The zebrafish as a model for tissue regeneration and bone remodelling

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Chapter 1

Chapter 1: Introduction

Summary

Bone development and regeneration involves a balance between breakdown and synthesis of bony elements in the skeleton. These two processes are carried out by osteoclasts and osteoblasts, and are influenced by endocrine factors, ageing and drug treatment. The disturbance in this harmonious balance leads to the diseases such as osteoporosis and osteoarthritis. Osteoclasts are of hematopoietic origin while osteoblasts are mesenchymal cells. The process of regeneration allows an organism to regain the function of an organ or structure damaged by injury or disease. Regeneration has interested scientists for centuries. In recent decades this field has gained great attention from biomedical researchers. The main purpose of this interest is to find a way where cells, tissues and structures lost due to mechanical injuries, disease or ageing can be restored through regenerative medicine. Lower animals like urodeles, amphibians and many invertebrates have retained their regenerative capabilities to a remarkable extent throughout evolution, whereas humans and most other vertebrate species seem to have lost much of regenerative abilities for lack of potential mechanism underlying this process. Among other organisms, many species of fish have also retained extensive regenerative capabilities even at adult stages. The zebrafish *Danio rerio*, a teleost, is known to retain regenerative capacity to a great extent in larval and adult stages, and can regenerate heart, notochord, lens, retina and many other organs in addition to scales and fins. Here, I review some key literature concerning bone and tissue regeneration in the vertebrates, with special emphasis on the zebrafish model, and the relevance of these processes to selected diseases in humans.

Zebrafish as an experimental model for developmental biology

Zebrafish or *Danio rerio* is a fresh water teleost fish endemic to the areas of India, Pakistan and Nepal [1]. The total body length at maturity can reach up to 3-4 cm [2]. The presence of black and white stripes on the skin, gives an appearance which is comparable to the zebra hence named as zebrafish [3]. Adult fish has a streamlined laterally compressed body with one dorsal fin, one ventral fin, a notched caudal fin and two pectoral fins which help in swimming. The mouth protrudes outward and dorsally but is devoid of any teeth; however there is a row of pharyngeal teeth in the 5^{th} pharyngeal arch to chew the food [4]. It has long been used as a pet, but its importance as an experimental animal was only realized after initial studies done by Creaser et al [5]. It has a life expectancy of 3-4 yrs. The young fish reach maturity and start to reproduce at about 3 months of age. Zebrafish is known for high fecundity, each female fish can lay around 200-300 eggs per mating, the eggs are fertilized externally. These eggs can be placed in Petri plates until they hatch and reach larval stage where they begin to feed, at around 5 days post fertilization [4].

Zebrafish eggs are highly suitable for developmental research [6]. One of the marvels of nature is that the eggs are virtually transparent and have a very fast rate of development. The most exciting feature for developmental biologists is that all the morphogenetic movements can be easily observed under a dissecting microscope. In contrast to the other experimental animal models the fast rate of development leads to the formation of most of the organs by 38 h post fertilization [3]. The embryos develop inside a transparent external chorion from which they hatch at around 2 -3 dpf and immediately start swimming freely. The embryos at this stage carry all of their nutrients in the form of yolk cells enclosed in a round yolk sac therefore no food is needed for first 5 days. Young larvae at 5 dpf can then be supplied with baby fish food and raised in medium-sized containers with constantly flowing water (for further protocols, see [7]).

The large number of eggs per batch makes it the most appropriate experimental animal for large scale screenings (for examples, see [8,9]) Short generation time that is 3 months, allows the development of transgenic fish

lines. External development of zebrafish embryos without parental care makes it most suitable for behavioural studies [10-13]. Another great advantage of using zebrafish as an experimental model for achieving genetic studies is that the sequence of zebrafish genome is available. In addition, the transparency of embryos and larvae is also ideal for whole mount staining procedures, thus making it possible to analyze in whole mount organism.

The Developmental Biology of Zebrafish Bone

Bone Metabolism

Normal bone development and turnover is a fine balance between bone breakdown (catabolism) and bone formation (anabolism). The chief cells involved in these complementary processes in the vertebrates are as follows [14]:

- **Osteoblasts**, which are derived from the mesenchymal stem cells, and which give rise to bone-depositing osteocytes;
- **Osteoclasts**, derived from mononucleated precursor cells of the haematopoietic lineage, and which fuse to form multinucleated cells.

In human beings, there is a very high rate of bone turnover during childhood, in which formation exceeds resorption. Formation and resorption are in approximate balance in young adulthood, but with ageing there is a net loss of bone [15].

Throughout life, bone Remodelling takes place in foci containing osteoblasts, osteoclasts and their precursors, and the processes are coupled and in the healthy person, are in equilibrium. Both of these cell types arise from distinct cell lineages and maturation processes. Osteoblasts arise from mesenchymal stem cells, while osteoclasts differentiate from hematopoietic monocyte/macrophage precursors [14]. If the balance between the activities of these two cell types is disturbed, this can lead to loss of bone density (osteoporosis) or to increased bone formation (osteopetrosis). Loss of bone is also seen in osteoarthritis and rheumatoid arthritis [16,17].

Studies on osteoclasts in mammals

Osteoclastogenesis is dependent on cytokines, of which two main are RANK-L (receptor activator of nuclear factor-κB ligand) also known as TRANCE (TNF-related activation-inducing cytokine) and MCSF (macrophage colony stimulating factor [18-20]. For a summary of genes involved in bone Remodelling, see Table 1. MCSF (macrophage colony stimulating factor) is considered critical for the proliferation of osteoclast progenitors, whereas RANK L controls the differentiation process directly by activating RANK. It has been established that RANK works in close cooperation with certain other receptors like OSCAR (osteoclast-associated receptor) and TREM 2 (triggering receptor expressed on myeloid cells) [15]. Stimulation of RANK and of the immunoglobulin-like receptors cooperatively phosphorylates ITAM (Immunoreceptor tyrosin based activation motif).

Authors of [21] studied the differentiation of osteoclast precursors into osteoclasts in mice. MCS-F stimulated the proliferation of macrophage precursors and their expression of osteoclast markers (RANK and TRAP; tartarate resistant acid phosphatase) *in vitro*, a macrophage cell line behaved also in this way. During this process the cells changed from spindle-shaped to round (pre-osteoctyes) and finally to multinucleated. The macrophage marker CD14 was simultaneously down regulated. Also down regulated was OPN (osteopontin) which is characteristic of M-CSF dependent cells but this expression weakens after differentiation into pre-osteoclasts. Carbonic anhydrase 2 is expressed in the mature osteoclasts but not in the precursors, its expression is noticed in the cytoplasm and the inner surface of the ruffled border [22]

Studies on osteoclasts in fish

Matrix degradation by osteoclasts is a key process in both normal bone turnover and the bone disease osteoporosis [23]. Osteoclasts are classically described (at least in mammals) as multinucleated giant cells of the myeloid (monocyte-macrophage) lineage [15,24]. They display a characteristic ruffled border where proteases and hydrogen ions are secreted, allowing for bone resorption and formation of ‘resorption pits’ in the bone surface [25]. Osteoclast morphology varies between mammals and teleosts (bony fishes),

and also between different groups of teleosts [24]. In the skeleton of young zebrafish, for example, osteoclast activity is carried out by both mononucleated and multinucleated cells [26]. In fact, there is an ontogenetic progression from mono- towards multinucleated osteoclasts [26]. In juvenile zebrafish, bone resorbing cells in the developing lower jaw are at first mononucleated. In thin skeletal tissues such as the neural arch, mononucleated cells are predominant in adults [26]. In rainbow trout, scale resorption is predominantly carried out by mononucleated osteoclasts [27]. Although in mammals these mononucleated cells are often just regarded as osteoclast precursors, in fish mononucleated osteoclasts are active bone resorbing cells [28,29].

One family of osteoclast proteases are the matrix metalloproteinases (MMPs). They are involved in the breakdown of extracellular matrix by osteoclasts, but also by other cell types like fibroblasts [30]. MMPs are multi-domain enzymes that require zinc as cofactor for proteolytic activity. Extracellular matrix turnover occurs in a wide range of physiological processes, including embryonic development and morphogenesis, bone resorption and tissue regeneration. Moreover, MMP-mediated breakdown of the extracellular matrix has been implicated in disease processes including cartilage destruction in osteoarthritis [31]. The importance of MMPs in bone development is underlined by studies on *mmp-2* and *mmp-9* null mice, which suffer from bone abnormalities, osteoporosis and osteopetrosis respectively [32]. In view of their role in physiological and pathological processes, MMPs are important targets in pharmaceutical research and drug development.

In bone turnover, secreted MMPs participate in the breakdown of collagen, which in turn allows osteoclast attachment [33]. Furthermore, *MMP-9* is associated with osteoclast migration through the collagen matrix [34]. Matrix metalloproteinases may also break down residual collagen left by cathepsin K after the pH rises in the resorption pit [35]. MMP-2 and *MMP-9* (gelatinase A and B, respectively) are particularly active against gelatins (denatured collagens) and intact collagen types I and IV. In the bone of dermal origin, matrix degradation is thought to rely more on MMPs and less on cathepsin K [36]. MMP-2 has also been described to play a crucial role in formation and maintainance of the osteocytic canalicular network whereas MMP-9 is active in early calvarial bone development and in orthodontic tooth movement [37]. Regenerating fins of adult zebrafish express *mmp-2* and regeneration can be

inhibited by the MMP inhibitor GM6001 [38]. More recently, MMPs have also been implicated in angiogenesis and liberation of growth factors [39,40].

TRAP and cathepsin K *in situs* in the newly-hatched medaka (stage 40-44 of [41] show a specific labelling of cells [42]. They found strong scattered expression in the pharyngeal region and teeth. The labelled cells were both mononuclear and multinucleated with typical ruffled borders. Expression of *mmp-9* gene has been studied in different embryonic and adult stages of zebrafish, it is expressed in the myeloid cells [43]

Table 1 Genes expressed by osteoclasts or their precursors

Gene	Expression (any vertebrate)	Ref.	Genbank Accession Number for Zebrafish
TRAP	multinucleated osteoclasts	[21,42,44]	17049327, 10934646, 11148180
CD14	Macrophages (NOT osteoclasts).	[21]	10934646
CD43	pro-osteoclasts	[21]	10934646
Cathepsin K	multinucleated osteoclasts	[21,42,45]	17049327, 10934646, 9028530
RANK/OPGL/TRANCE	Pre osteoclasts	[46-49]	
Oscar	Myeloid cells-Mature DC	[50]	15155468
Calcitonin receptor (ctr)	Inhibits osteoclast activity.	[42,51,52]	17049327, 10704727, 17535751
Carbonic anhydrase II (CAII)	Mature osteoclasts,	[21,22,53]	10934646, 16418777

Mmp-9	Myeloid cells + mono- and multi-nucleated osteoclasts	[21,43] [40,54]	16815100, 10934646,
Vacuolar-type proton ATPase	Pre osteoclasts	[55]	17273786
osteopontin (OPN)	M-CSF dependent bone marrow macrophages	[21]	10934646

Bone turnover and Disease

Parathyroid hormone and menopause

Primary hypothyroidism and hyperthyroidism affects bone turnover. Decreased bone mass and high bone resorption occurs when there is an up-regulation in thyroid hormones and suppression in thyroid stimulating hormone. This effect was observed even in patients with subclinical hyperthyroidism [56].

In women facing menopause, bone turnover is up-regulated with increased activity of osteoclasts and osteoblasts. The net effect of imbalance between bone deposition and resorption is bone loss [57,58] and in most of the cases the primary reason for this imbalance is loss of estrogen. Estrogen deficiency directly or indirectly increases the number of osteoclasts. In a study it is reported that an alternative mean by which TNF alpha modulate post-menopausal osteoporosis may be FSH (follicle stimulating hormone) arising as a powerful inducer of osteoclastic bone resorption [59].

Effects of ageing on bone metabolism

With increasing age the balance between bone deposition by osteoblast and bone resorption by osteoclasts is lost. In childhood there is more bone deposition and less bone resorption, after 20 yrs of age there is a balance between deposition and resorption but later to 25 yrs less deposition and more resorption occurs [15]. As a result of this imbalance there is more bone

resorption, leading to greater incidence of osteoporotic conditions at old age. This weakening of calcified skeleton in elderly population is similar between males and females [58].

Osteoporosis

This group of disorders results in increased fracture risk because of reduced bone mass and poor bone quality [60]. A variety of medical conditions such as diseases or use of certain medications that adversely affect skeletal health leads to secondary osteoporosis. Studies suggest that 20 to 30 % of osteoporotic cases are secondary [61].

There are three main causes of osteoporosis namely ageing, oestrogen deficiency and glucocorticoid use [62,63]. Osteoporosis can be treated by inhibiting resorption using estrogens or bisphosphonates (the latter inhibit osteoclast function) [64]. There is a continuing search for drugs that stimulate bone formation (anabolic agents) or better still, stimulate formation and suppress resorption at the same time. One potential target is Cas-interacting zinc finger protein (CIZ), which inhibits bone formation without affecting resorption. Therefore inhibitors of this gene might be effective therapeutics [60].

Ciz $^{-}/^{-}$ mice have increased bone mass. Calcitonin is also widely accepted as an Osteoclast inhibitor, studies in teleost and mammals suggest that calcitonin suppresses the activity of osteoclasts [51,52].

Glucocorticoid-induced osteoporosis (GIOP) is a major clinical problem given the widespread use of steroids [65]. It is the most common cause of secondary osteoporosis and the association between glucocorticoid use and increased fracture risk is well established. These agents even at low doses, can cause severe reduction in bone formation and can to a lesser extent increase bone resorption [61]. Multiple oral corticosteroid bursts over a period of years can produce a dosage-dependent reduction in bone mineral accretion and increased risk for osteopenia in children with asthma [66].

Osteoporosis also occurs in ageing men but its progress is relatively slower, occurring due to prolonged continuous drop in free serum testosterone. Androgens have a direct effect on the bone cells including osteoclasts [67].

Osteopetrosis

This disease results in a loss of distinction between cortex and trabeculae of the bone. Resorption and skeletal Remodelling are dysfunctional resulting in abnormally shaped bone [68]. The unique feature of this disease is the accumulation of cartilaginous bars surrounded by bone throughout the marrow cavity. This results in a structurally compromised bone explaining the easily breakable bones of osteoporotic patients [69]. Most forms occur due to dysfunctional ion transporting proteins in the osteoclast which results in failure of acidification of the resorptive microenvironment [68].

Pyknodysostosis

Mutation in cathepsin K gene in humans promotes osteoclast dysfunction resulting in a sclerotic bone disease distinct from osteopetrosis. The cathepsin K dysfunction disorder is described by diffuse skeletal sclerosis short stature and distinct cranio-facial abnormalities in addition to the dissolution of terminal phalanges of hands and feet. Pyknodisostotic patients also experience increased fracture risk [70,71].

Silica Nanoparticles for drug delivery

In addition to studying the expression of markers, we also wanted to see whether we could perform functional studies for manipulating cell differentiation. For this purpose, we chose Mesoporous silica nanoparticles (MSNPs) as new drug delivery system (DDS) to deliver osteoclastogenic compounds such as MCSF and RANK-L in zebrafish embryos. These were already under development in our research consortium, and so this provided an interesting opportunity to incorporate them into this study. They have been used for cell culture studies [72], but testing their suitability in zebrafish provided us with an interesting possibility to develop an *in vivo* delivery system. MSNPs are a new DDS the toxicity of which in the zebrafish embryos needs to be studied carefully. The immunotoxicity of MSNPs is not known in zebrafish embryos previously. Therefore in order to use as DDS we needed to carefully determine the toxicity and then use them to deliver compounds into zebrafish embryo model.

Nanoparticles are being synthesized as a next generation of materials used for a variety of applications such as electronic devices, clothes, sunscreens and cosmetics [73]. There is variety of nanomaterials such as metal nanoparticles, nanoshells, Fullerenes, quantum dots, polymer nanoparticles, dendrimer, liposomes [74-84]. In future, nanomaterials may be applied for disease diagnosis and for drug delivery targeted at specific sites for example useful to treat cancer cells [85].

Nanomaterials in Cell culture

Studies of uptake of hydrophobic silica nanoparticles on human breast cancer cells (MCF-7) and rat neural stem cells (NSCs; [72] elucidate the capacity to carry proteins unchanged into the cytosol. *In vitro* studies with HeLa cells has demonstrated the uptake efficiency and uptake mechanism of mesoporous silica nanoparticles (MSNs) can be manipulated by the surface functionalization of nanoparticles [86]. MSNs can be efficiently employed as carriers for intracellular drug delivery in cell cultures [87]. Previous studies have shown that non-phagocytic eukaryotic cells can endocytose latex beads up to 500 nm in size. Particles around 200 nm in size or smaller are taken up with highest efficiency, whereas very little uptake has been observed for particles larger than 1 μm [87].

Toxicity *in vivo* systems

In zebrafish the toxicity assessment of gold and silver nanoparticles shows a higher toxicity of silver compared to gold [88]. The visual inspection of heat map to rank the high content data was ranked for toxicity of nanoparticles as QD1>ZnO.Pt>SiO_2> Ag>AL_2O_3=Au [89]. It is also found that Fullerenes C60 cause oxidative stress in juvenile large mouth bass [90]. Silica nanowires were found to be more toxic as compared to silica nanoparticles in zebrafish embryos when administered in surrounding water [91]. *In vivo* biodistribution and toxicity depends on nanomaterial composition, size surface functionalization and route of exposure [92].

It has been found that toxicity of nanoparticles may vary with size, structure and composition of nanoparticles [93,94]. Mice have also been used for studying nanoparticles biology [95]. Organs that can take up nanostructures in

mice include the spleen, lymph node, and bone marrow [96]. All of these organs contain large concentrations of macrophages, involved in the uptake and metabolism of foreign molecules and particulates [96]. It has been observed that surface-modified nanostructures that are coated with the polymer polyethylene glycol (PEG) are capable of avoiding reticulo-endothelial system uptake [97]. In order to understand the fate and interaction of nanomaterials with the immune cells further studies are needed.

Regeneration in zebrafish

The adult zebrafish as some other teleost fishes possess an ability to regenerate some parts of the body such as heart, liver, fins and scales [2,98]. This ability makes it possible to study various processes involved in regeneration to be useful for regenerative medicine. The purpose is to help understand the mechanisms and establish methods to increase the regenerative capability in humans where it is only found in the form of wound healing.

Osteoclasts on regenerating zebrafish scales

Zebrafish scales have the ability to regenerate quickly when removed from the skin, or damaged by abrasion [99]. Scales can be studied to get a better understanding of the underlying mechanisms of skeletal development, such as matrix formation and degradation, cell differentiation and mineralization [54,99-101].

Elasmoid scales are a component of the dermal skeleton and are composed of a collagen matrix mineralized with hydroxyapatite crystals on the exterior (episquamal) side [102]. Concentric ridges (*circuli*) and grooves (*radii*) radiate from the central *focus* to the edges of the scale giving it the specific form for zebrafish [103]. The scale matrix is synthesized and shaped during ontogeny and regeneration by the scleroblasts [104]. Scleroblasts are subdivided in osteoblasts and osteoclasts, based on their scale forming and resorbing properties, respectively in more recent literature [27].

The external layer is synthesized first, followed by the elasmodine layer which has a similar arrangement to that of mammalian lamellar bone [105,106]. New scale begins to be deposited immediately after the removal of the old one within the scale pocket on the skin [107]. It takes about four weeks to fully

develop the regenerated scale to the original thickness, although there are still some structural differences between the normal and regenerated scales.

Both in mammals and in teleosts, staining of tartarate-resistant acid phosphatase (TRAcP) activity demonstrates bone surfaces that are being actively resorbed or have been resorbed [24]. Indeed, mononuclear and occasional multinuclear osteoclasts, positive for TRAcP but also the osteoclast marker *cathepsin K*, were found on the episquamal side of scales of different fish species [100,108]. Multinucleated osteoclasts resorbing the scale matrix have also been identified by means of electron microscopy in fish [27,107,109].

Adult Zebrafish fin regeneration

Studies have been done to ascertain the process of regeneration in the zebrafish in addition to the generally used regeneration models such as anurans. However, most of the studies are focused on the regeneration of adult tissues in this vertebrate model [2,13,110-118]. The regeneration in the zebrafish caudal fin occurs in two steps; wound healing and blastema formation. Blastema is a tissue critical for appendage regeneration; it consists of proliferative stem cells which lead to the reconstruction of the lost tissue [119,120]. To achieve proper shape, size and structure of a regenerating tissue the blastema is regulated by surrounding influences in teleost species. In adult-amputated zebrafish fins, the blastema develops at the site of each fin ray in turn driving regenerative events [115]. There is some compartmentalization of proliferating and non-proliferating regions in the adult zebrafish regenerating fin tissue. Mesenchymal compartmentalization is found to be critical for regeneration with a role of epidermal influence on the position and size [111,114]. There are eight different cell groups in the regenerating tail of adult zebrafish of which four cell types in the wound epidermis, three cell types in blastema and one is *mmp9* expressing cell type [117].

Caudal fin regeneration in larval zebrafish

It has been previously reported that the molecular mechanisms involving mesenchymal and epithelial cells in the tissue repair are the same in larval and adult fin primordia [121]. Larval fin repair occurs through the formation of blastema and wound epithelium where there are a large number of

proliferative cells present. Cell proliferation leads from the distal to proximal region in embryos as well as adults. It is suggested that neutrophils and macrophages are not deemed essential for regeneration [110]. According to [121] the cells in the regenerates are epidermal or mesodermal.

The normal zebrafish embryonic fin fold extends posteriorly surrounding the dorsal and ventral sides of tail; this morphology is achieved by 28h post fertilisation [121] or between *prim-6* and *prim-16* of [122]. Till 6 dpf the fin is a simple structure composed of epithelial and mesenchymal cells with no cartilage and fin rays differentiated yet. Within three days of amputation the larvae retain the complete form and structure of the lost part of the tail [121].

Glucocorticoids and regeneration

Glucocorticoids (GCs) are the steroid hormones secreted by the adrenal glands under stress conditions, and these hormones are also involved in the regulatory mechanisms of development, bone turnover, cellular apoptosis in general, metabolism, and circadian rhythm regulation [123]. Generally GCs are anti-inflammatory and immuno-suppressive agents widely used clinically to treat autoimmune diseases, allergies, prenatal lung maturation, and transplant rejection [124-126].

The secretion of glucocorticoids is a classic response to stress. It has been found by [127] after reviewing a large data of studies done on the role of glucocorticoids in various mechanisms studied. Depending on physiological endpoint in question glucocorticoid effect falls into different categories with mediating, suppressive or preparative actions. The regulation of actions of GCs is mediated by the glucocorticoid receptor (GR) which belongs to the nuclear receptor superfamily [128]. These genes are known to be highly conserved between species. Zebrafish is known to possess one GR gene. The main endogenous glucocorticoid in rodents is corticosterone while in humans and zebrafish it is cortisol [129] review).

Dexamethasone is a synthetic analogue of the glucocorticoid class of steroid hormones 20 to 30 times more potent than *hydrocortisone* and 4 to 5 times more potent than *Prednisolone* [130-132]. This hormone is commonly used to treat certain inflammatory and autoimmune diseases such as osteoarthritis, to

reduce allergic response, to treat oncologic conditions and high altitude sickness due to neural oedema. Dexamethasone has been found to have some negative effect on wound healing by affecting collagenization, epithelization, and fibroblast content in mice even after single dose of 1 mg/kg [133]. Corticosteroids are known to reduce inflammation, which in turn affects cell migration, proliferation, and angiogenesis [134]. GCs mostly have a suppressive action on the immune and inflammatory response even at basal levels of glucocorticoid concentrations. There is also some evidence of permissive actions of glucocorticoids playing important roles. Therefore it can be assumed that the GCs present already permissively activate the immune response as the first response to a number of stressors, whereas stress induced GCs later suppress increased immune activation later. Corticosteroids are potent drugs that are extensively used for the treatment of inflammatory and autoimmune conditions. Same drugs however are also responsible for causing numerous side effects on many body systems [135].

Genes involved in regeneration of caudal fin

RAR γ

Retinoic acid is a signalling molecule for vertebrate pattern formation both in developing and regenerating tissue [136]. RARγ mRNA is the prominent RAR transcript found in normal regenerating tissue. RARγ is expressed in the distal ends of blastema of regenerating adult fin tissue [112].

Wnt3a

Wnts are secreted glycoprotiens that play an important role in body patterning, cell proliferation, cell differentiation and tumour formation [137]. The function of this protein in body patterning occurs during early embryogenesis. Wnt3a is a ligand shown to activate β-catenin signalling. Wnt3a is a candidate for mediating the function of Wnt /β-catenin signalling during limb regeneration in newt [138]. Wnt 3a has also been shown to have expressed in the fin regenerates in adult zebrafish, found in the regenerating fin epidermis. β-catenin expression thus is most likely important in maintaining fin epidermis and not necessary for regeneration [139]. Wnt signalling plays a role in the maintenance and renewal of stem cells which are cells that help repair the tissue damage [137].

Msxb

Expression of the transcription factor msxb reflects the growth rate of the blastema regulated by cues along proximodistal axis; *msxb* expression may also be required for the higher growth rate of proximal blastema cells [140].

Hoxd11

Transcription factors like *msxb* and *hoxd11* are regeneration-specific in the proximal blastema more than the distal. Mesenchymal cells of the blastema express the genes. It is known that their information on patterning resides in mesenchymal cells rather than the epidermis that covers them [140]. *Msxb* in conjunction with *hoxd11* may be involved in converting positional information into various rates of cell proliferation such that distal blastema grows slower than proximal.

Aims and Scope of this Thesis

This thesis examines the cells, derived from the haematopoietic system, involved in Remodelling and regeneration of various tissues in zebrafish. We hypothesised that precursors for osteoclasts are found in early (5 dpf) embryos. If this hypothesis is proven correct, then it would have the practical benefit of indicating the potential of the zebrafish as an embryonic model for bone Remodelling. As there was no information on any such cells are present in zebrafish embryos that early in development, the first steps were to:

1. Look for the expression of genes known to be expressed in osteoclasts.
2. See whether we could activate expression of these genes with functional studies

In chapter 2 we examined the hypotheses that early zebrafish embryos contain cells expressing osteoclast markers. To do this, we examined the expression patterns of a panel of markers known to be involved in bone Remodelling in other models. To further examine the hypothesis that these cells might be osteoclasts, we examined whether we could activate expression of cathepsin K using vitamin D3 and dexamethasone.

In chapter 3 we activated TRAcP positive cells in zebrafish embryos by injecting MCSF and RANK-L loaded onto Mesoporous Silica Nanoparticles. This was a

further functional test of our hypothesis that cells expressing osteoclasts markers in zebrafish embryos might be osteoclasts.

In chapter 4 we further characterised the cells expressing osteoclast markers by looking at the adult regenerating zebrafish scale model. This was in order to look for similarities between the well-characterised adult osteoclasts and the hypothesised osteoclasts in the embryo.

In chapter 5 we examined whether glucocorticoid exposure in embryonic stages has long lasting effects on wound healing and regeneration. If so, this would support the hypothesis that cells involved in wound healing and regeneration are present in early embryos. Glucocorticoid exposure was used to mimic stress.