The zebrafish as a model for tissue regeneration and bone remodelling

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Chapter 4: Matrix metalloproteinases in osteoclasts of ontogenetic and regenerating zebrafish scales

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Abstract

Matrix metalloproteinases (MMPs) are key enzymes in the turnover of extracellular matrix in health, disease, development and regeneration. We have studied zebrafish scale regeneration to ascertain the role of MMP-2 and *MMP-9* in these processes. Scales were plucked from the surface of anaesthetised adult male zebrafish, and the scales that regenerated in the scale pocket were recovered at various time points after plucking. Analyses consisted of (i) *mmp-9 in situ* hybridisation; (ii) *MMP-9* + TRAcP double-staining; (iii) qRT-PCR for *mmp-2* and *mmp-9*; (iv) zymography for gelatinolytic activity and (v) a hydroxyproline assay. We found that *mmp-9* positive cells were confined to the episquamal side of the scales. Ontogenetic scales had irregular clusters of mono- and multinucleated *mmp-9* expressing cells along their lateral margins and radii. During regeneration, *mmp-9* positive cells were seen on the scale plate, but not along the lateral margins. Double staining for TRAcP and *MMP-9* revealed the osteoclastic nature of these cells. During early scale regeneration, *mmp-2* and *mmp-9* transcripts increased in abundance in the scale, enzymatic MMP activity increased and collagen degradation was detected by means of hydroxyproline measurements. Near the end of regeneration, all of these parameters returned to the basal values seen in ontogenetic scales. These findings suggest that MMPs play an important role in Remodelling of the scale plate during regeneration, and that this function resides in mononucleated and

multinucleated osteoclasts which co-express TRAcP and *mmp-9*. Our findings suggest that the fish scale regeneration model may be a useful system in which to study the cells and mechanisms responsible for regeneration, development and skeletal Remodelling.

Introduction

The elasmoid scales of zebrafish (*Danio rerio*) can regenerate when removed from the skin, e.g. by abrasion or experiment. Like regenerating fin rays [197], 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 mineralisation.

Elasmoid scales are a component of the dermal skeleton. They are composed of a collagen matrix that is mineralised on the external (episquamal) side with hydroxyapatite crystals [102].The episquamal side of the scale possesses concentric ridges (*circuli*) and grooves (*radii*) radiating from the central *focus* to the edges of the scale. Each radius is covered by a dermal space with cells and blood vessels embedded within a loose matrix [198]. Scleroblasts synthesise and shape the scale matrix during ontogeny and regeneration [104]. The external layer is synthesised first, followed by the elasmodine layer, composed of type I and V collagen fibres in a plywood-like arrangement [105]. The collagens of the elasmodine layer are similar in arrangement to mammalian lamellar bone [106] and mineralise slowly from the external layer [199]. When a zebrafish scale is plucked from its scale pocket, formation of a new scale is initiated immediately [107]. Already after two days, a new mineralised scale plate can be seen, but it takes up to four weeks for a new scale to grow to the size and thickness of the removed scale. As a consequence of this rapid reformation, the focus of early regenerating scales is less structured than that of ontogenetic scales. The typical grooves and radii appear late in scale regeneration, which is believed to be the result of basal plate Remodelling [101,200]. Note that in this context, the term ‘ontogenetic’ scale is used for the scales that developed during the early ontogeny of the fish, in contrast to the scales that regenerate after plucking.

The scale compartment constitutes a significant, readily accessible calcium source of fish as it can contain up to 20% of the total calcium in the body [201]. Fish withdraw calcium from their scales in periods of high calcium demand, rather than from their axial skeleton as mammals do [202-204]. However, mobilisation of scale calcium demands the same active and controlled mineralisation and demineralization. Scales are covered with a monolayer of cells, originally called scleroblasts, on both the mineralised and un-mineralised side [103]. More recent literature subdivides the scleroblasts in osteoblasts and osteoclasts, based on their scale forming and resorbing properties, respectively [27,205,206].

This is substantiated by the classical osteoblast marker alkaline phosphatase (ALP), found in hyposquamal scleroblasts [108]. Both in mammals and in teleosts, staining of tartrate-resistant acid phosphatase (TRAcP) activity demonstrates bone surfaces that are being actively resorbed or have been resorbed [146]. 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 [27,109].

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 (monoctye -macrophage) lineage [15,146]. They display a characteristic ruffled border where proteases and acid 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 [146]. 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. 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 even 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 is 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 *mmp2* and *mmp9* 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 [35]. 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* (gelatinases A and B, respectively) are particularly active against gelatins (denatured collagens) and intact collagen types I and IV. In 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 maintenance of the osteocytic canalicular network, whereas *MMP-9* is active in early calvarian bone development and in orthodontic tooth movement [37,207,208]. 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].

Given the involvement of matrix metalloproteinases in bone Remodelling and fin regeneration, we predict the presence of MMP2 and MMP9 in scale cells. We furthermore predict an increased MMP activity in scale regeneration associated with scale matrix Remodelling. In the current study, we investigated

both expression of *mmp* genes and actual activity of MMP enzymes in the process of scale regeneration.

Materials and methods

Animals and scale collection

Experimental procedures were approved by the ethical committee of the Radboud University. Wild type adult male zebrafish (*Danio rerio*) of approximately one year old were kept at 26 °C in 1.5 litre tanks under 12 h light: 12 h dark cycle. Fish were fed twice a day with commercially available food (Tetramin, Tetra, Melle, Germany). Prior to scale harvesting, fish were anesthetised in 0.05% v/v 2-phenoxyethanol. Scales were carefully removed under a microscope from the left side of the body using watchmaker's forceps. When necessary, fish were euthanised using an overdose of 2-phenoxyethanol (0.5% v/v) and then scales or skin were collected.

To induce regeneration, approximately 50 scales were removed under anaesthesia from the left side of a fish. For analysis of gene expression and enzyme activity, ontogenetic (non-plucked) and regenerating scales were taken from the same fish (right and left sides of the body respectively). Fish were sacrificed for scale collection on days 4, 5, 6, 8, 11 and 14 (note that scales before 4 days of regeneration are too small to collect). At these time points, 40 ontogenetic (right side) and 40 regenerating (left side) scales were collected for RNA isolation and zymography. Additional fish were used for *in situ* hybridisation and histological analysis on days 2, 4 and 8 of regeneration.

In situ hybridisation

Primers were designed based on the *Danio rerio mmp-9* sequence (Table 1). The probe sequence was amplified by PCR, cloned in a TOPO vector (Invitrogen, Carlsbad, USA) which was used to transform competent cells. Samples of positive clones were then sent for sequencing to Macrogen Inc. (Seoul, South Korea). Linearization of template was done using enzyme Xho1. The PCR product was cleaned using Wizard SV Gel and PCR Cleanup system (Promega, Leiden, The Netherlands).

Skins samples were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4, 4°C). Samples were subsequently dehydrated in a graded series of RNAse-free methanol solutions to 100%, and then stored at -18°C. Prior to hybridisation, the skin samples were cut into 25mm^2 pieces and impaled on Drosophila pins (Watkins & Doncaster, Cranbrook, UK) to prevent the tissue from curling during incubation. The skin pieces were rehydrated through a graded series of methanol and processed for *in situ* hybridisation using standard protocols adapted with minor changes from [161].

Table 5 Sequences of the primers used to quantify relative gene expression levels and develop the in-situ hybridisation probe.

Gene	Accession nr.	Sequence (5'→ 3')
β-actin	AF057040	Fw: CTTGCTCCTTCCACCATGAA
		Rv: CTGCTTGCTGATCCACATCT
40s	AY648802	Fw: GTGTGGTGACCAAGATGAAGATG
		Rv: ACGGTTGTACTTGCGGATGTAA
mmp-2	NM_198067	Fw: GAGAACCAACAGACAAGCCATTG
		Rv: TCTGATCTGGGCTACAGCATCA
mmp-9	NM_213123.1	Fw: CCAGTTGTGCCAACGAAACC
		Rv: GAAGGGACCACCTGAGTTGTG
mmp-9 ISH	NM_213123.1	Fw: CACAGCTAGCGGATGAGTATCTGAAGC (pos. 253)
		Rv: AATGGAAAATGGCATGGCTCTCC (pos. 1135)

Histological sections

After *in situ* hybridisation, the skin pieces were embedded in Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany), according to the manufacturer's instructions. In some cases, the tissue was first decalcified in Osteosoft® (Merck KGaA, Darmstadt, Germany) for 24 h before embedding. Sections were cut at 5µm, counterstained with neutral red and cover-slipped with Permount™

(Fisher Scientific, Fair Lawn, New Jersey). The imaging was done with Nikon eclipse E800M equipped with DSF1 camera.

Plasma membrane staining

Whole-mount prefixed adult zebrafish scales were stained with Concanavalin A (ConA) FITC conjugate Type 4 (Sigma-Aldrich, St. Louis, USA) for membrane glycoproteins [209]. Scales were incubated in Con A FITC (25 µg/ml) for 10 min and then counterstained with DAPI nuclear stain (5 µg/ml for 2-3 min; Invitrogen, Carlsbad, USA). Confocal imaging was done using a Zeiss observer LSM 500.

Quantitative analysis of *mmp-9* positive cells

The total number of *mmp-9* positive cells was counted in the serial sections of whole mount *in situ* hybridised scales on skin. Each section was 5µm thick and the total number of serial sections selected for counting was the same for each group (control, 2day regenerated and 4day regenerated). The *mmp-9* positive cells were counted under a Nikon eclipse E800M microscope. Cell numbers were expressed relative to ontogenetic scales and statistically tested by means of a Mann-Whitney *U* test.

Anti zf-MMP9 and TRAcP double staining

Ontogenetic and regenerating scales (8 days) were fixed for 30 min in 4% paraformaldehyde in PBS at 4^{o}C and subsequently washed with PBS. Whole scales were incubated for 1 hour with block buffer (1% normal donkey serum in PBS) and subsequently incubated overnight at room temperature with zebrafish anti-*MMP-9* (Anaspec, Fremont, USA) at a dilution of 1:100 in block buffer. Next, scales were rinsed three times with PBS and incubated at room temperature with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, USA) in blockbuffer at a dilution of 1:200 for 1 h. Scales were again rinsed three times with PBS and *MMP-9* was visualised with Vectastain ABC kit (Vector Laboratories, Burlingame, USA) according to manufacturer's instructions for staining with nickel-*diaminobenzidine (Ni*-DAB). Scales were subsequently stained for TRAcP activity according to the method described by van de Wijngaert & Burger (1986) [210].Nuclei were stained with haematoxylin.

Dissected skin parts, with scales embedded, were subjected to TRAcP staining only.

qRT-PCR

Total RNA was isolated from regenerating scales and ontogenetic scales using Trizol (Invitrogen, Carlsbad, USA) according to manufacturer's instruction and subsequently treated with DNase I (Invitrogen). cDNA was synthesised using Superscript Reverse Transcriptase II enzyme (Invitrogen) according to manufacturer's instructions. Thus obtained cDNA was 10x diluted in ultrapure water for quantitative PCR.

Quantitative PCR was done according to Gorissen and co-workers [211]. Primer sequences for the different target genes are listed in table 1. The expression levels of the housekeeping genes *β-actin* and *40S* were combined in an index using the software tool *BestKeeper* [212]. Expression of *mmp* genes was corrected for primer efficiency and reference genes, and plotted relative to expression in ontogenetic scales collected on day 0.

Gelatin Zymography

Activity of putative matrix metalloproteinases 2 and 9 was detected with gelatin zymography. Ten ontogenetic and 10 regenerated zebrafish scales were cultured for 20 h in 100 µl MEMα (Invitrogen). Zymography was done according to Bildt and co-workers [213]. Relative MMP activity was calculated from band intensity with Quantity One software (Bio-Rad, Hercules, USA) and related to 2 ng human recombinant proMMP-2.

GM6001 exposure

GM6001 (ilomastat) from Millipore (Billerica, USA) was dissolved in DMSO at a concentration of 1 mg/ml. From two groups of six fish each, approximately 50 scales were removed from the left side of the fish. To specifically investigate MMP activity during scale plate Remodelling, GM6001 exposure (100 nM) was started at day 2 in one group while the other group was exposed to the vehicle. Water was replaced every other day. On day 4 and day 7, three fish from each tank were sacrificed.

Western Blot

Medium from overnight scale cultures was concentrated approximately fivefold by vacuum drying. Samples were loaded on a SDS-PAGE gel according to standard procedures. Proteins were transferred to an Immobilon-P PVDF membrane (Sigma-Aldrich) and used for Western blot with anti-*MMP-9* (Anaspec) at a dilution of 1:1000. Biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, USA) was used as second antibody at a dilution of 1:1500. The Western blot was developed with Vectastain ABC kit (Vector Laboratories) according to manufacturer's instructions for staining with nickel-*diaminobenzidine* (*Ni*-DAB).

Hydroxyproline assay

Twenty scales (ontogenetic or 6 days regenerating) were taken from 6 fish and cultured overnight in 200 µl MEMα. Collected culture medium was mixed with 400 µl 100% ethanol and allowed to precipitate overnight. Samples were centrifuged 15 min at 1710g and supernatants were collected. Pellets were washed with 200 µl 70% ethanol and supernatants were added to previously obtained supernatants. Samples were dried in a hot stove and then resuspended in 50 µl 2M NaOH. Samples were autoclaved for 30 min and hydroxyproline was measured according to the method described by Reddy and Enwemeka [49].

Results

mmp-9 gene expression in ontogenetic scales

In ontogenetic (non-plucked) scales of adult male zebrafish, *mmp-9* expression was confined to cells on the episquamal side, along the radii and margins of the scale (see Fig. 1 A, B and D for whole-mounts and Fig. 1 C and E for histological sections). Scleroblasts on the hyposquamal side showed no hybridisation; the *mmp-9*-positive cell population was confined mainly to the episquamal surface of the scale and included both mononucleated cells (Fig. 1 C and D) and multinucleated cells (Fig. 1 B, E). Fig. 1 F shows a superimposed confocal image

of plasma membranes stained with concanavalin A FITC conjugate. No separate plasma membranes were seen dividing the cytoplasmic mass of cells similar to the *mmp-9* expressing cell in Fig. 1 B. However, the nuclear membranes of the five nuclei were distinct. The extent and position of these marginal cells varied between individual scales on the same fish, and between scales from different fish, and were absent in some scales. Despite this irregular distribution, the differences in expression as a result of scale regeneration are far more pronounced.

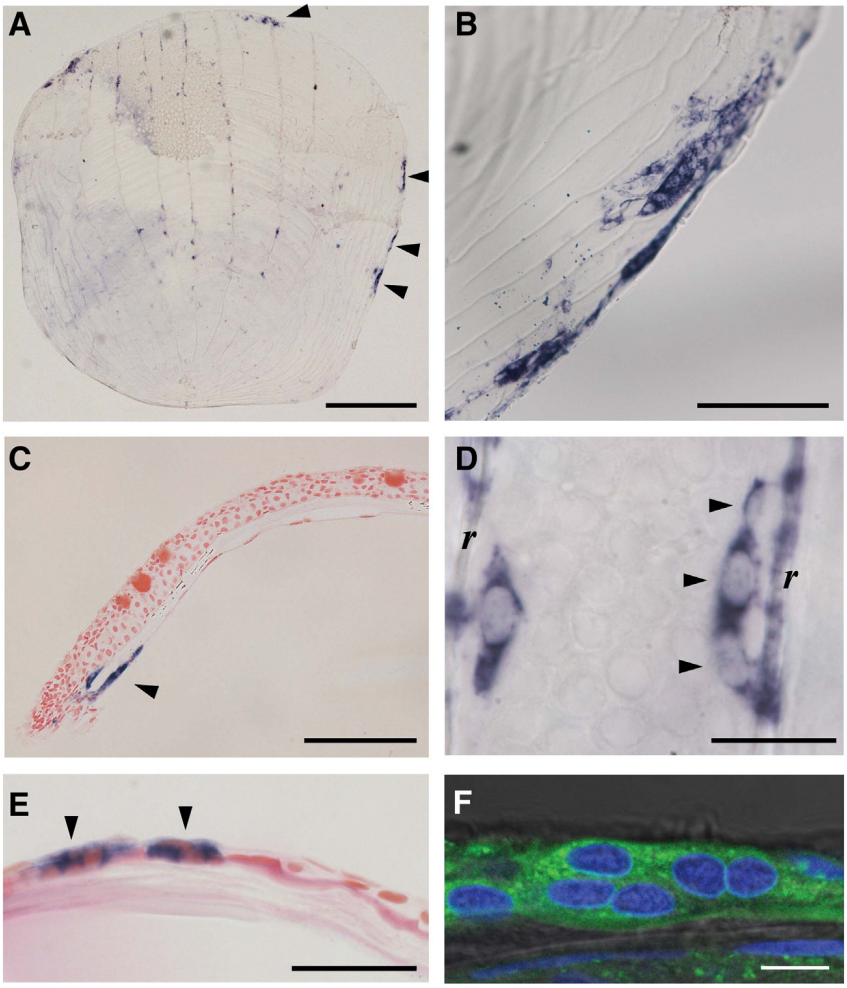

Fig. 1. *In situ* hybridization of zebrafish *mmp-9* on whole ontogenetic scales and in histological sections. **A** whole mount ontogenetic scale, arrowheads indicate the

positive clusters on the scale margins; bar = 300 µm. **B** Another ontogenetic scale showing *mmp-9* positive cells on the margin and radii of the scale; bar = 100µm. **C** Section of an ontogenetic scale, showing the *mmp-9* positive marginal cells (arrowhead); bar = 100 µm. **D** Multinucleated cell(s) expressing *mmp-9* on the radii (*r*) of an ontogenetic scale; bar = 10 µm. **E** Section of an ontogenetic scale with a multinucleated cell (arrowheads) on the episquamal side (towards top of picture); bar = 50 µm. **F** Superimposed Z-stack confocal image of ConA-FITC (green) stained cell on scale margin with nuclei counterstained with DAPI (blue); bar = 10 µm.

mmp-9 gene expression in regenerating scales

In sectioned whole mounts of 2 days regenerated scales, *mmp-9* transcripts were present in cells scattered on the episquamal, mineralised side of the newly-formed scale matrix (Fig. 2 A and B). These cells were predominantly mononucleated. However, after 4 days of regeneration, *mmp-9* expressing cells were more abundant in sections (Fig. 2 C). The 4 days regenerated scales possess aggregates of cells which appear by light microscopic observations to be multinucleated in sections. In the sections of 4 days regenerated scales, the collagenous matrix was thinner than that of ontogenetic scales and radii had not yet formed. In both 2 and 4 days regenerated scales there were no multinucleated marginal aggregates as seen in ontogenetic scales. In the sections of 8 days regenerated scales, *mmp-9* expression was similar to that of 4 day regenerated scales (Fig. 2 D). There were single cells expressing *mmp-9* all over the entire scale. Multinucleated *mmp-9* expressing cells were also present (Fig. 2 E). Quantification of the number of positive cells reveals that there are fewer *mmp-9* positive cells on day 2, but their numbers are increased on day 4 (Fig. 3).

MMP-9 & TRAcP double-staining

Staining on scales embedded in the skin clearly depict TRAcP postive cells along the margins of all scales (Fig. 4 A). Ontogenetic scales show positive staining for TRAcP activity on the episquamal side, predominantly along the radii (Fig. 4 B). At higher magnifications, *MMP-9* positive cells can also be detected (Fig. 4 C and E), some of which were located in close vicinity of resorption pits. Some mononuclear osteoclasts along the the radii show colocalisation of *MMP-9* and TRAcP (Fig. 4 D). On regenerating scales, the TRAcP activity appears increased and irregularly spread compared to ontogenetic scales (Fig. 4 E). Mononuclear

osteoclasts that both express *MMP-9* and secrete TRAcP were seen along the grooves of the scale (Fig. 4 F). At more irregular areas of TRAcP staining, multinuclear osteoclasts with *MMP-9* immunoreactivity appeared to be present as well (Fig. 4 G).

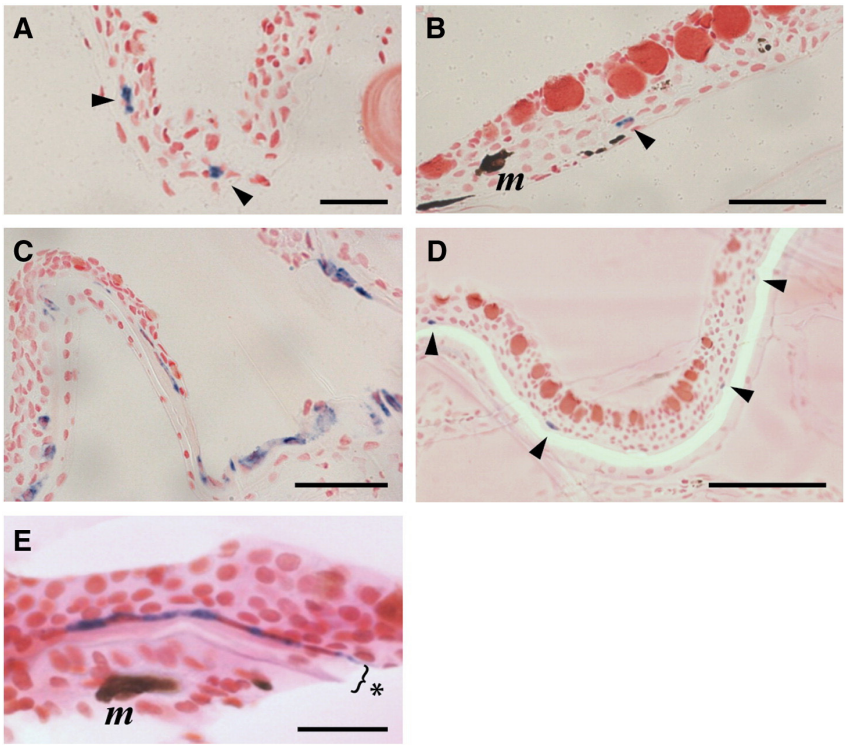

Fig. 2. Regenerating zebrafish scales, hybridised *in situ* as whole mounts with *mmp-9* probe, and then sectioned. **A, B,** Sections through the skin with 2 day regenerating scales. Mononucleated cells positive for *mmp-9* are situated on the newly-deposited matrix (arrowheads); *m* = melanocyte; bars in A and B = 50 µm. **C** Section through the skin and scale at 4 days regeneration. Numerous positive cells are distributed along the whole scale; bar = 100 µm. **D** Section through the skin with an 8 day regenerating scale, showing mononucleated *mmp-9* positive cells (arrowheads) at the radii; bar = 100 µm. **E** Section through 8 day regenerating scale showing *mmp9* positive cells lying on the episquamal surface of the newly-formed scale matrix (indicated by the bracket and asterisk). *m*, melanocyte; bar = 25 µm.

qRT-PCR profiling of mmp-2 and *mmp-9* expression

Expression of the *mmp-2* and *mmp-9* genes in ontogenetic and regenerated scales is illustrated in Fig. 6. Note that scales could not be collected earlier than 4 days of regeneration because of their small size. In 4 day regenerating scales, *mmp-2* expression is increased compared to ontogenetic scales (Fig. 5A). On days 5 and 8 of regeneration, *mmp-2* expression is significantly increased (by as much as fourfold). Expression of *mmp-9* is already up-regulated significantly after 4 days, and remains up-regulated until day 8 (Fig. 5 B). After 2 weeks, expression of both *mmp* genes has returned to levels seen in ontogenetic scales.

Zymographic analysis

The medium from an overnight culture of scales demonstrates the presence of several molecular species with gelatinolytic activity (Fig. 6). To identify the molecular species and normalise the MMP activity, human recombinant MMP-2 and -9 were loaded in lanes 1 and 2, respectively. The largest molecular species secreted by scale cells, can be identified as the inactive pro*MMP-9*, which has been activated after electrophoresis by autocatalysis. The gelatinase with a weight of approximately 77 kDa, has been confirmed to be active *MMP-9* with Western blot (Fig. 6). The other two clear bands are predicted to be MMP-2, the other matrix metalloproteinase with a preference for gelatin. Both the latent form (approximately 67 kDa) and the active form (approximately 59 kDa) of zebrafish MMP-2 are several amino acids smaller than their mammalian counterparts [214]. The faint and heavy bands located around 150 kDa are most likely MMP-dimers, which are normally observed in zymographies [215].

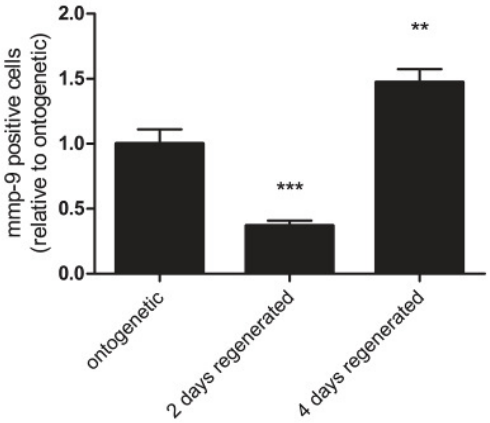

Fig. 3. Analysis of the number of *mmp-9* expressing cells in 2 and 4 days regenerating scales compared to ontogenetic scales. Significance compared to ontogenetic scales was calculated by means of a Mann-Whitney U test. Bars represent the mean value, error bars indicate S.D. (N=68-116, **: P<0,01; ***: P<0,001).

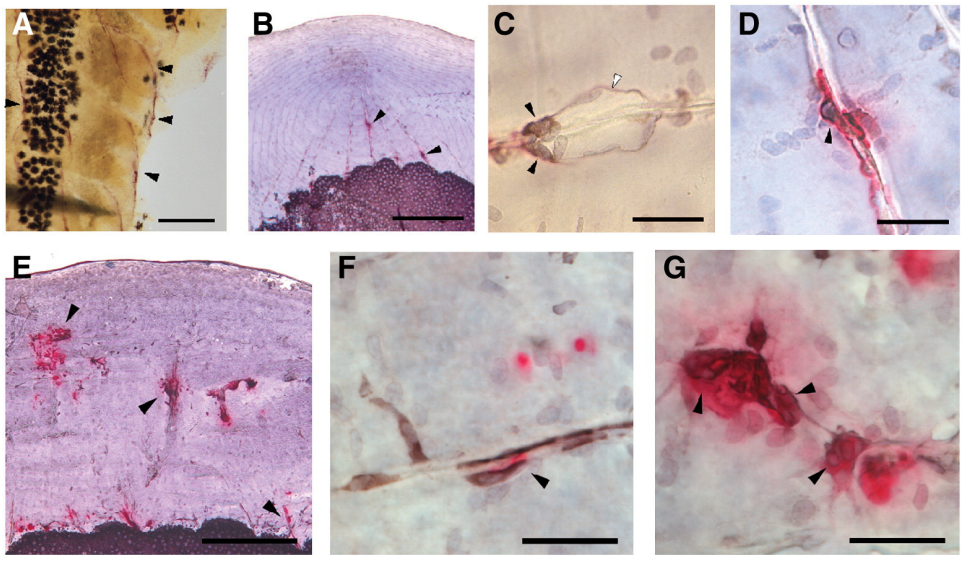

Fig. 4 Whole skin TRAcP staining (A) and *MMP-9*immunocytochemistry (black) combined with enzymatic TRAcP staining (red) in ontogenetic (B, C, D) and 8 day regenerating scales (E, F, G). **A** Overview of ontogenetic scales in the skin with stained for TRAcP. Positive cells are found along the margins (arrow heads), scale bar = 500 µm.

B overview of an ontogenetic scale with TRAcP staining along the radii (arrows); bar = 250 µm **C** *MMP-9* positive cells (black arrows) with minimal TRAcP staining in a typical resorption pit (white arrow) along a radius; bar = 50 µm. **D** mononucleated osteoclast with *MMP-9* immunoreactivity (arrow) along a radius; bar = 50 µm. **E** overview of a regenerating scale with irregularly distributed TRAcP positive areas (arrows); bar = 250 µm **F** Typical *MMP-9* positive cells with TRAcP secreted into the surrounding matrix (arrow) ; bar = 50 µm. **G** Typical multinuclear cells (arrows) positive for TRAcP and *MMP-9*. Note that nuclei are smaller in these cells; bar = 50 µm.

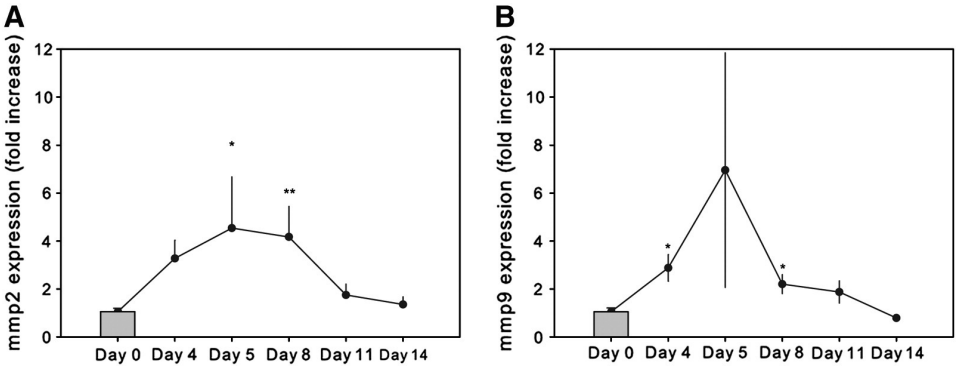

Fig. 5. rtQ-PCR. **A** expression of *mmp-2* in ontogenetic and regenerating scales. **B** expression of *mmp-9* in ontogenetic and regenerating scales. The solid bar represents the expression in ontogenetic scales. Significance was calculated by means of a Mann-Whitney U test. Points represent the mean value, error bars indicate S.E.M. (N=5, *: P<0,05; **: P<0,01).

The total amount of secreted gelatinases is increased in regenerating scales. Especially the activity of the lightest molecular species (active MMP-2) had increased, and the inactive pro*MMP-9* disappeared (Fig. 7A). An analysis of the intensity of the bands sheds more light on the changes in gelatinases expressed in ontogenetic and regenerating scales (Fig. 8). As mentioned above, no bands of 87 kDa could be detected in the regenerating scales. Significantly more of the putative active MMP-2 and *MMP-9* were present in the culture medium of regenerating scales. The amount of latent MMPs remained the same (67 kDa), or decreased (87 kDa).

The zymographic analysis of the scales from fish exposed via the water to GM6001 show clear differences between exposed fish and the control group (Fig. 7B). Although the scales have not been subjected to GM6001 during

culture, the *in vivo* GM6001 exposure resulted in bands of lower intensity compared to the control group.

Hydroxyproline assay

The modified amino acid hydroxyproline was used as a measure of matrix degradation. In culture medium of ontogenetic scales, hydroxyproline could not be detected. However, it could be detected in the culture medium of 6 day regenerating scales at a level of 0,2 ± 0,17 ng hydroxyproline per scale, which indicates increased matrix degradation in regenerating scales.

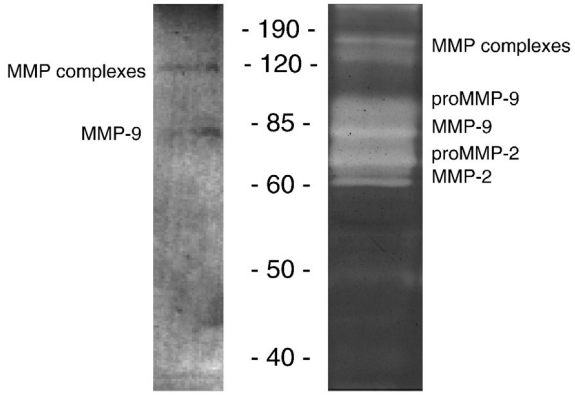

Fig 6. Anti-zf*MMP-9* Western blot (left) and zymogram (right) of the same medium sample from an overnight scale culture. The same band of 77 kDa is observed on the Western blot for *MMP-9* and the zymogram. MMP complexes are also observed.

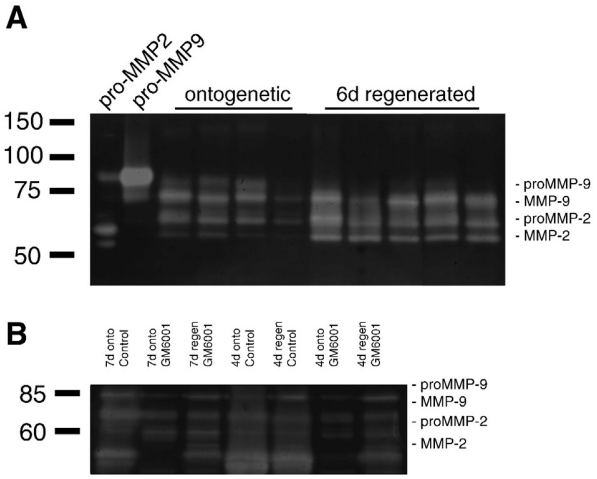

Fig. 7. **A** Zymographic analysis of medium from overnight scale cultures. The positive controls (human recombinant proMMP-2 and pro*MMP-9* are shown in the first two lanes. Lanes 3 to 6 show the gelatinolytic activity of MMPs excreted by ontogenetic scales. Lanes 7 to 11 show an increase in gelatinolytic activity of MMPs secreted by scales after 6 days of regeneration. **B** Zymographic analysis of 4 and 7 day ontogenetic and regenerating scales from fish exposed to GM6001 or vehicle. Gelatinolytic activity was attenuated in all groups of fish exposed to GM6001.

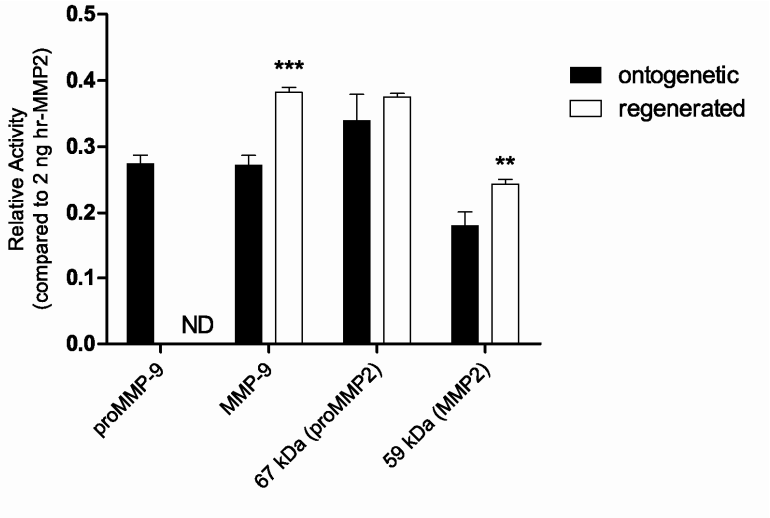

Fig 8. Semi-quantitative analysis of gelatinolytic activity on ontogenetic (closed bars) and regenerating (open bars) zebrafish scales. ND, not detectable. Bars represent mean values, error bars indicate S.E.M. Increase in the putative active MMPs tested for significance by means of a Mann-Whitney U test, N=4-5 (** P<0,01; ***P<0,001).

Discussion

We have shown both by *in situ* hybridisation and immunocytochemistry the presence of mononucleated and multinucleated *mmp-9* positive cells on the episquamal side of adult zebrafish (regenerating) scales. Plasma membrane staining and TRAcP - *MMP-9* double staining identified these cells as osteoclasts. We found an increase in expression of *mmp* genes, cell abundance, activity of MMPs and hydroxyproline levels during scale regeneration. These results combined confirm that MMPs and anticipated osteoclasts play an important role in scale resorption and Remodelling.

Although many other types of cells, such as fibroblasts and macrophages, are known to express *mmp-9* [216], the epidermis that covers the scale is completely devoid of *mmp-9* expressing cells. The wound that results from scale removal closes within two h [101]. This is an important notion as it confirms that gene expression and enzymatic activity reported here is not from inflammatory cells, but exclusively from scale cells.

The *in situ* hybridisation study revealed both mono- and multinucleated cells expressing *mmp-9* transcripts. To provide a better picture of the nature of these cells, scales were stained for plasma membranes and TRAcP. Double staining for TRAcP and *MMP-9* shows that these two osteoclasts markers are usually co-expressed. This is found for both the marginal and episquamal cells and defines these positive cells as osteoclasts. Indeed, mononucleated osteoclasts have been described in other thin zebrafish skeletal elements [26]. Inside the multinucleated aggregates, we did not see plasma membranes which prove that they are indeed multinucleated osteoclasts. They were found on both ontogenetic and regenerating scales. Our finding of mono- and multinucleated osteoclasts, expressing both *MMP-9* and TRAcP, provides further insight into the process of scale regeneration [101,200]. This is significant because TRAcP is considered to be a marker for osteoclasts able to resorb bone, as judged from "resorption pits" seen next to these cells.

The expression of *mmp-9* that we have found in marginal cells of ontogenetic scales is possibly related to the normal growth of scales that continues throughout the fish's growth. The irregular distribution of positive cells along the margins of ontogenetic scales shows that growth does not take place along the entire margin at the same time but is probably confined to different spots. Another explanation for these cells could be that they repair the normal wear-and-tear of individual scales. Cells expressing *mmp-9* transcripts are also found along the radii, where most areas of scale resorption are found. The hypothesis that radii are primary sites of calcium and phosphorus recruitment is supported by the presence of blood vessels above the radii enabling transport of those minerals [198]. Since we found no staining of cells on the hyposquamal surface, it is reasonable to conclude that hyposquamal scleroblasts, which have osteoblast-like characteristics [108], do not express *mmp-9*.

Both *in situ* hybridisation and quantitative PCR show that *mmp* genes are significantly up-regulated in regenerating scales from day 4 onwards. Interestingly, on early regenerating scales (2 days), only a few, mononucleated *mmp-9* positive cells are present on the new scale. At this point in regeneration, the first collagen matrix is deposited and has just started to mineralise (de Vrieze, unpublished data). There are no marginal *mmp-9* positive cells during early regeneration, likely due the complete new-formation of the scale. The increase in *mmp-2* and *mmp-9* expression is at its maximum around day 5. At this time, the scale plate has formed and the focus is already mineralised (de Vrieze, unpublished data). Histology revealed that on day 4 and 8, both mono- and multinucleated osteoclasts are present on the scale matrix. This coincides with the increased *mmp* expression and the initiation of scale plate Remodelling. After 10 to 14 days, expression of the *mmp-2* and *mmp-9* genes declines and returns to levels seen in ontogenetic scales. The scale plate is now formed and mineralised to its full extent, and will be remodelled to its original design [101].

To further establish the role of MMPs in scale regeneration, we investigated here the secretion of these proteins by scale cells. Our results show that the increase in secreted MMP activity in the medium by means of gelatin zymography correlates with the up-regulation in gene expression during scale regeneration. A significant increase is observed in putative active forms of the two gelatinases. The amount of latent proMMP in the medium remains the same or decreases, indicating that more MMPs are activated. The inhibition of MMP activity by *in vivo* exposure to the MMP inhibitor GM6001, further underlines the parallels between zebrafish and mammalian MMPs. The preferred substrates of active MMPs are gelatin, a product of collagen degradation, and to a lesser extent, native collagen. The switch to higher MMP activity indicates an increase in gelatin degradation and thus an increase in scale matrix degradation. As there are other substrates for MMPs [39], additional roles of MMPs in scale regeneration cannot be excluded by our findings. In mammalian bone development, *MMP-9* also regulates bioavailability of growth factors [40]. Nothing is known about the presence of growth factors on scales, but collagen and its degradation products (e.g. gelatin) are present to a large extent in the scale matrix [105]. The release of

hydroxyproline from regenerating scales confirms that the scale matrix is indeed degraded during regeneration as a result of Remodelling. Our data suggest that matrix proteolysis is an important function of matrix metalloproteinases during scale regeneration.

Conclusions

The gelatinases MMP-2 and *MMP-9*, expressed in mono- and multinucleated osteoclasts, play a pivotal role in the regeneration of zebrafish scales. These enzymes are specialised in degradation of extracellular matrix, and are likely to be involved in the Remodelling and organization of the scale surface, probably by shaping the radii and circuli. In mammalian bone of dermal origin, MMPs also function in the osteoclastic degradation of matrix. As a result of these parallels, scales may offer a valuable model to study the underlying mechanisms of osteoclastic bone resorption [217]. Their small size, short regeneration time and possession of cells that express important osteoblast and osteoclast markers, could make them particularly suitable for applications such as high throughput *in vitro* assays.