The zebrafish as a model for tissue regeneration and bone remodelling
Sharif, F.

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Chapter 2: Expression Patterns of Genes Associated with Bone and Tissue Remodelling in Early Zebrafish Embryos

Abstract

Genes involved in bone and tissue Remodelling in the vertebrates include matrix metalloproteinase-9 (*mmp-9*), receptor activator of necrosis factor κ-β (*rank*), *cathepsin K* and tartrate-resistant acid phosphatase (TRAcP). We examine whether these markers are expressed in cells of zebrafish of 1-5 days post fertilization. We also examine adult scales, which are known to contain mature osteoclasts, for comparison. *In situ* hybridisation, histochemistry and serial plastic and paraffin sectioning was used to analyse marker expression. We found that *mmp-9* mRNA, TRAcP enzyme and *cathepsin K* GFP protein were expressed in haematopoietic tissues and in the cells scattered sparsely in the embryo. *Cathepsin K* and *rank* mRNA were both expressed in the branchial skeleton and developing pectoral fin. In these skeletal structures, histology showed that the expressing cells were located around the developing cartilage elements, in the parachondral tissue. In a transgenic zebrafish line with GFP coupled to *cathepsin K* promoter, *cathepsin K* expressing cells were found around pharyngeal skeletal elements. We exposed prim-6 zebrafish embryos to a mixture of 1µM dexamethasone (DEX) and 1µM Vitamin D3. These compounds, which are known to trigger osteoclastogenensis in cell cultures, led to an increase in intensity of *cathepsin K* GFP expression around the skeletal elements as well as in haematopoietic tissue. Our findings suggest that cells expressing a range of osteoclast markers are present in early larvae. If this is confirmed, it could lead to establishing a zebrafish embryonic model for studying bone biology and disease.

Introduction

Bone and tissue Remodelling are normal processes in adult animals, and in developing embryos. The genes involved include enzymes that break down extracellular components, and cell signalling molecules involved in the activation of specialised cells involved in Remodelling [15]. There are two different kinds of cells associated with bone Remodelling, namely osteoclasts and osteoblasts which work in harmony in a normal healthy system [15]. Any irregularity or abnormality in the Remodelling process leads to certain pathological conditions such as osteoporosis [141].

In some previous studies, mature osteoclasts have only been detected in developing zebrafish after 20 days post fertilization (dpf) [26]. These cells are initially mononucleated, although multinucleated osteoclasts are also present in the adult zebrafish [26]. A recent review [142] has shown that the bone of zebrafish is osteocytic or cellular as opposed to the bone of most of the other advanced teleost fishes which are acellular or anosteocytic. Furthermore, those authors concluded that the osteocytes of zebrafish are mesenchymal analogues of the osteoblasts of mammals. The multinucleated osteoclasts of zebrafish display similar features to that of mammalian multinucleated osteoclasts, for example formation of Howship's lacunae and staining positive for TRAcP (tartarate resistant acid phosphatase) enzyme [26]. The presence of two types of active osteoclasts (mono- and multi-nucleated) is not restricted to zebrafish but is common for many other teleost species such as Goldfish (*Carassius auratus*) and Carp (*Cyprinus carpio*).

Resorption by large multinucleated cells is lacunar, whereas resorption by mononucleated cells is shallow and non-lacunar in many teleost species [143,144]. Therefore evidence from humans and other mammalians as well as teleosts suggests that a significant number of active osteoclasts are mononucleated [142,143]. In addition to a number of similarities with human and mammalian bone resorption there are differences in the regulation of mammalian to fish osteoclasts. The calcium homeostasis is based on the gills which are the target organs for calcitonin function and the site of osteoclast origin in teleost fish is not the bone marrow [145], therefore it is considered that osteoclasts are formed in the head kidney and spleen [99,146].

Two molecules which are essential for initiating osteoclastogenesis are macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B (NF-κB) RANK ligand (RANK-L). Both of these are expressed by stromal cells related to osteoblasts. RANK is itself expressed on osteoclast precursors [147]. Cathepsin K, *MMP-9*, carbonic anhydrase 2 and calcitonin receptor are expressed strongly in multinucleated osteoclasts and weakly in pre-osteoclasts [21].

Another important enzyme in this context is tartrate resistant acid phosphatase (TRAcP) which is expressed in activated murine and teleost osteoclasts [26]. TRAcP is involved in hydrolysis of various substrates including components of bone matrix [148,149]. Increased levels of TRAcP activity may be detected in clinical disorders involving bone lysis, such as osteoporosis; and also in osteoarthritis and rheumatoid arthritis [150,151].Loss of function studies in mice reveal an important role for TRAcP in skeletogenesis [148]. As is the case with the archetypal multinucleated mammalian osteoclasts, the mono- and multinucleated osteoclasts of teleosts secrete TRAcP at the site of active bone resorption [28,144,152,153]. Thus TRAcP-staining can be specific for osteoclastic bone resorption (and also for sites where osteoclasts were previously active) [26,28,143]. Ballanti et al., [154] regard expression of this marker as one of the best ways to identify osteoclastic cells.

RANK (also known as TNFRSF11A, TRANCE-R) is a member of TNF receptor super family. Together with its ligand, the TNF-family molecule RANK-L (TRANCE, or osteoclast differentiation factor, ODF) it is a key regulator of bone Remodelling, and is essential for the development and activation of osteoclasts [46]. RANK is expressed by osteoclast progenitors, mature osteoclasts, chondrocytes and mammary gland epithelial cells [47,48]. Mice with a genetic mutation of RANK have a complete block in osteoclast development, but after the administration of RANK the osteoclasts develop normally in those mice [49]. It is therefore established that the RANK-L expressed by osteoblasts and its receptor RANK expressed in osteoclast precursors are essential for osteoclastogenesis.

Cathepsin K a cysteine protease expressed by osteoclasts and synovial fibroblasts is responsible for removing the organic matrix, mainly fibrilar type-1 collagen, and solubilisation of the inorganic component (hydroxyapatite) [155].

Cathepsin K is released as a pro-enzyme in the resorption pit having an acidic pH. Inhibition of cathepsin K by specific inhibitors results in the accumulation of undigested collagen fibrils in lysosomes within osteoclasts [155]. Cathepsin K expression is elevated in the synovium of rheumatoid arthritis patients [156]. Expression of cathepsin K is also localized within a range of cells at the site of cartilage erosion in rheumatoid arthritis [156,157].

Members of the matrix metalloproteinase (MMP) family of genes are important for the Remodelling of the extracellular matrix (ECM) in a number of normal biological processes such as embryonic development, morphogenesis, reproduction, tissue resorption and Remodelling [158]. They also perform the same function, i.e. ECM Remodelling in some pathological processes, including cancer metastasis, and rheumatoid arthritis. Finally, MMPs are present in some snake venoms, where they may be involved in lysis of tissue in the prey [159,160].

It is important to understand the processes underlying the Remodelling process, since disease can arise if these are dysregulated. Therefore, as a step towards establishing a zebrafish model for bone diseases, we have characterized the expression of panel of osteoclast-associated markers in early stages of zebrafish development. Early stages are particularly desirable for establishing a model because they are free from the legal restrictions that apply to the use of adults, and are therefore more suited to high throughput assays.

Experimental procedures

Animals

Danio rerio (zebrafish) was used as the animal model for expression profiling of selected genes and proteins. All experimental procedures were conducted in accordance with the Netherlands Experiments on Animals Act that serves as the implementation of" Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC, and were performed only after a positive recommendation of the Animal Experiments Committee had been issued to the licensee. Spawning of *Danio rerio* took place at 26$^\text{o}$C in aerated 5 litre tanks, in a 10h: 14h light: dark cycle. In each mating setup, two

females and one male fish were placed together. The eggs are usually laid after first light in the morning. They were collected within the first hour, sorted and distributed in Petri dishes, filled with egg water (14 gm 'Instant Ocean®' salt in 100 ml of demi water).

The eggs were cleaned and transferred to 9cm Petri dishes at a concentration of 60 eggs per dish. They were maintained at 28°C in atmospheric air in a climate cell, also with a 10h: 14h light: dark cycle. The Petri dishes were checked 4 h and 8 h for dead and unfertilized eggs which were removed and discarded. After continued incubation in the Petri dishes at 28°C embryos were harvested each morning from day 1 to day 5 and fixed as follows. Eggs were immersed in 4% buffered paraformaldehyde (PFA) at 4°C overnight and then dehydrated in an ascending series of Methanol staring from 25% to 100% and finally stored at -20°C in 100% methanol. One and two day old embryos were dechorionated before fixation.

Cloning of genes and synthesis of probe

The NCBI genbank was searched for homologous sequences with the Blast X algorithm using zebrafish query. Only for cathepsin K (Goldfish, *Carassius auratus*, AB236968) and *MMP-9* (Common carp, *Cyprinus carpio*, AB057407) did we find similar enough sequences for our goal and aligned them with their zebrafish counter parts. These alignments were used to design PCR primers based on conserved regions. The other primers were designed only using, mostly multiple, Zebrafish sequences. Primers are shown in Table 2

Table 2 Nucleotide sequence of primers used for PCR

Primer	Nucleotide sequence 5`-3`	Position
Receptor activator of necrosis factor Kappa B		
RANK F1	TGGCGGAAGGAAAGATTCCTC	157
RANK F2	TGTGGCTCTGACCGCAGTCC	243
RANK R1	CGCAGTCCGGCTGACTCTG	1071
RANK R2	CTGGGACTTTGCTGCAGTAGATGC	1060

Cathepsin K

CTS K F1	GATGAGGCTTGGGAGAGCTGGAA	180
CTS K F2	GACGATTTGGGAGAAGAACATGCTG	256
CTS K R1	TTTCGGTTACGAGCCATCAGGAC	1013
CTS K R2	CCCTTCTTTCCCCACTCTTCACC	1063

Matrix
metalloproteinase 9

MMP 9 F1	TTCGTGACGTTTCCTGGAGATGTG	207
MMP 9 F2	CACAGCTAGCGGATGAGTATCTGAAGC	253
MMP 9 R1	TGGCTCTCCTTCTGAGTTTCCACC	1120
MMP 9 R2	AATGGAAAATGGCATGGCTCTCC	1135

The PCR parameters consisted of 5 min of denaturation, followed by 40 cycles of denaturation 95° C, 10 sec, annealing (10 sec), and extension 60 second ending with 10 min of extension at 72°C. The PCR products chosen for cloning had the following primer combinations: CTSK F1 & R2, *MMP-9* F2 & R2 and RANK F2 & R2.The PCR products were cleaned using Wizard SV Gel and PCR Cleanup system (Promega: Leiden, the Netherlands).

The ligation, cloning and transformation of plasmids in competent cells were done with the TOPOTA PCRII kit from Invitrogen (Breda, the Netherlands). White colonies of transformed cells were grown and checked by PCR. Samples were then sent for sequencing to Macrogen. All the sequence analysis showed a strong homology with the reference sequences. Linearization of template was done with restriction enzymes XbaI, XhoI, HindIII or BamHI, depending on the direction of the gene and potential restriction sites present in the product, and cleaned with Wizard SV Promega columns. T7 or SP6 RNA-polymerases (Roche) were used to synthesis the digoxigenin labelled RNA probes. The probes were

stored at -20°C. Also sense probes were prepared for all of the genes, and used as negative control in *in situ* hybridization (ISH). The genbank accession numbers of our PCR products are shown in Table 3.

Table 3 Genbank accession numbers of the genes cloned and used for *in situ* hybridization in this study.

Gene	Genbank accession number
MMP-9	HM239640
CTS-K	HM239643
CTS-K-1b	HM239644
Rank	HM239645
TRACP	HM239646

In Situ Hybridization (ISH)

Fixed and dehydrated embryos (see above) were rehydrated from methanol by passing through descending concentration of methanol, namely 75%, 50%, and 25% methanol. They were then washed twice in 1x phosphate buffered saline (PBS) with 0.2% Tween 20 (PBST) for 10 min each. In some cases, embryos of 2-5 dpf were then bleached at room temperature with hydrogen peroxide until pigmentation had completely disappeared (10 to 12 min). One day old embryos were found to be too delicate for bleaching. Then they were washed twice in PBST for 5 min. Embryos were treated with Proteinase-K (10 µmg/ml) for incubation time that was varied according to the stage 1 dpf 10 min, 2 dpf 15 min 3 dpf 20 min 4 and 5 dpf 40 min. Further processing of embryos was conducted, with minor modifications, according to Xu Q and D.G.Wilkinson [161].

Adult fish scales

We fixated adult zebrafish skin with scales in 4% PFA at 4°C overnight and stored them in 100% methanol after dehydration. *In situ* hybridization was carried out as described above for embryos except that no bleaching was done and, proteinase-k treatment was done for 10 min.

Transgenic *Cathepsin K* larvae

Cathepsin K transgenic GFP labelled zebrafish published recently [162] were kindly provided by Prof. Stefan Schulte-Merker . The eggs were obtained from one adult pair and were cleaned and sorted as described above (see 'Animals'). At the *prim-6* stage [122] the transgenic embryos were continuously exposed to a mixture of 1µM (DEX) (Sigma Aldrich Switzerland) and 1 µM vitamin D3 (cholecalciferol; SERVA electrophoresis GmbH, Germany) for 4 days. Controls were treated in the same way except that DEX and vitamin D3 were not added. At 5 dpf, control and treated larvae were observed alive under confocal microscope.

TRAcP enzyme staining

Normal zebrafish embryos and adult scales were preserved in 100% methanol after rehydration through a graded series of methanol in PBST. TRAP enzyme kit (Sigma-Aldrich 387A-1KT, Chemie GmbH, Steinheim, Germany) was used for staining the embryos and scales according to the user instructions.

Sectioning

Sections were prepared from the whole mount ISH larvae. The larvae were dehydrated in graded ethanol, and embedded in wax using Histoclear as the intermediate reagent. Some sections were prepared with Technovit embedding after dehydration.

Imaging

Imaging of *in situ* embryos and sections was done using Nikon eclipse E800M equipped with DSF1 camera. For *in vivo* confocal imaging of the transgenic larvae, they were immobilized in 1% low melting-point agarose (SIGMA) after anesthesia (0.04% Tricaine). Imaging was done using a Zeiss observer LSM 500.

Results

In situ hybridization results for *cathepsin K*, *rank* and *mmp-9* are shown in Figs. 1-9.

Cathepsin K

Hybridization patterns of *cathepsin K* are illustrated in Figs. 1 A-H and Figs. 2 A-D. Embryos hybridized with sense probe showed no specific signal (Figs.1 B). With antisense probe (Figs. 1 A, C-H and Figs. 2 A-D), there was expression as follows. At 1 dpf, hybridization was seen in a longitudinal stripe of adaxial mesoderm cells along each side of the trunk (Figs. 1 A), the stripe being interrupted at intersomitic boundaries (Fig. 1 C). Histological sections (Fig. 2 A) showed that this staining was paranotochordal, extending laterally from the notochord towards the epidermis. Hybridization was also seen in the pectoral fin buds (Fig. 1 A).

At 2 dpf, specific expression in whole mounts gave an appearance of skeletal elements in the head, (trabeculae cranii, branchial arches, and lower jaw) and in the cleithrum and pectoral fin buds (Fig. 1 D, E, and F). Histological sections showed that expression was perichondrial (Fig. 2 B). By 3 dpf the expression in the pectoral fin was reduced and by 4 dpf was faint (Figs. 1 G and H). At 5 dpf, expression in the branchial arches was indistinct in whole mounts but was still visible in histological sections (Fig. 2 B), where specific expression was again seen in what we assume to be the perichondrium of the branchial arches. However, as with the pectoral fin and the lower jaw, there was no staining in the cartilage itself.

 To confirm the specificity of the probe we examined expression in adult scales. Here, the hybridization was specific and distinct. Large clusters of heavily-stained cells were localized at the margins of the scales (Fig. 2 C). At higher magnifications these clusters could be seen to have many nuclei, the dark staining being localized to the cytoplasm (Fig. 2 D).
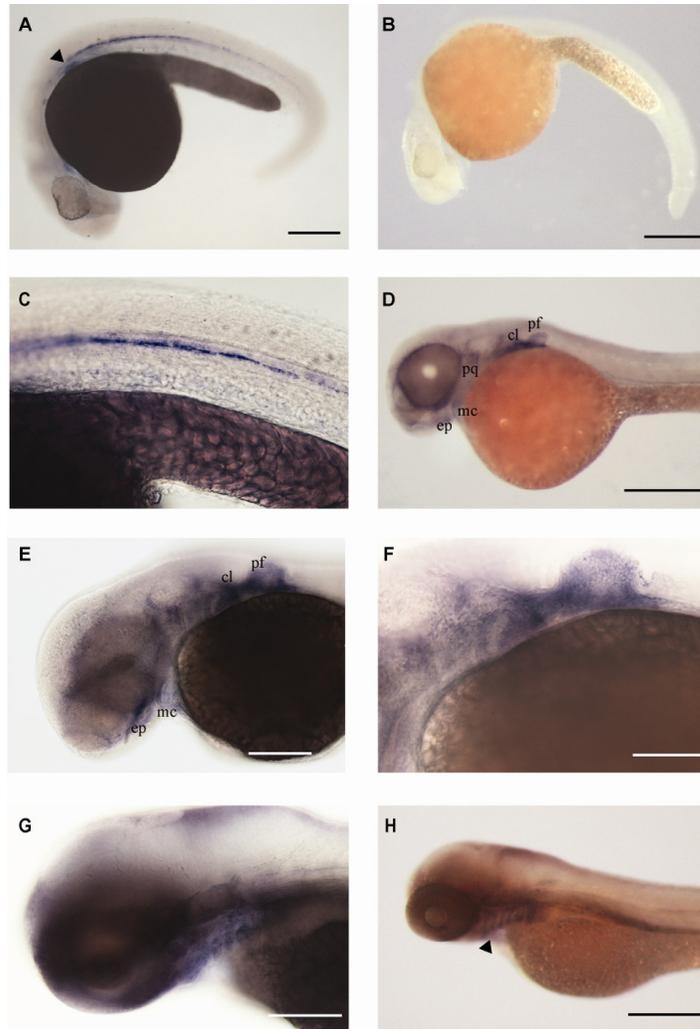
Fig 1 *Cathepsin K* gene expression by *in situ* hybridization in zebrafish embryos, scale bar=200μm unless otherwise mentioned. **A** Expression in rostral blood island, lateral sides of notochord and pectoral fin bud (arrow head), in 1 dpf embryo. **B** Sense control of A with no specific expression. **C** Detail of Fig 1 A showing expression in the lateral region adjacent to the notochord, (arrow heads) scale bar=100 μm. **D** 2 dpf embryo with expression in the Meckel's cartilage (mc), ethmoid plate (ep), cleithrum (cl) palatoquadrate (pq) and pectoral fin bud (pf). **E** Detail of Fig D scale bar=150 μm. **F** Detail of Fig E showing expression in the pectoral girdle and fin bud, scale bar=50 μm. **G** 3 dpf zebrafish embryo showing expression in the pharyngeal arches, scale bar=200 μm. **H** 4 dpf larva with expression in the pharyngeal arches (arrow head).
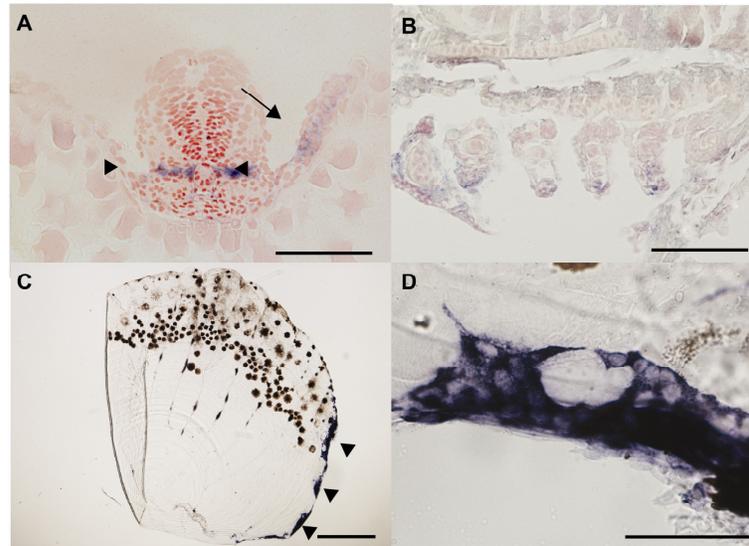
Fig 2 *Cathepsin K* gene expression by *in situ* hybridization in zebrafish larvae and scales. **A**. Histological section through the posterior region of 1 dpf embryo expressing *cathepsin K* gene in the cells of adaxial mesoderm, (arrow heads) and pectoral fin bud (arrow) scale bar=100 μm. **B** Section through pharyngeal arches of 5 dpf larva showing expression around the cartilaginous elements within the arches, scale bar=50 μm. **C** Whole mount adult scale showing expression at the margin, scale bar=300 μm (arrow heads). **D** Putative multinucleated cell expressing *cathepsin K* in the marginal region of a scale, scale bar=20 μm.
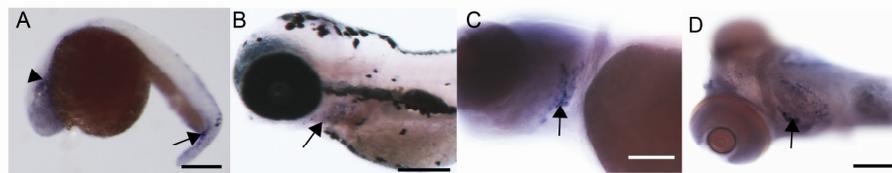

Fig 3 *Rank* gene expression in zebrafish embryos. **A** 1 dpf embryo expressing *rank* gene in the rostral blood island (arrow head) and ventral blood island (arrow) in addition to staining in the jaw region, scale bar=300 μm. **B** 3 dpf larva expressing *rank* in the pharyngeal arches, (arrow) scale bar=150 μm. **C** 4 dpf larva expressing *rank* in the pharyngeal arches (arrow), scale bar= 200 μm. **D** Ventral view of 4 dpf larva with expression in the pharyngeal arches (arrow) and anterior end of the Meckel's cartilage, scale bar= 200 μm.

rank

Hybridization patterns with *rank* antisense (Figs 3 A-D) and sense probes (Fig. not shown). Sense controls showed no hybridization. At 1 dpf, diffuse expression was observed in the head region, around the yolk sac and in the tail in the region of the ventral blood island (Fig. 3 A) where *mmp-9* and *cathepsin-k* expression was also seen. At 2 dpf expression was observed in the cleithrum and pectoral fin bud. At 3 and 4 dpf *rank* expression was seen in the pectoral fins and the branchial arches (Fig. 3 B and C). At 5 dpf, expression was seen only in the branchial arches (Fig. 3 D). Histological sections through the branchial arches of 5d old larvae confirmed that expression was in the loose mesenchyme but not the cartilage elements (Fig. 4 A), the same being true of the pectoral fin (Fig. 4 B). In adult scales, staining was observed in the edges of the scales only (Fig. 4 C).

mmp-9

Hybridization patterns with *mmp-9* antisense probes are illustrated in Fig. 5. At 1 dpf, hybridization was seen in the ventral blood island (also the site of *rank* and *cathepsin-k* expression. Expression of *mmp-9* was also observed in the posterior extension of yolk sac. At 2 dpf, hybridization was seen in numerous, scattered cells in the head, pharyngeal region, on the yolk sac, near the ventral aorta and around the gut (Fig. 5 A and B). At 3 dpf, there was hybridization in individual cells scattered in the tissue including pharyngeal region and caudal hematopoietic tissue (Fig. 5 C). At 4 and 5 dpf a few cells expressing *mmp-9* were found scattered on the rostral part of the body, but were less numerous than at earlier stages.

Fig 4 *Rank* gene expression in zebrafish larvae and scales. **A** Histological section through pharyngeal arches showing *rank* expression in the arches (arrow), scale bar=50 µm. **B** Section through pectoral fin of 2 dpf embryo showing *rank* expression in the tissue around the cartilage (red) scale bar= 100 µm. **C** Whole mount adult scale with *rank* expressing cells on the margin, scale bar=100 µm
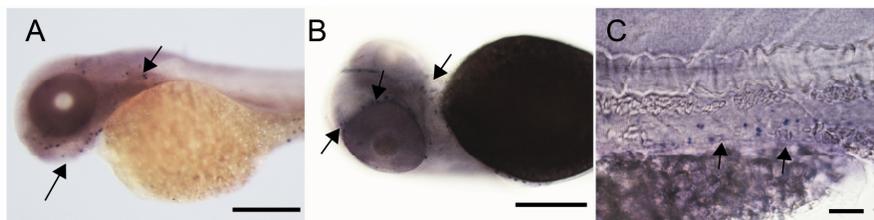

Fig. 5 *mmp-9* gene expression in zebrafish embryos; **A** 2 dpf embryo with numerous cells expressing *mmp-9* gene in the head region (arrows), scale bar=150 µm. **B** 2 dpf embryo, ventral view, scale bar=100 µm. **C** 4 dpf larva with expression in the ventral blood vessel in the posterior region of the body, scale bar=30 µm.
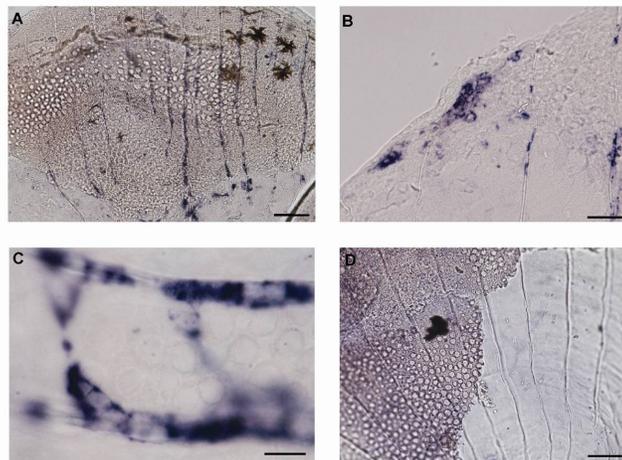
Fig. 6 Whole mount *in situ* hybridization on adult scales with *mmp-9* gene. **A** Expression in the radii of the scale, scale bar=100 μm. **B** *mmp-9* expressing cell aggregates at the margins of a normal scale, scale bar=100 μm. **C** Multinucleated cells expressing *mmp-9* in the radii, scale bar=40 μm. **D** Scale hybridized with sense probe with no specific staining, scale bar=200 μm.

We hybridized adult zebrafish scales as positive controls for *mmp-9* expression. There was expression in the radii of the scales in mono- and multinucleated cells (Fig. 6 A). Specific and strong expression was observed in the margins of the scales similar to that of *cathepsin K* expression (Fig. 6 B). Higher magnification shows expression in the multinucleated cells within the radii (Fig. 6 C). There was no specific expression in the margins or radii of the scales hybridized with the *mmp-9* sense probe (Fig. 6 D).

TRAcP enzyme staining

Immunohistochemical staining with TRAcP enzyme was done on whole mount zebrafish larvae (Fig. 7A-D). In 1 dpf embryos, strong and specific enzyme staining was seen in cells in the ventral blood island. At 2 dpf expression in the cells in the heart and pericardium was visible (data not shown). Stained cells were also present in the ventral fin fold and along the caudal haematopoietic tissue or CHT. In 3 dpf embryos, cells scattered very sparsely over the body expressed TRAcP staining (Fig. 7 A and B). There were numerous cells stained with TRAcP enzyme in the tail region around the notochord. At 4 dpf there was staining at the rostral end of Meckel's cartilage, in the pectoral fin and in the pericardial region. In the posterior region of the body, stained cells were present in the ventral vein. Similar to 4 dpf the expression in 5 dpf larvae was in the Meckels cartilage, pectoral fin (Fig. 7 C) and along the ventral blood vessels and near the somites (Fig. 7 D).

TRAcP enzyme staining was also done on adult zebrafish scales as positive controls. Staining was observed at the lateral margins of the scales (Fig. 8 A) as well as very dense staining in some scales in the mid region along the grooves of normal scales (Fig. 8 B). However no TRAcP staining was seen in the radii of the scales in our specimens. With the same staining multinucleated cells within the mid region of the normal scale were seen (Fig. 8 C).
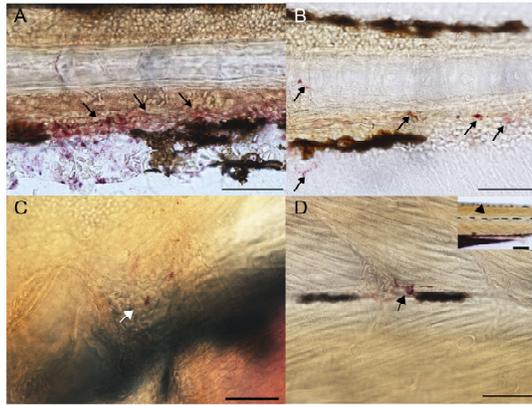
Fig. 7 TRAcP histochemical staining. **A** 3 dpf larva showing expression in the caudal hematopoietic tissue and blood cells (arrows) scale bar 50 µm. **B** Tail region of 4 dpf larva with expression in individual cells (arrows), scale bar 50 µm. **C** 5 dpf larva with expression at the pectoral fin (white arrow). Scale bar = 50 µm. **D** 5 dpf larva expressing TRAcP in a single cell on the left flank of caudal body region (arrow) inset showing D at low magnification. Scale bar = 550 µm
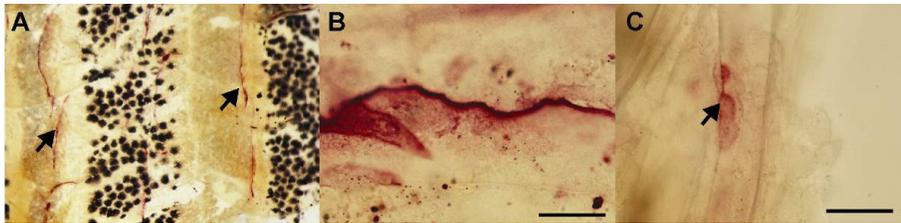

Fig 8 TRAcP histochemical staining on zebrafish scales. **A** Scales on the skin of adult zebrafish expressing staining in the lateral margins (anterior to the top and dorsal to the right), scale bar= 200 µm.**B** Groove on a single scale with TRAcP staining, scale bar=20 µm. **C** Putative multinucleated cell stained with TRAcP enzyme, scale bar=20 µm.

Transgenic Cathepsin K Larvae

GFP labeled *cathepsin K* transgenic zebrafish larvae showed expression in the pharyngeal region in the scattered cells (data not shown). After exposing the embryos continuously with DEX and vitamin D3 for 4 days, the expression was more strongly seen around the pharyngeal skeleton, in np (nasal placode), Mc (Meckels Cartilage), Bh (basihyal), cb (ceratobranchial), pf (pectoral fin) (Fig 9 A-

D) . In the lateral view *cathepsin K* expression was clearly seen in the pharyngeal arches, ceratobranchials, pectoral girdle and pectoral fin. There was also expression around the otic vesicle (Fig 9 C and D). However, a weaker *cathepsin K* expression in the control transgenic larvae was observed (Fig 9 E and F). There was an interesting *cathepsin K* expression in the scattered cells within the ventral vein near the caudal body region of the treated larvae similar to TRAcP and *mmp-9* (Fig 9 G and H).
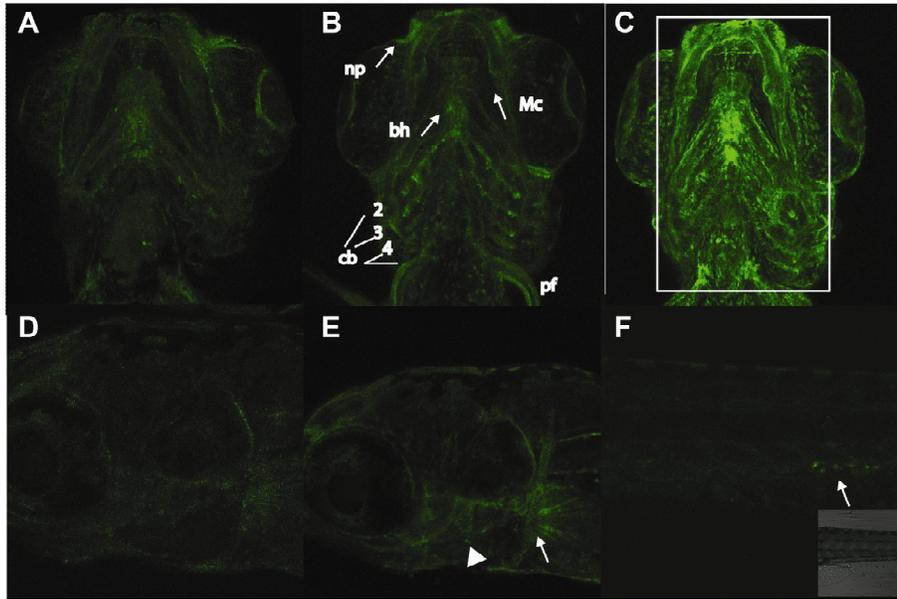

Fig 9 GFP labelled *cathepsin-k* transgenic zebrafish embryos. **A** Confocal image showing 5 dpf larva with expression in pharyngeal skeleton (ventral view, anterior side upwards). **B** DEX and vitamin D3 treated 5 dpf larva showing increased *cathepsin-k* transgenic expression in bh (basihyal), cb (ceratobranchials 2-5), Mc (Meckel's cartilage), np (nasal placode), pf (pectoral fin). **C** DEX and vit D3 treated 5 dpf larva showing increased *cathepsin-k* expression, rectangle shows the area marked for fluorescence intensity assay. **D** Confocal image showing left lateral view (anterior directed to the left) of the 5 dpf control larva with expression around ov (otic vesicle), cb (ceratobranchial), pf (pectoral fin). **E** Lateral view of DEX and vitamin D3 treated larva showing the skeletal elements with strong GFP signal, pharyngeal arches (arrow head) and pectoral girdle (arrow). **F** Caudal region of a treated larva showing the ventral vein with scattered cells expressing GFP *cathepsin-k* (arrows)
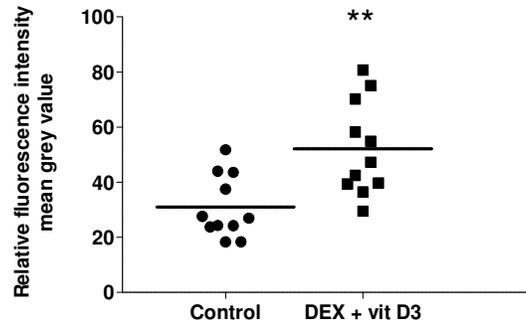
Fig. 10 Measurement of relative fluorescence intensity showing significant increase in case of DEX + vitamin D3. Lines represent standard error mean. Each of the dots represents one control larva, and square represents each treated larva.

Discussion

We studied the expression in zebrafish embryos and larvae of a panel of genes that are associated with bone and tissue Remodelling. Differences in expression were seen between all of the genes and are summarized in Table 4. Similarities in expression were evident between *cathepsin K* and *rank*, on the one hand, and *mmp-9* and TRAcP on the other. *Cathepsin K* transgenic expression in normal embryos was associated with both type of expression as observed in *cathepsin K* and *rank* as well as *mmp-9* and TRAcP.

Table 4 Summary of expression patterns of osteoclast markers in zebrafish development found in this study.

Marker	Expression
cathepsin K	pharyngeal skeleton and pectoral fin, and in CHT in case of (transgenic larvae)
rank	pharyngeal skeleton and pectoral fin
mmp-9	scattered cells on whole body and CHT
TRAcP	scattered cells on whole body and CHT (at 5 dpf in Meckel's cartilage)

Cathepsin K and rank

In 1 dpf embryo hybridized with *cathepsin K* probe, adaxial staining was seen. We cannot say however, whether this expression represents either the slow muscle precursors or sclerotomal elements which are known to occur in this region [163]. *Cathepsin K* and *rank* expression was more restricted to the cells associated with pharyngeal skeleton and pectoral fin skeleton [164,165] in embryos older than 1 dpf. It is known that by 5 dpf osteogenesis occurs where cartilaginous skeleton transforms into calcified tissue [166]. Also this calcification or bone deposition occurs from outside in, that is the exterior part of bone is remodelled to deposit bone [167].

We found expression of *cathepsin K* in the sections around pharyngeal arches, similar to the expression of *osterix* (SP7) in the perichondrium around the cartilage of a 4 dpf zebrafish embryos by (Kimmel et al 2010) [167] Therefore it is possible that this expression of *cathepsin K* mRNA may be due to osteoclasts. It is also important to note here that we found strong similarities in the expression of *cathepsin K* and *rank* genes in the larvae as well as in the adult scales. In the adult zebrafish scales the *cathepsin K* [168] and *rank* genes are both expressed in the marginal regions. This positive expression suggests that same genes are also expressed in the adult tissues which are known to carry osteoclasts [169]. However the scale-margin expression of *cathepsin K*, but not *rank*, was found in multinucleated cells.

Cathepsin K reporter line showed a similar pattern of *cathepsin K* to the *in situs*, with cells scattered along the pharyngeal arches. Further work is being carried out at present to document the expression pattern of GFP *cathepsin K* in detail. When the embryos were exposed at *prim-6* stage with 1µM of DEX and vitamin D3 for 4 days and then observed with *in vivo* confocal microscopy we found that there was much stronger expression of GFP *cathepsin K* gene along the skeletal elements. GFP expression was seen in all the skeletal elements which are known to have developed by this stage, compared to very weak expression in controls.

Initial observations therefore suggest that there was activation in the GFP *cathepsin K* expression in DEX and vitamin D3 treated embryos. It was also found that GFP *cathepsin K* positive cells were present in the tail region along

the ventral blood vessel. This expression in the ventral blood vessel is similar to the expression of TRAcP positive cells as well as *mmp-9* positive cells in normal untreated larvae. It can be therefore concluded that the *mmp-9* mRNA, TRAcP enzyme and GFP *cathepsin K* protein expressing cells are not only found around the skeletal elements but are also found scattered in other parts of body specially blood vessels, which is logical considering hematopoietic origin of osteoclasts.

mmp-9 and TRAcP

In the present study, *mmp-9* and TRAcP enzyme were both expressed in cells sparsely scattered all over the body. Unlike *cathepsin K* and *rank*, *mmp-9* and TRAcP expression was neither specifically associated with the pharyngeal arches nor the pectoral fin. Expression of *mmp-9* has been reported in cells scattered mostly on the head region and the posterio-lateral trunk, in embryos from 2-5 dpf [170,171]. The expression of *rank* in 1 dpf embryos in the ventral blood island and rostral blood island, and *mmp-9* in the ventral blood island is also interesting as this is the site of haematopoiesis in the early embryonic stages of zebrafish development [172].

TRAcP expression was observed in the skeletal structures such as Meckel's cartilage only in 5 dpf larvae, but not in larvae younger than 5 dpf. Previous researchers have argued that TRAcP expression is a definitive marker for active osteoclasts [142]. It is also worth considering here that the enzyme histochemistry does not seem to penetrate deep into the calcified tissue in whole mounts as much as GFP *cathepsin K* expression. Further work is required to determine the nature of the cells expressing the markers characterized here.

In adult scales the *mmp-9, cathepsin K* and TRAcP expression is similar except for additional expression in the radii of the scales in the case of *mmp-9* hybridization. One possibility is that mononucleated cells expressing *mmp-9* in the radii of the scales are non-activated cells of the osteoclast lineage, whereas the multinucleated radial and marginal aggregates are mature osteoclasts. Nonetheless, the fact that marginal, multinucleated cells in the adult scales express *mmp-9*, *cathepsin K* and TRAcP is suggestive of an osteoclastic lineage as we found in other studies recently published for *mmp-9* and TRAcP expression in adult zebrafish scales [42,168,169]. However here we for the first

time present expression of *cathepsin K* in the same cells and *rank* in mononucleated cells.

Conclusions

Our results show that genes associated with osteoclasts are expressed in early zebrafish development and in the multinucleated cells expressing *mmp-9* and *cathepsin K* genes, and TRAcP enzyme, in adult scale osteoclasts. Our data with the transgenic *cathepsin K* larvae suggests an association with the larval pharyngeal skeleton; this is also comparable to our expression data of *cathepsin K* expression with *in situ* hybridisation. The TRAcP enzyme, *mmp-9* mRNA and *cathepsin K* GFP transgenic line also show expression in the blood cells found in the ventral vein. The expression of *cathepsin K* in the reporter line was upregulated by DEX and Vitamin D3 treatment. Together these findings raise the possibility that osteoclast-like cells are present at early stages of zebrafish development. Further work is underway to determine the function of these cells in development.