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# Simulated microgravity upregulates gene expression of the skeletal regulator Core binding Factor α1/Runx2 in Medaka fish larvae in vivo

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**Abstract**

Long-term space flight results in significant bone loss in humans. However, it remains to be shown how microgravity affects the expression of genes involved in modeling and remodeling of bone material in vivo. For these analyses, animal models are instrumental to study alterations at the molecular and cellular level. Although it is not known at present, whether fish loose bone in microgravity, they show many experimental advantages to approach these questions in vivo. Here, we report for the first time that living Medaka larvae can be used in hypergravitation and clinorotation experiments to study the effect of altered gravity on gene expression in a whole-animal situation. Living Medaka larvae at 1 day post-hatching were exposed to hypergravity and simulated microgravity for 24 hours (h) and the level of mRNA expression of skeletal regulators was determined by real-time RT-PCR. No effect of altered gravity was observed on the expression of *osteoprotegerin* (*opg*) genes that regulate osteoclast formation in humans. However, clinorotation resulted in a significant increase of expression of *core binding factor* α1 (*cbfa1/runx2*), a crucial regulator of osteoblast formation. Exposure to hypergravitation for 24 h on the other hand had no effect on *cbfa1/runx2* expression. This shows that *cbfa1/runx2* responds to reduced gravity by expression level changes in vivo. Furthermore, it demonstrates that Medaka provides a valuable experimental model to study molecular mechanisms for compensating microgravity induced bone loss.


*Keywords:* Animal models; Medaka; *cbfa1/runx2*; Osteoprotegerin; Clinorotation; Cartilage; Skeleton; Bone loss

## 1. Introduction

Small laboratory fish like the zebrafish or the Medaka (*Oryzias latipes*) have recently been established as useful animal models for a large number of human diseases. This includes, e.g., muscular dystrophy (Bassett and Currie, 2004), spinal muscular atrophy (McWhorter et al., 2003), cardiovascular disease (North and Zon, 2003) and many others (see Rubinstein, 2003 for review). Fish models also offer a number of experimental advantages to gain insights into the pathogenesis of human skeletal disorders (Wagner et al., 2003). For example, several zebrafish mutants have been identified in mutagenesis screens that develop skeletal defects (Fisher et al., 2003). The molecular

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characterization of these mutants identified a number of skeletal genes implicated in bone formation in fish, including *collagen type 1a* (Fisher et al., 2003) and *tfap2a* (Barrallo-Gimeno et al., 2004; Knight et al., 2004). Also, efficient transgenic and gene knock-down techniques have been developed for functional analysis of genes in vivo (Nasevicius and Ekker, 2000; Thermes et al., 2002). Similar to zebrafish, the Medaka also produces large numbers of rapidly developing and nearly transparent embryos that allow easy observation of skeletal development in situ (Wagner et al., 2003; Iwamatsu, 2004). Furthermore, efficient large-scale mutagenesis screens have been performed in Medaka to isolate embryonic mutations (Furutani-Seiki et al., 2004) and genome sequencing projects have been established (Naruse et al., 2004; Sasaki et al., 2004). Thus, fish offer a large number of unique experimental features that allow studies on the regulation and function of regulatory genes implicated in the complex networks of cartilage and bone formation.

In mammals, Cbfa1/Runx2 is an essential transcription factor required for the differentiation of osteoblasts (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997) and the maturation of chondrocytes (Inada et al., 1999; Kim et al., 1999; Stricker et al., 2002). Thus, it is a central regulator of bone and cartilage formation. Human patients with *cbfa1/runx2* haploinsufficiency develop severe cleidocranial dysplasia (Mundlos et al., 1997). The *cbfa1/runx2* genes have also been identified in Medaka and zebrafish (Inohaya and Kudo, 2000; Flores et al., 2004). In these species, *cbfa1/runx2* is co-expressed with other known skeletal regulators in regions of skeleton formation, suggesting a role during bone and cartilage development of fish (Inohaya and Kudo, 2000; Wagner et al., 2003; Flores et al., 2004). However, functional data in fish analyzing these roles in detail are lacking so far.

Interestingly, clinorotation experiments using cell cultures of man and mice have shown that expression of *cbfa1/runx2* and other skeletal genes is modulated by altered gravity at the transcriptional level (Kanematsu et al., 2002; Nakamura et al., 2003; Ontiveros and McCabe, 2003; Zayzafoon et al., 2004). Due to experimental limitations, however, no in vivo data are available for the gravity regulation of these genes at the organismal level. Recently, it has been shown that living fish embryos respond to altered gravity and can be successfully used to monitor gene expression changes after clinorotation (Gillette-Ferguson et al., 2003). Here, we show that *cbfa1/runx2* expression is significantly altered in Medaka larvae that have been subjected to simulated microgravity by clinorotation. To the best of our knowledge, this is the first report on altered expression of a skeletal gene in simulated microgravity in vivo.

## 2. Material and methods

### 2.1. Staining of cartilage and bone in Medaka

For visualization of cartilage and bone, Medaka embryos and larvae were fixed overnight in 4% paraformaldehyde at 4 °C and subsequently washed in PBS. To stain cartilage, fish were incubated in 0.1% Alcian Blue dissolved in acidic ethanol for at least 1 day and destained in acidic ethanol for at least 6 h at room temperature (for details see Barrallo-Gimeno et al., 2004). To stain calcified bone, fish were incubated in 1% KOH containing 6.25% of an Alizarin Red stock solution (1 g Alizarin Red in 100 ml 0.5% KOH) for 30 min and destained in 1% KOH for 30 min at room temperature (for details see Gavaia et al., 2000). Stained fish specimens were mounted in glycerol or 2% methyl cellulose and images were taken with a Zeiss photo microscope.

### 2.2. Hypergravity and clinostat rotation experiments

Medaka (*Oryzias latipes*) larvae at 1 day post-hatching (1 dph) were subjected to hypergravity (3*g*) for 24 h using the MuSIC (Multi-Sample Incubation Centrifuge) equipment available at the Microgravity User Support Centre (MUSC) of the German Aerospace Center (DLR) in Cologne, Germany. For clinorotation experiments, 1 dph larvae were subjected to simulated microgravity for 24 h using a 2D fast rotating clinostat, also available at MUSC. Head and trunk of the larvae had a diameter of approximately 1 and 0.5 mm, respectively. The average body length was 5 mm. To simulate microgravity, larvae were clinorotated at 60 rpm in vessels of 3 mm diameter resulting in compensated gravity with residual centrifugal forces up to $10^{-3}g$. Control larvae were kept at 1*g* for 24 h under identical conditions. The experiments were performed in the dark to exclude any effects of the dorsal light reflex on the swimming behaviour of the fish. After incubating the larvae at different gravity conditions, they were briefly anesthetized on ice and then immediately sacrificed by decapitation. To stabilize endogenous RNAs for subsequent expression analysis, the larvae were placed into RNA*later* reagent (Qiagen) and stored at −20 °C for further analysis.

### 2.3. Analysis of gene expression in Medaka larvae

For expression analysis, 30 complete larvae were pooled for probe and control, respectively, and total RNA was isolated using the RNeasy Mini Kit (Qiagen). Using Oligo(dT) primers (Invitrogen) and Superscript III reverse transcriptase (Invitrogen), cDNA was synthesized from total RNA. Gene expression in probe and control was determined via real-time RT-PCR (iCycler