Effects of Variation in RUNX2 Tandem Repeats on Jaw Development

E. Yang (1), D. Chu (2), G. Kandapillai (2), Y. Piao (1), R. Schneider (1,2)
1) UCSF School of Dentistry, 2) UCSF Department of Orthopedic Surgery

UCSF School of Dentistry

Background

- Size-related jaw anomalies are common and pose a wide scope of clinical challenges with skeletal malocclusion. Currently, the only treatment options are surgery and orthodontics. Molecular-based therapies that regulate jaw length non-invasively can be generated by identifying mechanisms that control jaw length (Ryoo et al., 2010).
- Neural crest mesenchyme (NCM), which gives rise to the jaw skeleton, controls expression of the osteogenic transcription factor, RUNX2.
- RUNX2 expression is thought to regulate jaw length by causing undifferentiated proliferating NCM to begin osteogenic differentiation and terminate proliferation.
- RUNX2 contains a variable tandem glutamine (Q) and alanine (A) repeat region **(FIGURE 1)**. The Q:A ratio influences RUNX2 transcriptional activity and correlates with facial length in some species (Fondon et al., 2004).

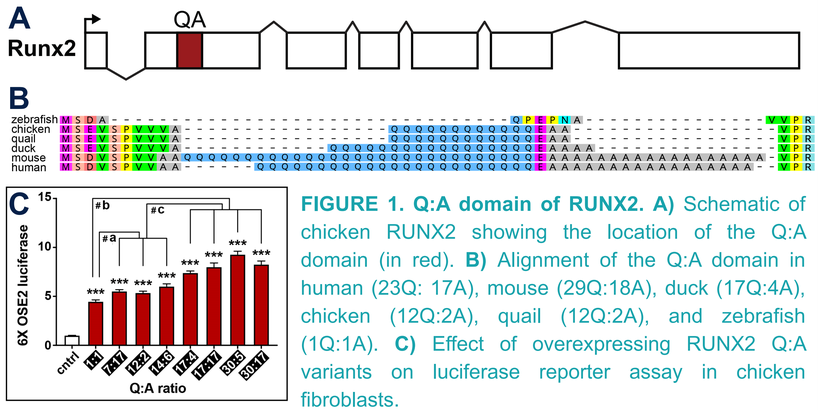

FIGURE 1. Q:A domain of RUNX2. A) Schematic of chicken RUNX2 showing the location of the Q:A domain (in red). **B)** Alignment of the Q:A domain in human (23Q: 17A), mouse (29Q:18A), duck (17Q:4A), chicken (12Q:2A), quail (12Q:2A), and zebrafish (1Q:1A). **C)** Effect of overexpressing RUNX2 Q:A variants on luciferase reporter assay in chicken fibroblasts.

Hypothesis

Here we test the hypothesis that changes in the ratio of glutamines (Q) to alanines (A) within the tandem repeat region of RUNX2 will differentially regulate target genes, which will affect osteogenesis and jaw length.

Literature Cited

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Methods

- Plasmids containing RUNX2 Q:A variants (1Q:1A, 17Q:4A, and 30Q:17A) were overexpressed (OE) by electroporating HH8.5 chicken embryos using inducible, integrating plasmids (Chu et al., 2020) **(FIGURE 2)**.
- For RNA seq RUNX2 Q:A variants were electroporated into chicken embryos. At HH28 the lower jaws were harvested for RNA.
- To determine the effects on osteogenesis, samples were stained with Alizarin red, which stains mineralized bone, and cleared **(FIGURE 2D)**.
- Jaw length was quantified bilaterally through a landmark-based morphometric analysis using Fiji **(FIGURE 2D)**.
- Statistical significance was determined using paired t-test comparing the lengths of the control (ctrl) and overexpressed sides of the mandible.

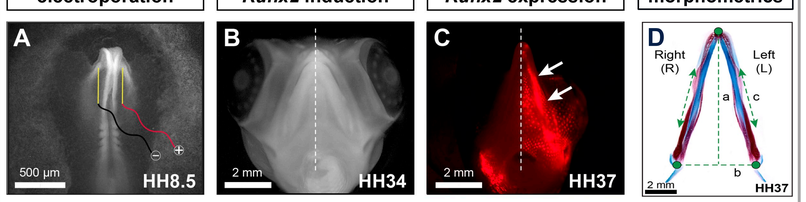

FIGURE 2. ***In ovo* electroporation to overexpress RUNX2. A)** Presumptive NCM was electroporated unilaterally in embryonic stage (HH) chicken embryos with RUNX2 Q:A overexpression constructs. **B)** At HH34, embryos were treated with dox for 48 hours. **C)** At HH37, specimens were screened by epifluorescence for positive unilateral RFP signal (arrows). **D)** Landmark-based morphometric analysis method of HH37 chicken mandible (Fish et al., 2014).

Results (ongoing experiments and analyses)

- RNA-seq of chicken embryos overexpressing empty vector (EV), 17Q:4A, and 30Q:17A were collected at HH28 and show that the different Q:A variants differently affect transcription **(FIGURE 3B and C)**. Currently working on sequencing 1Q:1A.

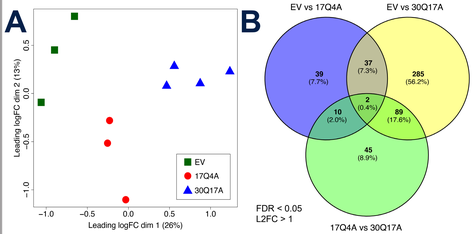

FIGURE 3. Effect of RUNX2 Q:A repeat on transcriptional regulation. A) Multidimensional scaling plot of EV, 17Q:4A, and 30Q:17A **B)** Venn diagram of differentially expressed genes (FDR < 0.05 and L2FC > 1) between EV, 17Q:4A, and 30Q:17A.

- Chicken embryos overexpressing 1Q:1A,17Q:4A, and 30Q:17A variants exhibited significantly shorter mandibular jaw length on the OE side compared to the control side (P=0.0008, 0.0034, and <0.0001, respectively), while the empty vector OE embryos showed no difference between the control and OE side (P=0.4431) **(FIGURE 4)**.
- As the Q:A repeat increases in size the mean of differences becomes more negative (greater difference between control and OE), although not significantly.

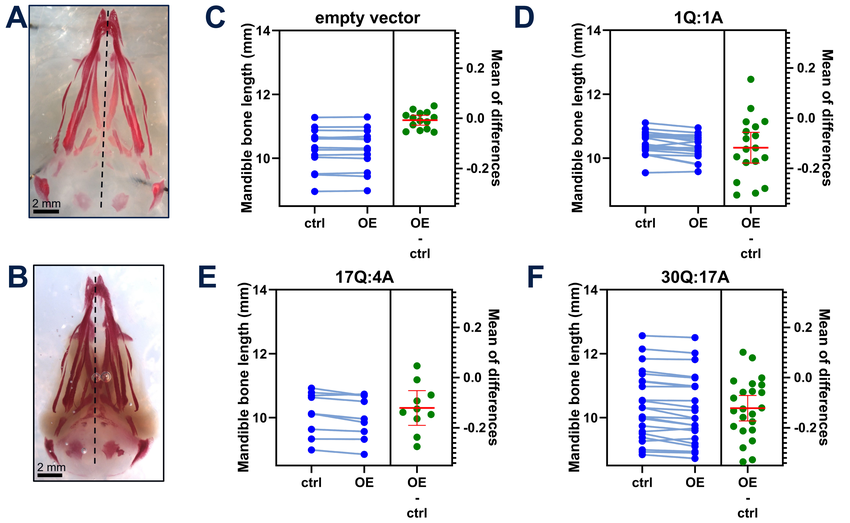

FIGURE 4. Paired plots comparing control and OE side lengths in chicken embryos. Wildtype control vs. **A)** empty vector control, **B)** 1Q:1A, **C)** 17Q:4A, and **D)** 30Q:17A. The blue line connects the blue dots, which represent ctrl and OE sides of each sample. The green dots represent the difference in jaw length of the ctrl and OE side. Empty vector (n=14), 1Q:1A (n=18), 17Q:4A (n=10), and 30Q17A (n=23). **E)** Alizarin Red stained HH37 control chicken embryo. **F)** Alizarin Red stained HH37 chicken embryo overexpressing 1Q:1A.

Conclusions and Next Steps

- Different RUNX2 Q:A variants differentially regulate the transcriptome. Next steps include performing: Gene ontology (GO) analysis on differentially expressed genes to identify pathways in regulation of jaw length, ChIP-seq of RUNX2 Q:A variants to identify if direct targets of RUNX2 are differentially bound by RUNX2 Q:A variants, and sequencing 1Q:1A samples for RNA-seq.
- The paired analyses reveal that altering the RUNX2 Q:A tandem repeat motif is sufficient to modulate jaw length *in ovo*, with 1Q:1A, 17Q:4A, and 30Q:17A producing significant shortening.

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