

## Short Communication

# Conditional Knockout of the MicroRNA 17-92 Cluster in Type-I Collagen-Expressing Cells Decreases Alveolar Bone Size and Incisor Tooth Mechanical Properties

(alveolar bone / MiR17-92 cluster / incisors / lower jaw)

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**Abstract.** To test the role of the miR17-92 (miR) cluster in dental bones, we evaluated the incisor tooth phenotype by micro-CT in 5- and 12-week-old conditional knockout (CKO) mice deficient in the miR17-92 cluster in type-I collagen-expressing cells and bone strength by finite element analysis. The incisor teeth of CKO mice showed a 23–30 % reduction in tissue volume and bone volume. Accordingly, the stiffness and failure load of incisor teeth assessed by finite element analysis showed an 18–40 % decrease in CKO compared to wild-type mice. A positive correlation between bone parameters and strength data suggests that the decreased mechanical properties of incisor teeth are due to decreased tissue volume and bone volume. Subsequently, we found that the width of alveolar bone was reduced by 25 % with a 16 % increase in periodontal ligament space, suggesting that the CKO mice are more susceptible to tooth movement. Since alveolar bone is populated primarily by osteoblast lineage cells, it is likely that the reduction in periosteal expansion of alveolar bone in the lower jaw of CKO mice results from decreased periosteal bone formation. Overall, our phenotype analysis demonstrates that the miR17-92 cluster is essential for development and maintenance of tooth strength by regulating its tooth size.

## Introduction

Bone strength in the lower jaw (mandible), including the alveolar bone and teeth, are very important for grind-

ing food materials, preventing tooth movement and protecting against osteoporotic fracture. Bone strength is determined by the size of the bone, its density and its quality (Seeman, 2008; Takaishi et al., 2013). An increase in bone strength in the bones of the lower jaw (alveolar bone) and teeth is mediated by osteoblasts, periosteal cells, ameloblasts, and odontoblasts, whose cellular processes (proliferation, differentiation and survival) play an important role in promoting bone formation and thereby bone strength. These cellular processes are mediated by multiple genes and signalling pathways, which are regulated by factors at the transcriptional and post-transcriptional level. Therefore, identifying key factors that regulate anabolic genes at the protein level is important for development of strategies to improve alveolar bone, tooth strength, and to diagnose individuals at risk for dental bone disease.

MicroRNAs (miR) are a family of small, non-coding RNAs that regulate gene function post-transcriptionally either by degrading or stabilizing messenger RNA. In particular, the role of miRs in dental bone physiology and odontogenesis is apparent because of increased osteoclast activity contributing to osteoporotic fracture in the lower jaw, which contains teeth and alveolar bone (Gao and Zheng, 2013; Gama et al., 2015). In this regard, studies have identified, using a microarray approach, differential expression levels of miR that lead to alveolar bone and tooth destruction caused by an increased inflammatory response induced by osteoclastogenesis (Gao and Zheng, 2013). More specifically, others have shown that miR146a plays an important role in dental follicle cell differentiation (Chen et al., 2014). In addition, it has been shown that miR143 and miR145 play an important role in controlled odontoblast differentiation and dentin formation (Liu et al., 2013). While data from these studies show that there is an association between miR expression and the function of the dental bones in the jaw, the role of all key miR and the phenotypic changes that their loss produces has yet to be elucidated.

In a recent study, we found that conditional disruption of the miR17-92 cluster in type-I collagen-expressing osteoblast lineage cells resulted in decreased periosteal

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Abbreviations: BV – bone volume, CKO – conditional knockout, IFN- $\gamma$  – interferon  $\gamma$ , miR – microRNAs, PDL – periodontal ligament, TRAP – tartrate-resistant acid phosphatase, TV – tissue volume, UTR – untranslated region, WT – wild-type.

bone formation and mineral apposition rates in long bones, suggesting that the miR17-92 cluster is essential for osteoblast differentiation/function (Mohan et al., 2015). Consistent with this finding, another study also reported that knocking down the miR17-92 cluster affects osteoblast differentiation and cortical bone mass (Zhou et al., 2014). Additionally, a third study has shown that the expression level of miRs in the miR17-92 cluster is increased during odontogenesis (Jevnaker and Osmundsen, 2008). Since similar regulatory pathways are predicted to control osteoblast and odontoblast differentiation, we hypothesized that the miR17-92 cluster was also essential for dental bone development and maintenance. To test this, we evaluated the dental phenotype of miR17-92 cluster conditional knockout (CKO) mice and control mice to determine whether the miR17-92 cluster also played a role in dental bone development.

## Material and Methods

The miR17-92 cluster CKO mice were generated for skeletal phenotype analyses by crossing miR17-92 floxed mice with transgenic Cre mice in which Cre expression is driven by the regulatory regions of the *Colla2* gene as described (Mohan et al., 2015). Dental bones collected from 5- and 12-week-old CKO and control mice were used to evaluate differences in the alveolar bone, tooth length and incisor size at a resolution of 10  $\mu\text{m}$  by micro-CT, a high resolution tomography image system (Scanco Medica Viva CT40, Brüttisellen, Switzerland). Parameters including tissue volume (TV,  $\text{mm}^3$ ), bone volume (BV,  $\text{mm}^3$ ), and molar tooth length (mm) were evaluated as described earlier (Mohan et al., 2015). Furthermore, the scanned cross-sectional area of incisor teeth from wild-type (WT) and CKO mice were converted to a finite element mesh with a Poisson's ratio of 0.3 and scale factor -1. This was subjected to a mathematical simulation of high friction compression test using a young modulus of 10,000 MPa, according to the manufacturer software instruction (Scanco finite element analysis software, version 1.16) to evaluate the tooth strength (Ulrich et al., 1998; Boutroy et al., 2008). Parameters such as stiffness (N/mm) and failure load estimation (kN) were calculated in the WT and CKO mice as described earlier (Pistoia et al., 2004), where failure is estimated as the force at which 2 % of the bone elements exceeded 7,000 microstrain, using a tissue level effective strain.

Additionally, we also performed histomorphometric analysis of the lower jaw of CKO and WT mice. The lower jaw samples fixed in 10% formalin were decalcified in 14% EDTA (pH7.1) and then embedded in paraffin for sectioning as described earlier (Mohan et al., 2015). Five  $\mu\text{m}$  longitudinal serial sections of the lower jaw were prepared and sections were stained with toluidine blue. Periodontal ligament ( $\mu\text{M}$ ) and alveolar bone width were analysed by light microscopy (Olympus, Center Valley, PA) in the WT and CKO sections under 100X magnification. Subsequently, the lower jaw longi-

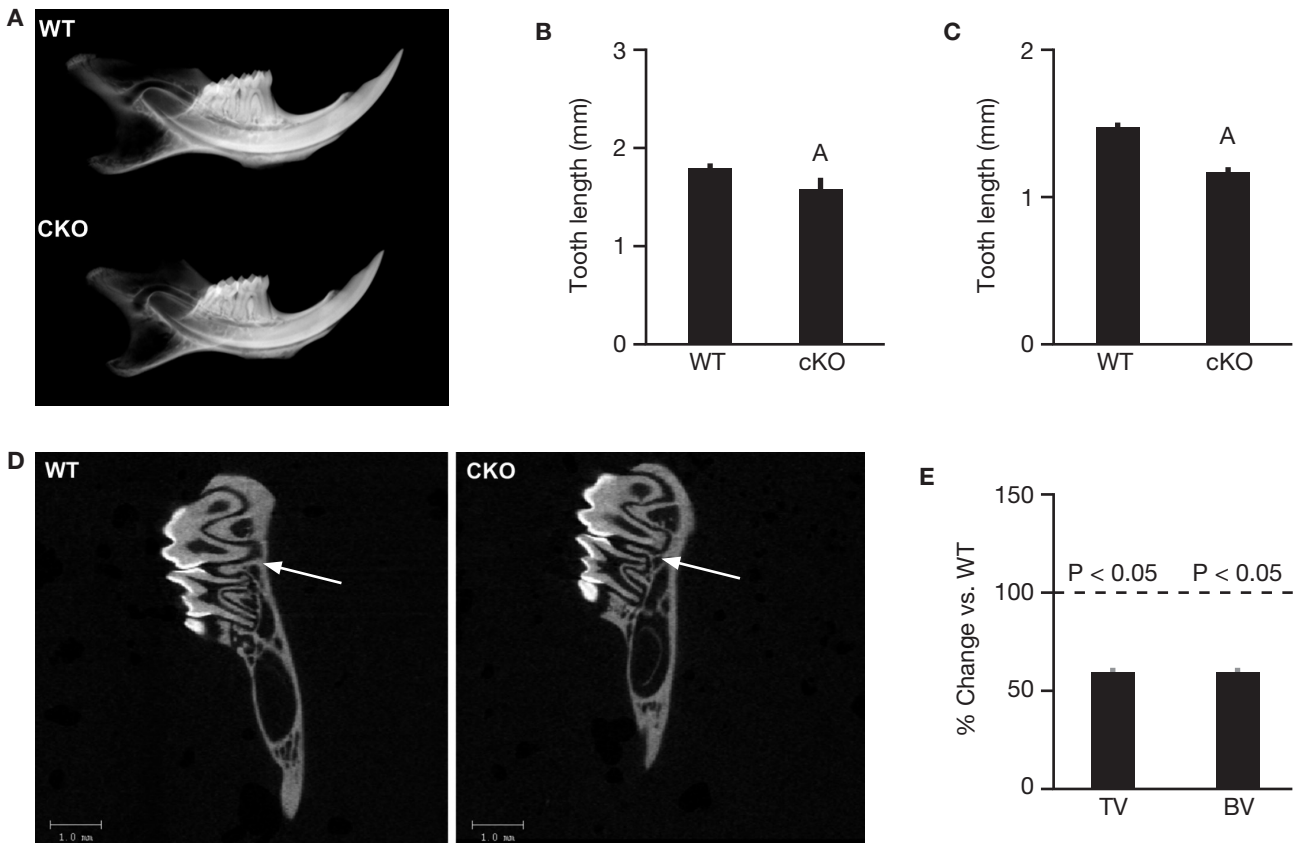
tudinal paraffin sections from 5-week-old miR17-92 cluster-deficient mice were stained for tartrate-resistant acid phosphatase (TRAP) and analysed using an Olympus microscope (BX60) with OsteoMetrics software (Decatur, GA) (Mohan et al., 2015). Data in this study are presented as the mean  $\pm$  SE. The values are expressed as absolute values or as percentage changes in the CKO vs. WT mice for the various measurements. The Student's *t*-test was used for micro-CT and finite element analysis to determine whether the differences were statistically significant ( $P < 0.05$ ). SPSS (version 21) statistical software was used to perform the analyses.

## Results and Discussion

The role of the miR17-92 cluster in dental bones is not well established because mice with disruption of both alleles of the miR17-92 cluster result in a smaller embryo and the animals die immediately after birth due to anomalies in the lung and heart. We recently reported that mice deficient in the miR17-92 cluster in type-I collagen-expressing cells had a 28–30 % reduction in the cross-sectional area of long bones due to decreased periosteal expansion (Mohan et al., 2015). Since collagen type-I Cre is also used for evaluation of the role of genes expressed in ameloblasts and odontoblasts, we evaluated the dental phenotype of the miR17-92 CKO mice. For phenotype analysis, we primarily focused on the incisors because in the murine model, the incisors tend to grow continuously throughout life (Goldberg et al., 2014). There is only one generation of teeth, and therefore anomalies present in the animal's teeth remain throughout their life.

A scout view of the lower jaw using micro-CT analysis illustrates that mice deficient in the miR17-92 cluster in type-I collagen-expressing cells showed an overall reduction in the size of the lower jaw when compared to WT mice (Fig. 1A). Subsequently, quantitative analysis by  $\mu\text{CT}$  in 5- and 12-week-old mice revealed a 23–30 % reduction in incisor tooth tissue volume (TV) and bone volume (BV), respectively, when compared to the teeth in WT littermates (Table 1A). Furthermore, our study also found that the molar length was reduced by 16–18 % in the 5- (Fig. 1B) and 12- (Fig. 1C) week-old CKO compared to WT mice. Significantly, our  $\mu\text{CT}$  data suggests that the miR17-92 cluster is at least in part involved in regulating the TV, reflecting the tooth cross-sectional area of incisors and to some extent the longitudinal tooth growth.

Previously, we reported a reduction in body weight ( $15\% \pm 0.91$ , mean  $\pm$  SE) in 4-week-old female miR17-92 cluster CKO mice. However, the change in size phenotype in both teeth and other parts of skeleton are larger than the body weight changes. Further studies are needed to examine whether the mechanisms by which the miR17-92 cluster influences body weight and bone size are similar or different. Additionally, we found that the incisor phenotype changes observed in pubertal mice (5 weeks) between CKO and WT mice were also main-



**Fig. 1.** (A) Micro-CT image of lower jaw of representative 12-week-old WT and CKO mice. Quantitative analysis of molar tooth length in a (B) 5-week- and (C) 12-week-old WT and CKO mouse. (D) Longitudinal 2D micro-CT image of the lower jaw with an arrow indicating alveolar bone. (E) Quantitative analysis of alveolar bone parameters by micro-CT in 5-week-old WT and CKO mice

Values are mean  $\pm$  SE, A –  $P < 0.05$  vs. WT, N = 3.

tained throughout the postnatal growth period (12 weeks) (Table 1A), providing strong evidence that miRs in the miR17-92 cluster are important for odontogenesis.

Since mice deficient in the miR17-92 cluster displayed a significant reduction in the TV of incisor teeth and since TV plays an important role in determining

**Table 1.** Micro-CT analysis of (A) incisor tooth parameters in 5- and 12-week-old WT and CKO mice and finite element analysis of (B) mechanical properties of incisor tooth in 5-week-old WT and miR17-92 CKO mice

A			
Genotype	Parameters	5 weeks	12 weeks
WT	TV	0.33 $\pm$ 0.005	0.44 $\pm$ 0.017
	BV	0.32 $\pm$ 0.006	0.43 $\pm$ 0.018
CKO	TV	0.26 $\pm$ 0.008*	0.31 $\pm$ 0.005*
	BV	0.24 $\pm$ 0.005*	0.30 $\pm$ 0.005*

Values are mean  $\pm$  SE, \* –  $P < 0.05$  vs. WT, N = 3

B			
Age	Genotype	Stiffness (N/mm)	Failure load (kN)
5 weeks	WT mice	6.62 $\pm$ 0.11	3.24 $\pm$ 0.24
	CKO mice	5.38 $\pm$ 0.02*	1.89 $\pm$ 0.11*

Values are mean  $\pm$  SE, \* –  $P < 0.05$  vs. WT, N = 3

tooth strength in addition to bone density, we predict that the miRs in the miR17-92 cluster may, at least in part, play an important role in the mechanical strength of incisors. To test this, we evaluated the mechanical properties of incisors using the finite element analysis program from Scanco. We found that stiffness, a measure of rigidity, and a measurement related to bone size, failure load, a measure of tooth breaking strength, were significantly lower (18–40 %) in mice deficient in the miR17-92 cluster in type-I collagen-expressing cells compared to WT mice (Table 1B). Furthermore, we also performed a correlation analysis between mechanical strength properties (stiffness and failure load) vs. TV and BV to elucidate whether the decrease in incisor mechanical strength is due to TV and BV measured by  $\mu$ CT. The results show a significant correlation between TV, BV stiffness and failure load ( $r = 0.80$ ,  $P < 0.05$ ). Data from this study demonstrates that the decreased incisor mechanical properties in miR17-92 cluster deficient mice are likely due to a decrease in TV and BV.

In addition to the incisors, we tested whether alveolar bone is affected by the miR17-92 cluster deficiency. Alveolar bone plays multiple functions in the upper and lower jaw. In particular, this bone prevents the tooth from drifting during orthodontic movement. It also serves as a support for attachment of periodontal liga-

ments. In our study, we found that the alveolar bone size was smaller in the CKO mice compared to WT mice (Fig. 1D) by micro-CT. Quantitative analysis revealed a 40 % reduction in alveolar TV and BV (Fig. 1E). Consistent with these findings, histomorphometric analysis also revealed a 25 % reduction in alveolar bone widening in the CKO compared to WT mice. In terms of a mechanism for this reduction, we tested whether the decreased alveolar bone size was due to increased bone resorption from increased osteoclast activity by measuring TRAP staining.

The results from our study showed that there was no difference in the TRAP staining (data not shown) between CKO and WT mice. Previously, we have reported that the miR17-92 cluster deficiency in osteoblast lineage cells reduced periosteal bone formation and the mineral apposition rate. Since alveolar bone is populated primarily by osteoblast lineage cells (Sodek and McKee, 2000; Javed et al., 2010), it is likely that the reduction in periosteal expansion of alveolar bone in the lower jaw of CKO mice results from decreased periosteal bone formation. Additionally, studies have shown that increased periodontal ligament (PDL) space is in part responsible for orthodontic tooth movement and pathogenesis. The PDL space can increase if there is a decrease in alveolar bone size contributing to widening of the PDL. Accordingly, in our study, we found a 16 % increase in the widening (at the alveolar crest fibre site) of the PDL due to a significant reduction in alveolar bone size, suggesting that these mice were more susceptible to orthodontic tooth movement, a prediction that needs to be rigorously tested in the future.

In terms of genes contributing to the reduced periosteal bone size in the miR17-92 cluster CKO mice, we have shown in our recent study that periostin expression was reduced in the periosteum of long bones of miR17-92 CKO compared to WT mice (Mohan et al., 2015). Studies have shown that periostin is an important regulator of periosteal bone formation and that mice deficient in periostin were reported to have reduced periosteal bone size, reflected by decreased TV at the cortical site (Kawanami et al., 2009; Bonnet et al., 2013). Additionally, using the EMBI-BI pairwise sequence alignment program, we found that the periostin 3' untranslated region (UTR) contains a miR20a seed sequence, and miR20a is part of the miR17-92 cluster (Fig. 2).

#### SITE-1

<i>Postn</i>	658	AAAAGUGAGUUUAG	671
<i>mRNA</i>		. .   .	
miR20a	1	<b>UAAAGUGC</b> CUUAUAG	14

#### SITE-2

<i>Postn</i>	607	UAAAGAGAAU - UACUGCAG	624
<i>mRNA</i>		. . .       .	
miR20a	1	<b>UAAAGUGC</b> CUUAUAGUGCAG	19

Fig. 2. MiR20a seed sequences (highlighted in bold underlined font) binding to 3'UTR of periostin

In general, miR are known to bind to the 3'UTR of target mRNAs leading to their degradation. However, miR can also bind to and stabilize mRNAs to prevent them from being degraded. For example, miR29 has been reported to bind to the 3'UTR of interferon  $\gamma$  (IFN- $\gamma$ ) and stabilize IFN- $\gamma$  mRNA (Savan et al., 2011). Another study has shown that miR369-3 binds to the 3'UTR of tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) mRNA and stimulates mRNA translation (Vasudevan et al., 2007). Therefore, in our study, we hypothesize that miR20a regulates periosteal bone size by stabilizing the periostin mRNA by preventing it from being degraded. Further studies are necessary to test this speculation and to identify the involvement of other miR in the cluster or miR20a targets in regulating bone size.

In conclusion, our phenotype analysis demonstrates that the miR17-92 cluster is essential for maintaining normal tooth strength by regulating its size.

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#### Conflict of interest

There is no conflict of interest.

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