The zebrafish as a model for tissue regeneration and bone remodelling

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Chapter 6: Summary and Discussion

The aim of this thesis was to investigate the expression, and function of genes associated with Remodelling and regeneration in the zebrafish model species. Since the zebrafish is emerging as a promising low cost and easy to maintain experimental animal model with most of its genome sequence available, it will be very useful to carryout high throughput drug and compound analyses, once specific disease models are established and validated.

Here, we studied the role of cell populations, defined by their expression of markers, in bone regeneration and Remodelling in zebrafish embryos and adult zebrafish scales. We also examined how these processes are disrupted by behavioural stress using the case of zebrafish embryonic caudal fin regeneration. We used mesoporous silica nanoparticles to carry cytokines, known to activate hematopoietic cells into osteoclasts, into tissues of the living embryo. Finally we studied the regeneration of the caudal fin of zebrafish embryos with a special emphasis on the effect of glucocorticoids on regeneration and wound healing. Glucocorticoids in this case mimic stress conditions in the embryos, thus helping understand the effect of early exposure to stress on wound healing and tissue Remodelling.

In chapter 2 we studied the expression of a panel of genes that are associated with bone and tissue Remodelling, namely: *mmp-9*, *cathepsin K, rank* and TRAcP in zebrafish embryos and larvae. Differences in expression were seen between the genes, but there were two identifiable groups based on similarities in expression. The groups were *cathepsin K* and *rank*, on the one hand, and *mmp-9* and TRAcP on the other.

We found strong similarities in the expression of *cathepsin K* and *rank* genes in the larvae as well as in the adult scales. In larvae both these genes were associated with the skeletal structures. In the adult zebrafish scales the *cathepsin K* [168] and *rank* genes are both expressed in the marginal regions. However the scale-margin expression of *cathepsin K*, but not *rank*, was seen in multinucleated cells.

Unlike *cathepsin K* and *rank*, the *mmp-9* and TRAcP expression was neither specifically associated with the pharyngeal arches nor the pectoral fin. The expression of *rank* and *mmp-9* in 1 dpf embryos is interesting as it is found in the site of hematopoietic in the early embryonic stages of zebrafish development such as the ventral blood island and rostral blood island. Previous researchers have argued that TRAcP expression is a definitive marker for active osteoclasts [142]. TRAcP expression was observed in the skeletal structures such as Meckel's cartilage in 5 dpf larvae, but not in larvae younger than this. However, it was strongly expressed in the scattered cells all over the embryos (2-5 dpf) more specifically in the caudal hematopoietic tissue. This TRAcP expression was similar to *mmp-9* expression by *in situ* hybridization in embryos and early larvae.

In cathepsin K GFP transgenic zebrafish embryos, it was found that after a continuous exposure to a combination of dexamethasone (DEX) and vitamin D3, there was a stronger and much clearer expression all over the pharyngeal skeleton in 5 dpf zebrafish embryos, compared to the controls exposed to only egg water. There was also expression in individual scattered cells near the tail region of 5d old embryos. This expression in scattered cells is similar to TRAcP and *mmp-9* expression, and therefore suggests that the TRAcP and *mmp-9* in addition to cathepsin K GFP protein are most probably expressed in the same population of cells which may also be osteoclasts.

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 scale 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 presented expression of *cathepsin K* in the multinucleated cells and *rank* in the mononucleated cells.

In chapter 3 we used a relatively new drug delivery system (DDS) with the aim of delivering the compounds related to osteoclastogenesis into zebrafish

embryos. We used mesoporous silica nanoparticles (MSNPs) to carry the cytokines MCSF and RANK-L into the living zebrafish embryos. This was to see whether we could activate existing immune cells into TRAcP expressing cells in early larvae, which are not otherwise known to have osteoclasts at that stage.

Before we preceded with osteoclast activation experiments we considered it important to first look at the possible toxicity of MSNPs in the zebrafish embryos, and also to assess the safe concentration of injected particles. Therefore, we tested the toxicity, and capacity for drug delivery, of MSNPs injected into the living zebrafish embryo. We found that there was no significant difference between the toxicity of MSNP injections, and of buffer-only injections, as measured by several parameters (mortality, cell death, gross malformations). Injection of fluorescent MSNPs led to an influx of immune cells at the site of injection that persisted for 2-3 days. However, the same type of influx was also seen in control embryos, suggesting that it is not the MSNPs that are responsible for the immune response, but a reaction of the injection procedure itself. It has previously been shown that trauma to zebrafish embryos (e.g. a fin clip or mechanical wounding with an injection needle) causes an influx of macrophages and neutrophils to the wound site [180-183].

MSNPs are very small in size with a very high surface area, which makes them very attractive systems to carry the drugs. We found no significant toxic effects of injected MSNPs in the living embryos. This means that at low concentrations, MSNPs are very good delivery systems in the whole organism.

We observed very little overlap in expression with confocal imaging of lyz: DsRED2-labeled cells and MSNPs suggesting that very small amount of the particles are actively phagocytosed by neutrophils. With L-plastin immuno-labelling, which stains leucocytes, more overlap with the fluorescent signal of the particles was observed, suggesting that part of the MSNPs may be phagocytosed by immune cells. Other studies have shown that, in the presence of artificially provided essential cytokines like MCSF and RANK-L, immune cells are activated into TRAcP positive osteoclasts [196].

Further work is required to clarify the differentiation status of the TRAcP positive cells. Furthermore, the presence of osteoclast-like cells in zebrafish larvae could lead to a disease model for bone disorders and for the study of

effects, for example, of anti osteoporotic drugs. What is clear from our findings in chapter 3 is that MSNPs can be used for drug or compound delivery in the zebrafish embryo with no excess of gross toxic effects or immune responses attributable to the nanoparticles themselves.

Out of the panel of markers studied in chapter 2 we further investigated the role of genes such as matrix metalloproteinases in chapter 4. These genes are known to be involved in matrix degradation and have been found to be expressed in mammalian osteoclasts. We studied 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. Together, these results suggest that MMPs and osteoclasts play an important role in scale resorption and Remodelling. It was suggested by our *in situ* hybridisation study that both mono- and multinucleated cells express *mmp-9* transcripts in zebrafish scales. Our finding of mono- and multinucleated osteoclasts, expressing both MMP-9 and TRAcP, provides further insight into the process of scale regeneration.

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

In chapter 5 we used a larval zebrafish caudal fin model of generation to investigate the lasting impact of glucocorticoid exposure in early developmental stage on the capacity to regenerate tissue at later time points in life. We report for the first time that a history of glucocorticoid exposure is associated with lasting effects on tissue regenerative capacity in larval zebrafish. These findings are in agreement with previous studies reporting a link between early-life stress and impaired wound healing in human in adulthood.

We found that susceptibility to DEX-induced delay in wound healing/tissue repair is seen not only shortly after injury (i.e. 2 days post caudal fin amputation) but also appear to be an effect that may endure much longer throughout the lifespan, since a similar phenomenon is also observed when injury occurs much later (11 days post caudal fin amputation). Taken together, these findings suggest that DEX exposure during early sensitive periods of development appears to cause permanent alterations in the cellular/molecular immune processes that underlie the early phases of wound healing.

Increased cell death studied by acridine orange and TUNEL labelling suggested that there is significantly higher cell death in the regenerated tissue in the DEX treated larvae as compared to the controls. This increased cell death may be in the immune cells as well as other cells within the blastema tissue. As blastema has pluripotent stem cells in the phases studied here, so it is possible that this aberrant regeneration is a result of increased cell death in the pluripotent stem cells within the blastema. This apoptosis in blastema tissue can be an explanation of restricted tissue regeneration. Future studies are required to assess whether blocking (morpholino or pharmacological antagonism) would have prevented the effects observed here.

Conclusions

- Genes associated with osteoclasts are expressed in early zebrafish embryos (Chapter 2)
- Cells expressing osteoclast markers could be induced by the injection into zebrafish embryos of nanoparticles loaded with cytokines (Chapter 3)

- The same genes are expressed in embryonic, as well as adult contexts (Chapters 2 and 4)
- These cells are involved in adult tissue Remodelling in scale regeneration in zebrafish (Chapter 4)
- Mimicking stress in early life can have a deleterious or delaying effect on the Remodelling or regeneration of tissues in the zebrafish at later stages (Chapter 5)
- The zebrafish could be a useful model for studying the processes involved in bone Remodelling and regeneration (General conclusion from this thesis)