reported studies focusing on cell apoptosis induced by exogenous hydrogen peroxide (Qu X et al., 2014).

Moreover, fewer cells were detected undergoing cell death if they were treated with 672 for 24 hours before H_2O_2 exposure. The quantitative results are displayed in Figure 2.7 bottom, the columns show the percentage of cells undergoing apoptosis (positive with FITC) upon hydrogen peroxide treatment. It is obvious that the subpopulation of cells categorized in Q2 and Q3 is smaller in 672 pre-dosed wells compared to the DMSO control upon hydrogen peroxide treatment. This observation suggests that 672 can prevent cells apoptosis induced by exogenous H_2O_2. However, the population of early stage apoptotic cells ($FITC^+/PI^-$) was not altered much and only $FITC^+/PI^+$

cells were observed to decrease significantly. This might be caused by the enhanced permeabilization of plasma membrane brought by H_2O_2 treatment resulting in that PI diffuses through the membrane and binds to DNA hence increased the signal.

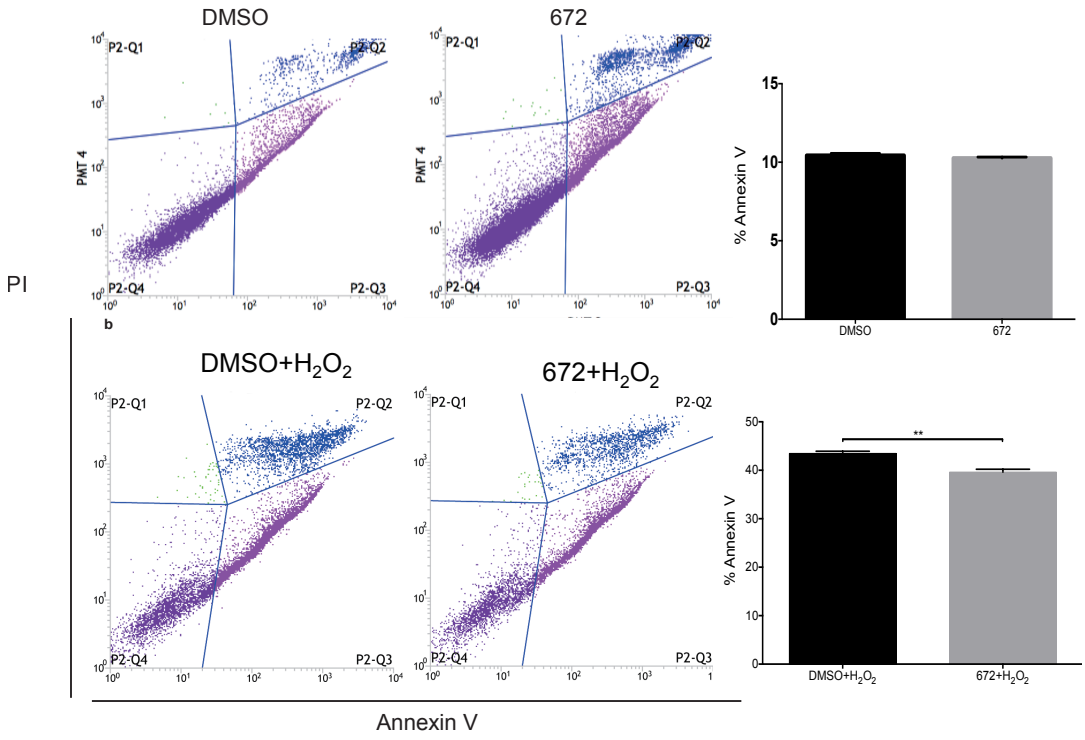
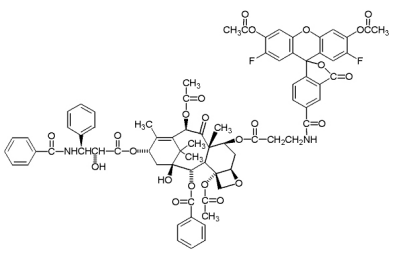


*Figure 2.6 Top: cells treated with DMSO (Left) or 672 (Right) do not show difference on the proportion of Annexin V-positive (P>0.5). Bottom: cells positive with Annexin V are reduced in 672 pre-treat wells in response to 300 μM H_2O_2 (Left) and quantified number (Right). ** P<0.01. n=3.*

3.3 Time-lapse microscopic analysis

Time-lapse microscopy is time-lapse photography applied to microscopy. Microscope image sequences are recorded and then viewed at a greater speed to give an accelerated view of the microscopic process. This technology allows the observation of any microscopic object over time. After this technology being adapted into cell research, it has been widely used in cell biology to observe artificially cultured cells studying the cell expansion,

morphological changing and polarization (Noctor SC et al., 2004). By analyzing the sequential time-lapse pictures, it is hoped that recording the time needed for the division of single cells could provide direct evidence supporting the idea that the selected compound might have positive effect on cell proliferation. After trying to locate a specific cell on the series pictures, indistinct boundary between cells and the location shift due to the cell expansion and mobility made it difficult to accurately track the repertoire process of cell division.



To improve cell identification, tubulin tracker was utilized to label the cytoskeleton of cells. Tubulin tracker is an uncharged compound (as shown on left) without fluorescence and can easily pass through the membrane of cells.

After incubating cells with tubulin tracker for 30 min allowing the dye getting into cells, the lipophilic blocking group in the compound will be cleaved by non-specific esterases in the cell and then become charged, which will show green fluorescence (Fig 2.7 Left).

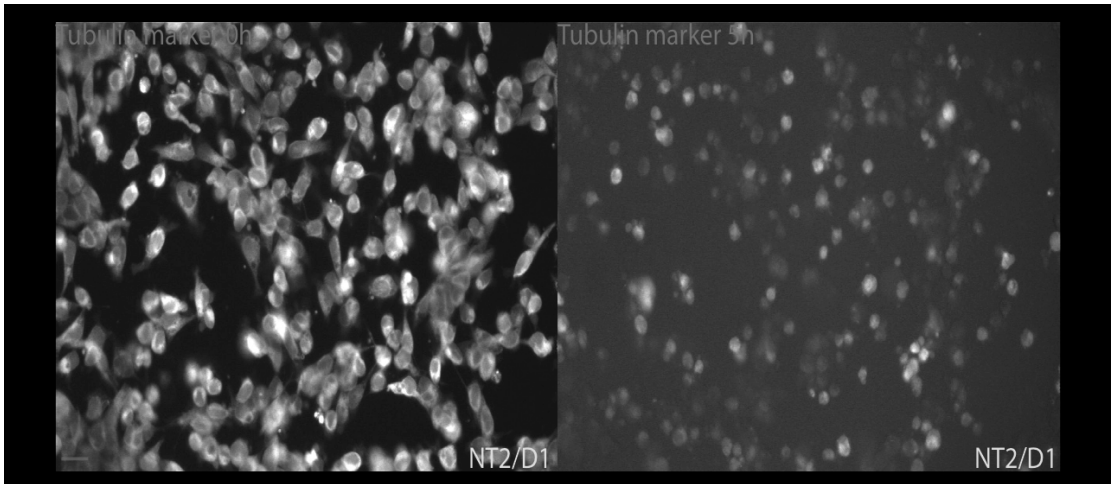


Figure 2.8 Tubulin Tracker (Life technology) labels polymerized tubulin green-fluorescent staining in live cells (left), which fades out after few hours incubation (right).

As shown in the picture (Figure 2.7 Left), cells could be recognized easily, which permits the tracking for cell division. However, it has been reported that mitosis can be disrupted when tubulin bound with Taxol, the main component of tubulin tracker (Díaz et al., 2003). Thus, as a consequence, cells were believed to be stopped at G2 phase during cell cycle. Nonetheless, Figure 2.1 (Right) shows that after 6 hours culturing in incubator, green fluorescence of tubulin marker was observed to have faded out. Taken all, time-lapse microscopy might be suitable for recording cell cycle of cells, but the method of live imaging still needs some improvement. And analysis evidently showing the doubling time difference in different culturing medium also require a stable marker for labeling cells.

3.4 Clonogenic assay on hES cells

One of the most important characters of pluripotent stem cells is having the capacity to self-renewal. Clonogenic assay allows a single stem cell to adhere

to the culturing matrix and expand to form colonies, which serves as a reliable model for analyzing if exogenous factors can alter the attachment or expansion of stem cells (Hannan S et al., 2013).

Though there is a possibility that our compounds can enhance the proliferation of EC cells in culturing condition, it is still not clear if some or all of these compounds work in a similar manner like Y27632 on promoting cell adhesion. Clonogenic assays are employed as a reliable test because it shows if the cells can survive and proliferate under duress, therefore it will be direct evidence if compounds share similar mechanism with Y27632.

Due to the high efficient attaching feature of EC cells in lab culturing comparing to hES cells, clonogenic assay performed on EC cells will be difficult to assess whether cell attachment or expansion are influenced by treatment of compounds. To specifically address this issue, human embryonic stem cells seeded as individual single cells were used in this assay to investigate the mechanism of action of 689 and 672. To avoid the possibility that cell clumps might attach and expand on feeder cells, hES cells were enzymatically dissociated with TrpLT into single cells, then washed with fresh medium and counted. Then same amount of single cell suspension was plated in medium with desired concentration of compounds or positive control Y27632 on 6 well plates coated with cellstart. Three different hES cell lines (mshef14, mshef 13 and mshef11) were tested using the same method, as different cell lines might behave differently upon chemical treatment.

The number of attached cells or small colonies formed following seeding using mshef14 is present in Figure 2.8. It is obvious that cell seeding was altered by dosing with most compounds, especially Y27632. Much more cells were seeded in Y27632 well could be seen after 16 hours following seeding, but not in the wells dosed with our compounds. In 672 or Cells attached are much less comparing to control well on 16 hours post seeding in 672 and 1173 wells (Fig 2.9 a1). Moreover, in medium containing both 1μM 689 and Y27632, cells can seed better suggesting that Y27632 play a dominant role in enhancing cell adherent (Fig 2.9 a1). When cells were pre-dosed with Y27632, seeding efficiency was improved dramatically (around five fold, Fig 2.9 a1 and b1). But 689 pre-treated cells do not show obviously enhanced seeding efficiency. However, 672 seems to have limited positive effect upon pre-dosing rather than during seeding (Fig 2.9 b1 and b2). Seeded cells maintaining in medium containing compounds can expand better than control group because more newly formed small colonies were observed in 689, 1170 (an analog of 689) or 672 wells (Fig 2.9 a1). This finding was not observed in pre-dosed cells seeded in fresh medium (as method 2.4; Fig 2.9 a2 and b2). Though seeding efficiency could be improved by being dosed with Y27632, cells are believed to have problems to expand if Y27632 was persistently present after seeding, since no more colonies form up after over-night seeding. Above all, Y27632 was shown to promote cell-matrix adherent and enhance single cell seeding. Our compounds did not show similar influence on seeding, but promoted cell colony expansion following cell attachment.

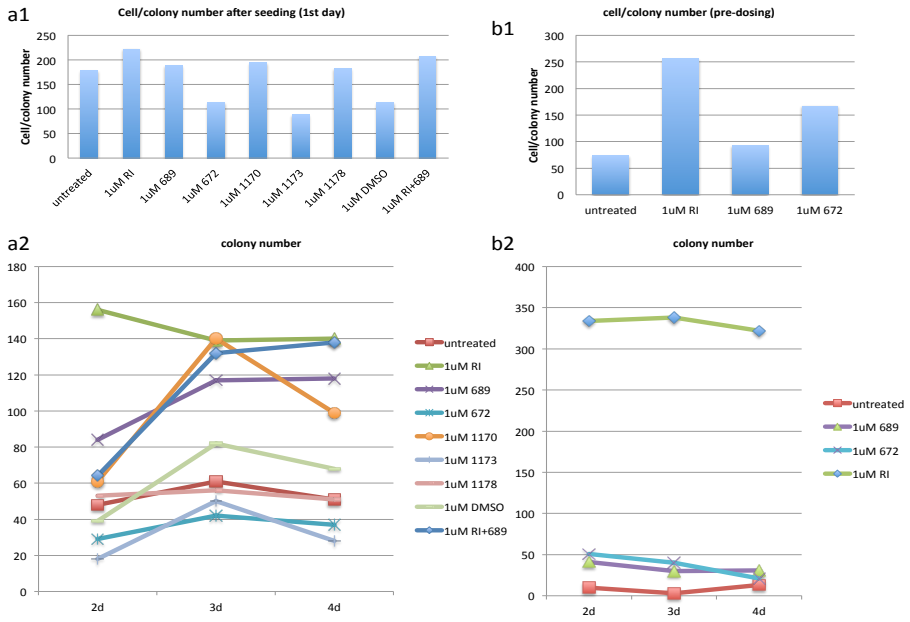


Figure 2.9 Clonogenic results of mshef 14 cells: a1 & a2: number of cells or colonies attached 16 hours after seeding in medium contains compounds and number of colonies following attachment. b1 & b2: number of cells or colonies attached 16 hours after seeding in fresh medium (pre-dosing in suspension for 3 hours then washing with PBS) and number of newly formed small colonies observed following attachment.

To confirm this finding, cell clumps were mechanically cut by pipette tip and seeded on cellstart-coated plates containing medium with compounds. After feeding with medium containing the selected compounds or Y27632, each colony was imaged everyday following seeding (Fig 2.10). Image J was utilized to assess the size (handy select the colony along the edge and read out the area) of colony during expansion. By comparing the ratio of cell colony enlargement after 48 hours dosing, it seems that colonies are easier to expand when being treated with 689 and 672 than Y27632. However, t-test does not suggest there is an obvious difference between the increased ratio of control and 689 cells, which might be due to variability brought by the irregular shape of colonies (Fig 2.10).

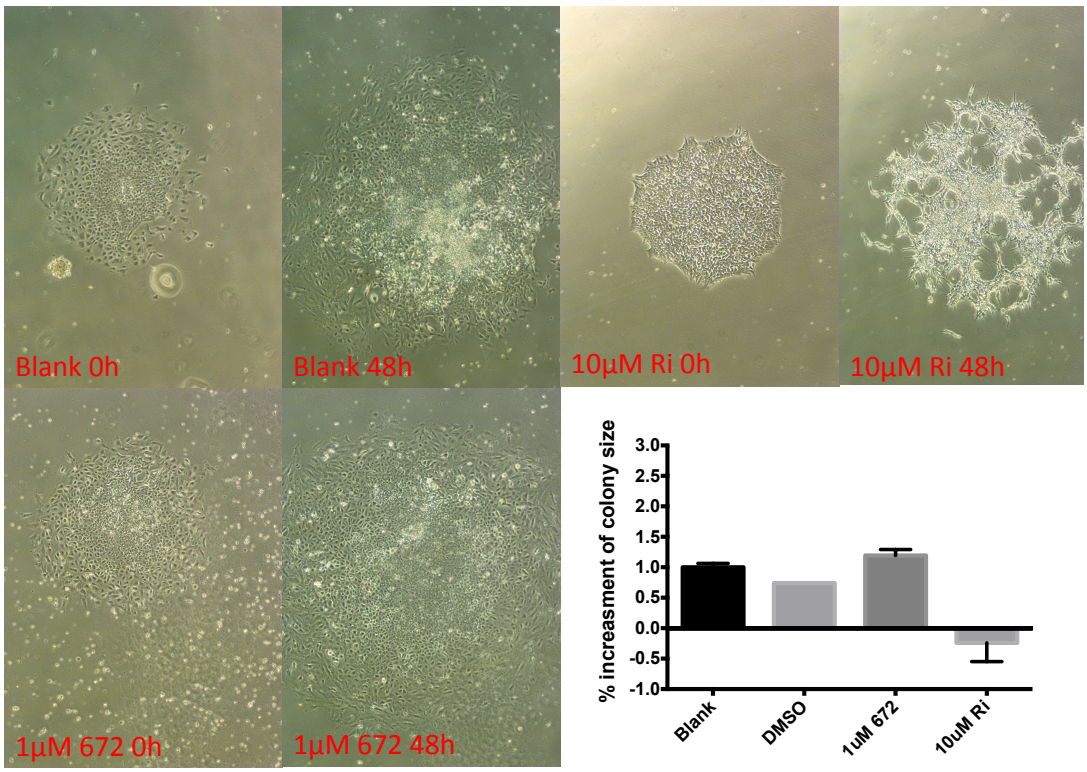


Figure 2.10 Pictures of the same cell colony before and after being treated with compounds. Columns represent the ratio of size changing of cell colony in different conditions. Significant analysis suggests there is no difference between control and treatment (P>0.05, n>3).

Mshef 13 was observed to be very sensitive to Y27632 (Fig 2.11). Although the cell seeding could be enhanced greatly as above, they have serious problems on forming colonies if Y27632 persistently present after seeding, and a large amount of cells were observed to round up under microscopy (possible dead) after 40 hours maintaining in medium with Y27632. Moreover, 689 and 1170 were also found to have no effect on cell attachment but have profound impact on the colony formation after adherent. 672 did not show influence on mshef 13 as there is no difference of number of cell colonies was found between 672 and control wells.

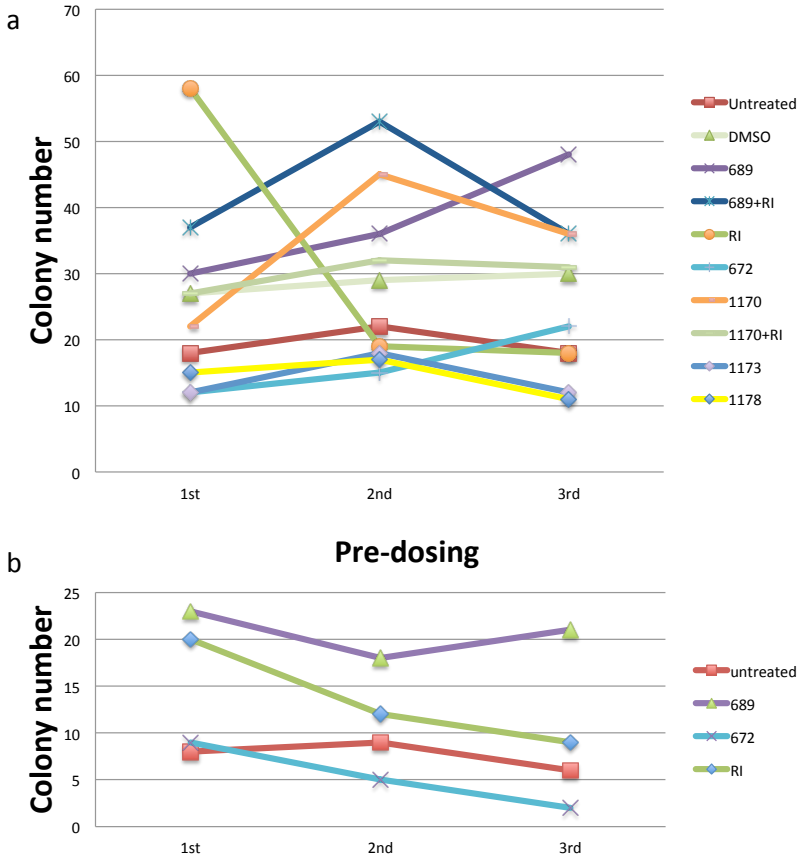


Figure 2.11 Clonogenic *results of mshef 13 cells: number of newly formed small colonies observed after seeding in medium contains compounds (a) and fresh medium (pre-dosing, b).*

Mshef 11, as the most reliable clinical level hES cell line in our lab, can be routinely cultured and passaged on cellstart coated flask and maintains all pluripotent features. When a single cell suspension was plated into medium, no enhancement was found on seeding in 689, 672 or 1170 wells but in Y27632 well (Fig 2.12 a-c). After being seeded, cells forming colonies are easier in the present of 689 and 672, which suggests that 689 and 672 may benefit the expansion of cell colony (fig 2.12 a & c). In competition assay, 689 and 672 are thought to act in different mechanism of Y27632 since fewer cells attached onto plates in 689/Y27632 and 672/Y27632 co-dosed wells in

contrast to Y27632 only (Fig 2.12 b). Given no obvious difference was detected between 689, 672 and 689/672 group, it is possible that 689 and 672 might work similarly when promoting colony expansion.

In addition, the pluripotency of stem cells upon the treatment with small-molecule compounds were also assessed. Tra-1-60 antibody was applied to cells after culturing with medium containing compounds for 4 days (after numbering the number of colonies), followed by Alex488 conjugated secondary antibody staining. 10 colonies in each well were imaged. It was noted that when cells treated with Y27632 continuously, they can attach better but quite a lot colonies formed did not show stem-cell characters. This finding is promised with previous report that Y27632 is able to induce differentiation (Hotta et al., 2009). Most colonies in control and 689 or 672 well were positive for Tra-1-60 but not in Y27632 wells after 5 days treatment (Fig 2.12 d). As Tra-1-60 expression will be decreased when cells undergoing differentiation, we assume that 689 and 672 might help hES cells expanding and maintaining pluripotency.

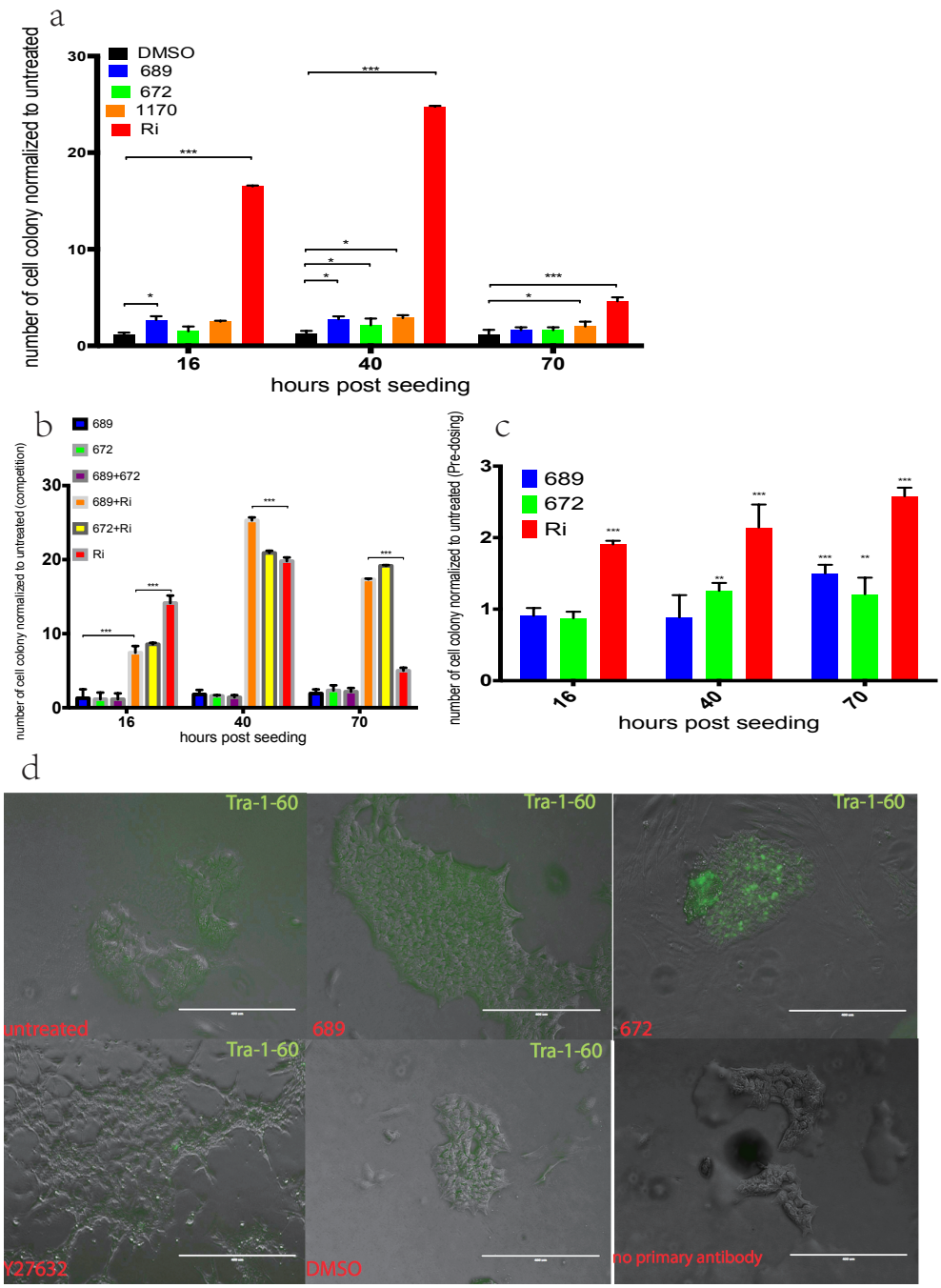


*Figure 2.12 a-c) number of newly formed small colonies observed in corresponding conditions normalized to untreated condition * P<0.05; ** P<0.01; *** P<0.001. n=3; d) fluorescent images of cell colonies stained with Tra-1-60 (green).*

3.5 Quantitative real time polymerase chain reaction (qPCR)

To analyze if the expression level of any interest genes could be altered by dosing cells with the selected compounds, EC cells cultured in medium containing desired concentration of compounds were suspended and the same amount of cells was used for isolating RNA. After testing primers of each interest gene and a smooth standard curve was gained as described in method 2.5, few vital genes involved in cell proliferation and cell programed death marker (apoptosis). Also oxidative stress modulations were analyzed for hunting possible target signaling. Total RNA of $4x10^3$ EC cells dosed with DMSO or compounds for 2 days was extracted and analyzed as described in methods 2.5. Each experiment was performed triplicated from RNA isolation.

During a cell cycle, Proliferating cell nuclear antigen (PCNA) was detected to be expressed in most cells when they entering S phase. As a widely used marker for cell proliferation, PCNA has demonstrated to be directly involved in DNA replication (Lee and Gye, 1999). As shown in Figure 2.12, relative level of PCNA was analyzed against GAPDH (house keeping gene) and no altering was detected by treating cells with selected compounds.

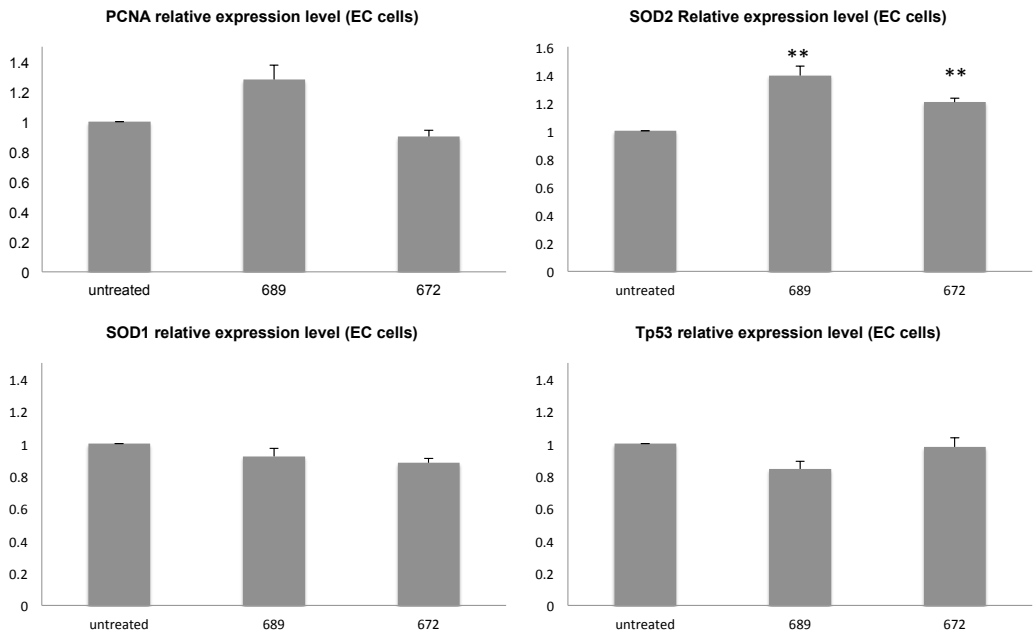


*Figure 2.13 Data shows the results of quantitative real-time PCR analysis of the relative expression level of PCNA, SOD1, SOD2 and tp53 normalized to the level of GAPDH in response of 1 μ M 689 or 672 treatment. ** P<0.01. n>3.*

It was suggested by the separate study that 689 might be able to prevent cell damage through up-regulating SOD2 in human mesenchymal stem cells (Mohanty et al., 2012). Relative SOD1, SOD2 and tp53 expressing level were analyzed to answer the question if function of compounds is relative with oxidative stress or cell death prevention. The results suggest SOD2 gene is believed to be upregulated by 689 (which is promising with previous study) and 672. But the level of the other two genes was shown unchanged (Fig 2.12).

4. Summary

In the initial assays using EC cells, the hit compounds 689 and 672 are believed to have the abilities to significantly increasing the cell number. Small molecules were then dosed onto hES cells and the results confirmed that 689 and 672 are able to increase the cell colony number during the treatment. When using PI staining in cell sorting, no significant difference was found among any proportions of cell cycle phases upon the treatment with compounds. But EdU incorporation and apoptosis analysis revealed that 672 could boost the proliferation of EC cells, as well as preventing cell death induced by exogenous hydrogen peroxide. Although SOD2 relative expression was detected to be up-regulated by 689 and 672, it is still not clear if SOD2 is the molecular target for compounds.

Moreover, it is also found that 689, 1170 and 672 can benefit cell colony formation after single cell seeding on cellstart coated plates. Due to the fact that the influence of compounds can be only detected when compounds were added in culturing medium, which suggests the mechanism these compounds act might be involved in the process of oxidative hemostasis modulation rather than cell adherent. Since compounds do not share similar functions as Y27632, it is still necessary to explore the possible target signal pathway of these compounds.

III. *In vivo* screening

The fish caudal fin is a widely employed experimental model for studying appendage regeneration in vertebrate animals. The structure of the caudal fin in adult fish consists of fibroblasts coupled with segmented bony fin rays (Akimenko et al., 2003). Following amputation, blastema will be formed near the tip of fin rays and cells in the region will proliferate to replace the missing ones. Similarly, epithelial cells around the stump will contract and seal the wound in zebrafish larvae. From 3-48 hours post amputation, proliferating cells within the region adjacent to the stump will provide cells that needed for fulfilling the amputated part of fin fold (Yoshinari and Kawakami, 2011). Given that the larvae fin regeneration needs shorter time than adult fish and it is easier to set the same amputation location on fish larvae (pigment gap), here we used larvae instead of adult fish to reveal the possible influence the compounds may have upon the progress of fin regeneration.

1. Materials

Fertilized eggs were obtained from wild type (AB) zebrafish (University of Sheffield, UK) for all of the experiments.

Chemicals used are the same as described in the previous chapter and information for all reagents used is shown in Appendix.1.

2. Methods

All embryos were raised and used in the laboratory according to standard procedures (Westerfield, M., 2007). Details for each experimental protocol are described below.

During washing steps in staining assays, samples were kept in corresponding buffer or reagents in eppendorf tubes and the tubes were left on a horizontal rotator at room temperature.

2.1 Alcian blue staining and toxicity test

Zebrafish eggs were collected then kept at 28 ^{o}C in plastic petri dishes, each containing 20mL E3 medium (45 eggs per dish). After 48 hours, 40 fish embryos were transferred into fresh E3 medium containing different concentrations of the selected compounds or DMSO, in the incubator at 28 ^{o}C. The number of live embryos was counted every 24 hours (dead embryos were removed when checked every 6 hours by poke testing with tweezers for mobility and reaction).

Alcian blue staining was performed using the protocol as previously described (Neuhauss et al., 1996). Larve at 3dpf were treated with compounds for 24 hours and then stored in 100% methanol at -20ºC until use. Following several washes with PBST, samples were stained overnight in stain solution (5 mL 10M HCl, 0.5 g Alcian Bule, 495 mL H_2O). After rinsing several times using fresh PBST, samples were bleached by the bleaching solution (0.5 mL 30% H_2O_2, 5 mL 10% KOH, 44.5 mL H_2O) for 0.5 hour, at 37^{o}C. Embryos were

then digested with 30% saturated borate (18 mL saturated borate, 32 mL H_2O, 25 mg trypsin) for 30 min at 37^{o}C until the brain was clearly visible. Samples were then rinsed with PBST and 50% glycerol and stored in 100% glycerol. Following imaging, comparisons for size and morphology of cartilage in the head of each treated larva were made with controls.

2.2 Efficacy of small molecules in fish fin regeneration

In this *in vivo* screening, the caudal fin regeneration of zebrafish larvae was used as a model of tissue regeneration. Fish larvae were collected and amputated at 48 hours post fertilization.

2.2.1 Fin amputation of zebrafish larvae.

45 larvae were anesthetized using tricaine (2 mL in 20 mL E3 medium; Tricaine methanesulfonate, Sigma) in a plastic petri dish. After incubating at room temperature for 5 minutes, all fish were checked for being fully anesthetized. At a time, 10 larvae were placed on a strip of masking tape stuck to the bottom of a petri dish lid with minimum medium, using a plastic pipette. Using a microsurgical stab blade (5.0 mm, Surgical Specialties corporation) amputation was performed, in the pigment gap near the caudal fin fold, under a Leica SP3 dissection microscope (Fig 3.1).

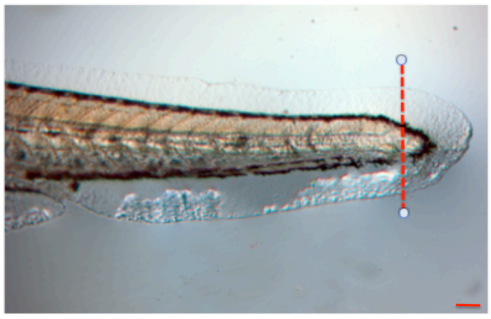


Figure 3.1 Site of fin amputation used in this project. Amputation (red dashed line) was performed in the pigment gap and all efforts possible were taken to ensure amputation was performed in the center of the gap. Scale bar 50 μm.

Few drops of fresh E3 medium without Tricaine were added onto the larvae following amputation. Then, all larvae were transferred to fresh E3 medium containing dissolved compounds or DMSO, in new petri dishes, which were then stored in a 28°C incubator until the next experimental step.

2.2.2 Fixation and storage.

Once the designated time point was reached following amputation and treatment, larvae were anesthetized using tricaine as above. They were then transferred into 1.5 mL eppendorf tubes (Treff Lab, Switzerland) with a minimum amount of E3 medium. Then, 500 µL of 4% paraformaldehyde (PFA) in PBS was added into each tube. Larvae were fixed in PFA overnight at 4^{o}C. After fixation, they were subjected to a methanol series in PBST (one wash in 30% & 60% methanol and 3 washes in 100% methanol). Larvae were then stored in 100% methanol at -20°C for storage up to a year.

2.2.3 Sample imaging and measuring the length of fin regrowth

Fish larvae were amputated, treated with compounds and then anesthetized as described above. Each larva was then mounted on a glass slide and covered by glass cover slip (20x20mm; Scientific Laboratory) with four pieces of plasticine stick in four corners for avoiding samples being squashed. Samples were then imaged under a dissection microscope coupled with a ProgRes C14 camera. Captured images were imported into the ProgRes C14 imaging software, and balance and exposure were adjusted for the best fitness. For recording the length of the regrown fin, a rectangle was drawn

from the anus to the distal point of the fin on the image. The width of the rectangle was recorded as the length of fin (Fig 3.2).

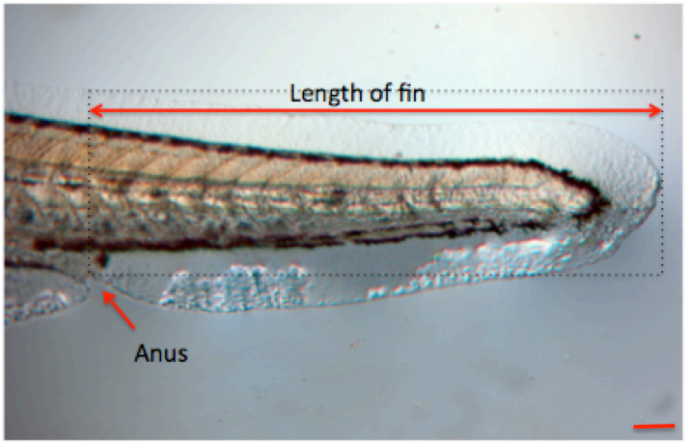


Figure 3.2 A rectangle was draw from the anus to the distal point of the fin, and the width of rectangle was recorded for comparing. Scale bar 50 μm.

2.3 Exploring for mode of action of selected compounds

2.3.1 Immunohistochemistry

Following amputation in pigment gap, larvae (at least 40 in each group) were treated in E3 medium containing compounds or DMSO. Then, all samples were fixed at 6, 12, 18, 24, 36 or 48 hours post amputation in 4% PFA in PBS, overnight at 4°C. This was followed by two washes in PBST for 5 min each and one wash each in methanol/PBST (30%, 60% and 100%) for 10 min at RT. Once samples turned pink (after being maintained for 2 hours in 100% methanol) they were transferred into fresh 100% methanol and kept at −20 °C.

Larvae were then rehydrated by subjecting them to the reverse methanol in PBST series (100%, 60% and 30%) and 5 washes in PBST. Then, the samples were digested with 10 µg/mL Proteinase K at RT for 9 min. Proteinase K action was stopped by refixing the larvae with 4% PFA for 20 min at RT followed by 4 washes in PBS for 5 min each at RT. Larvae were blocked in block solution (PBST with 10% NBCS) for 2 hours at room temperature, and then incubated overnight in 1 mL of fresh block solution with Anti-Phosphorylate Histone H3 antibody (PHIS, 1:200; Calbiochem, Germany) or Anti-Caspase 3 antibody (1:500; Abcam) at 4°C.

Following the overnight incubation with the primary antibody, samples were briefly rinsed in PBST, followed by 6 washes in PBST, for 20 min each. Samples were then incubated in block solution, for 2 hours, as described above. They were then incubated overnight in fresh block solution with Anti Rabbit ALEX-488 antibody (1:1000; Invitrogen) or alkaline phosphatase Anti-rabbit antibody (1:1000; Vector laboratory) at 4°C.

Once again, following overnight incubation samples were briefly rinsed in PBST, followed by 6 washes in PBST, for 20 min each and then fixed in 4% PFA, for 30 min. After rinsing once more in PBST for 10 min, samples were stored in Vectashield with DAPI (for Anti- Phosphorylated Histone H3 antibody staining), or following the staining procedure for whole-mount *in situ* hybridization described in 2.3.4 (for Anti-caspase-3 antibody).

2.3.1.1 Imaging for antibody staining.

In Anti-Phosphorylate Histone H3 antibody staining, samples (20 embryos were taken out randomly from each group for imaging) were mounted for imaging as detailed above and imaged on the Olympus FV1000 with SIM-scanner on a BX61 confocal microscope. Images were acquired using two-channel imaging with multiple Z points. Laser lines of different wavelength were used to excite specific probes: 488 nm laser for ALEXA 488 and 405 nm laser for DAPI. Exposure time, image sharpness and grey levels were adjusted to reach the best fitness. 10-15 Z points (maximum between points was 4 nm) imaging program were performed on each sample. All images were then transferred into the Image J software (open source) to create an extended focus projection of the Z stack for analysis.

In anti caspase-3 antibody staining, imaging was performed as in 2.2.3.

2.3.1.2 Collection and analysis of PHIS data

To analyze the fluorescence images, a particle analysis program in the Volocity software (PerkinElmer Inc) was used. All particles (fluorescent dots) within the 300μm wide rectangle (drawn from the distal point of caudal fin, as shown in figure 3.3) were measured, and the number of green dots in areas ranging from 50 to 3000 μm^2 was counted for comparing.

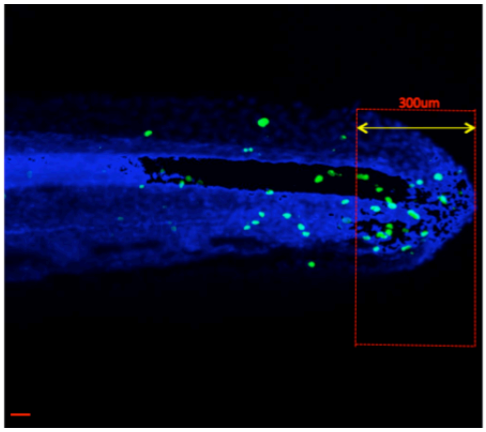
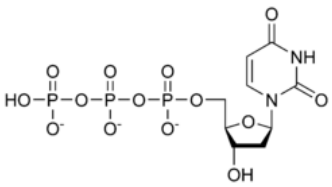


Figure 3.3 All particles (green) within the 300 μm wide rectangle (drawn from the distal point of caudal fin were measured, and then the number of green dots were counted for comparing. Scale bar 50 μm.

2.3.2 *In situ* apoptosis (TUNEL) detection during fin regeneration

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is used for detection and quantification of apoptotic cells. Cleavage of genomic DNA during apoptosis will cause double stranded as well as single stranded DNA to break, which can be characterized by labeling free 3'-OH terminal with modified nucleotides dUTP (structure as shown below) in an enzymatic (Terminal deoxynucleotidyl transferase, TdT) reaction. Due to the high specificity in the binding of TdT to the breakdown fragments of DNA, only cells undergoing programmed cell death will show the 3'-OH end, therefore can be labeled with fluorescent-conjugated dUTP in tissues. This allows this technique has been widely used as programmed cell death marker in *in vivo* studies. In zebrafish, TUNEL staining is also reported to successfully label the apoptotic cells in different organs (Barbosa J et al., 2015).



Larvae at 48 hpf were dechorionated. Following amputation in pigment gap as described above, larvae (10 in each group) were treated with E3 medium

containing compounds or DMSO. Then, all fish larvae were fixed at 6, 12, 24 or 48 hours post amputation in 4% PFA in PBST overnight at 4°C. They were then washed twice in PBST for 5 min each and one wash in methanol/PBS (30%, 60% and 100%) for 10 min at RT. Once samples turned pink (after being maintained for 2 hours in pure methanol) they were transferred into fresh 100% methanol and kept at −20 °C.

Larvae were then rehydrated by subjecting them to the reverse methanol in different concentrations in PBST (100%, 60% and 30%) and 5 washes in pure PBST. Then, the samples were digested with 10 µg/mL Proteinase K at RT for 10 min. Proteinase K action was stopped by refixing samples with 4% PFA for 20 min at RT followed by 4 washes in PBS for 5 min each at RT. Larvae were permeabilized in acetone/ethanol (1:2) for 7 minutes at −20°C, washed in PBST three times for 5 min each and then run through Apop fluorescein *in situ* apoptosis detection kit (S7110 Millipore, USA) following the protocol recommended by the manufacturers. Samples were then stored in Vectashield with DAPI.

2.3.2.1 Imaging for TUNEL staining.

Samples (15 larvae were taken out randomly from each group for imaging) were mounted for imaging as described above and imaged on the Olympus FV1000 with SIM-scanner on a BX61 upright confocal microscope. Two-channel imaging with multiple Z points was used for the acquisition of the images. The fluorescence probe was excited using a 488nm laser line, and the DAPI counter stain was excited using 405 nm laser line. Exposure time,

gain and grey levels were adjusted in order to achieve the sharpest image quality. Z stacks were captured using 5-20 Z points with a minimum distance between of 2 nm. Once captured, images were imported into the Image J software (open source) to create an extended focus projection of the Z stack for analysis.

2.3.2.2 Data collection and analysis

To perform the analysis, Volocity software (PerkinElmer Inc) was used using particle analysis software. All particles were measured, and then the number of green dots in areas ranging from 50 to 1000 μm^2 was counted.

2.3.3 Exogenous hydrogen peroxide treatment

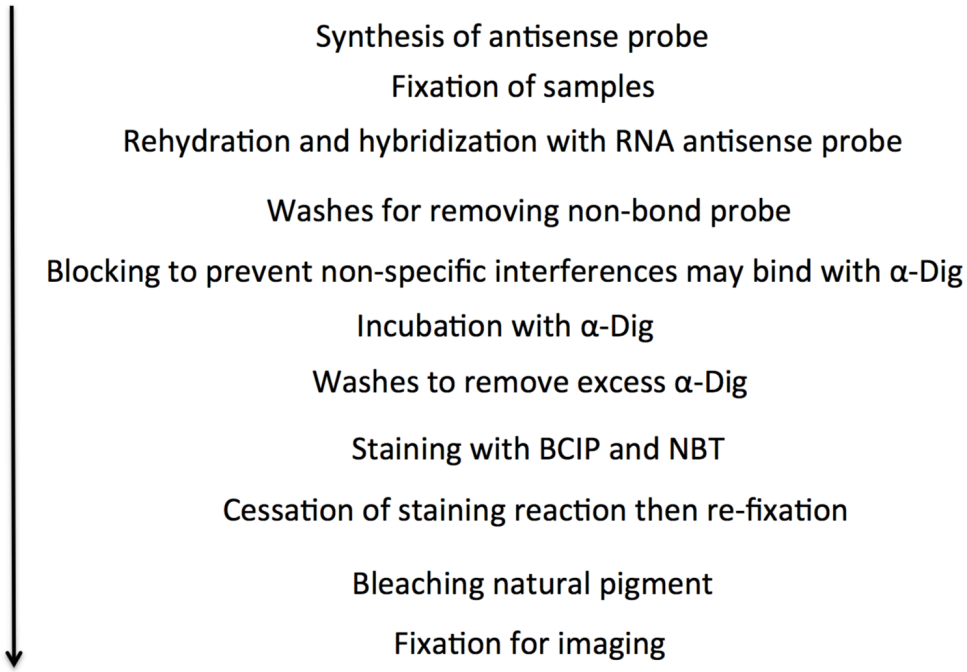
20 fish at 48 hpf were treated with E3 medium containing the selected compounds or DMSO for 6 hours. Then, hydrogen peroxide (Fisher scientific, USA) was added into the medium to the desired concentration for pre-treatment assay. In the rescue assay, increasing concentration of hydrogen peroxide was put in together with the selected compounds or DMSO. After 6 hours, samples were anesthetized and fixed following the protocol mentioned above. Imaging and analysis were done following the same protocol as in 2.3.2.

2.3.4. Whole-mount in situ hybridization

Whole mount in situ hybridization (ISH) was successfully applied to zebrafish in 2008 (Thisse et al., 2008) and remains one of the most robust methods to analyze gene expression in tissues (Rattner et al., 1978). The principle of ISH is based on the affinity between an antisense RNA to the mRNA sequence from the gene of interest (Jin and Lloyd, 1997). During the fixation of samples, mRNA from the sample is retained, which permits the binding of antisense RNA probe to the mRNA in the cells from where this gene was originated. During the probe synthesis, the reaction was supplied with Dig tagged NTPs, which allows it to bind to the target mRNA in the sample and to be recognized by the anti-Dig antibody. The alkaline phosphate conjugated with the antibody can react with the staining reagents BCIP (5-Bromo-4 chloror-3'-indolyphosphate p-Toluridine salt) and NBT (Nitro-Blue Tetrazolium), which form a blue precipitation in the cells where the target gene was located. Thus, after being incubated with antisense RNA probe, anti-Dig antibody and then the staining reagents, cells could be imaged. From the images, blue staining in the samples can show where and how much the gene of interest was.

2.3.4.1 Procedure of *In Situ* hybridization (ISH)

The protocol for ISH experiments can be described using the following flow chart:



2.3.4.2 Antisense RNA probe synthesis

To synthesize the anti-sense RNA probe, the DNA fragment containing the gene of interest was copied out from the cDNA of tissue samples in a PCR reaction. This DNA sample was then transcribed to RNA tagged with T3/T7 using designed primers. Primers were designed using Primer 3 software or used reported ones as shown in Appendix.2. A solution containing the DNA template diluted in nucleic acid-free water, Transcription buffer DIG labeled NTP mix (Boehringer), RNAse inhibitor (Promega) and appropriate polymerase (T3 or T7) and primers were incubated for 2 hours at 37°C. Following this, DNA was removed by incubating with RNAse-free-DNAse at 37°C for another 30 minutes. Products were then filtered through a sigma spin filter and dissolved in 14 µL of RNAlater solution (Sigma, USA), which was used as the RNA probe. Electrophoresis was performed after each PCR reaction to make sure each transcription was successful.

2.3.4.3 Washing, incubation and staining of ISH

Embryos (10 in each group) at 48 hpf were mechanically dechorionated. Following amputation in pigment gap as described above, larvae (10 in each group) were treated with E3 medium containing compounds or DMSO. Then, all samples were fixed at 6, 12, 24 or 48 hours post amputation in 4% PFA in PBST overnight at 4°C. They were then washed twice with PBST for 5 min each and one wash each in methanol/PBS (30%, 60% and 100%) for 10 min at RT. Once samples turned pink (after being maintained for 2 hours in pure methanol) they were transferred into fresh 100% methanol and kept at −20 °C.

Larvae were then rehydrated by subjecting them to the reverse methanol in PBST series (100%, 60% and 30%) and 5 washes in PBST. Then, the samples were digested with 10 µg/mL Proteinase K at RT for 10 min. Proteinase K action was stopped by refixing with 4% PFA for 20 min at RT followed by 4 washes in PBS for 5 min each at RT. Samples were then pre-hybridized for 2 h with the hybridization solution (+) without probe at 70°C for blocking unspecific binding. Following this, samples were transferred into 200µL of fresh hybridization solution (+) containing 1-4 µL of anti-sense RNA probe in each tube. Tubes were left overnight at 70 °C for hybridization.

Samples were then washed for 10 min each, at 70°C, using the 2xSSCT/ hybridization solution (-) series (25% 2X SSCT/75% Hyb-, 50% 2X SSCT/50% Hyb-, 75% 2X SSCT/25% Hyb-) for removing unbound probes. After washing once with 2xSSCT for 10 min and twice with 0.2xSSCT, at 70 °C for 30 min, samples were cooled down to RT. This was followed by washing through the

PBST/0.2xSSCT series (25% PBST/75% 0.2X SSCT, 50% PBST/50% 0.2X SSCT, 75% PBST/25% 0.2X SSCT) at RT for 10 min each. After this, samples were washed twice in PBST, at RT. After incubating for 3 hours at RT in blocking buffer containing BSA and sheep serum to avoid non-specific binding of antibody, samples were transferred to fresh blocking buffer containing anti-DIG antibody (1:10000; Roche, USA) and left overnight at 4°C.

Then, after a brief wash with PBST, samples were washed a further 6 times in PBST for 15 min each and then incubated in staining buffer for 1-3 hours at RT. Staining was stopped by rinsing in PBST with 1mM EDTA for 15 min then re-fixed in 4% PFA for 30 min. Following the methanol/PBST series (30%, 60% and 100%) washing, samples were maintained in 100% methanol for 2 hours at RT. After reversing the methanol/PBST series (100%, 60% and 30%), embryos were washed in PBST twice for 5 min each and bleached by bleaching solution (hydrogen peroxide) up to 10 min at 37°C. Then samples were washed in PBST three times for 5 min each and soaked in 50% glycerol for 10 min. Finally, samples were stored in 100% glycerol in dark. 10 Embryos from each group taken out randomly were imaged on microscopes as detailed in sections 2.2.3.

2.3.5 Quantitative real time polymerase chain reaction (qPCR)

Zebrafish embryos at 48 hpf were dechorionated then divided into four groups (20 in each). All fish were kept in E3 medium containing the selected compounds or DMSO at 28^{o}C. After 12 hours, all smaples were anesthetized in tricaine, and then washed twice in PBST. Total RNA from 15 larvae in each

group was extracted and analyzed using the protocol described in previous chapter section 2.5.

3. Results and Discussion

3.1 Toxicity screening

By observing the morphology and reaction upon physical stimulation, it is possible to find out if small molecule compounds have an effect on the health of fish larvae. After 48-hours fertilization, fish embryos were transferred into fresh E3 medium with different concentrations of compounds or DMSO in an incubator at 28°C and checked every 12 hours for their mobility and reaction by poking with tweezers. The number of healthy embryos was recorded every 24 hours, as shown in Figure 3.4.

3000165

Liveability	1.11uM	3.33uM	10uM
24h	100%(42/42)	91.1%(41/45)	0%(0/42)
48h	100%(42/42)	88.89%(40/45)	/

3000689

Liveability	1.11uM	3.33uM	10uM
24h	100%(43/43)	93.3%(42/45)	88.6%%(39/44)
48h	100%(43/43)	86.67%(39/45)	81.8%(36/44)

3000432

Liveability	1.11uM	3.33uM	10uM
24h	100%(42/42)	/	100%(42/42)
48h	95.2%(40/42)	/	100%(42/42)

3000672

Liveability	1.11uM	3.33uM	10uM
24h	100%(41/41)	/	100%(41/41)
48h	100%(41/41)	/	100%(41/41)

Control

Liveability	1st	2st
24h	100%(22/22)	100%(41/41)
48h	100%(22/22)	97.56%(40/41)

Figure 3.4 Number of healthy fish larvae upon chemical treatment.

3000165 showed toxicities on zebrafish larvae in the concentration above 1.1 μM by both visual (bended body) and physical inspection (can not swim away upon tweezers poking) as described in methods 2.1. The larvae treated with 165 at 1 μM were observed with bended body and no fish larvae could survive at a concentration of 165 higher than 10 μM. However, the other three compounds are believed to be safe to larvae since all larvae developed normally as those in control group and no obvious abnormalities were detected.

3.2 Alcian Blue staining

Sulphated proteoglycans (PGs) are secreted from chondrocytes during chondrogenesis (Neuhauss et al., 1996). The polysaccharide chains of PGs is a highly negative charged molecule due to the presence of sulphate and carboxylic groups in uronic acids. Alcian blue, a cationic dye, can form strong electrostatic interactions with the negatively charged polysaccharide, therefore, is widely used for staining cartilage and bone system in animals.

During its development, the skeleton of the pharyngeal cartilage of zebrafish, is amenable to staining by Alcian blue after 3 dpf (Fig 3.5), which makes this method suitable for studying early development of zebrafish. From the image taken, it is assumed that 432, 689 and 672 do not have an influence on the cartilage development since no obvious size difference was observed in the cartilage of the jaw between treated and untreated fish. On the other hand, 165 was also shown to be toxic to zebrafish because the size of cartilage was observed to be smaller in the 165 treated group when compared to control

(Fig 3.5). Thus, it would be necessary to exclude 165 from further *in vivo* screening.

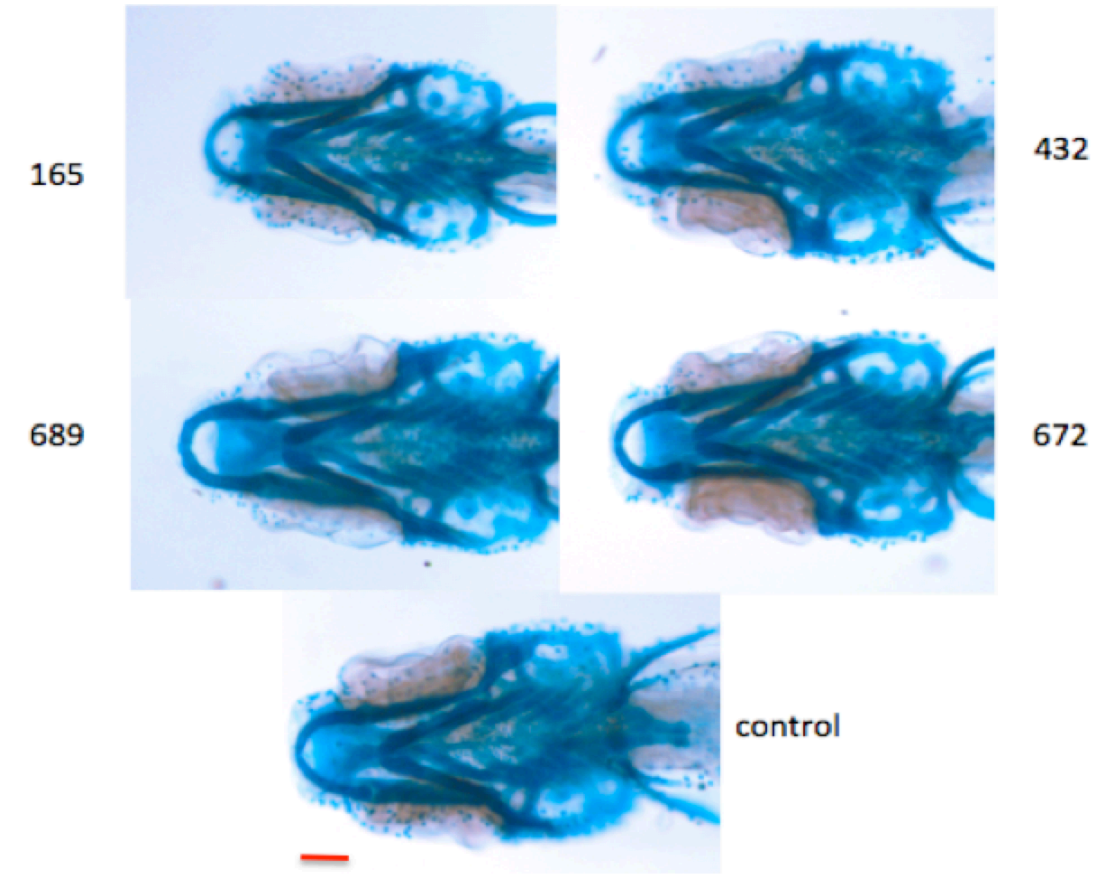


Figure 3.5 Comparison of head length following compound treatment. Alcian blue staining shows that the size of cartilage in zebrafish larvae is not influenced by treatment with compounds (except 165). Scale bar 50 μ m.

3.3 Comparison of Length of regrown fin

In zebrafish larvae, following amputation, it was observed that epithelial cells around the stump contracts to seal the wound. From 3-48 hours post amputation, cells within the region adjacent to the stump proliferated to provide cells needed for regenerating the amputated part of fin fold. The origin of cells that form the blastema is still unclear. It has been various speculations

for that including a hypothesis that blastema cells may represent only a single cell type or multiple cell types that have the potential to form all cell types of the fin (Tu and Johnson, 2011; Tornini V & Poss KD, 2014). However, it is well accepted that the regeneration of caudal fin after amputation is blastema-dependent. As our compounds showed a profound effect on promoting stem cell proliferation, zebrafish larvae at 48 hpf were amputated in pigment gap then treated in E3 medium with desired concentration of compounds to test if compounds have any effect on fin regeneration. During the treatment, it was quite interesting to consistently observe longer fins in some chemical-treated groups. The length of regrown fin was measured as the protocol described in methods 2.2.

For non-biased results, all sample tubes were re-labelled with randomly coded name by Dr. Henry Roehl before images were taken then decoded after measurement. The results are presented in Figure 3.6. The average length of regrown fin in each group (n>40) is represented by columns.

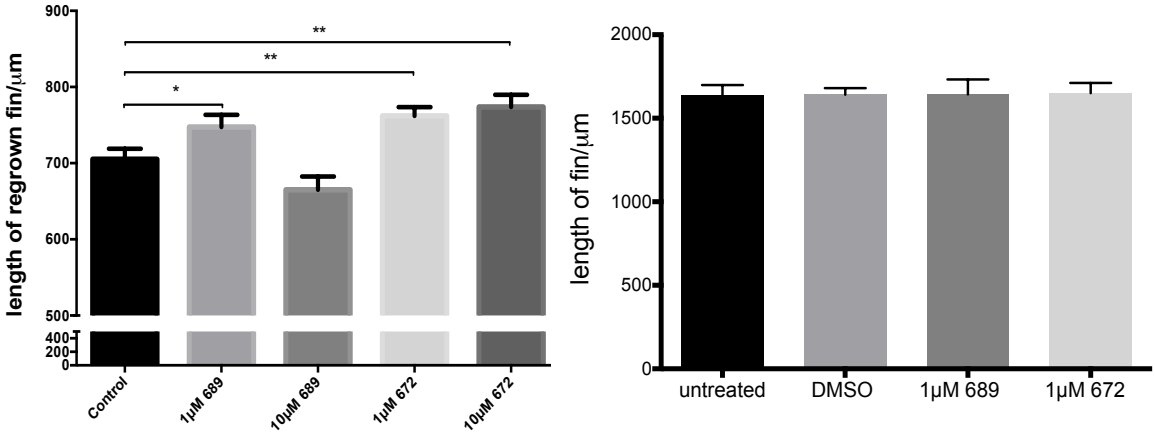


*Figure 3.6 Left: length of fin regrowth in zebrafish after treating with compounds following amputation in pigment gap. Stastical significance is determined using one-way ANOVA versus control. *<0.05; ** p<0.01. n>40. Right: length of fin was not observed different between untreated and treated fish larvae (P>0.05. n=15).*

One-way ANOVA significance analysis suggested that 672 and 689 might have a positive influence on fin regeneration. When fish were treated with 672 after amputation, *p* values of both 1 and 10 μM dosing groups results normalized to control group are smaller than 0.05. This indicates that the fins were more effectively regenerated in 672-treated group than the DMSO control. Similar observations can be also seen when fish were treated with 689 in 1 μM (Fig 3.6). Overall, it is exciting to note that 689 and 672 both show positive influences on fin regrowth. However, since there have not been any chemicals reported to benefit the regrowth of caudal fin in zebrafish larvae, if these compounds directly improved the fin regrowth or not still require further investigation. Also, to address whether the improved fin regeneration was due to the promotion of blastema cells or the prevention of cell death upon treatment with compounds, further investigation on this model is needed.

3.4 Cell proliferation analysis

Based on the positive efficacy result (3.3), it will be necessary to find out how compounds improved the process of fin regeneration. After blastema formation in the wound plane, progenitor cell-like cells in blastema will proliferate and contribute to the precursor cells of different cell types needed for replacing the missing parts (Münch J et al., 2013). Hypothetically, if the process of fin regrowth synchronizes with the dividing efficiency of blastema cells, more cells undergoing mitosis near wound plane would be expected to be detected in fish during the enhanced fin regeneration by our compounds.

During mitosis, the assembly of chromosomes is strongly affected by the highly compact chromatin fibers, which are composed of nucleosomes with an octamer of core histones (any two of H2A, H2B, H3 and H4) being wrapped by DNA. As a study focused on mitosis suggested (Hans & Dimitrov, 2001), histone H3 phosphorylation at serine 10 is believed to have a vital role during cell division and de-condensation of chromatin fiber for transcription. Inspired by this, an anti-phosphorylated histone H3 (PHIS) antibody was then chosen in this project to assess cells that underwent mitosis, and a fluorescent-conjugated secondary antibody was used for visual identification of those cells. Since antibody does not bind nuclei acid, PHIS staining serves as a quantitative fluorescence-based assay with less background interference in *in vivo* studies (Love NR et al., 2013).

Following amputation in pigment gap as described in methods 2.2, larvae at 48 hpf were kept in E3 medium containing 1 μM 689, 672 or DMSO. After being incubated with anti-phosphorylated histone H3 antibody and anti-rabbit secondary antibody ALEX 488, samples were imaged on the Olympus FV1000 confocal microscopy. Volocity software (PerkinElmer Inc) was used as a particle analysis software. All particles within the 300μM width rectangle (draw from the distal point of caudal fin) were measured, and then the green dots in areas ranging from 50 to 3000 μM^2 were counted as positive cells (as described in methods 2.3.1).

In un-amputated fish, positive cells of PHIS staining were barely detected, which suggested that the mitotic cells observed around the wound plane were

caused by fin regeneration. As shown in Figure 3.7, columns height represent the average number of mitotic cells detected in samples fixed at different time points post amputation. In the control group, the population of positive cells increases gradually from 24 hpa until 40 hpa, which suggests that fin regrowth occurs most efficiently during this period of time. 689 and 672 seem to positively influence the mitotic processes of the cells during fin regeneration. It was noted that the increase of mitotic cells starts earlier in 689 and 672 treated fish comparing to the DMSO control. Furthermore, compounds 689 and 672 might not work via the same mechanism since 689 shows the ability to enhance mitosis after 24 hours post amputation, but opposite effects of 672 were observed. However, due to the biological variability of results within the same test group (15 fish in each group were imaged and analyzed), One-way ANOVA significant test showed no significance between the DMSO control and compounds treated fish on the average number of mitotic cells at any time post amputation. This indicates that 672 and 689 might do not enhance the proliferation of blastema cells.

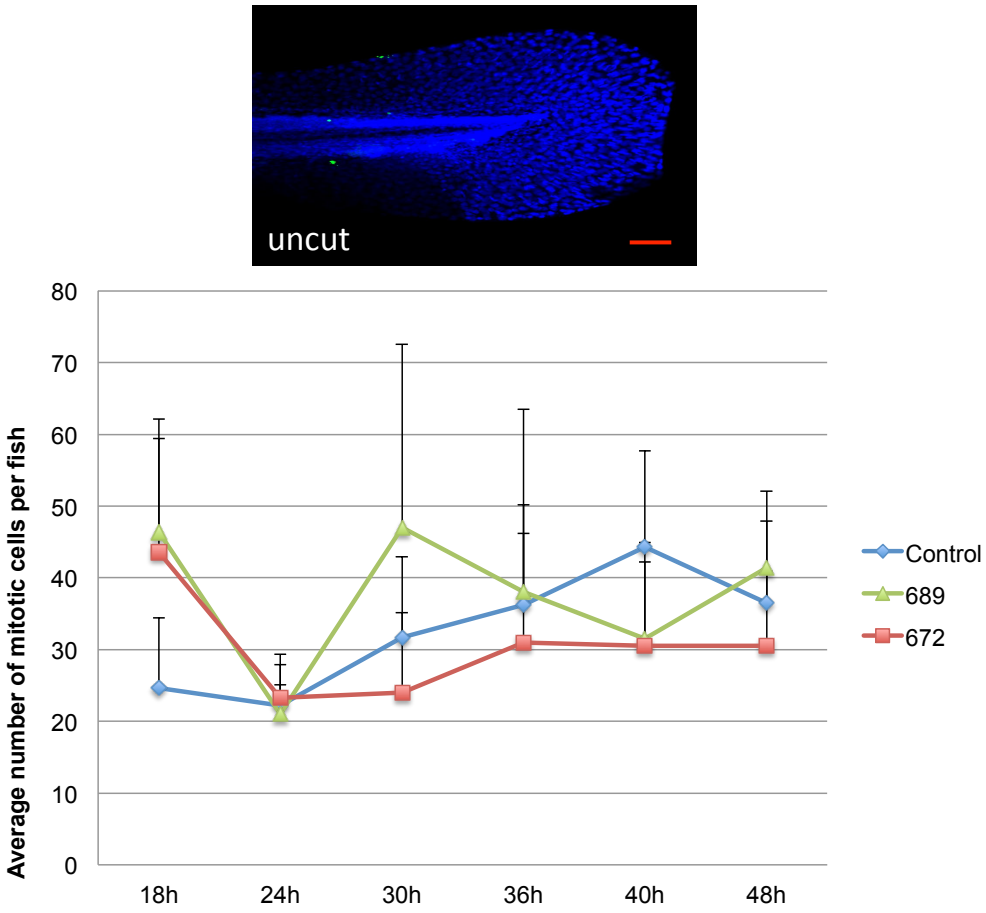


Figure 3.7 Top: Confocal image of PHIS staining of uncut fish larvae, no positive cells were observed. Bottom: Data shows the average number of dots with secondary antibody (green) within the regrown fin in each larvae treated by compounds by 18 to 48 hours following amputation on 2dpf respectively, representing the efficiency of mitosis during fin regeneration. P>0.05. n=15.

3.5 Apoptosis/ cell survival analysis

Upon 689 or 672 treatment, no significant alteration on cell proliferation was detected during fin regeneration, but improved fin regrowth was observed. Therefore, it was assumed that small-molecule compounds might prevent cell death instead of promoting cell division. To prove this hypothesis, assays

analyzing the level of programmed cell death were performed on zebrafish larvae.

3.5.1 Anti-caspase3 antibody staining for analyzing apoptosis during fin regeneration

Caspases, a family of proteases, control the final step in the cell programed death by working together with multiple other signaling pathway involved (Shalini S et al., 2015). Caspase-3 is one of the downstream regulatory molecules in this family. An antibody that specifically recognizes caspse-3 can also recognize the cells undergoing apoptosis in tissues. After being incubated with antibody, samples were stained and imaged as described in methods 2.3.1. As seen in Fig 3.8,the cells are positive for caspase-3 staining. But it is difficult to quantify the intensity of staining, and a more suitable assay is needed to address this issue.

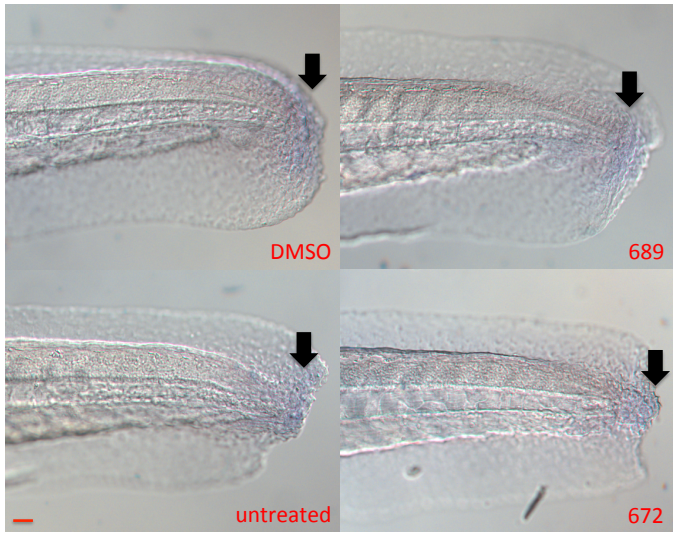


Figure 3.8 Anti-Caspase3 antibody staining on amputated zebrafish (arrow headed).

3.5.2 *In situ* apoptosis detection during fin regeneration by TUNEL staining

To improve the understanding of the mode of action of our compounds during fin regeneration, 20 zebrafish larvae at 48 hpf were amputated and treated following the method mentioned in 2.2.1. Following this, all larvae were fixed at 6, 12, 24 or 48 hours post amputation. After TUNEL apoptosis detecting, samples were imaged (Figure 3.9 a) and analyzed as described in methods 2.3.2.

All fluorescent particles within the caudal fin were recorded, and then the total number of fluorescent particles in an area from 50 to 1000 μM^2 was counted. As shown in Figure 3.9 b, the dots represent the number of apoptotic cells observed in the treated and non-treated fish. By comparing the average number of positive cells in each group with the DMSO control, it is possible that 689 and 432 might function via preventing apoptosis during regeneration, since the average number of apoptotic cells during fin regeneration in the fish treated with these two compounds are less. (Fig 3.9 b 12h and 48h). However, the same issue in PHIS analysis occurred because the biological variability in each group results in that the data in general looks a bit dispersed and there is no significant difference was detected consistently. If dosing with these two compounds is able to prevent cell death, then the decrease of TUNEL labeled cells is supposed to be detected consistently during fin regrowth. Moreover, a certain level of programmed cell death has been revealed to be crucial to the initiation of appendage regeneration (Tseng

A et al., 2007). Thus, it remains unclear whether 689 or 672 can protect cell from apoptosis during fin regeneration.

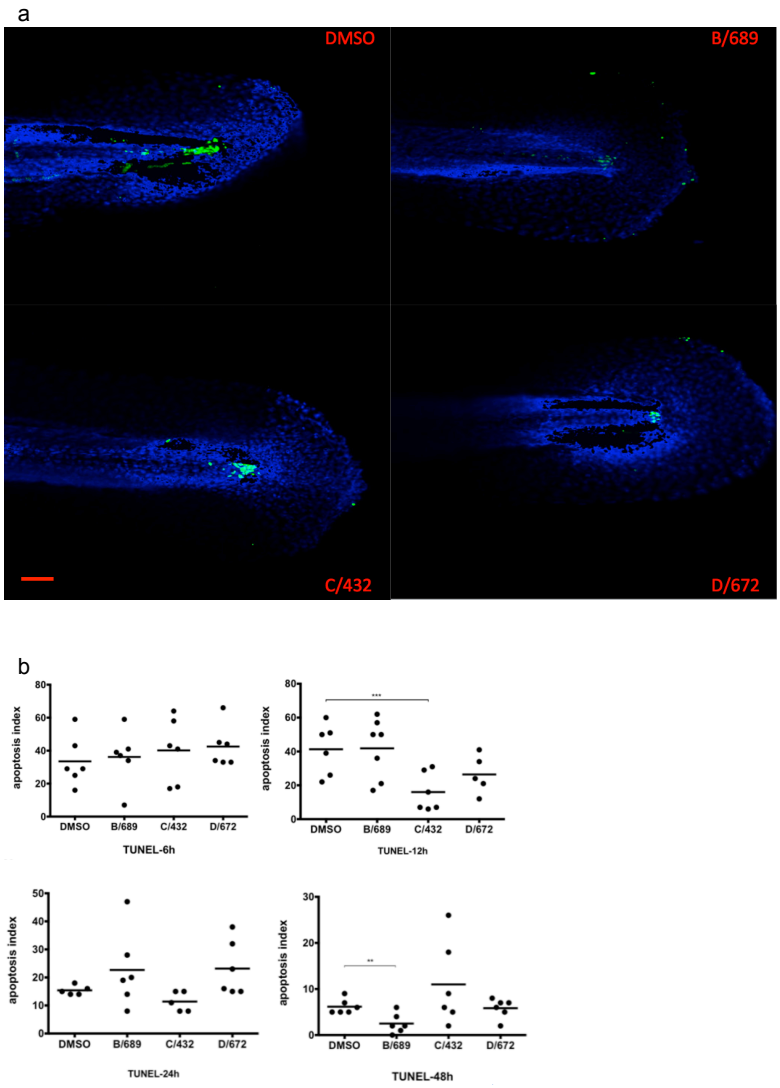


*Fig 3.9 TUNEL staining analysis of apoptosis (green dots) during 6 hpa tp 48 hpafin regeneration in zebrafish (A). The data (B) apoptosis index represents number of dots (green) in the regrowing fin region per fish larvae fixed on 6-48 hpa. Stastical significance is determined using one-way ANOVA versus control. ** p<0.01, ***<0.005. n>10. Scale bar 50 μ m. n=15.*

3.5.3 Exogenous H_2O_2 induced apoptosis can be prevented by 689 or 672 pre-treatment.

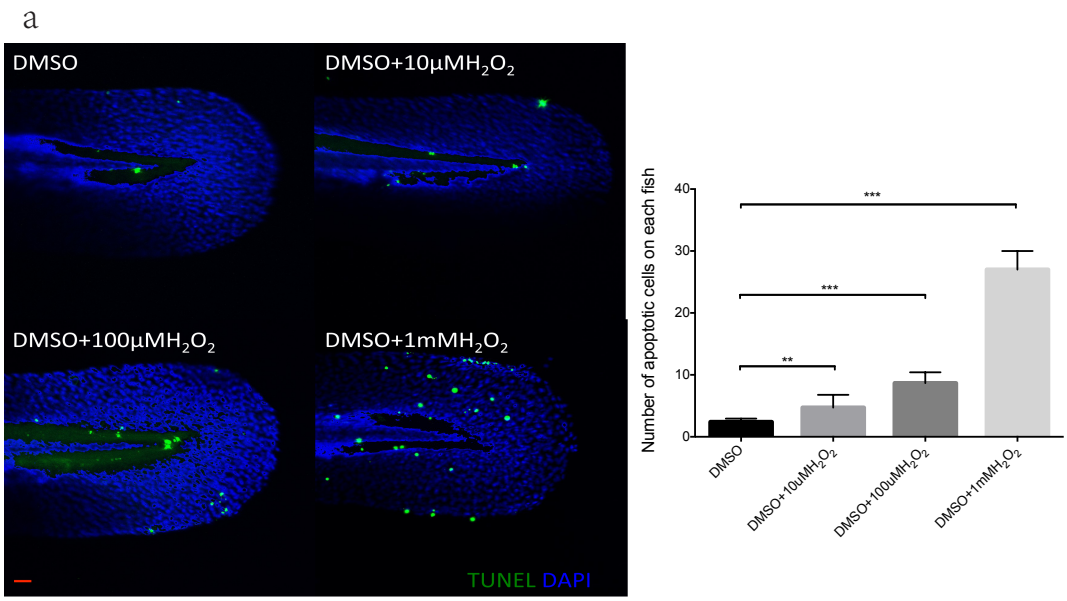
Since no significant decrease of apoptotic cells was detected during regeneration upon dosing them with compounds, which may be due to the biological variability of samples in the same group, we decided to use exogenous H_2O_2 induced apoptosis instead.

Hydrogen peroxide (H_2O_2), as a reliable reagent, is able to stably induce cell apoptosis both in cells (Qu X et al., 2014) and in animal models (Love NR et al., 2013). Un-amputated fish larvae were kept in fresh medium containing ascending concentration of H_2O_2 to find out if a stable level of cell apoptosis can be generated. Fish larvae at 48 hpf were treated in E3 medium containing 10-1000μM hydrogen peroxide for 6 hours then fixed. The fish were subject to the same TUNEL apoptosis analysis as described in methods 2.3.2. Data is shown in Figure 3.10 a (Left), confocal images showed the TUNEL positive cells (ALEX 488, Green) and Nuclei (DAPI, Blue). All green particles within the caudal fin were counted, and the number of apoptotic cells were demonstrated as columns in Figure 3,10 a (Right). Results showed that the number of cells undergoing apoptosis increased gradually with the ascending hydrogen peroxide concentrations. This indicates that H_2O_2 is able to induce cell apoptosis in fish larvae in a concentration-dependent manner.

Based on the fact that this model can robustly generate a certain level of apoptosis, larvae were treated with DMSO, 689 or 672 for 6 hours prior to the treatment with H_2O_2 in the medium (final concentration of 100 μM) for another

6 hours. All larvae were subjected to TUNEL *in situ* apoptosis analysis as described in methods 2.3.2. Quantitated results (Fig 3.10 a & b Right) showed that the number of cells undergoing apoptosis is significantly decreased in the samples pre-treated with 689 or 672.

Since pre-dosing with 1 μM 689 or 672 may prevent apoptosis induced by exogenous hydrogen peroxide (Fig 3.10 b), it will be necessary to test whether they can rescue these apoptotic cells to the same extent when they are applied alongside with H_2O_2 instead of 6 hours prior to H_2O_2 treatment. As shown in Figure 3.10 b (Left), apoptotic cells were barely detected in 689 and 672 treated fish, which suggests that if 689 or 672 exist together with 100 μM H_2O_2 in medium, apoptotic cells are significantly decreased in number in contrast to the number of apoptotic cell in H_2O_2 treated fish (Fig 3.10 b Right). Taken together, both 689 and 672 were shown to protect cells from programmed death induced by H_2O_2, which suggested that these compounds might be able to prevent cell death by modulating oxidative homeostasis during fin repair resulting in the improvement of fin repair.



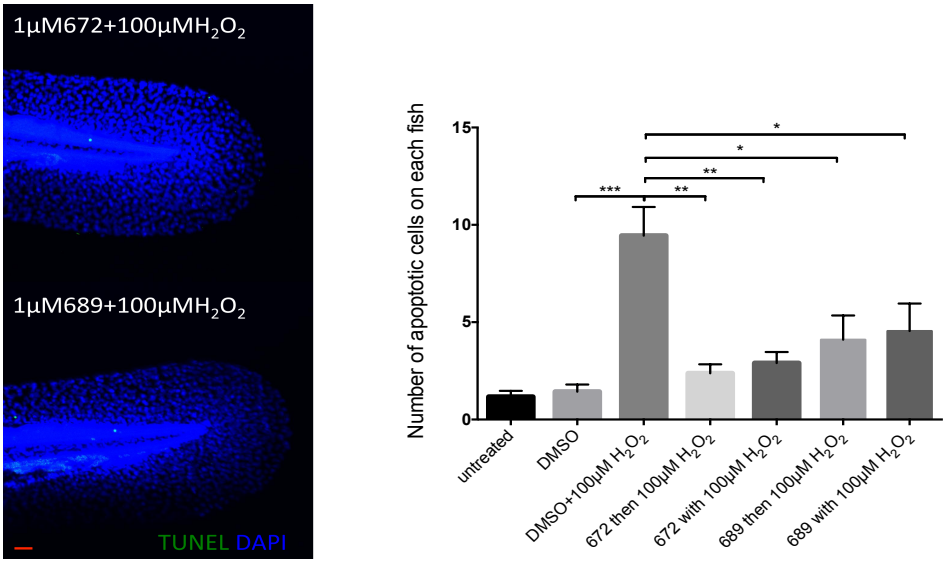


*Figure 3.10 a) Exogenous hydrogen peroxide shows stable induction of apoptosis on zebrafish (Left: confocal images; Right: quantified data). Pre-dosing fish with 689 or 672 is found with reduction of the number of apoptotic cells , similar result was also shown on samples dosed with compounds and hydrogen peroxide together (b). * P<0.05; ** P<0.01; *** P<0.001. n=15.*

3.6 Whole Mount *in situ* hybridization

No significant effect on increasing the population of dividing cells in fin regeneration was observed, which might be because the progenitor cells were not regulated directly. Experiments analyzing the level of PCNA gene expression in zebrafish have shown that PCNA works similarly in zebrafish as in mammal, and so has been widely used as a marker to detect the proliferating cells in zebrafish (Lee and Gye, 1999). So, an established PCNA probe (primers shown in Appendix.2, Roehl lab unpublished data) was used in the first *in situ* experiment for analyzing the effect of compounds on gene regulation. By comparing the area of staining in each fish treated with the selected compound for different period of time with DMSO control (Fig 3.11), it seems that from 24 hours post amputation, PCNA level in control fish (Right panel) decreased significantly, but not so much in 1 μM 672 treatment group (Left panel).

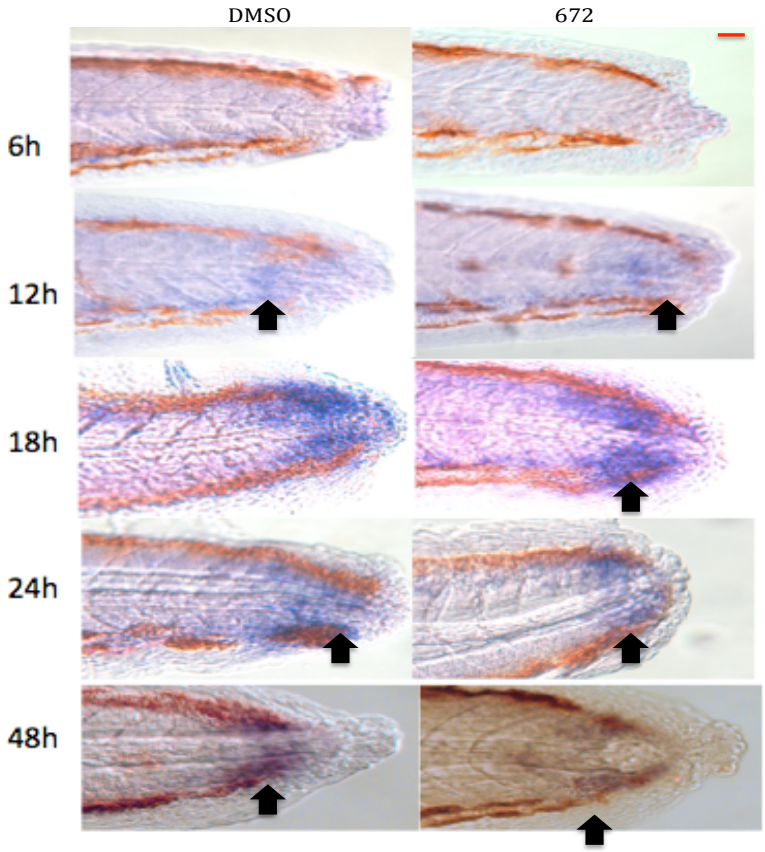


Figure 3.11 PCNA in situ hybridization staining (blue, arrow headed) of zebrafish fixed on 6-48 hours post amputation then treated with 672 (Right) or DMSO (Left). Scale bar 50 μ m.

The second experiment on *in situ* hybridization was carried out on Aldh1a2 (raldh2) gene, which encodes the major enzyme for embryonic RA synthesis. In zebrafish larvae, RA signaling has been revealed to have a vital role during the process of caudal fin repair post-amputation (Mathew et al., 2009). Thus raldh2 is used to recognize the formation of blastema by regenerating cells in zebrafish larvae (Begemann et al., 2001). The same primers reported by Begemann et al were used to assess the gene expression during fin regeneration upon treatment with 672. In Figure 3.12, images show the expression of raldh2 in fish during fin regeneration. By being applied with

raldh2 RNA probe, it is observed that expression of this gene was possibly brought forward by treating with 1 μM 672 (Right panel) in contrast with DMSO control (Left panel).

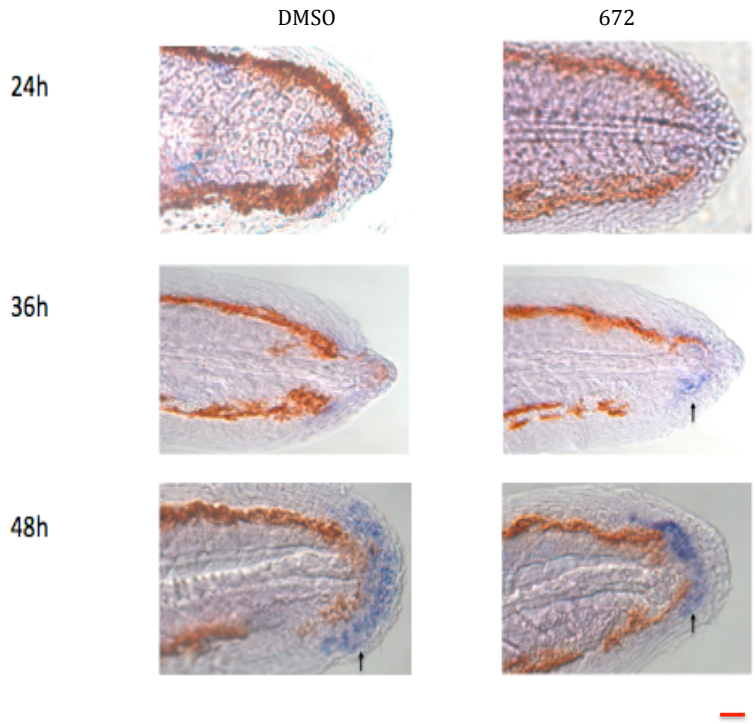


Figure 3.12 raldh2 in situ hybridization (blue, arrow headed) of zebrafish fixed on 24-48 hours post amputation then treated with 672 (Right) or DMSO (Left). Scale bar 50 μm.

Moreover, to find out if p53 is involved in the mechanism of 672 acts in, Mdm2 in situ hybridization was employed as a reflection of p53 activity. E3 ubiquitin-protein ligase (Mdm2) is an important negative regulator of the p53 tumor suppressor. The function of Mdm2 protein is believed to be an E3 ubiquitin ligase recognizing the N-terminal trans-activation domain (TAD) of the p53 tumor suppressor as well as inhibiting the activation of p53 transcription (Oliner et al., 1992). The anti-sense RNA probe used the same one reported before (Thisse et al., 2004). As the peak expression of Mdm2 during fin

regeneration shows up around 30hpa (data shown in Chapter VI), fish treated with 672 or DMSO were fixed at 30hpa and applied with anti-sense Mdm2 RNA probes (Fig 3.13). Comparing the coverage and density of Mdm2 expression in in situ staining pictures, it is difficult to draw a concrete conclusion on whether this gene expression level was modulated by treating larvae with the selected molecule from this qualitative rather the quantitative data.

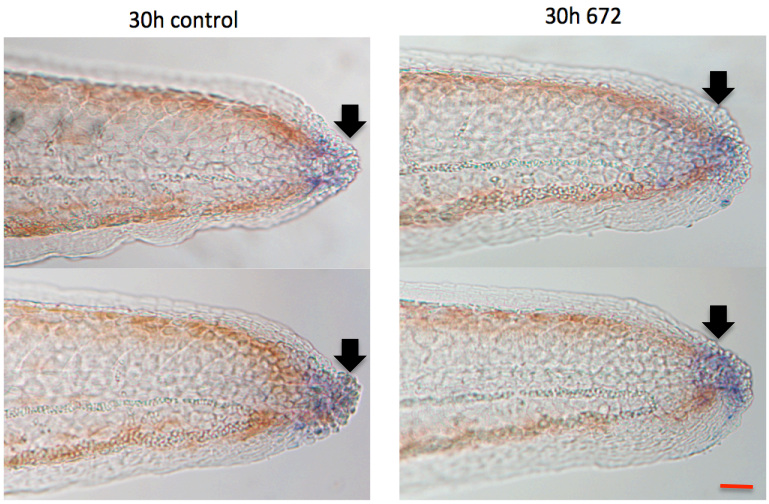


Figure 3.13 Mdm2 in situ hybridization (blue, arrow headed) of zebrafish fixed on 30hpa then treated with (672 Right) and DMSO (Left). Scale bar 50 μm.

The fourth *in situ* experiment was investigating whether 672 and 689 can upregulate SOD2 expression in zebrafish, as 689 has been observed to upregulate this gene in human mesenchymal stem cells. A fragment of DNA (675bp) was copied out from genomic cDNA using the primers shown in Appendix.2. After the transcription reaction as described in method 2.3.4.2, synthesized anti-sense RNA probe was applied to the larvae treated with 672, 689 or DMSO. The in situ staining of SOD2 was observed to be weak

especially in caudal fin area (Fig 3.14 top). However, as shown in Figure 3.14, the expression level of SOD2 in hindbrain area is strong enough for detecting. By comparing the staining in the hindbrain, it is believed that 689 and 672 up-regulated the expression level of SOD2 during 24 hours treatment (Fig 3.14).

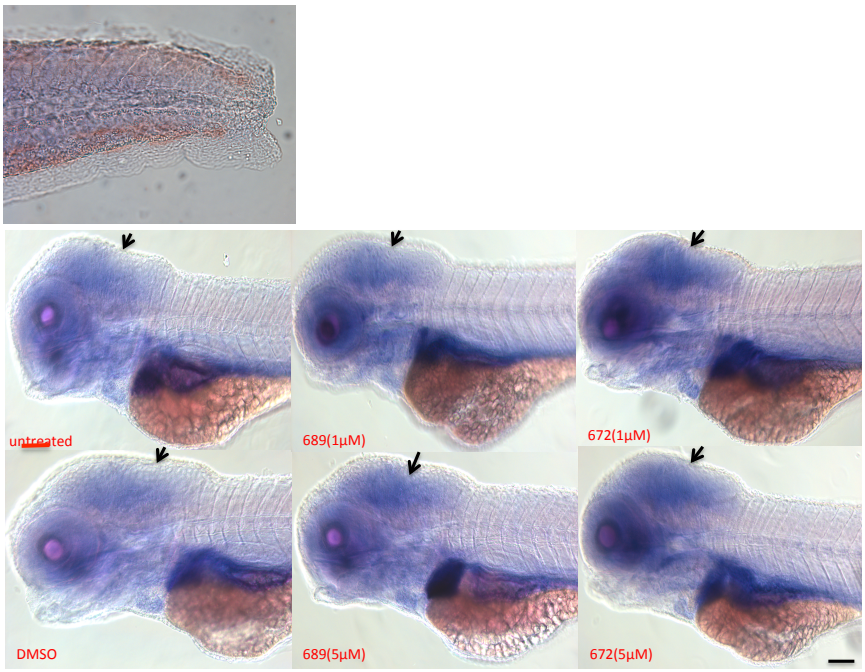


Figure 3.14 Top: SOD2 expression was not observed in caudal fin. Bottom: SOD2 in situ hybridization on 2dpf zebrafish larvae treated by compounds for 24 hours. Scale bar 50 μ m.

3.7 qPCR analysis

In situ hybridization provided some useful information on the expression profile of certain genes. However, given that the accurate quantification of the amount of staining is rather difficult, quantitative real-time PCR was therefore employed to verify the observation in *in situ* staining.

Total RNA was extracted from 15 zebrafish larvae in each group treated with the selected compounds or blank control, then the relative RNA level in each

group was assessed following the same protocol mentioned in Chapter II section 2.5. As seen in Figure 3.15, columns show the relative expression level of corresponding gene normalized to a house keeping gene β-actin (McCurley A et al., 2008).

Treating the larvae with the selected compound was believed to be with no influence on the relative PCNA expressing level (Fig 3.15 top left) even though its expression level seems to be slightly altered.

To investigate whether compounds influenced p53 level in zebrafish embryos, tp53 gene expression level was analyzed in each group treated with 1 μM 689 or 672 for 12 hours respectively. The amount of tp53 expression in each group was shown to be similar with blank control, suggesting that compounds may not alter p53 expression level (Fig 3.15 bottom right).

Up-regulation of SOD2 gene in zebrafish was observed in the present of 1 μM 689 and 672 (Fig 3.15 top right) but no alteration of SOD 1 was observed (Fig 3.15 bottom left), which is in consistent with results previously obtained. This finding suggested that increased ability of these compounds in scavenging to reactive oxygen species might be the mechanism of action of these chemicals.

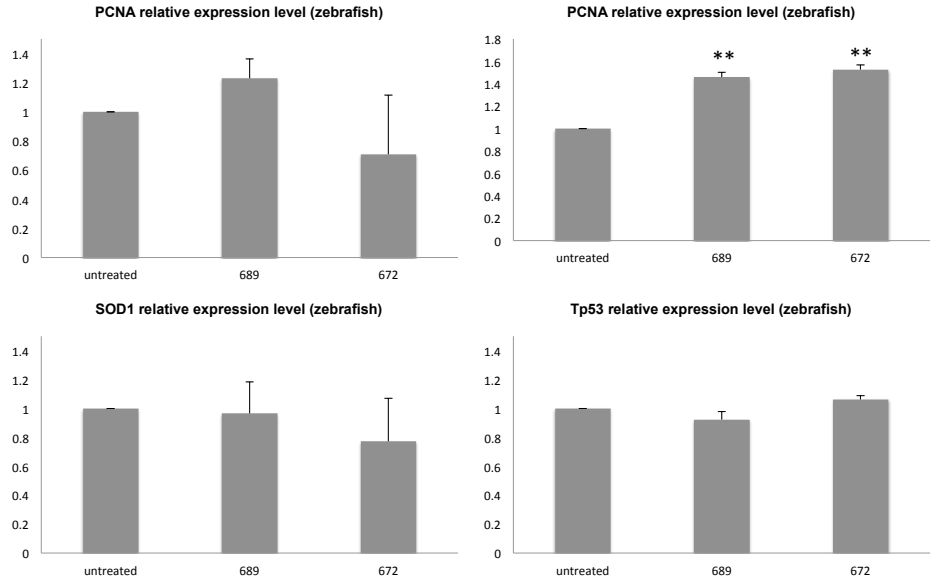


*Figure 3.15 quantitative real-time PCR analysis the relative expression level of PCNA, SOD1, SOD2 and tp53 in zebrafish treated by 689 or 672. ** P<0.01. n>3.*

4. Summary

As a captivating subject, regeneration widely occurs in the animal kingdom, and the principle of it is actually indeed fundamental, which is the most attractive aspect of this field. But since the capacity of regeneration in mammals is limited, it is an immensely challenging task for human beings to master the regenerative power to cure diseases and deal with the trauma from wounds. The aim of studying regenerative medicine is to develop methods for better understanding of how other organisms use their genetic toolkit to achieve natural regeneration. Nonetheless, all of the regeneration relies on the cell source - proliferating stem/progenitor cells. Some evidence

has been provided in Chapter I supporting this opinion. However, very little is known about the origin of blastema, which limits the potential of zebrafish to become a widely used vertebrate model for studying the appendage regeneration. Further investigation is required to address if any of these genes or chemicals could influence any specific signaling pathways involved in the process of development or regeneration, especially during the procedure of blastema formation.

Small molecules were screened using wild type zebrafish larvae for investigating their function in fin regeneration. After selecting the suitable concentration to treat fish larvae, Alcian blue staining, PHIS staining, TUNEL staining, Whole-mount in situ hybridization and qPCR were performed.

Toxicity test and Alcian Blue staining showed that compounds do not impact the natural development of the bone system in zebrafish. Physical examination of fin regrowth post amputation showed that they produced moderate acceleration in the fin regeneration. PHIS staining and TUNEL staining were employed to investigate whether compounds accelerate cell proliferation or preventing cell death.

The data collected from PHIS staining provided insignificant evidence that mitosis is enhanced when exposed to compounds during the regeneration of caudal fin. Moreover, fishing for the target gene was not successful since expression of PCNA was not regulated in zebrafish in in situ hybridization assay. To analyze cell apoptosis in zebrafish, TUNEL staining was carried out

and the results showed that pre-treating with 689 or 672 could prevent the apoptosis induced by exogenous hydrogen peroxide, which might be via up-regulating SOD2 leading to ROS scavenge (qPCR and in situ hybridization). SOD2 has been illustrated to be involved in preventing cell death by controlling ROS level in cells (Mohanty et al., 2012; Kammeyer A et al., 2015). However, the mode of mechanism of these compounds act upon during the fin regeneration is still inclusive since the exact role of SOD2 scavenging ROS leading to the prevention of cell programed death is yet to be validated. Taken the above, it is very possible that SOD2 might not be the direct target for 689 and 672, but rather that the positive effect of the compounds on SOD2 might be the consequence resulted from up or down regulation of other molecules in the pathways involving SOD2. Based on this hypothesis, exploring the exact molecular targets for these compounds was carried out in this project. Since SOD2 expression level is believed to be modulated by compounds both in cells and in fish, it is necessary to show how the targets of these compounds influence the expression level of SOD2

IV. Target exploration

Based on the data gained so far, 689 and 672 are believed to increase the population of stem cells *in vitro* by beneficiating cell expansion, as well as helping fish larvae fin regeneration, both of which might be because they have positive influence on SOD2 that modulates the oxidative stress leading to preventing cell death induced by hydrogen peroxide. The direct targets for these chemicals are yet to be explored because SOD2 was reported to function as a mitochondrial enzyme to catalyze the breakdown of ROS into H_2O_2 but not the further degradation (Lee et al., 2009). More importantly, while the mode-of-action of compound 689 is through SOD2 reported by our group in previous studies (Mohanty et al., 2012), the mode-of-action for compound 672 is completely unknown. It is a novel compound that is different with compound 689, yet is able to promote cell proliferation via modulating SOD2 function, which has not been reported in any previous work. Thus, the exact molecular targets of these two small molecules still need to be explored. The link between biological activities of these compounds and molecular targets involved in SOD2 modulation were carried out in parallel using different assays described below.

Although, these compounds were designed based on the structure of cellular prion protein and can bind prion protein hypothetically, based on the previous work carried out by Miss Knight, 689 and 672 did not show any affinity to PrP^C protein. In the thermal shifting assay, T_m change of PrP^C upon the treatment of 689 or 672 was only minus 1-2 degree (data not shown), which suggests these compounds do not bind PrP^C specifically. Moreover, another Surface

Plasmon Resonance (SPR) based direct binding assay confirmed that there is no direct interaction between these compounds and PrP^C (data not shown). Therefore, despite that PrP^C is involved in regulating the oxidative stress at cellular levels which might be linked to SOD2 modulation, PrP^C itself as a direct target for these two compounds can be excluded from further investigation.

1. Materials and Methods:

Both human EC and ES cells were maintained and harvested following the same protocol described in chapter II.

All reagents used are shown in Appendix.1.

1.1 High-throughput RNAi screening

As data shown in previous chapters, 672 and 689 are believed to up-regulate SOD2 expression. Given that SOD2 transcription might not be enhanced directly by 672 or 689 treatment, SOD2 relative genes are likely to be involved in the function of our compounds. Informatics analysis of genbank (http://www.ncbi.nlm.nih.gov/genbank/) indicated 27 genes that potentially could regulate SOD2 expression and these were tested by a primary RNAi screening assay using both human EC and ES cells.

1.1.1 Gene Silencing

Homo sapien siRNA was acquired from Dharmacon and used at a working concentration of 30 nM and four different siRNA oligos aiming the same gene were used as a combination to ensure the knockdown of the target gene.

siRNA was delivered using Dharmafect 1 reagent (Dharmacon) according to manufacturer's instructions (0.06 µL for 30 nM reaction). Silencing was carried out by bathing cells in DMEM (10% FCS) or Nutristem containing siRNA (30 nM) for 1 hour at room temperature after seeding. Then after 24 hours culturing in incubator, additional equal volumes of fresh medium was added in.

1.1.2 Procedure of RNAi Screening

After siRNA being added into 384-well plates as designed using a liquid handling robot (Hamilton), EC cells were seeded at 1000 cell per well using multidrop reagent dispenser (Thermo). In case of hES cells (Mshef 11), 2500 cells were seeded on Cellstart coated 384-well plates in 10 µL Nutristem, and siRNA was added into each well after attachment over two nights. Following the incubation with siRNA for 1 hour at room temperature and overnight at 37^{o}C, cells were treated with compounds (689 or 672) or DMSO at a concentration of 1 µM for 2-3 days. Cells were then washed with cold PBS and then fixed with ice cold MeOH by microplate washer (BioTek). Following the incubation with 1:1000 Hoechst dye in PBS for 10 min at room temperature, cells were rinsed 2 times with PBS then left in 50 µL PBS. The whole-view image of each well was acquired under an ImageXpress Micro wide-field, high-content screening microscope (Molecular Devices, CFI S Plan Fluor N.A. 0.60 2× objective (Nikon)). Microscopy images were analyzed by an automated data application "Transfluor" (Molecular Devices), parameters were optimized to enable detection of nuclei between 8 and 16 µm in size and

15 gray levels above local background intensity. The same experiment was repeated at least three times (12 wells set up as the same condition in each time) using different batches of cells.

Random wells in each plate were also incubated with an anti-SSEA3 antibody (1:200) and alexa-conjugated secondary antibody (1:1000) for assessing the pluripotency of cells after handling.

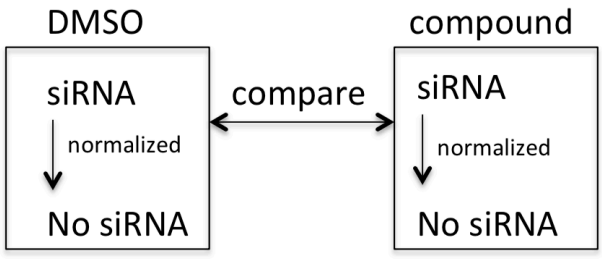
1.1.3 Statistical Analysis

Total cell number of cells in each well was analyzed using robust z-score (the number of standard deviations from the mean) method as a measure of the signal window, which shows explicit data on the strength of each silenced gene relative to the rest of the sample distribution. Median absolute deviation (MAD) instead of mean is used as described as Birmingham et al., 2009 to show the difference caused by the silencing of corresponding gene.

$$MAD = median_i (|X_i – median_j (X_j)|)$$

$$Z'\text{-factor} = (X_i – median_i)/MAD$$

To avoid the false positive caused by siRNA off-target effects, cell number in each siRNA knockdown wells was normalized to the wells without siRNA but with DMSO to obtain a z-score as a control. Then this normalized z-score was compared with the z-score of the same siRNA but compound treated wells by Prism 6 (GraphPad). $p<0.05$ was considered to be significant.



1.2 Kinase profiling

This assay was performed by MRC international kinase profiling center using the protocol previously established (Hastie et al., 2006).

1.3 Insulin function analysis on stem cell growth

E8 basal medium (A1517001, GIBCO) was used to assess the growth of stem cells in insulin-free medium, as E8 does not contain insulin or any other growth factors. Recombinant human insulin (Sigma I9278, 5 μg/mL) was used as a rescue reagent. Cell suspension was seeded into E8 medium. E8 medium containing insulin, 672 or both were added into each well after two days (hES) or overnight (EC) attachment. Nutristem or DMEM (10% FBS) were used as control. Cell population was quantified following the protocol motioned above and compared to optimize the growth condition in different circumstances after 2-3 days treatment. One-way ANOVA was applied for significance analysis using Prism 6 (GraphPad).

1.4 Thermal shifting of Insulin Receptor

A long isoform of recombinant human insulin receptor protein (11081-H08H-250) and Insulin-like growth factor 1 receptor protein (Life Tech, 10164-H08H-50) were purchased from life technology Normal PBS was used as the buffer and SYPRO orange as the dye. 5 μg of protein and 1-10 μM of 672 were added in 96-well plates at a final volume of 20 μL volume each. Experiments were performed on Agilent qPCR machine following the instruction provided by manufacturer. Data was analyzed on Prism 6 (GraphPad) for melting curve of each condition using Melting Boltzmann by nonlinear regression, and the T_m value of each condition was calculated.

2. Results and Discussion

2.1 RNAi screening showed 672 is ERK1 dependent while the action of 689 involves CYP4F12.

RNA interference (RNAi) technology utilizes small RNA molecules to inhibit gene expression through destructing specific mRNA to block this gene post transcription. RNAi is an important defence mechanism in biological realm to kick out parasitic nucleotide sequences, which was firstly observed in bacteria. After this phenomenon being introduced into genetic research by Andrew Fire and Craig C. Mello in 1998 to knock down interest gene (Fire A et al., 1998), it has been widely adopted to genetic studies.

As a reliable approach to suppress the gene of interest, RNAi has widely used for gene silencing. Due to the sequence homology between siRNA and the target mRNA, cell based genome-wide screening could be successfully performed to identify function of certain gene in regulating cell behavior, morphology and pluripotency etc. (Boutros et al., 2004). Utilizing this valuable research tool to selectively suppress specific genes efficiently (55%-90%), it is possible to identify of the signaling or even specific gene required for the normal bioactivity of our compounds.

Three types of small RNA molecules are commonly used in mammalian models. Three type of RNAi short hairpin RNA (shRNA), small interfering RNA (siRNA) and bi-functional shRNA are mainly used. After the successful high-throughput screening of genomic dsRNA on drosophila for identifying

Parkinson-relative genes, human genome-wide siRNAs have been synthesized and are commercially available to specifically suppress a large amount of interest genes individually (Human siRNA Libraries, Dharmason). Furthermore, the same library of genomic-wide siRNA has been revealed to be with reliable knockdown effect using four different siRNA oligos aiming the same gene in previous report (Ivatt RM et al., 2014). Thus, the most challenging task in this project will be the delivery of dsRNAs into cells and avoiding erroneous result.

After the transfection with siRNA and with or without the compounds' treatment, cells were fixed and analyzed as the methods described in 1.1. As shown in figure 4.1, the columns represent the average of normalized z-score (quadruplicated of each condition) in corresponding siRNA-silencing condition: the left one shows the score in the wells treated with DMSO, while the right column is for 672. It is noted that when SOD2, erk1, egr1 or sirt1 was silenced, the decrease of cell population in DMSO treated wells was significantly lower than in 672 treated wells. This observation suggested that the normal level of these genes might be not crucial for cell growth, but is related to the normal activity of 672.

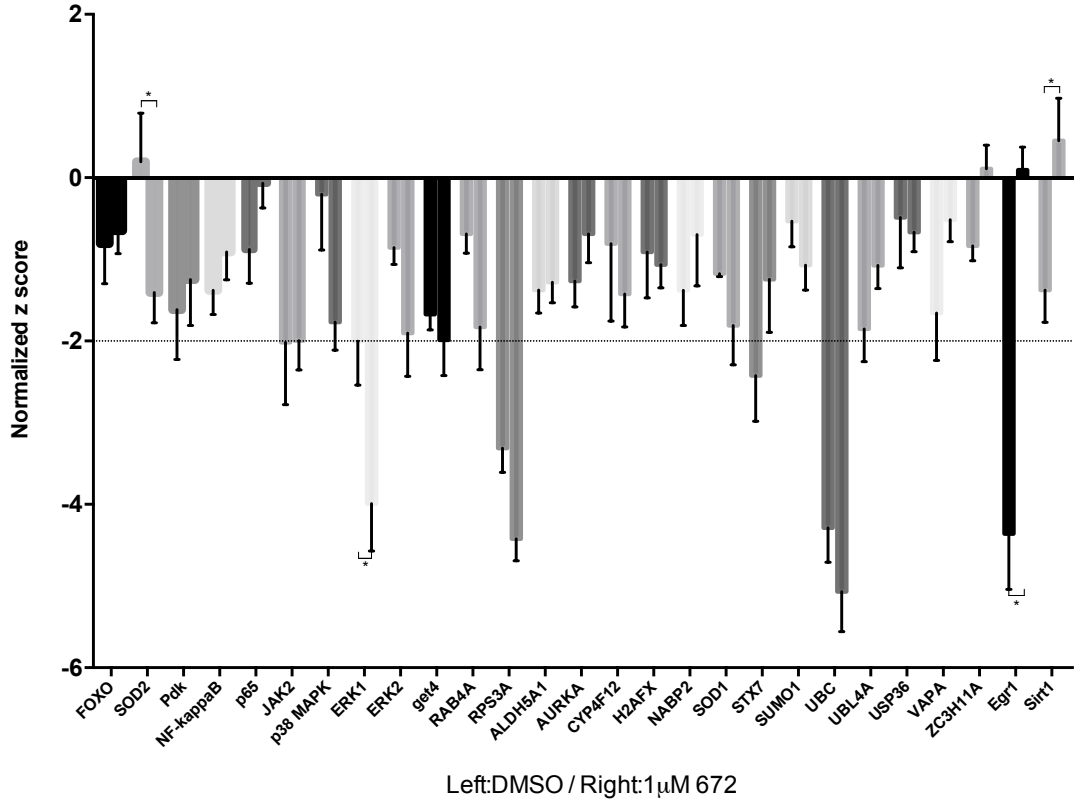


*Figure 4.1 RNAi screening of the genes could potentially regulate SOD2 expression reveals that when erk1 is silenced, growth of EC cells is disrupted in DMSO condition but significantly inhibited when 672 in present. * P<0.05. n=12.*

SOD2, as previous described, is able to prevent cell death by modulating ROS level. Given that egr1 and sirt1 are transcriptionally upstream of SOD2, these two genes might also be involved in 672's mechanism. ERK1, one of the key kinases in MEK/ERK signaling pathway, is one of the most important cellular homeostasis kinases modulating cell proliferation, differentiation and survival (Li, J et al., 2007). Knockdown of this gene showed the most significant difference between DMSO and 672 on EC cell growth, which indicates that if ERK1 was silenced, the enhancement of cell growth brought by 672 could be disrupted.

As a key kinase signaling in response of FGF pathway, MEK/ERK signaling plays a vital role in transferring extracellular stimulation through membrane and then regulates cell survival, proliferation and fate determination in most types of cell (Bottcher and Niehrs, 2005; Kang S et al., 2014). Many subtypes and steps of this cascade are conserved across different research models and were demonstrated to be responsible for responses in mammalian cells in parallel (Cano & Mahadevan, 1995).

However, only ERK1-silencing showed significant disruption on cells treated by 672, other MAPK kinases like ERK2 or MAP3K1. To test if 672 can enhance the phosphorylation of ERK1 or JNK, anti-phosphorylated ERK antibody and anti-phosphorylated JNK antibody staining were used (Figure 4.2). In pictures, cells were shown blue (nuclei, DAPI) and red (phosphorylated ERK) or green (phosphorylated JNK). No significant difference in the level of ERK phosphorylation was observed between DMSO and 672 treated cells. It is supported by a previous study showing that ERK has no direct contribution to the proliferation of hES cells (Li, J et al., 2007). Given all, it was reasonable to assume that 672 is only ERK1 dependent, but do not enhance the phosphorylation of ERK1.

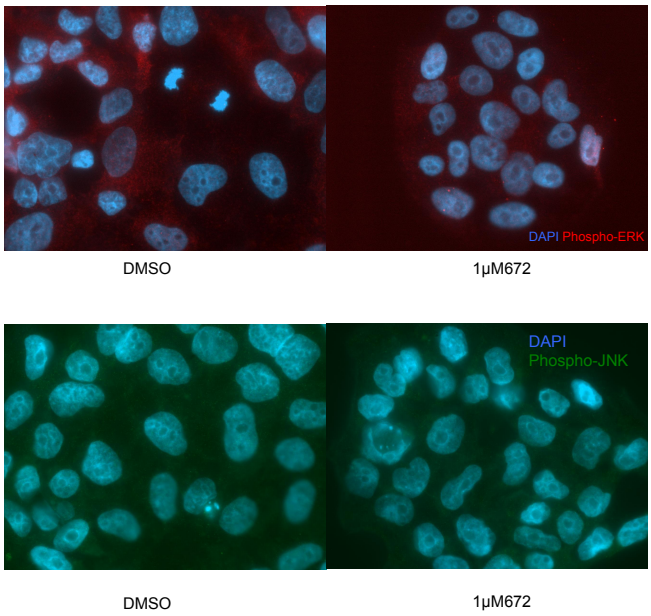


Figure 4.2 Anti Phospho-ERK and Anti Phospho-JNK antibody staining do not suggest that the phosphorylation level of these two kinases is altered by 672 treatment.

In the parallel assay, 689 was shown to have significant impacts on ROS modulation. When CYP4F12 or SOD1 was silenced, significantly disruption on the growth of EC cells was observed in the wells treated with 689 (Figure 4.3). It was assumed that 689 works in modulating oxidative reaction through ROS level regulation, which is very different with 672.

CYP4F12 is an enzyme belonging to the cytochrome P450 superfamily. The cytochrome P450 proteins are mono-oxygenases and involved in multiple reactions such as drug metabolism, cholesterol or other lipids synthesis as a catalyzer both exogenously and endogenously (Hsshizume et al., 2001). 57 members of the P450 family enzymes have been identified in Human Genome Project (Cauffiez et al., 2006). However, the knowledge of most of them remains limited due to its complex nature. Current understanding of P450 family suggests that this family of enzymes is with a great potential as a

target for studying xenobiotic toxicity, carcinogenesis and relevant pathology (Groves JT, 2015).

Moreover, SOD1, as one of the most important components modulating the cellular oxidative metabolism, was shown to be involved in the detoxification of reactive superoxide radicals by converting them to oxygen and hydrogen peroxide, which allowing further degradation (Brown et al., 1997). It is also believed that PrP^{C} is able to regulate the incorporation of Cu^{2+} into SOD1, which permits the normal function of SOD1 (Brown et al., 1998). Furthermore, the enhancement on engraftment of mesenchymal stem cells to bone morrow in mice and the upregulation of SOD2 brought by treating cells with 689 were both revealed to be PrP^{C} – dependent (Mohanty et al., 2012). Thus, although 689 was observed to be with no binding affinity with PrP^{C}, it is still possible that 689 might be able to interact with prion signaling pathway, which overlaps with SOD leading to the modulation of intracellular ROS level.

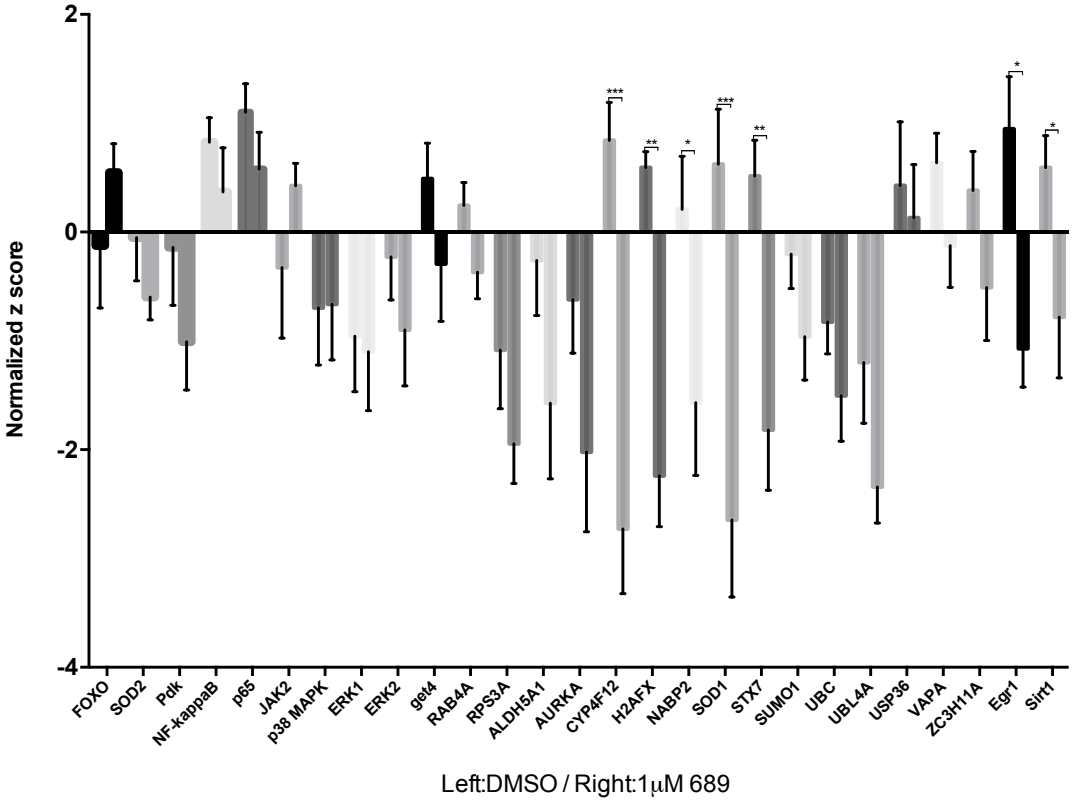


*Figure 4.3 RNAi screening of the genes could potentially regulate SOD2 expression reveals that when CYP4F12, H2AFX, NABP2, STX7 or SOD1 are silenced, growth of EC cells is not regulated in DMSO condition but significantly inhibited when 689 in present. * P<0.05, ** P<0.01; *** P<0.001. n=12.*

To verify the finding gained on EC cells, hES cells were used following the same protocol described in method 1.1. Due to the difficulties in attachment of hES to plastic, single-suspended hES were initially seeded into cellstart-coated 384-well plates. After two days attachment, siRNA and DF1 were added into wells respectively at designated concentration. The RNAi results on ERK1 were consistent across the two sets of the experiments using different type of cells (Figure 4.1 and 4.4 for 672 and Figure 4.3 and 4.5 for 689), which strongly supports the hypothesis that the enhancement of 672 on stem cell's proliferation is ERK1 dependent, while 689 is able to modulate ROS level through P450 family.

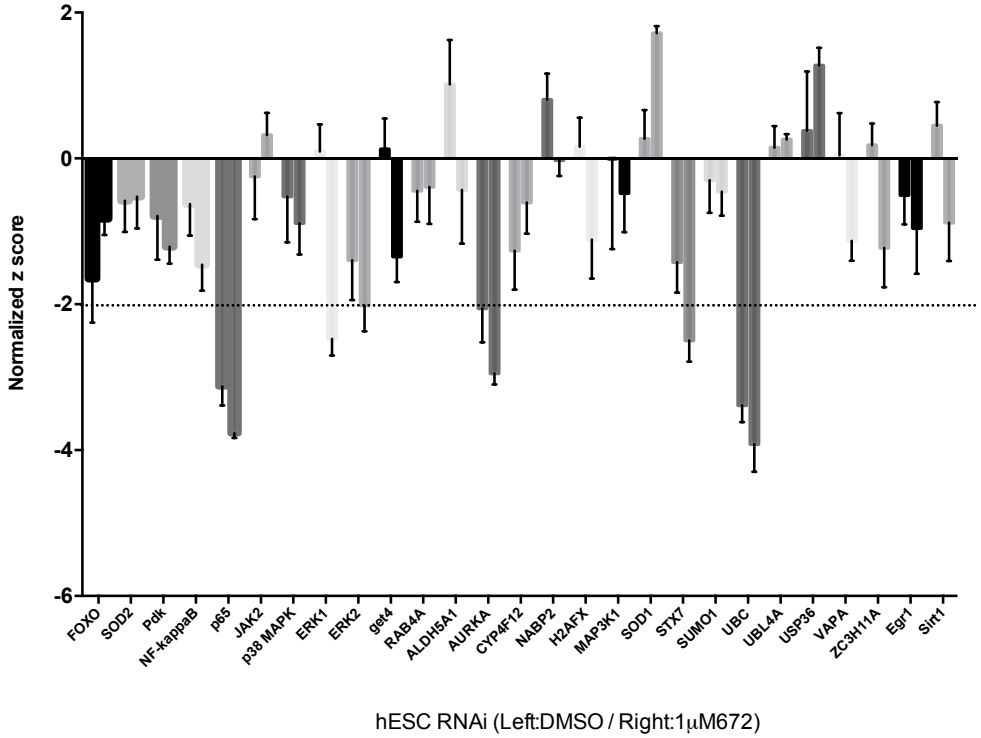


*Figure 4.4 RNAi screening of the genes could potentially regulate SOD2 expression reveals that when erk1 is silenced, growth of ES cells is disrupted in DMSO condition but significantly inhibited when 672 in present. * P<0.05. n=12.*

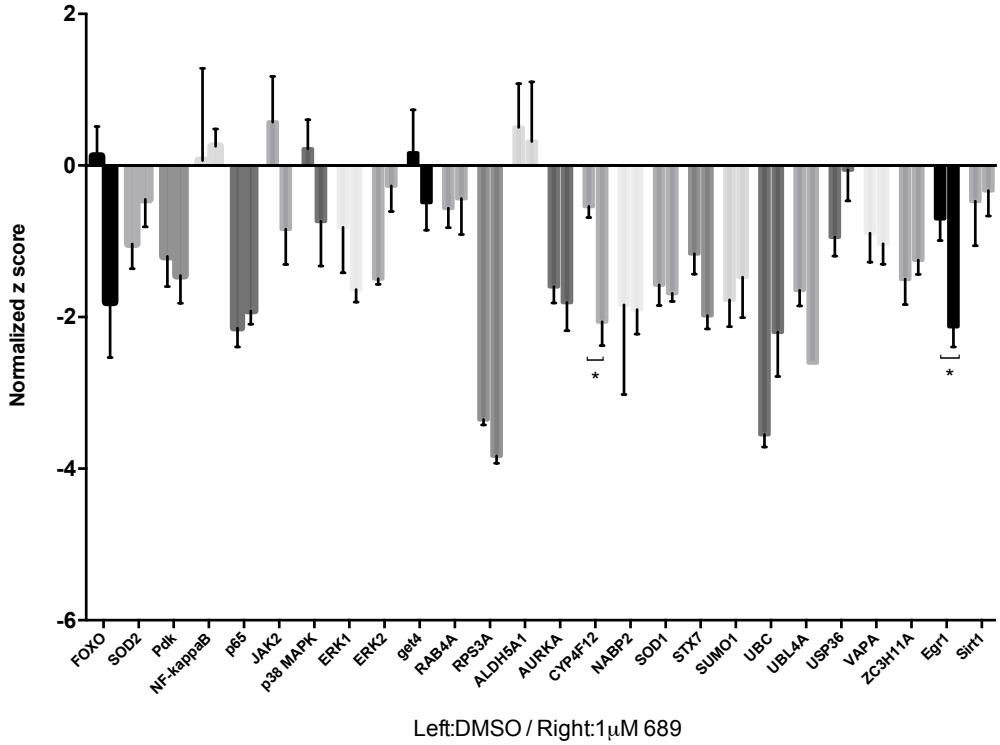


*Figure 4.5 RNAi screening of the genes could potentially regulate SOD2 expression reveals that when CYP4F12 is silenced, growth of ES cells is disrupted in DMSO condition but significantly inhibited when 689 in present. * P<0.05. n>12.*

To exclude the possibility of false positive result caused by RNAi off-target effects, five different non-coding RNAi were randomly plated on 384-well plates together with the experimental siRNA. The data suggests that the assay is reliable because none of the five non-coding RNAi was observed to have significant effects on the growth of cells (-1<z<1; Fig 4.6).

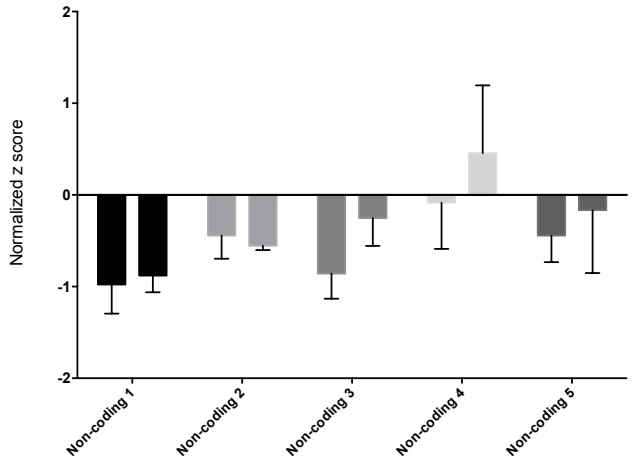


Figure 4.6 Five different non-coding RNAi do not alter the growth of EC cells (Left:DMSO; Right:672). P>0.05. n>12.

To test if cells were differentiated or remained as pluripotent stem cells during the experiment, random wells were incubated with SSEA-3 antibody after the RNAi screening. Fluorescence images showed that most hES cells are positive with SSEA-3 (Fig 4.7), indicating that the treatment with siRNA and compounds did not induce the differentiation of stem cells.

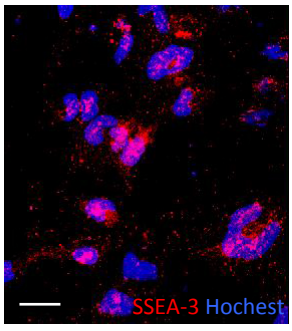


Figure 4.7 Fluorescence images of hES cells stained with Anti-SSEA3 antibody (Red) and Hochest (Blue).

2.2. Kinase profiling shows 672 might interact with insulin receptor complex

Based on the RNAi screening result, 672 was suggested to be ERK1 dependent. It was assumed that this compound may act through regulating kinase signaling. To verify this hypothesis, kinase-profiling assay was employed to determine whether 672 can interact with a recombinant kinase. Hypothetically, if 672 has binding affinity with any kinase or its relevant substrate, the phosphorylation of the kinase on its substrate should be disrupted.

Radiolabeled ATP and an appropriate acceptor peptide or protein substrate were utilized to measure the activity of 121 key protein kinases. Phosphocellulose paper immobilized with acceptor peptides or proteins was immersed in phosphoric acid to allow them to be positively charged. During the protein kinase reaction, peptides lose the binding and being washed off along with all ATP. Negatively charged ATP from kinase reaction does not adhere to the paper and is washed away as well (Traxler, 1997). Only the radiolabelled substrates will be left on the paper and be quantified to assess the reaction efficiency as shown in Figure 4.8.

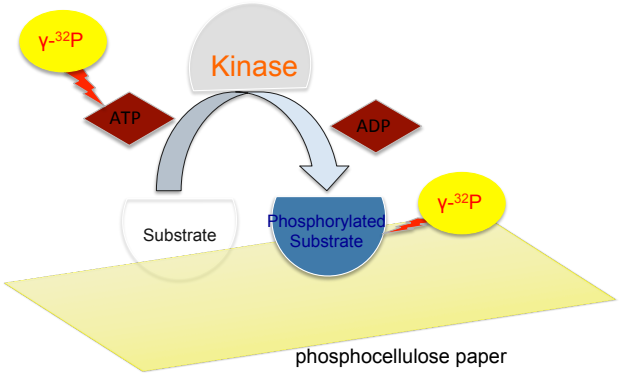
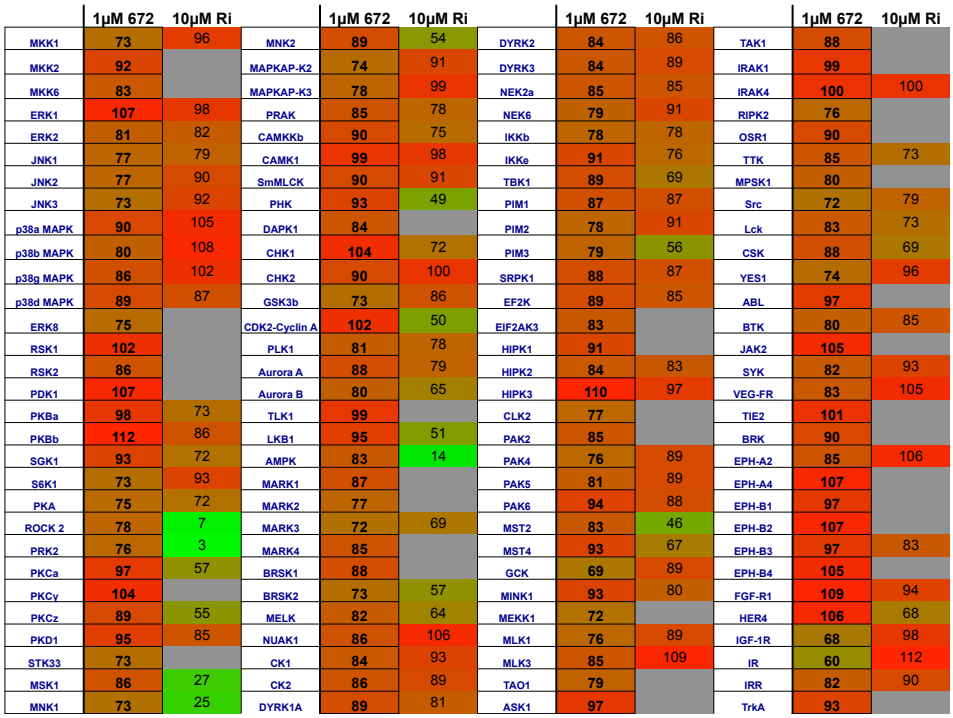


Figure 4.8 Principle of kinase profiling.

121 key kinases were performed as the method described in previous studies against appropriate substrate or peptide (Hastie et al., 2006). Quantification of radiolabeled substrate left on the paper was taken as the relative kinase activity.

After normalizing against the DMSO control (Fig 4.9), it was very interesting to find out that 672 was not observed to interact with any MAPK kinases or JNK kinases. However, the phosphorylation of Insulin Receptor (IR) on its substrate was observed to be with only 60% when treated with 672.

As one of the most important metabolism regulators, insulin signaling is considered as a complex and highly integrated network in response to extracellular cue then stimulate multiple intracellular pathways regulating cell metabolism, growth and differentiation (Vigneri et al., 2010). Insulin receptor (IR), a transmembrane tetrameric glycoprotein with intrinsic tyrosine kinase on intracellular side, is able to autophosphorylate then phosphorylates a large series of protein kinases and result in cell survival (AKT) and proliferation (ERK) regulation in response of insulin stimulation. Although the understanding of the role that IR plays in pluripotent cells is still in its infancy, recent studies revealed that induced pluripotent stem cells (iPSC) derived from fibroblasts of patients with IR mutations had defects in proliferation (Iovino et al., 2014). It was demonstrated that, compared with normal fibroblast cells, iPSCs with mutant IR showed only about 50% doubling efficiency. Interestingly, the activity of ERK1 was also observed to be reduced significantly in IR-mutant iPSCs in the same study.



	1μM 672	10μM Ri		1μM 672	10μM Ri		1μM 672	10μM Ri		1μM 672	10μM Ri
MKK1	73	96	MNK2	89	54	DYRK2	84	86	TAK1	88	
MKK2	92		MAPKAP-K2	74	91	DYRK3	84	89	IRAK1	99	
MKK6	83		MAPKAP-K3	78	99	NEK2a	85	85	IRAK4	100	100
ERK1	107	98	PRAK	85	78	NEK6	79	91	RIPK2	76	
ERK2	81	82	CAMKKb	90	75	IKKb	78	78	OSR1	90	
JNK1	77	79	CAMK1	99	98	IKKe	91	76	TTK	85	73
JNK2	77	90	SmMLCK	90	91	TBK1	89	69	MPSK1	80	
JNK3	73	92	PHK	93	49	PIM1	87	87	Src	72	79
p38a MAPK	90	105	DAPK1	84		PIM2	78	91	Lck	83	73
p38b MAPK	80	108	CHK1	104	72	PIM3	79	56	CSK	88	69
p38g MAPK	86	102	CHK2	90	100	SRPK1	88	87	YES1	74	96
p38d MAPK	89	87	GSK3b	73	86	EF2K	89	85	ABL	97	
ERK8	75		CDK2-Cyclin A	102	50	EIF2AK3	83		BTK	80	85
RSK1	102		PLK1	81	78	HIPK1	91		JAK2	105	
RSK2	86		Aurora A	88	79	HIPK2	84	83	SYK	82	93
PDK1	107		Aurora B	80	65	HIPK3	110	97	VEG-FR	83	105
PKBa	98	73	TLK1	99		CLK2	77		TIE2	101	
PKBb	112	86	LKB1	95	51	PAK2	85		BRK	90	
SGK1	93	72	AMPK	83	14	PAK4	76	89	EPH-A2	85	106
S6K1	73	93	MARK1	87		PAK5	81	89	EPH-A4	107	
PKA	75	72	MARK2	77		PAK6	94	88	EPH-B1	97	
ROCK 2	78	7	MARK3	72	69	MST2	83	46	EPH-B2	107	
PRK2	76	3	MARK4	85		MST4	93	67	EPH-B3	97	83
PKCa	97	57	BRSK1	88		GCK	69	89	EPH-B4	105	
PKCγ	104		BRSK2	73	57	MINK1	93	80	FGF-R1	109	94
PKCz	89	55	MELK	82	64	MEKK1	72		HER4	106	68
PKD1	95	85	NUAK1	86	106	MLK1	76	89	IGF-1R	68	98
STK33	73		CK1	84	93	MLK3	85	109	IR	60	112
MSK1	86	27	CK2	86	89	TAO1	79		IRR	82	90
MNK1	73	25	DYRK1A	89	81	ASK1	97		TrkA	93	

Figure 4.9 Kinase profiling data suggests that 672 does not interact with MAPK kinases but might have affinity with IR and Ri (Rock inhibitor) has profound inhibition on ROCK2.

2.3 Activated IR signaling is crucial for the proliferation of stem cells

To find out if normal level of IR activity is crucial for the growth of stem cells and if the mechanism of 672 is associated with this pathway, medium without insulin or any other growth factor was used. As data shown in Figure 4.10.1 and 4.10.2, columns represent the average population of cells in different conditions. It is obvious that growth of both human EC (Fig 4.10.1) and ES (Fig 4.10.2) cells was shown to be inhibited in basal medium without any growth factors comparing to normal culturing medium (DMEM with FBS for EC and Nutristem for ES).

However, this growth disruption was observed greatly rescued in basal medium supplied with human recombinant insulin (5 μg/mL), which was further improved when both insulin and 672 co-exist in basal medium. It seems that 672 can only boost the growth of both human EC and ES cells when insulin is present, indicating that insulin-dependent IR signaling is crucial for the proliferation of stem cells and the activity of 672 relies on the stimulation of this signaling pathway triggered by insulin.

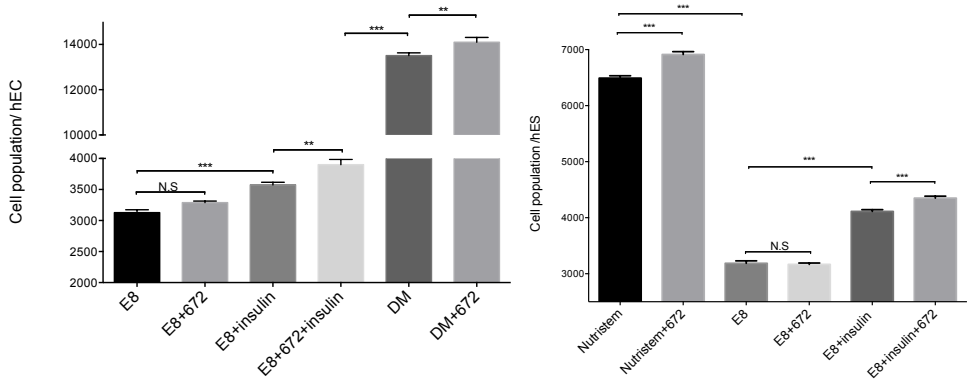


Figure 4.10.1 (Left) and Figure 4.10.2 (Right) show human EC or ES cells can not grow properly in E8 basal medium (without insulin). This situation can be partially rescued by adding in human recombinant insulin, and further improved when 672 in present.

2.4 Thermal shifting analysis suggests that 672 might interact with the IRS-1 complex

Thermal shifting is a simple but reliable and quick assay for analyzing the affinity between chemical and protein. As the principle graph shown (Fig 4.11), when a protein is heated up, it will melt and expose its hydrophobic core which is able to bind the fluorescent dye. This results in the alteration of the fluorescent level. When equilibrium between folded and unfolded state is

reached, the temperature at that point is called the melting temperature (T_m). But if any protein is bond to the compound, the stability of protein could be altered (either more stable or less stable) and melting temperature will shift with an either increased or decreased T_m (Mascini M et al., 2006).

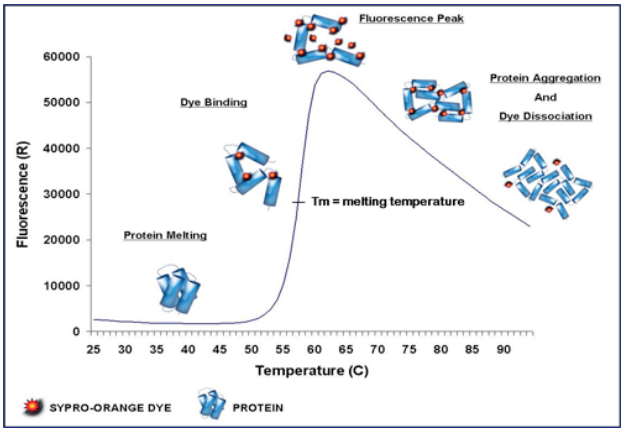


Figure 4.11 Principle of Thermal shifting assay (U.S. Department of Energy Office of Science | UChicago Argonne LLC).

Based on all the data gained so far, it is very likely that 672 acts through IR signaling (cell growth analysis) in ERK1 dependent manner (RNAi) by interacting with the phosphorylation of IR on IRS-1 (as shown in kinase profiling result). Moreover, given that the inhibition on IR activity by 672 was only 40%, which might due to the fact that the substrate of IR used in kinase profiling was a small fragment of peptides and 672 does not bind IR directly (thermal shifting, Fig 4.12). IRS-1, as a main intracellular substrate in response to the stimulation of the binding of insulin to IR or IGF (Insulin growth factor) to IGF-1R, is believed to be a potential target of 672. Since the protein of IRS-1 is not commercially available, to prove this hypothesis, the genes coding IRS-1 and its relative/co-operative protein were silenced by

siRNA respectively to investigate if 672 works through IR signaling through interacting with IRS-1.

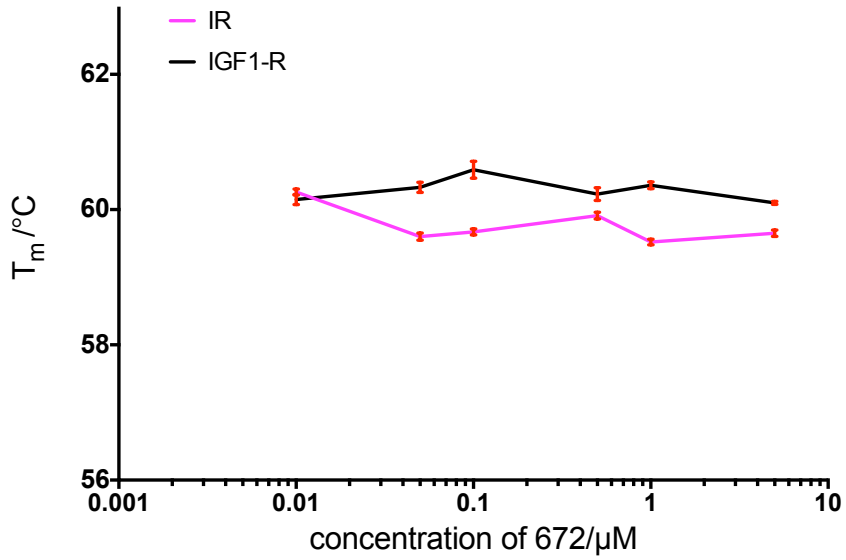


Figure 4.12 Thermal shifting analysis of affinity between IR/ IGF-1R and 672 does not suggest 672 can bind any domain of IR.

In response to Insulin/IGF-1 stimulation, the intracellular tyrosine kinase of IR/IGF-1R will stimulate IRS-1 (Vigneri et al., 2010; Skolnik et al., 1993), the phosphorylation of which on its tyrosine residues will associate and provide over 30 potential recognition sites for series of kinases, PI3K, Akt, GSK3 and MAPK .etc (Chardin et al., 1993; Pawson T, 1995). Heavily phosphorylated IRS-1 also provides these binding sites to adaptor proteins like Grb-2, Fyn, Nck, Crk (Gual et al., 2005). The recruitment of Grb-2 and Sos complex to IRS-1 onto the plasma membrane by Shc (a SH2– phosphotyrosine-binding domain adaptor) will stimulate the guanine nucleotide exchange factor Sos, which in turn leads to the exchange of GDP to GTP in Ras. Then the activated Ras will trigger the MEK-MAPK kinase cascades (Lawrence et al., 2007; Skolnik et al., 1993).

Although, IRS-1 silencing do not suggest that there was a further deduction of cell number in 672 treated wells comparing to the DMSO control, which might be due to the fact that IRS-1 is vital for cell growth. Taken that if IRS-1 is knockdown, total cell number decreased was significant (z<-3) in DMSO only condition and even worse in 672 (z<-4), it is not safe to say that 672 can interact with IRS-1 directly.

However, SOS, GRB2 and SCH-1 were shown to be dependent by 672 (Fig 4.13). The knockdown of these genes did not disrupt the growth of stem cells, but seriously inhibited the enhancement on the cell growth by 672. Given that regulation of MAPK pathway is one of the main roles played by Insulin/IGF-1 signaling pathway (Kang S et al., 2014), which strongly supports the hypothesis that 672 acts through Insulin/IGF-1 signaling in ERK-1-dependent manner by interacting with IRS-1 complex.

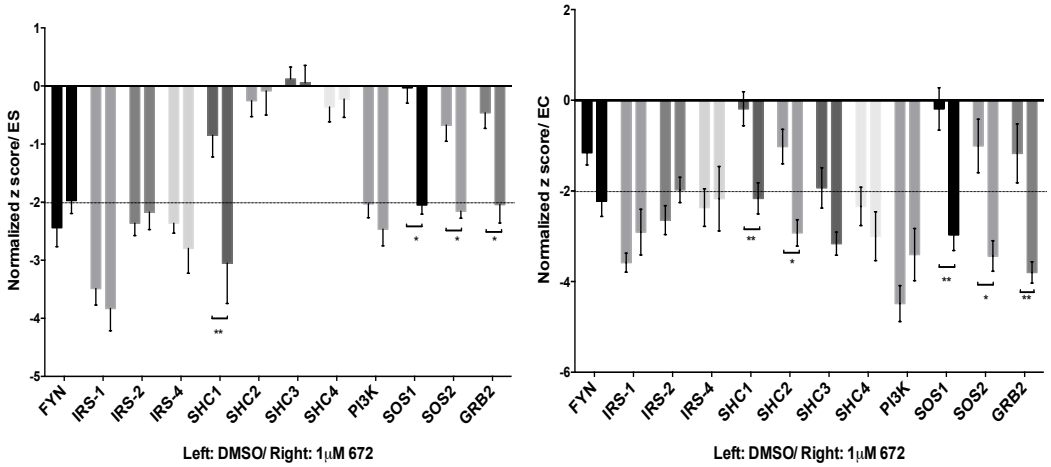


*Figure 4.13 RNAi silencing reveals that knockdown SHC1, SHC2, SOS1, SOS2 or GRB2 do not alter growth of EC cells but block the enhancement from 672. * P<0.05, ** P<0.01. n=12.*

3. Summary

RNAi screening proves that the bioactivity of 672 is ERK1 dependent, while kinase profiling and subsequent assays suggested that it is possible that 672 can interact with IRS-1 complex as shown in Figure 4.14. 689, as a regulator of oxidative suppressor, was shown to be P450 dependent.

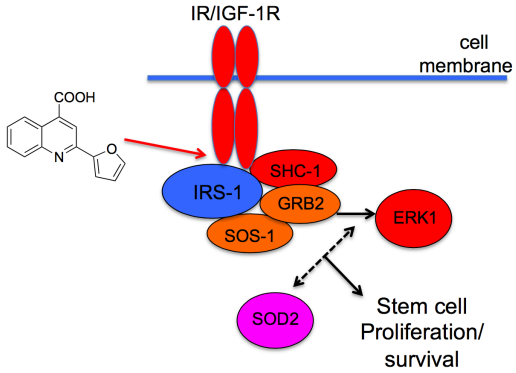


Figure 4.14 The potential mechanism 672 acts is promoting stem cell proliferation through interacting with IRS-1.

However, the role that IRS-1 plays in Insulin/IGF-1 signaling is still controversial, since the counter argument is also supported by the evidence that insulin can activate certain kinases inhibiting IRS-1 by inducing the phosphorylation of IRS-1 at specific sites (Baltensperger et al., 1993; Wary et al., 1996; Yin Y et al., 2014). Based on these observations, it is assumed that IRS-1 might serve as not only a positive but also negative modulator in Insulin/IGF-1 signaling. Taking that in antibody staining, the phosphorylation of ERK was not observed to be significantly enhanced by dosing cells with 672 (this may because that an antibody can only recognize one of phosphorylation sites of this kinase), how 672 interacts with IRS-1 leading to the promotion of stem cell proliferation remains as a topic that warrants further investigations in the future studies.

V. General discussion and conclusions

1. Screening of prion-aiming small molecules modulators of regeneration

Cellular prion protein (PrP^C) has been demonstrated to play a role in enhancing proliferation and promoting self-renewal of stem cells, as well as to be involved in cell-fate altering (Mouillet-Richard S, 2000; Zhang CC, 2006 and Miranda A et al., 2013). Although, the knowledge of how prion acts in modulating cell pluripotency is still unclear, the crucial status of this intracellular protein undeniably makes further investigation focusing on the interaction of prion and its corresponding molecules precious.

Small molecules have advantages because they do not cause immunological response and usually can pass membrane without permeabilizating the cell. And the low-cost, relative easier synthesis and easy storage also make this technology could be standardized. More importantly, since the effects on inhibiting or activating from chemicals can be adjusted by modulating the concentration and are often reversible. It is valuable to identify small-molecule compounds that can regulate prion and result in the modulation of stem cells.

Based on the screening data gained so far, it is very interesting to find out that several prion-aiming compounds showed consistent effect on promoting stem cells growth. Further cell proliferation analysis revealed that 672 can boost the proliferation of EC cells and cell colony expansion of hES cells. In the parallel work analyzing 689 function on human mesenchymal stem cells revealed that 689 can protect human mesenchymal stem cells (MSCs) from DNA damage

and promote cell cycle progression, also improved the engraftment ability of MSCs to bone marrow in mice (Mohanty et al., 2012).

Although no evidence has shown the affinity of these compounds with cellular prion protein (structure of which was aimed for designing all these small-molecule chemicals), some of them have shown reliable bioactivity. Since how prion works in regulating stem cells remains unclear, it is still too early to say that binding by chemicals cannot achieve the regulation of prion network. Thus, to answer if these compounds can work through the prion network, further investigation of the function of prion protein and its relative signaling pathways is demanded.

Prion-aiming small molecules were also observed with certain activity in zebrafish larvae fin regeneration. After setting the suitable concentration on treating fish larvae, Alcian blue staining, PHIS, TUNEL staining, Whole mount in situ hybridization and Quantitative real time polymerase chain reaction were used to explore the mechanism of the compounds. All data from these assays suggest two compounds 689 and 672 may play an active role in benefiting the regeneration of fin in zebrafish larvae. However, maybe due to the unavoidable variation among samples in each condition or the mild effect of compounds on animals, no significant impact was detected of these compounds on increasing the cell proliferation during the process of fin regrowth.

Moreover, similar to the *in vitro* result of analyzing the level of cell apoptosis, both these two chemicals 689 and 672 are observed to minimize exogenous hydrogen peroxide stimulated cell death. One of the cellular reactive oxygen modulators SOD2 is believed to be up-regulated by 689 or 672 treatment. It is noted, based on the gene expression data, the effect of 672 on SOD2 is better on zebrafish than cells, which is different with 689. This observation also supports that although both these two compounds show impact on the same gene SOD2, the mechanism of these two compounds maybe not the same one.

All regeneration relies on the cell source - proliferating stem/progenitor cells. Amounts of evidence have been provided in chapter I supporting this opinion. While, current knowledge about the origin source of balstema in animals remains plain. Since more and more genetic or chemical probes will eventually be developed for cell tracking in animal models, revealing the bioactivity of a specific molecule *in vivo* undeniably will become much easier and more reliable. Further investigation is required to address if these chemicals could influence any specific signaling pathways involved within the process of the formation and destruction of blastema during fin regeneration.

Given that no obvious enhancement of cell proliferation was detected during fin regeneration by dosing with compounds, which might because compounds did not have effect on blastema cells. However, since improved fin regrowth could be seen during the process of regeneration, it is more likely that the compounds might do not impact the cell cycle progression on blastema cells

but brought benefits to the cells instead (promoting ROS scavenging via up-regulating SOD2), which allows a more rapid fin regrowth.

2. Targeting exploration of compounds 689 and 672

During the exploration of the mechanism of these compounds work in using high-throughput RNAi screening analyzing the SOD2-relative genes, the hypothesis that these two chemicals do not share similarity in function was proven again. 672 was shown to be ERK1 dependent, while 689 was believed to have positive effect on regulating cell oxidative hemostasis through P450 family. However, when analyzing the phosphorylation activity of kinases *in vitro* to find out if 672 works as a kinase activity regulator, no MAPK kinases were shown disrupted on phosphorylating the substrates when 672 exists, but Insulin Receptor (IR). Nonetheless, no affinity was detected between the recombinant protein of IR and 672, which made the question more puzzling. But, taking both results into consideration, it was assumed that 672 may be with certain affinity with not the kinase (IR) but the substrate (IRS-1). In kinase profiling, Insulin Receptor Substrate (IRS)-1, as the main substrate of IR and Insulin-like Growth Factor (IGF)-1 was used as the substrate of IR.

This hypothesis was further proved in the assay analyzing cell growth in insulin-free basal medium. Knowledge of how Insulin/IGF-1 signaling plays in self-renewal or cell fate determination in human stem cells is still limited. Current understanding of it is that mammalian cells especially human stem cells' growth requires insulin supplement, and usually in a very high

concentration (Sigma Life Sciences Cell Culture Manual 2011-2014, p.116.). As one of the key modulators of cellular hemostasis across species, insulin-dependent Insulin/IGF-1 signaling can regulate multiple downstream kinases as signaling intermediates (Lee et al., 2013). It was observed that, the growth of stem cells was severely disrupted in basal medium without insulin, which was partially rescued by adding recombinant insulin in and even further improved when 672 exists. It is needed to mention that 672 cannot boost the cell growth in basal medium without supplementary of insulin, which indicates that 672 might work through IR/IGF-1 signaling and do require the activated Insulin/IGF-1 signaling.

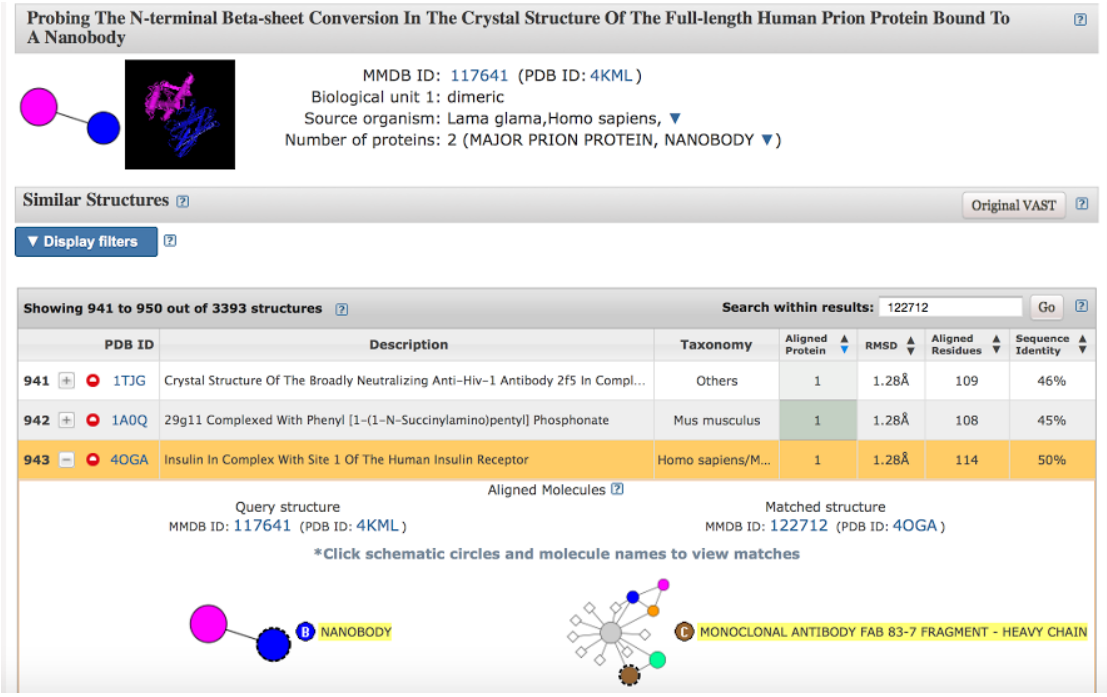
3. Compound 672 as a novel modulator for the proliferation of stem cells

Quinoline contained chemicals have been widely used as drugs dealing with auto-immuno diseases like arthritis (Hahn B et al., 2013) and systemic lupus erythematosus (SLE) (Sun S et al., 2007). As previous study designing chemicals aiming prion protein (Korth C et al., 2001), quinoline was also believed to be with certain affinity with cellular prion protein. In our library of small-molecule compounds aiming prion, quinoline is one of the eight chemical families. And 672, as the top hit in quinoline family also the whole library, shows profound effect on promoting stem cell growth.

As the analogues of 672, two small-molecule quinoline contained compounds H-1152 (Hwang et al., 2008) and CKI-7 (Rachidi N et al., 2014) have been reported to specifically inhibit ROCK II kinase and Casein kinase 1 (CK1)

respectively as discussed in chapter I section 1.7.2. Rock II and CK1 are both cytoplasmic serine/threonine kinases, indicating that the similar structure including quinoline between these two chemicals may have certain affinity with serine/threonine kinases. However, as an acid molecule, 672 does not share any similar structure with these two compounds but quinoline core. In addition, the conjugated system is larger in 672 comparing to these two chemicals, which also can explain why the effect of 672 was observed less intense than these two analogues. Thus, as a very simple structure with consistent bioactivity, 672 worth being further developed.

By comparing the structure between PrP^c and IR (Figure shown below), it is very interesting that these two membrane proteins do share some similar structures and series of amino acids. And since 672 shows a certain level of affinity with the substrate of IR in kinase profiling assay, the similar domain between PrP^c and IR provides an exploitable window for further study in order to reveal the function of the prion network in insulin resistant models.



On both molecular and cellular level, the mechanism of these small molecules gleaned from these studies will benefit our understanding of innate regeneration and help introducing this dramatic capacity to mammalians. The future of regeneration research is exhilarating and full of promise.

VI. P53 function during fin regeneration

Tissue repair and appendage regeneration are a complex process that have not been well revealed. Tumor repressor protein p53, as an important transcription factor, has been demonstrated with the ability inhibiting cell cycle progression for disrupting cell proliferation that will allow cells to repair damaged gene. Studies of tissue repair based on swine revealed *p53* are suppressed during proliferation and then back to normal during the whole regeneration process (Antoniades *et al*., 1994).

Function of p53 gene during the appendage regeneration was discussed in a recent work focusing on the limb regeneration of axolotl (Yun et al., 2013). Yun et al proposed that endogenous regulation of the activity of p53 is crucial in vertebrate limb regeneration. Data showed that the down-regulation of p53 activity is needed for cell cycle re-entry of somatic cells, which allows the formation of blastema. However, it is unknown whether mechanisms demonstrated in this study are similar during appendage regeneration in other organisms. Due to the limited knowledge of how p53 works during fin regeneration in zebrafish, the role of *p53* in zebrafish larvae during fin regeneration will be explored as a part of this program. Although, our compounds have been shown to be with no influence on the level of p53 expression, this work will help to reveal how the regulation of this gene modulates the nature occurring regeneration.

In the previous study using *in situ* hybridization performed by Miss Lloyd, Mdm2 and Gadd45ba (Gadd45) were analyzed as the read-out of p53

expression during fin repair. Mdm2, as a negative regulator of p53, can encode a protein inhibiting transcriptional activity of p53 and catalyzing p53 degradation (Manfredi, 2010). As shown in *in situ* pictures, Mdm2 expression level went up from 18hpa to 30hpa and then went down until 96hpa (Fig 5.1). *Gadd45*, as one of the p53-dependent genes, is always used to detect p53 expression as a replication of the p53 activity. Expression level of Gadd45 was seen from 18hpa until 72hpa (Fig 5.1).

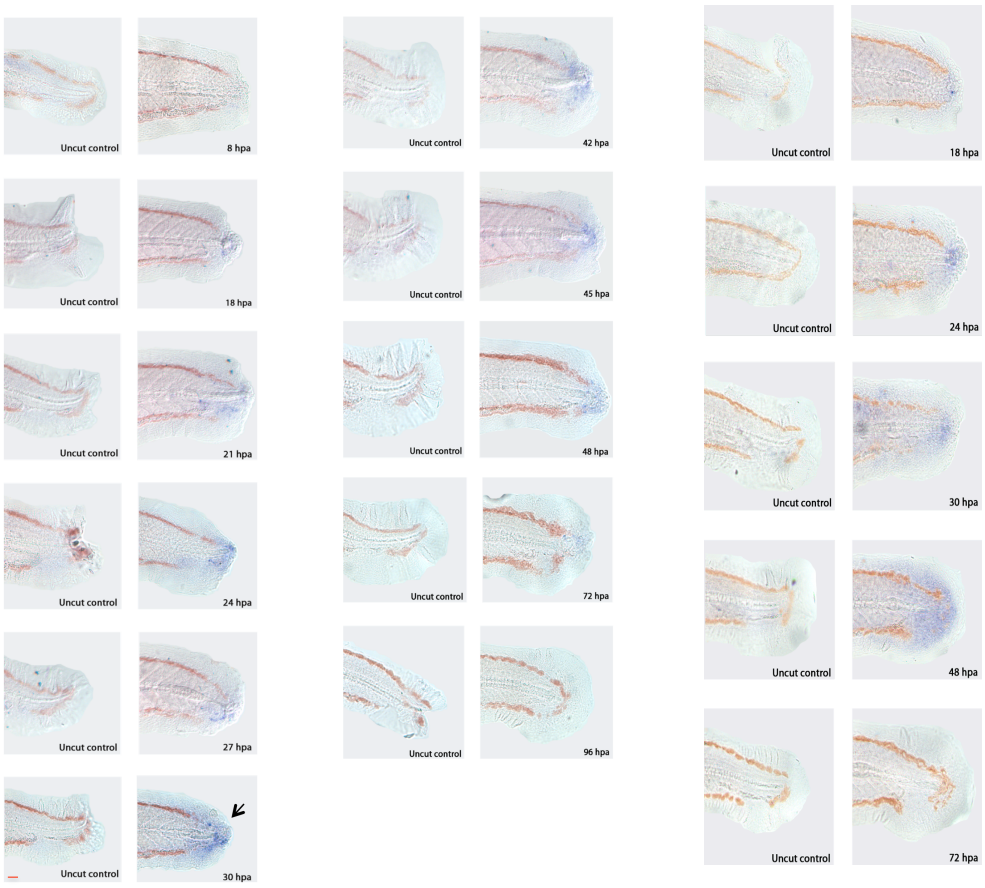
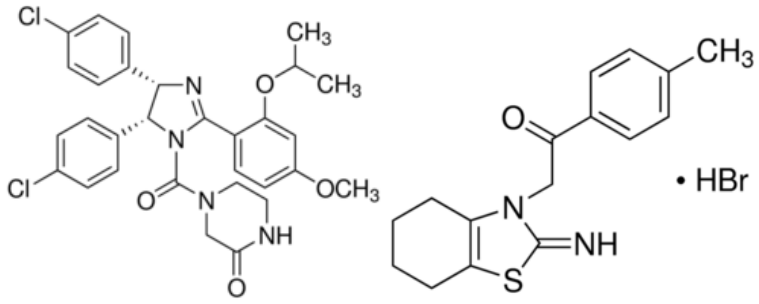


Fig 5.1 Left to Middle: In situ hybridization of Mdm2 at different time points from 8hpa to 96hpa post amputation in the pigment gap plane at 72hpf. A gradual increase in expression can be seen from 18hpa with peak expression seen at 30hpa (arrow) followed by a gradual decrease in expression until absence of expression at 96hpa. Uncut controls indicate absence of Mdm2 expression during these stages of normal development. Right: Gadd45 at different time points from 18hpa to 72hpa. Larvae were amputated in the pigment gap plane at 72hpf. Staining is seen from 18hpa with peak expression at 48hpa. Expression is absent at 72hpa. Uncut controls indicate absence of Gadd45 expression during these stages of normal development. (Pictures from Miss Lloyd)

Following Miss Lloyd's work, to demonstrate the p53 function during fin regeneration in zebrafish, modulation the activity level of p53 artificially is needed. Nutelin-3 (Left) and Pifithrin (Right) were used as



the stabilizer and inhibitor of p53 since Nutelin-3 can work as the agonist of Mdm2 and Pifithrin is able to block varies p53-dependent gene transcription like cyclin G, p21/waf1, and mdm2 (Manfredi, 2010). Moreover, as continuing treatment by 5μM Nutlin-3 and 1μM Pifithrin-α did not show an obvious alteration in tail morphology post amputation, it is still necessary to try different treatment methods (e.g. pulse treatment) on zebrafish.

Based on the previous work, there are three questions needed to be addressed in this study. Firstly, If chemical treatment can sufficiently modulate the activity of p53 in zebrafish? Secondly, is p53 regulation necessary in the initiating period of fin regeneration? Thirdly, if p53 level reversing in the late stage of fin regeneration is necessary as well?

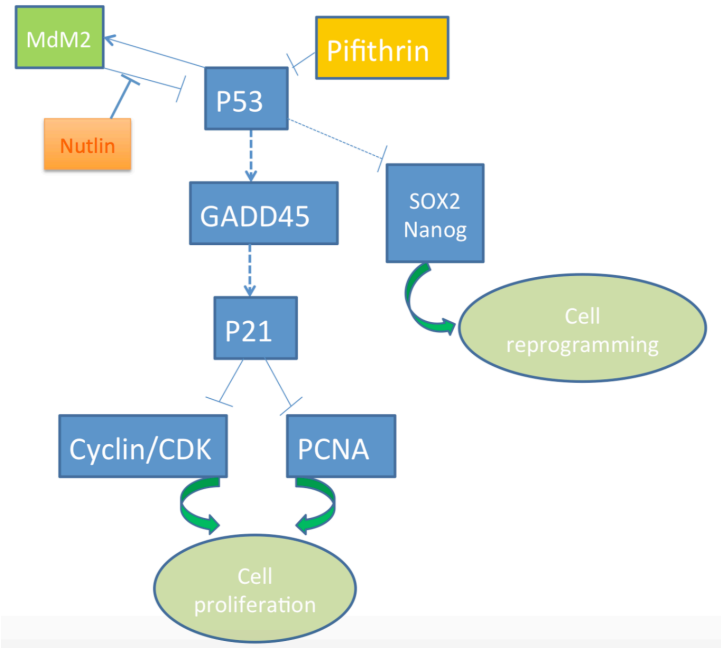


Figure 5.2 Hypothetical signaling map of p53 function in zebrafish fin regeneration (Kawamura et al., 2009; Li M et al., 2012; Levine A & Oren M, 2009; Smith M et al., 1994; Vousden K & Prives C, 2009; Yun M et al., 2013)

1. Method

Zebrafish husbandry and amputation are performed following the same methods described in previous chapter. Immunochemistry staining, *in situ* hybridization and TUNEL staining were taken out using the same protocol talked in Chapter III. Information of reagents used is shown in Appendix. 1. In situ hybridization of Msxc and MyoD used the anti-sense RNA probe synthesized in Roehl lab as reported before (Knight R et al., 2011).

1.1 P53 reporter fish line establishment

1.1.1 P53:Tag BFP vector construction

Multi-site gateway system was used to infuse entry clones p5e, pMe and p3e with destination vector together, which contains p53 binding site promoter, reporter and open reading frame respectively. P53 binding site was

generously provided by Dr Yun from UCL, and then ligase with p5e plasmid, which was further sequenced for verifying using primers (CTGCCAGGAAACAGCTATGAC/CTGATAGTGACCTGTTCGTTGC). Each clone was amplified by mini or midi prep using QIAGEN kit following the suggested protocol. And the final gateway reaction was mixed in the concentration based on the length of each with LR clonase II Plus enzyme (Invitrogen) to form P53:Tag BFP construct (Fig 5.3).

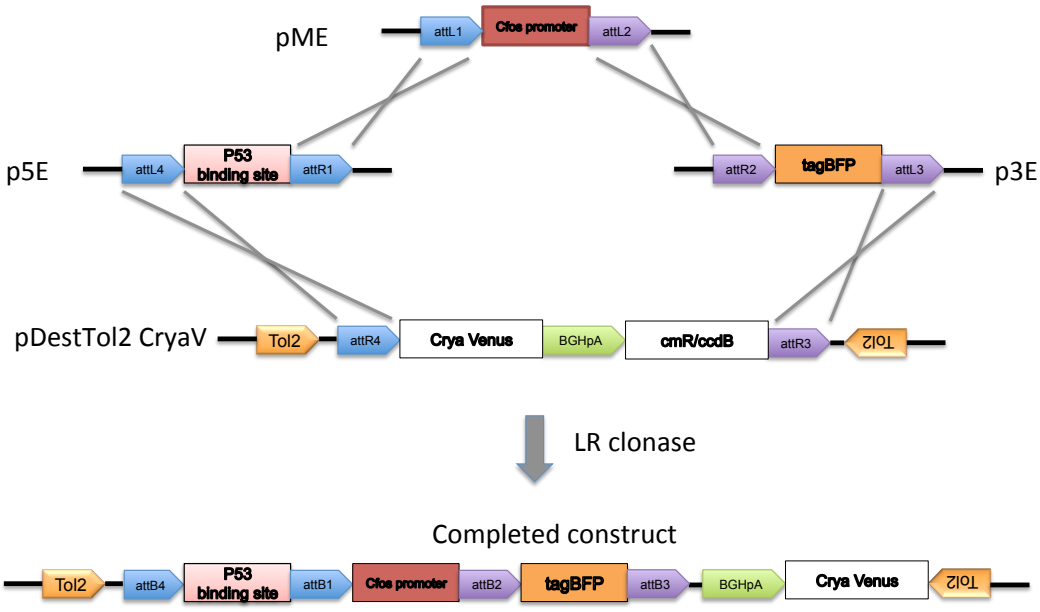


Figure 5.3 Plasmid constructed in Clonase Gateway reaction.

1.1.2. Injection and fish line raising

25-30pg P53:Tag BFP plasmid was injected into 1-cell stage wild type embryos. Embryos were then screened at 4 days after injection under a fluorescence dissection microscopy (Zeiss). Positive ones showing transgenesis marker (Crya Venus, green lens) were sent to be raised as the F0. After three months when F0 were sexually matured, they were pair mated

with wild type fish (AB). Embryos were then sorted again and positive larvae were kept as F1. F0 fish successfully passed transgene to F1 were labeled as founders.

1.2 Chemical treatment

All treatments were in E3 medium. Nutlin-3 and Pifithrin- α were dissolved in DMSO (Sigma) as 10mM stock. Larvae at 2dpf were treated with 1 μM Pifithrin- α or 5 μM Nutlin-3 in the dark for 4 hours then were removed to the fresh E3 medium in new petri dishes. Then larvae were anesthetized with tricaine and fixed at designated time points.

1.3 Quantitative PCR

RNA was isolated from Nutlin, Pifithrin or DMSO treated (2 hours) 2dpf fish larvae fixed at 3/6/18/24 hours post chemical dosing using the same method discussed in Chapter III for analyzing.

Primers used were

GAPDH: GATACACGGAGCACCAGGTT/GCCATCAGGTCACATACACG

MDM2: AACTCCCAACACAACCTT/GGGTCTCTTCCTGACTGGTG

GADD45: CCCTCCAGATCCATTTTACC/ATTCAGTTTGCTGAGCGCTG

2. Results and Discussion

2.1 Nutlin-3 and Pifithrin are able to modulate p53 protein activity

After being treated with Nutlin-3 or Pifithrin for 3-24 hours, fish larvae were then analyzed for the relative expression level of p53, mdm2, gadd45 and p21 by qPCR. Results claimed the stabilization on p53 by Nutlin-3 pulse treatment, given that the level of gadd45 lift after dosing then back to normal after chemical being removed. Morover, Nutlin-3 was supposed to act as the agonist of Mdm2 to prevent p53 degradation, level of mdm2 (went down) and p53 (went up) was observed to be altered by dosing larvae with Nutlin-3 based on the qPCR results (Fig 5.4).

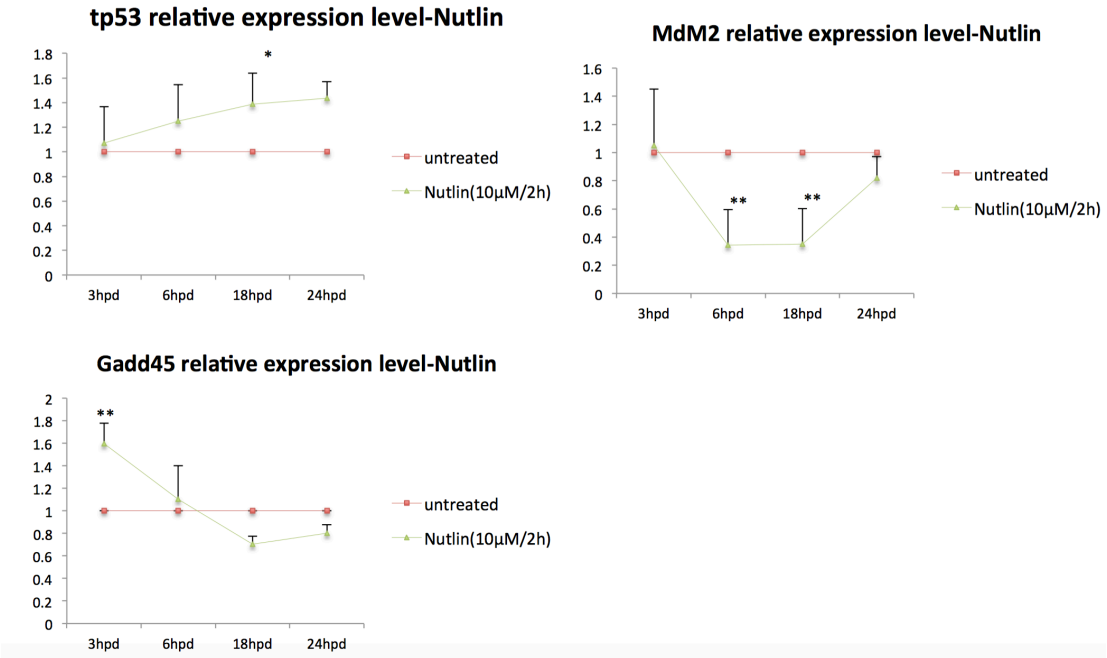


*Fig 5.4 qPCR data showing stabilization of p53 by Nutlin-3 pulse treatment. As Figure shows, level of GADD45 was observed upregulated on 3 hours post dosing (hpd) with Nutlin, and as a feedback, MDM2 went down when p53 level going up from 6hpd. * P<0.05, **P<0.01. n=3.*

Pifithrin, as a reliable inhibitor of p53 pathway, showed profound inhibition on the expression level of mdm2, gadd45 and p53. And the inhibition effect was reversible given that relative expression level of mdm2, p52, gadd45 and p21 returned to the similar level as control fish from 18 hours after pulse treatment (Fig 5.5). Taken all, Nutelin-3 and Pifithrin are reliable stabilizer and inhibitor of p53 and the pulse treatment of both is able to modulate the p53 activity at within 12 hours post treatment.

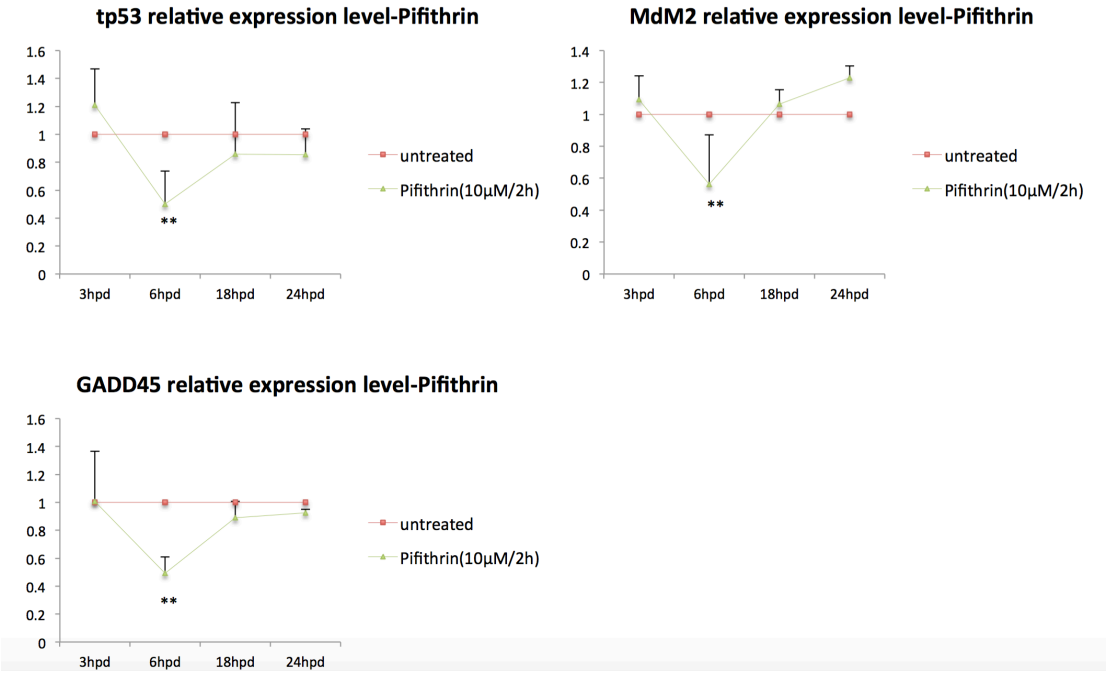


*Fig 5.5 qPCR data showing inhibition of p53 by Pifithrin treatment. The expression level of P53, MDM2 and GADD45 were all detected to be down regulated upon the treatment with Pifithrin at 6hpd. * P<0.05, **P<0.01. n=3.*

Although sequencing result was positive since p53-binding site was successfully ligase into P5e vector (Fig 5.6), establishment of the transgenic fish line was shown to be failed.

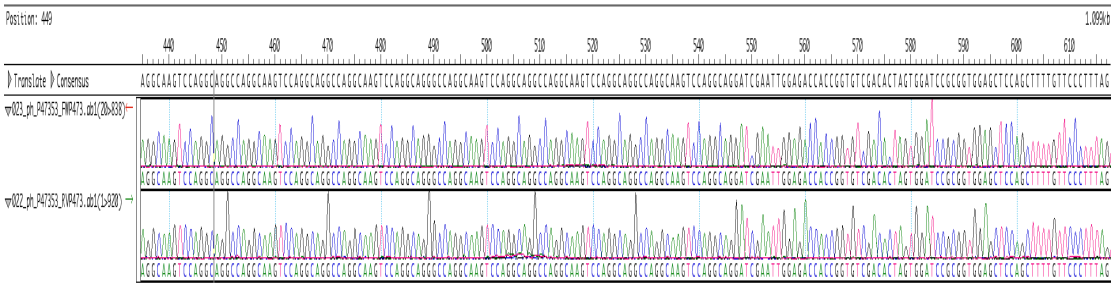


Fig 5.6 Tp53:tag BFP plasmid sequencing result shows the plasmid was successfully constructed.

Due to the undetectable of BFP expressed in F1 fish Larvae (Fig 5.7), which might because the commonly low-level of p53 expression, it was difficult to compare the intensity of fluorescence (blue) between control and chemical treated fish for revealing the p53 activity alteration by treatment with Nutlin-3 or Pifithrin.

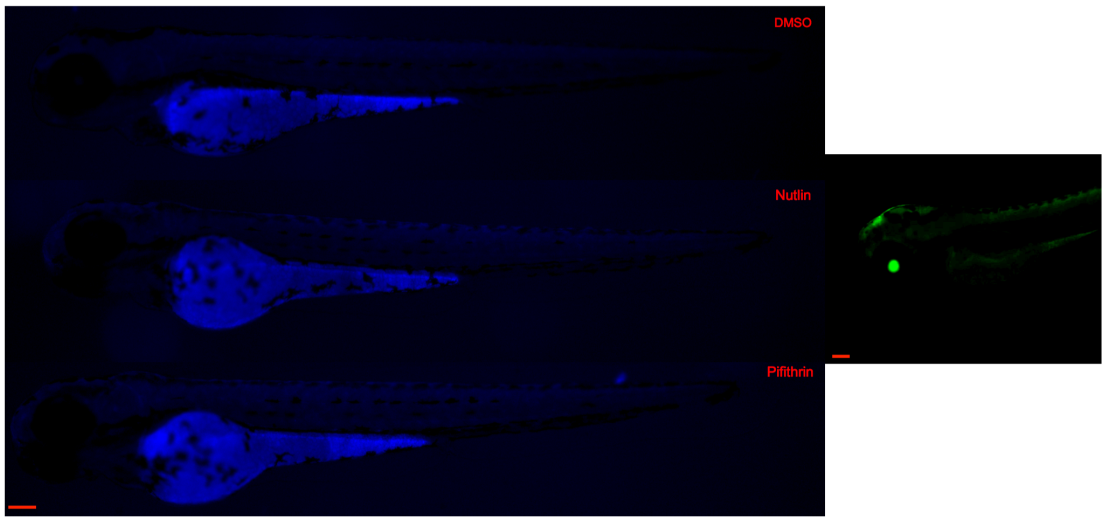


Figure 5.7 Florescence pictures of F1 positive larvae (right) treated with Nutlin (p53 stabilizer), Pifithrin (p53 inhibitor) or DMSO shows no significant difference of BFP intensity.

2.2 Down-regulation of p53 is needed for the proliferation of blastema cells

The impairment observed in Nutlin-3 treated larvae (Done by Miss Lloyd) suggests that the formation of blastema and the blastema cells proliferation may need an relative lower level of *p53* during the initial period of fin regeneration. *Msxc* was used as the marker labelling blastema cells because this gene has been shown to be vital in the fish blastema cells (Akimenko 1995, Odelberg et al., 2000). Knockdown of Msxc was demonstrated to drive pluripotent blastema cells to differentiate, which indicats that pluripotency maintaining requires certain level of Msxc expression in fish (Brockes and Kumar, 2002, Odelberg, 2004). In unpublished data from Roehl lab, *Msxc* expression level was observed to rise from 18hpa until 46hpa then go down until 72hpa, which suggests that the period between 18hpa and 48hpa is the time when blastema was formed. As shown in *in situ* pictures, MsxC is visually fainter in Nutlin-3 treated larvae compared to DMSO (Fig 5.8) suggesting stabilizing p53 in the initial period of regeneration may delay the formation of blastema.

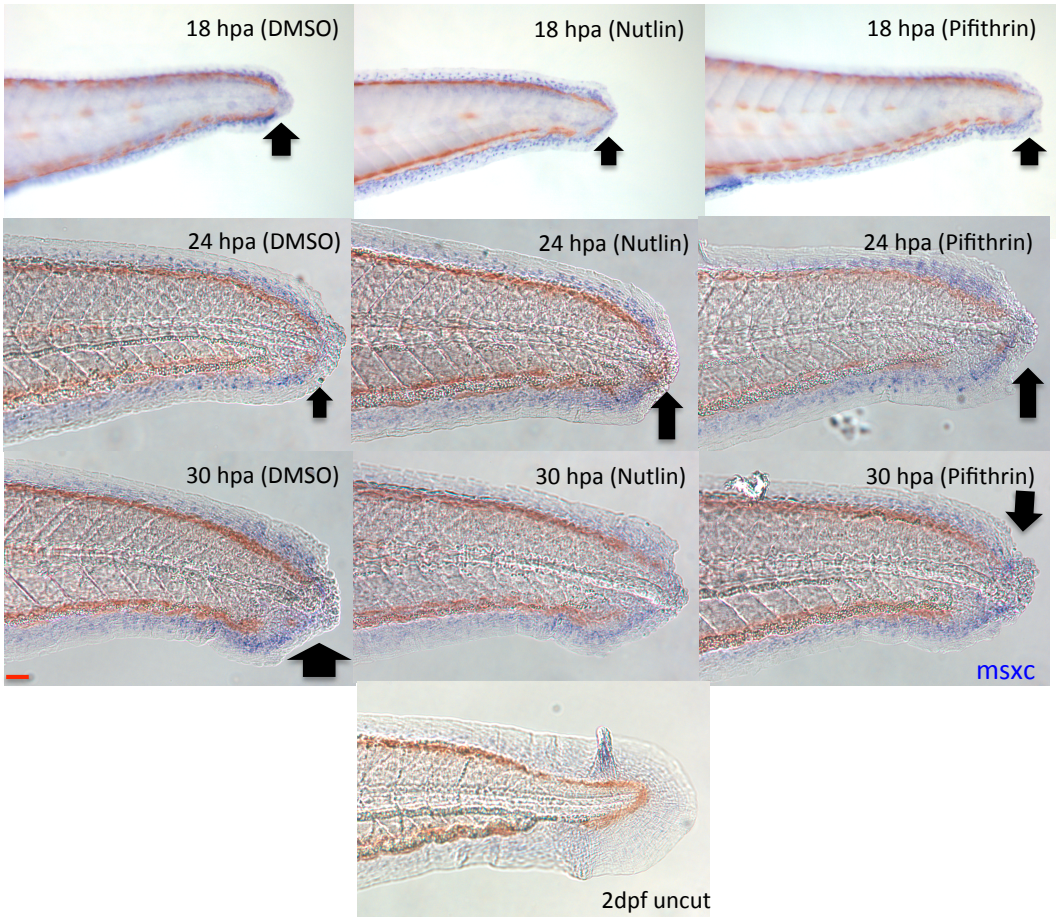


Fig 5.8 In situ hybridization shows stabilizing p53 using Nutlin in early stage of fin repair negatively altered the expression of one of the blastema marker MsxC (blue, arrow headed).

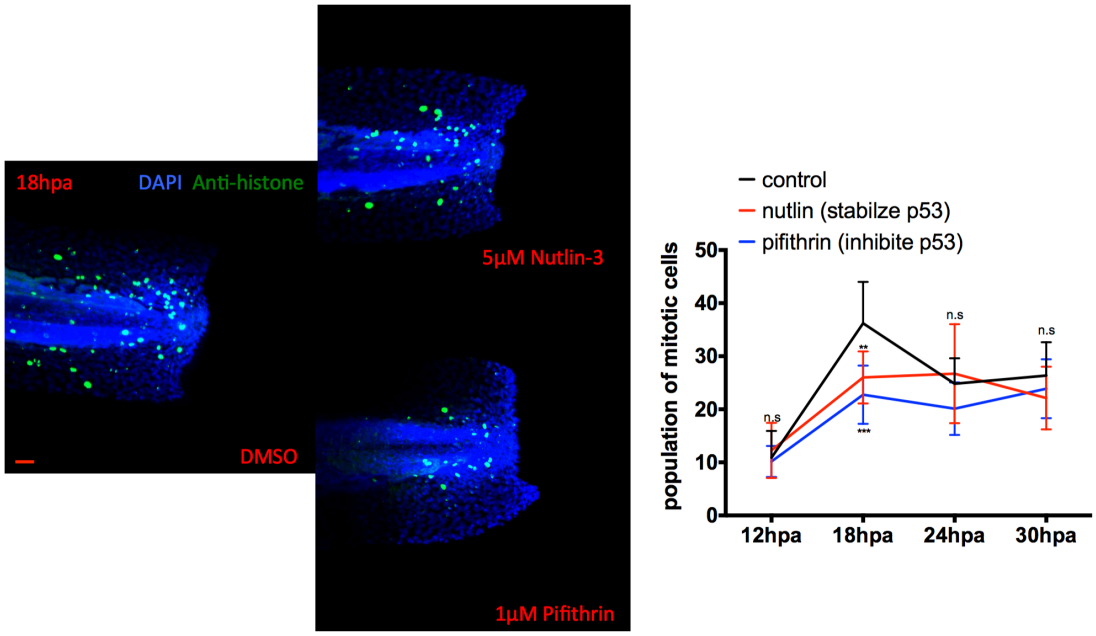


*Fig 5.9 Fish larvae were amputated then pulse treated by 5 μM Nutlin-3 or 1 μM Pifithrin or DMSO for 4 hours, then fixed at 12/18/24/30 hpa. All larvae were stained with antibodies against histone H3 (green) and DAPI (blue). 15 fishes from each group were imaged and the number of green dots (between 50- 3000 μm^2) within the caudal fin region (300 μm wide) in each fish was counted. Data (n>10) shows the average value ±SD, statistic significance was determined using T-test versus control. Ns p<0.1; **p<0.01; ***p<0.005. Scale bar 50 μm. Results suggest dosing with both two compounds significantly reduced mitotic cells on 18hpa.*

Using PHIS staining as described in Chapter III, dosing larvae with Nutelin-3 was believed to inhibit the proliferation of cells around the wound plane as shown in Figure 5.9. By stabilizing p53 using Nutlin-3, proliferation was thought to be weakened given that mitotic cells were significantly decreased in Nutlin-3 treated fish comparing to control at 18hpa.

Inhibiting the p53 activity by treating larvae with Pifithrin was believed to severely influence the fin regeneration because the proliferation was seriously abated in Pifithrin treated larvae. This observation suggests that p53, as a vital gene involved in cell homeostasis, is crucial to modulating the balance

between apoptosis and cell cycle. This finding was also proved again in apoptosis level analysis using TUNEL staining, which claims that apoptosis was also inhibited in Pifithrin treated larvae during fin repair but not in Nutelin-3 treated group (Fig 5.10).

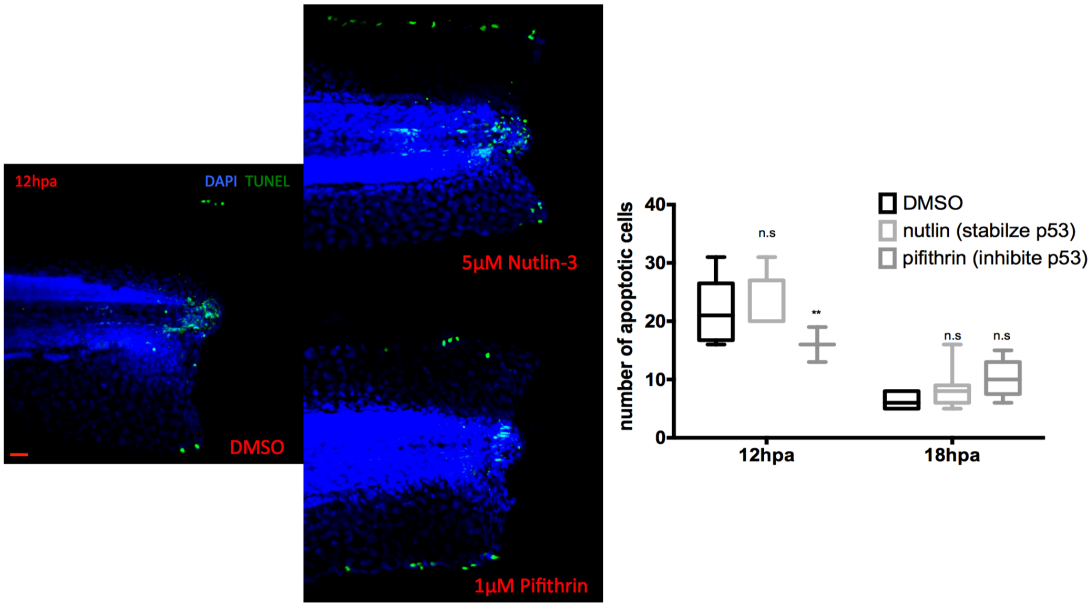


*Fig 5.10 Fish larvae were amputated then pulse treated with 5 μ M Nutlin-3 or 1 μ M Pifithrin or DMSO for 4 hours, then fix at 12/18/24/30 hpa. The level of apoptosis in all larvae was determined using the TUNEL in situ apoptosis kit (green, Millipore) and DAPI (blue). The bar shows the number of green dots (between 50- 1000 μm^2) within the region of caudal fin in each larvae during regeneration. Data (n>10) suggests apoptotic cells in Pifithrin treated larvae were significantly decreased on 12hpa. Statistic significance was determined using one-way ANOVA versus control. **p<0.01, n.s p>0.05. Scale bar 50 μ m.*

2.3 Normal p53 level is crucial for re-differentiation

Furthermore, as shown in the work done by Dr Yun (Yun et al., 2012), axolotl limb regeneration was observed to be improved when stabilising *p53* during the late stage of regeneration. This finding suggests that a normal or even relative higher level of p53 might be demanded for the late stage of limb regeneration.

The tissue replacement during fin repair requires different cell types, which means a certain type of precursor cells like muscle precursor cells could be used as the indicator showing the stage of re-differentiation. MyoD, one protein of myogenic regulatory factors, is widely utilized as the marker labeling differentiating muscle precursor cells or myoblast. As shown in *in situ* pictures, the staining of MyoD is weaker in Pifithrin treated fish larvae than DMSO or Nutlin-3 treated larvae (Fig 5.11). This observation strongly suggest that re-differentaition requires a relative normal or higher p53 activity.

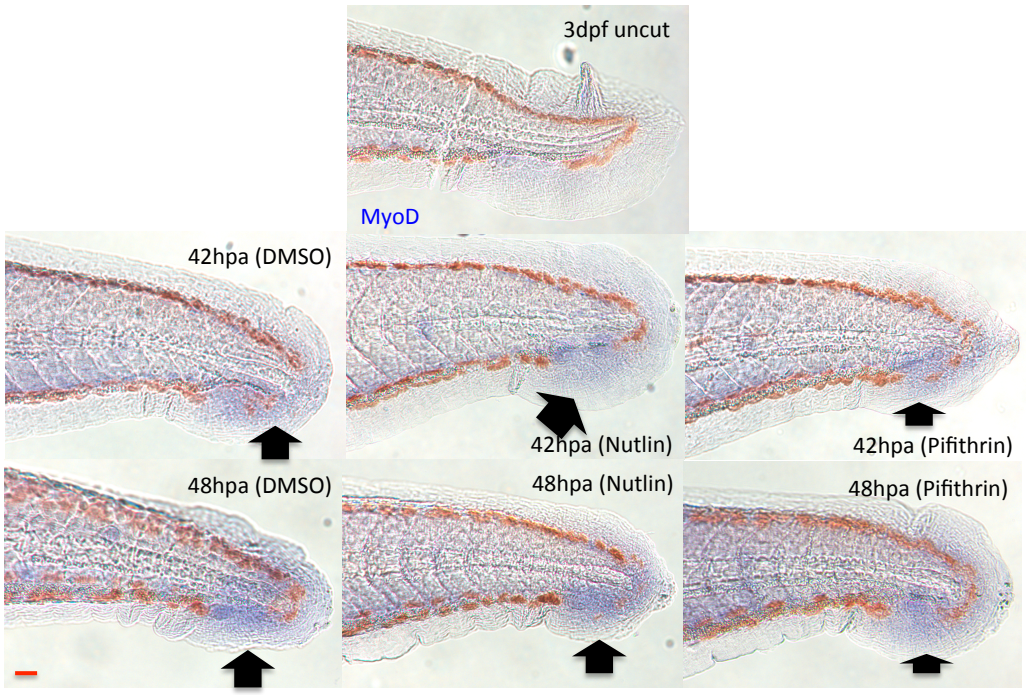


Fig 5.11 In situ hybridization pictures shows inhibiting p53 using Pifithrin in late stage of fin repair decreased the expression of a muscle precursor cell marker MyoD (blue, arrow headed).

3. Summary

As Yun demonstrated in the paper, tumor suppressor p53, as a key molecule involved in maintaining cellular haematosis, is also critical for limb regeneration in axolotl (Yun et al., 2012). To investigate if this mechanism is similar in zebrafish, larvae fin regeneration was utilized as the model analysing the regulation of p53 activity during vertebrate appendage regeneration. It is believed that dedifferentiation is essential for forming blastema in zebrafish fin regeneration. And as the niche of progenitor cells, a number of pluripotency makers were shown to be expressed in blastema (Odelberg, 2004). To manually modulate the p53 activity, Nutlin-3 (p53 stabilizer) and Pifithrin (p53 inhibitor) were applied on amputated fish larvae for exploring the consequence following losing natural p53 regulation during regeneration. A visually observed fainter staining of *Msxc* in Nutlin-3 treated fish comparing to DMSO strongly suggests that at least temporal low level of p53 is crucial for blastema formation (Fig 5.8). Moreover, significant fewer mitotic cells were detected in p53-stabilized fish (PHIS staining). Taking both results into consideration, the blastema formation during zebrafish fin regeneration requires the down regulation of p53 activity.

In axolotl, the level of Gadd45 was shown to increase in blastema cells but decrease in mesenchymal cells during limb regeneration (Yun *et al.,* 2012). In zebrafish larvae, an increase of *Gadd45* expression was also observed after blastema being formed, which indicates that *Gadd45* might be crucial to blastema cells for maintaining the pluripotency or processing differentiation (Fig 5.1). Given that inhibiting p53 by Pifithrin treatment at 48 hpa decreased

the expression of a reliable muscle precursor cell maker MyoD, which strongly suggests that a normal level of p53-dependent *Gadd45* is crucial for myogenesis.

However, Mdm2 can response to the alteration of p53 activity as a feedback regulation. So, it is possible that the up-regulation of Mdm2 observed during the initial period of fin regeneration may be not a true replication of p53 activity. Due to the fact that the fluorescence of transgenic line was too weak to be detected, it is still not clear how p53 activity regulates naturally during zebrafish fin regeneration. But, given that a certain low level of p53 was shown to be important in the period of blastema formation and cell proliferation post amputation, and Pifithrin inhibition on p53 resulted in impairment of fin fold (which might be due to the disruption of myogenesis), It is assumed that p53 regulation is crucial for fin regeneration in zebrafish. To investigate that whether p53 works as a balance mediating cell reprogramming and differentiation, establishing a transgenic fish line expressing detectable marker is needed in the future work.

Moreover, based on the fact that the regulation of an individual gene (p53) is able to influence the fin regeneration, how 672 or 689 improved the fin regrowth still needs to be assessed on gene level. Given that CRISPR-Cas system has been successfully adopted to zebrafish studies (Hwang WY et al., 2013), using this modern method knocking down/out genes coding IRS-1 or CYP4F12 will improve our understanding of how these compounds work in zebrafish.

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Abbreviations

Smed	*Schmidtea mediterranea*
Dj	*Dugesia japonica*
RB	Retinoblastoma protein
EGFP	Enhanced green fluorescent protein
RFP	Red fluorescent protein
hpa	Hours post amputation
dpa	Days post amputation
dpf	Days post fertilization
SSEA	Stage-specific embryonic antigens
TRA	Tumor rejection antigen
GCTM	Embryonal carcinoma antigen
FBS	Fetal bovine serum
MEFs	Murine embryonic fibroblasts
DMEM	Dulbecco's modified Eagle's medium
FGF	Epidermal growth factor
IGF-1	Insulin-like growth factor-1
CAMs	Surface adhesion molecules
ECM	Extracellular matrix
ROCK	Rho-associated protein kinase
PI3K	Phosphatidylinositol-3-kinase
iPSCs	Induced pluripotent stem cells
hES	Human embryonic stem
EC	Embryonic carcinoma
PLCk	Phosphoo-lipase Ck
MAPK	Mitogen-activated protein kinase
Sos	Guanine nucleotide exchange factor
Cyclin	Cell cycle check point
Xbra	Xenopus brachyury gene
msx	Muscle segment homeobox
RA	Retinoic acid
RAR	Retinoic acid receptor
shh	Sonic hedgehog
Gli	Glioma-associated oncogene homologue
APC	Adenomatosis polyposis coli
TACE	Tumor necrosis factor-alpha converting enzyme
NICD	Notch intercellular domain
FACs	Fluorescence-activated cell sorting
PI	Propidium iodide
PCR	Polymerase chain reaction
tricaine	Tricaine methanesulfonate
PFA	Paraformaldehyde
PHIS	Anti-Phosphorylate Histone H3 antibody
DF1	Dharmafect 1 reagent
IR	Insulin receptor
hpd	Hours post dosing

Appendix. 1

Solution	Composition	Source
0.2 x SSCT	1% 20x SSC, 0.1% Tween-20 in H_2O	Made in lab
2X SSCT	10% 20x SSC, 0.1% Tween-20 in H_2O	Made in lab
20x SSC	3M NaC1, 300mM Tri-sodium citrate	Made in Lab
Dimethyl sulfoxide *(DMSO)*		Sigma, USA
Anti-Histone H3 Rabbit antibody		Calbiochem, Germany
Anti Rabbit ALEXA 488 antibody	Alexa Flour 488 goat anti-rabbit IgG	Molecular Probes (Eugene, OR. USA)
Bleaching Solution	10% H_20_2, 0.5% 20x SSC, 5% Formamide in H_2O	Made in Lab
Block Solution for Immunohistochemistry	PBST with 10% NBCS	Made in Lab
Embryo medium (E3 medium)	1L stock contains 15mM NaCl, 0.5mM KCl, 1mM$CaCl_2$, 1mM $MgSO_4$, 0.7mM $NaHCO_3$ and 3 drops of methylene blue	Made in Lab
4% Paraformaldehyde (PFA)	4% Paraformaldehyde dissolved in PBS at 70^{o}C and stored at 4 ^{o}C.	Made in Lab
Phosphate Buffered Saline (PBS)	4 PBS tablets dissolved in 800 ml dH_2O, autoclaved and cooled before use	Made in Lab, PBS tablets from Sigma, USA
PBST	500ml PBS and 250ul 20% Tween-20	Made in Lab
Tricaine	500ml dH_2O, 2g Ethyl 3-aminobenzoatemethansulfonate and 5g Na_2HPO_4	Made in Lab
Vectashield with DAPI		Vector Laboratories

		Inc, Brulingame CA
Staining buffer	5ml 1M tris pH9.5, 2.5ml 1M $MgCl_2$, 1.66ml 4M NaCl, 250ul 20% Tween-20 in 50ml dH_2O	Made in Lab
Trizol reagent		Sigma, USA
High Capacity RNA-to-cDNA KIT		Applied Biosystems, USA
SYBR Green JumpStart Taq ReadyMix		SIGMA, USA
Methnol		Fisher chemical, UK
Glycerol		VWR, UK
Hybridization solution (+)	Heparin stock, formamide, tRNA, SSC, Tween, citric acid	Made in Lab
Hybridization solution (-)	formamide, SSC, Tween, citric acid	Made in Lab
Goitrogen 6-n-propyl-2-thiouracil (*PTU*)		Sigma, USA
Tubulin Tracker™ Green Oregon Green® 488 Taxol,		Molecular Probes
Rnase		Sigma, USA
TrypLE		Life Technology
Y27632		Abcam
Click-iT Plus EdU Flow Cytometry Assay Kit		Life Technology
Dharmafect 1		Dharmacon
RNAse inhibitor		Promega
Transcription buffer DIG labeled NTP mix		Boehringer
RNAlater		Sigma, USA
Hydrogen peroxide		Fisher scientific, USA
Nutlin-3		Sigma, USA
Pifithrin- α		Sigma, USA

Appendix. 2

PCNA (fish)	ATGGCGTGAAGTTCTCTGCT /TGCACTGGCTCATTCATCTC
Tp53 (fish)	GATGGTGAAGGACGAAGGAA/AAATGACCCCTGTGACAAGC
SOD2 (fish)	AGCGTGACTTTGGCTCATTT/TCTTCCGCTCTCCTTTTCAA
Ef1a (fish)	GATGCACCACGAGTCTCTGA/TGATGACCTGAGCGTTGAAG
Rpl13a (fish)	TCCCAGCTGCTCTCAAGATT /TTCTTGGAATAGCGCAGCTT
Pgk-1 (fish)	CACAAACCACAGGCAAAATG/TCTGGCATAGGGACTCCATC
Cyclin D (fish)	TGACTTGCCTTGACTTGTCG/GAAAAAGCAGGGAGCACTTG
Pea 3(fish)	CAGAGGAGGTGGCAAGACTC/GCTTCTGGCTCACACACAAA
β-actin (fish)	CGAGCAGGAGATGGGAACC/CAACGGAAACGCTCATTGC
SOD2 (homo sapiens)	CTAGGGTTCGCTCCAGTGAG /CATTGGGACAGAGGACCAGT
Tp53 (homo sapiens)	TCAACAAGATGTTTTGCCAACTG /ATGTGCTGTGACTGCTTGTAGATG
GAPDH (homo sapiens)	GGTCTCCTCTGACTTCAACA /AGCCAAATTCGTTGTCATAC
PCNA (homo sapiens)	TAGCCAAGGTCTCCCAGCTA/CAGGAAACACCAAAGGCAAT
SOD1 (homo sapiens)	ATGATCCCAGGTGTTTGAGC/AGCCTGTGTTTGGGGTGTAG
Mdm2 (in-situ)	GAC CCG GGG ATA CAG ATT CA/ GTC GGG TAC GTC CAC TCC AT
Raldh2(in-situ)	atactaatctcctggacctttctcc/catctttagaaccccagaagg
MsxC (in-situ)	ggtccgtagagtggtggaga/ggatccattaaccctcactaaagggaaacgcaggtaataacggcttg
Gadd45(in-situ)	CCGATGAGGAGGATCTGGAC/ TTCCAACCCCATTGCACATA