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Perspective

Osteoblast and osteoclast behaviors in the turnover of attachment bones during medaka tooth replacement

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ABSTRACT

Tooth replacement in polyphyodont is a well-organized system for maintenance of homeostasis of teeth, containing the dynamic structural change in skeletal tissues such as the attachment bone, which is the supporting element of teeth. Histological analyses have revealed the character of tooth replacement, however, the cellular mechanism of how skeletal tissues are modified during tooth replacement is largely unknown. Here, we showed the important role of osteoblasts for controlling osteoclasts to modify the attachment bone during tooth replacement in medaka pharyngeal teeth, coupled with an *osterix*-DsRed/*TRAP*-GFP transgenic line to visualize osteoblasts and osteoclasts. In the turnover of the row of attachment bones, these bones were resorbed at the posterior side where most developed functional teeth were located, and generated at the anterior side where teeth were newly erupted, which caused continuous tooth replacement. In the cellular analysis, osteoclasts and osteoblasts were located at attachment bones separately, since mature osteoclasts were localized at the resorbing side and osteoblasts gathered at the generating side. To demonstrate the role of osteoclasts in tooth replacement, we established medaka made deficient in *c-fms-a* by TALEN. *c-fms-a* deficient medaka showed hyperplasia of attachment bones along with reduced bone resorption accompanied by a low number of TRAP-positive osteoclasts, indicating an important role of osteoclasts in the turnover of attachment bones. Furthermore, nitroreductase-mediated osteoblast-specific ablation induced disappearance of osteoclasts, indicating that osteoblasts were essential for maintenance of osteoclasts for the proper turnover. Taken together, our results suggested that the medaka attachment bone provides the model to understand the cellular mechanism for tooth replacement, and that osteoblasts act in the coordination of bone morphology by supporting osteoclasts.

1. Introduction

To understand the dynamic system for maintaining the homeostasis of organ is one of the main fields in developmental biology. Tooth replacement in polyphyodont is a well-designed system to maintain the homeostasis of teeth; polyphyodont animals like fish can replace their teeth continuously throughout their life (Reif, 1982). In polyphyodont, the dentitions are usually organized with tooth families that are consisted of functional teeth and successional tooth germs (Reif, 1982), where old functional teeth are shed, and tooth germs move to take place of lost teeth for tooth replacement (Berkovitz, 2000; Bermis et al., 2005).

Like other non-mammalian vertebrates, the medaka fish is polyphyodont (Parenti, 1987). Medaka has thousands of functional teeth in the pharyngeal region, which are assembled as the multiple rows (Atukorala et al., 2010), while functional teeth are connected to their supporting skeletal tissues, called attachment bones, and successional tooth germs do not have any skeletal support. Moreover, tooth families and the tooth replacement are shown in medaka (Abduweli et al., 2014). These reports imply that attachment bones have to be continuously modified corresponding to the shedding and eruption of teeth during tooth replacement, however, the cellular mechanism showing the control of such a dynamic change in skeletal tissues is left to be unknown.

Previously, we found that many multinuclear osteoclasts are localized on attachment bones (Nemoto et al., 2007). Osteoclasts are bone resorbing cells acting with bone forming osteoblasts for the modification of skeletal tissues by bone modeling or remodeling, indicating the possibility that the modification of attachment bones during tooth replacement is achieved by osteoclasts on these bones, while their function coupled with differentiation has not been investigated.

Although recognition of skeletal tissues by osteoclasts for the

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modification is unknown, osteoclast differentiation that is regulated by surrounding cells in bone is found as follows. The interaction between osteoclasts and osteoblasts is important for osteoclast differentiation; the *in-vitro* study shows that when co-cultured with osteoblasts, macrophage-like osteoclast progenitors, driven by macrophage colony-stimulating factor (M-CSF) from bone marrow cells, differentiate into $TRAP^+$ mononuclear osteoclasts, and then into multinuclear osteoclasts by cell fusion (Takeshita et al., 2001). Also, the study about Runx2-deficient mice reveals that the osteoblast has a crucial role on osteoclast differentiation; only mononuclear TRAP-positive cells are detected in the periosteum during early skeletogenesis in the mice, though osterix-deficient mice have multinuclear osteoclasts (Nakashima et al., 2002).

Earlier, we found that during skeletogenesis of the vertebral body in medaka, osteoclasts have properties similar to those of mammals, as they are differentiated from $TRAP^+$ and $CTSK^+$ mononuclear cells into multinuclear osteoclasts (Kudo, 2011; Chatani et al., 2011). Furthermore, *c-fms* (the receptor of M-CSF)-deficient zebrafish show a reduced number of osteoclasts, resulting in a bone modeling defect, which indicates the essential function of M-CSF and *c-fms* in fish as well as in mammals (Chatani et al., 2011), suggesting that the basic molecular mechanism in differentiation of osteoclasts is common between mammals and lower vertebrata, and the interaction between osteoclasts and osteoblasts has a crucial role for osteoclast differentiation to develop the corrective skeletal structure in medaka as well.

Here, we demonstrated the cellular mechanism for the turnover of attachment bones during tooth replacement, which showed a high rate of systematic modification of bony tissues in a simple structure, along with visualization of osteoblasts and osteoclasts. To visualize osteoblasts and osteoclasts *in-vivo*, we previously developed a medaka *osterix* promoter-DsRed transgenic line for visualization of osteoblasts (Inohaya et al., 2010) and a medaka *TRAP* promoter-GFP transgenic line for that of osteoclasts (Chatani et al., 2011). Finally, we established a double transgenic line of *osterix*-DsRed and *TRAP*-GFP (Chatani et al., 2015) to examine the cooperation between osteoblasts and osteoclasts in the same animal *in vivo*. Our present results showed that osteoclasts, which had differentiated from *c-fms*$^+$ osteoclast progenitors, were exclusively localized on the developed attachment bones and that osteoblasts were mainly localized on the newly generated attachment bones. Osteoblast ablation induced disappearance of osteoclasts, which is a new finding confirming the communication between osteoblasts and osteoclasts in the turnover of attachment bones.

2. Materials and methods

2.1. Fish strains and maintenance

Cab, an inbred wild-type strain of the medaka (*O. latipes*), was used throughout this study. The fish were kept under a photoperiod of 14-h light/10 h dark at 28 °C. Eggs were obtained by random crossing and kept at the same temperature. Embryos were incubated at 30 °C after collection and then staged according to the Iwamatsu stages (Iwamatsu, 2004). For the larval experiment, larvae were incubated at 30 °C after hatching. For the breeding adult fish, larvae were incubated at room temperature after hatching.

2.2. Histological and histochemical examination of pharyngeal bones

Fish over 3 months old were used as adult fish. Adult fish were deeply anesthetized by using 3-aminobenzoic aced ethyl ester, and they were then sacrificed for isolation of their pharyngeal bones. These isolated bones were fixed overnight at 4 °C in (PFA) in phosphate-buffered saline pH 7.4 (PBS) containing 4% paraformaldehyde (PFA). The fixed bones were directly dehydrated by liquid resin (Technovit8100, Heraeus Kulzer, Wehrheim) and embedded in Technovit8100 with coagulant (Hater, Heraeus Kulzer, Wehrheim). Sections were cut at 4 μm, and fluorescence microscopy was used to detect the fluorescence of DAPI, GFP, and DsRed. Sections were stained with methylene blue for bright-field observation. Staining for TRAP activity was performed as previously reported (Chatani et al., 2011).

2.3. Bone staining

For *in vivo* staining of calcified bones, adult fish were treated with calcein (0.05%, Sigma) or Alizarin Complexone (0.025%, Wako Pure Chemical, Osaka, Japan) for 1 h at 30 °C. Larvae at the desired developmental stage were grown in medium containing the same concentration of calcein for 24 h or of Alizarin Complexone for 12 h, either one at 30 °C. After the staining solution had been washed out, the larvae were anesthetized by using 3-aminobenzoic aced ethyl ester and fixed with 4% PFA. The fixed samples were thereafter soaked in 50% glycerol, and pharyngeal bones were isolated from the samples. Alizarin red staining was performed as previously described (Ohisa et al., 2010).

2.4. Whole-mount RNA in situ hybridization

Samples were fixed in 4% PFA overnight at 4 °C. Whole-mount RNA *in situ* hybridization was performed as previously described (Nemoto et al., 2007). The digoxigenin-UTP-labeled RNA probe for *TRAP* was obtained as previously described (Nemoto et al., 2007). To synthesize *osterix*, *DSPP*, *MMP9*, and *c-fms-a* probes, we amplified the partial cDNA fragments of these genes by using the following sets of oligonucleotide primers: *osterix* F, 5′-ACTGTCAGGAGCTGGAAAGACTG-3′, and *osterix* R, 5′-AGATCTCCAACAATCCACTGCTG-3′; *DSPP* F, 5′-TTCGGAGCTTCAGAGCTCTGAGG-3′, and *DSPP* R, 5′-CCTCATCTGGCGTCATCAGTCCG-3′; *MMP9* F, 5′-CTGCTCTCCCAGCTGTTATTG-3′, and *MMP9* R, 5′-GAAATTTGCTGACCCGTATGA-3′; and *c-fms-a* F 5′-CTGAAGCTGATCCAGGATGC-3′; and *c-fms-a* R 5′-TCAGCAAAACTGATAGTTGTTAGTCTTC-3′.

A full-length cDNA fragment and the probe of *type X collagen* were obtained by methods previously reported (Yasutake et al., 2004, Nemoto et al., 2007).

2.5. Drug treatment

For osteoblast ablation, fish were kept in the dark at 30 °C during the metronidazole (Sigma) treatment, and the solution was refreshed once a day. The larvae were incubated with 5 mM metronidazole for 2 or 3 days, whereas the adult fish were treated with 5 mM metronidazole for 24 h at the 1st and 3rd day in the 3-day experiment or at 1st, 3rd, and 5th day in the 5-day one. For osteoblast ablation and restoration, larvae were incubated with 5 mM metronidazole for 2 days, and then they were put into water in a tank.

2.6. Semi-quantitative RT-PCR

Total RNA was extracted from left upper and lower pharyngeal bones by using TRIzol (Invitrogen). Total RNA of 0.5 μg extracted from each sample was used for synthesis of a cDNA library by using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Kusatsu, Japan). The following primers were designed based on the Ensembl genome browser:

GAPDH F, 5′-CTTGGATACACAGAGGACCAGG-3′, and *GAPDH* R,

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