SIX6: A new candidate gene for Pierre Robin sequence

2025 Research Aid Awards (RAA)

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FollowUp Form

Award Information

In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:

- *Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)*
- *Does this Final Report provide the level of detail, etc. that you would expect, if you were the reviewer?*

Title of Project:*
SIX6: A new candidate gene for Pierre Robin sequence

Award Type
Research Aid Award (RAA)

Period of AAOF Support
July 1, 2025 through June 30, 2026

Institution
University of Pittsburgh, Department of Orthodontics and Dentofacial Orthopedics

Names of principal advisor(s) / mentor(s), co-investigator(s) and consultant(s)
Dr. Alice Goodwin

Amount of Funding
$6,000.00

Abstract

(add specific directions for each type here)

See attached file.

Respond to the following questions:

Detailed results and inferences:*

If the work has been published, please attach a pdf of manuscript below by clicking "Upload a file".
OR
Use the text box below to describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis, and interpretation of results should also be attached by clicking "Upload a file".

supplemental.AAOF.pdf
According to our breeding scheme, mating Six6fl/+;mtHand2Cre males with Six6fl/fl females, we expected that 50% of the embryos would be controls (Six6fl/+ orSix6fl/fl), 25% mutants (Six6fl/fl;mtHand2Cre MUT), and the 25% heterozygotes (Six6fl/+;mtHand2Cre HET) collected at timepoints: E12.5, E14.5, E16.5, E18.5, and P1 (one day old pups). At E12.5, there were 16 (43%) controls, 1 (3%) mutant, and 20 (54%) heterozygous embryos. At E14.5, there were 26 (44%) controls, 3 (5%) mutants, and 30 (51%) heterozygous embryos. There were 26 (56%) control embryos, 4 (9%) mutant embryos, and 16 (35%) heterozygous embryos at E16.5. For E18.5, 20 (57%) controls, 3 (9%) mutants, and 12 (34%) heterozygous embryos were collected. Lastly, 14 controls (61%), 1 (4%) mutant, and 8 (35%) heterozygous mice were collected at one day old. Overall, the mutant embryos were less than the predicted outcome. Resorbed embryos were also genotyped to test for embryonic lethality of the mutants; however, the embryos were not mutant, but rather control or heterozygotes: 4 control embryos were resorbed at E12.5, 2 heterozygous embryos and 3 controls resorbed at E14.5, and 2 control embryos resorbed at 16.5.

RNAscope with probe targeting Six6 was performed on control tissue at E12.5 and E13.5 (N=3) to determine the expression of the Six6 gene. Six6 was expressed throughout the craniofacial tissues at E12.5 and E13.5, including the mandible, tongue, and palatal shelves, albeit at low levels. There was high expression of Six6 in the developing retina, as expected. To determine the expression pattern of Six6 protein and confirm the loss of Six6 in the Six6fl/fl;mtHand2Cre mutant embryo, immunofluorescence staining with antibody against Six6 was performed on Six6fl/fl;mtHand2Cre mutant and control embryos at E12.5 and E14.5. Six6 was highly expressed in the dorsal root ganglion as well as the trigeminal and hypoglossal nerve. There was also expression of Six6 throughout the mandible and tongue at E12.5 and E14.5, albeit at lower levels than the expression in neural tissue. Importantly, there was decreased Six6 expression in the mandibular mesenchyme at E12.5 and Meckel's cartilage at E14.5 in the Six6fl/fl;mtHand2Cre mutant compared to control, showing specific deletion of Six6 in the mandibular mesenchyme in the Six6fl/fl;mtHand2Cre mutant.

To determine if loss of Six6 resulted in compensation by other Six family members, RNAscope was performed with probes against Six1, 2, and 4 in Six6fl/fl;mtHand2Cre mutant and control embryos at E12.5 (N=1). Overall, the expression pattern of Six1,2, and 4 was similar in Six6fl/fl;mtHand2Cre mutant and control embryos. There may be a decrease in Six1 in the palatal mesenchyme with loss of Six6, however, more samples are needed to confirm this finding.

A qualitative analysis was performed using H&E staining to study the phenotypes of the mandible, tongue, and palate in Six6fl/fl;mtHand2Cre mutants compared to control at E14.5 and E16.5. At E14.5, the tongue looked more palatally placed and larger than the control (N=4). At E16.5, the mandible and tongue appeared similar, and there was no cleft palate present in the Six6fl/fl;mtHand2Cre mutant embryos compared to control (N=4). Measurements were made to determine subtle shape and size changes in the tongue and palate. The tongue area was measured in Six6fl/fl;mtHand2Cre mutant and control embryos. The average tongue area for the control samples was about 400,000 micrometers^2 and for the mutant samples was about 500,000 micrometers^2 with p value 0.6517 at E14.5. The controls had an average tongue area of about 590,000 micrometers^2 and the mutants had an average about 700,000 micrometers^2 with p value 0.0521 at E16.5. Thus, there was no statistically significant difference in tongue area at E14.5 or E16.5 in Six6fl/fl;mtHand2Cre mutant compared to control. To assess the position of the tongue relative to the skull, the distance from the dorsal surface of the tongue to the cranial base in the posterior sections and from the dorsal surface of the tongue to the middle and anterior sections was measured in Six6fl/fl;mtHand2Cre mutant and control embryos at E14.5 and E16.5. The tongue position for the controls was about 375 micrometers and about 350 micrometers for the mutants with a p value 0.715 at E14.5. At E16.5, the tongue position for the controls was about 480 micrometers and the average for the mutants was about 590 micrometers with a p value 0.0575. Thus, there was no statistically significant difference in tongue position between Six6fl/fl;mtHand2Cre mutant and control embryos. Although there was no overt cleft in the Six6fl/fl;mtHand2Cre mutant embryos, the palate area, width and height were measured at E16.5 to test for subtle changes in palate shape compared to control. The width was measured from one inflection point to the other inflection point at the inferior part of the palate while the height was measured from the apex of the palate to dorsum of the tongue. The average palatal width for the controls was about 1300 micrometers and the palatal width for the mutants was about 1400 micrometers with a p value 0.4557. The palatal area for the controls was about 125,000 micrometers^2 and about 145,000 micrometers^2 for the mutants with a p value 0.1849. The palatal height for the controls was about 140 micrometers and about 150 micrometers for the mutants with p value 0.5844. The palatal width, area, and height showed no statistically significant difference in Six6fl/fl;mtHand2Cre mutant and control embryos at E16.5.

MicroCT was used to measure the hemi-mandibles and position of the mandibles compared to the skulls of controls (N=2) and Six6fl/fl;mtHand2Cre mutant (N=1) when the mice were one day old (P1) (figures in the attached document show landmarks for measurements). The average lengths from the posterior of the skull to the anterior most point of the mandible were 9.376mm (control with ID 3577), 9.365mm (mutant ID 3578), and 8.960mm (control ID 3548). For the left mandible, the lengths were 5.447mm (control ID3546), 5.471mm (mutant ID3547), and 5.138mm (control ID3548). The average anterior widths (W1) for the left mandible were 0.9462mm (control ID3546), 0.980 mm (mutant ID3547), and 0.928mm (control ID3548). The average posterior widths (W2) for the left mandible were 1.227mm (control ID3546), 1.258mm (mutant ID3547), and 1.194mm (control ID3548). The average lengths of the right mandible were 5.388mm (control ID3546), 5.458mm (mutant ID3547), and 5.079mm (control ID3548). The W1 for the right mandible were 0.959mm (control ID3546), 1.006mm (mutant ID3547), and 1.134mm (control 3548). The W2 for the right mandible were 1.24mm (control ID3546), 1.272mm (mutant ID3547), and 1.199mm (control ID3548). It seems that the second control may have been a runt as the first control was similar in width and length to the mutant. More samples are needed to confirm these findings.

Overall, loss of Six6 in the mandible did not result in a PRS phenotype and there was no cleft of the secondary palate in these mutant embryos. Six6 does not seem to play an essential role in embryonic development in the mandible and tongue as hypothesized.

Were the original, specific aims of the proposal realized?*

Yes, the original specific aims were realized:
Specific Aim 1: Determine the expression pattern of Six6 during craniofacial development. The expression pattern of Six6 mRNA transcript and protein were determined at E12.5 and E14.5 using RNAscope and

immunofluorescence, respectively. Six6 mRNA was expressed throughout the developing palatal shelves, mandibular mesenchyme, and tongue at E12.5 and in the developing mandible and molar tooth buds at E14.5. Although broadly expressed, Six6 was observed at low levels. Six6 protein expression was observed in a similar pattern at these timepoints, and in addition, there was high Six6 protein expression in the trigeminal dorsal root ganglion and hypoglossal nerve. We also confirmed that the level of Six6 protein expression was decreased in the mandibular mesenchyme at E12.5 and Meckel's cartilage at E14.5 in the Six6fl/fl;mtHand2Cre embryos compared to control. Since the mtHand2Cre does not drive recombination in the trigeminal dorsal root ganglion, Six6 expression in this tissue was the same in mutant and control. The mRNA expression pattern of other Six family members, Six1, 2, and 4, was determined in Six6fl/fl;mtHand2Cre and control embryos at E12.5. Six1, 2, and 4 were expressed in the craniofacial complex as expected, and previously reported, and there did not appear to be any differences in expression in Six6fl/fl;mtHand2Cre embryos compared to control, however, additional samples are required to confirm this finding.

Specific Aim 2: Investigate the function of Six6 in craniofacial development. Six6fl/fl;mtHand2Cre and control embryos were generated and analyzed at E14.5, E16.5, and P1 to determine the role of Six6 in craniofacial development and whether loss of Six6 in the mandibular mesenchyme would result in a PRS phenotype with mandibular hypoplasia, retrusive tongue, and cleft palate. E14.5 and E16.5 Six6fl/fl;mtHand2Cre and control embryos were sectioned in the coronal plane and stained with H&E to determine the palate, tongue and mandible phenotype. There was no palatal shelf elevation delay or differences in the tongue or developing mandible at E14.5 in Six6fl/fl;mtHand2Cre embryos (N=3) compared to control. There was no cleft palate in the Six6fl/fl;mtHand2Cre embryos at E16.5. The secondary palate area, width, and height were also measured to determine if there were more subtle differences than an overt cleft, and there was no difference in these measurements between mutant and control. The tongue area and position relative to the skull were measured as well, and again there was no significant difference between Six6fl/fl;mtHand2Cre and control embryos. In order to assess the mandibular size, shape and position, microCT was performed on P1 Six6fl/fl;mtHand2Cre and control embryos. MicroCT was used instead of skeletal preps to generate more accurate measurements. Measurements on the width and length of the mandibles and position of the mandible relative to the skull in the antero-posterior dimension showed no difference between Six6fl/fl;mtHand2Cre and control embryos; however, additional samples are being collected to confirm this finding.

Were the results published?*

No

Have the results of this proposal been presented?*

Yes

To what extent have you used, or how do you intend to use, AAOF funding to further your career?*

Yes, the AAOF funding helped cover lab costs for my research as a resident and AAOF helped me with my travel and hotel expenses so that I could present my research at a national conference and at my residency program. I am fortunate to have received the AAOF funding award as it provided me with the resources to develop my research and critical thinking skills for my master's thesis and provide me with the opportunity to share a novel project with orthodontists, residents, and craniofacial researchers.

Accounting: Were there any leftover funds?*

If "yes", enter your best estimate and work with your grants manager to finalize financial reports and send refund payable to: AAOF
Attn: George
401 N. Lindbergh Blvd.
St. Louis, MO. 63141-7839

If "no", enter zero.

$0.00

Not Published

Are there plans to publish? If not, why not?*

Yes, these data will be published as part of my master's thesis at the University of Pittsburgh. In addition, the findings will be included in a publication including additional identified PRS candidate genes.

Presented

Please list titles, author or co-authors of these presentation/s, year and locations:*

"Six6: A New Candidate Gene for Pierre Robin Sequence (PRS)", Gina DeLeonibus and Alice Goodwin, oral presentation, AAO Annual Meeting, Orlando, FL, 2026
"Six6: A New Candidate Gene for Pierre Robin Sequence (PRS)", Gina DeLeonibus and Alice Goodwin, oral thesis defense, Pitt School of Dental Medicine, Pittsburgh, PA, 2026

Was AAOF support acknowledged?

If so, please describe:

Yes, AAOF support was acknowledge during the presentation.

Internal Review

Reviewer comments

The abstract was not attached. Please complete all the questions. Do not upload your MS thesis document. If it is a publication in a peer-reviewed journal, you can upload. Alternatively, use the text box to describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study.

Reviewer Status*

Approved

File Attachment Summary

Applicant File Uploads

- supplemental.AAOF.pdf

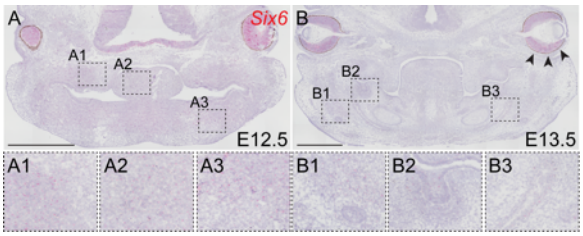


Figure 1: Coronal sections showing expression of *Six6* in mandible, tongue, and palate in control tissues at E12.5 (A) and E13.5 (B) (N=3/timepoint) with RNAscope. Each red dot represents a *Six6* mRNA transcript.

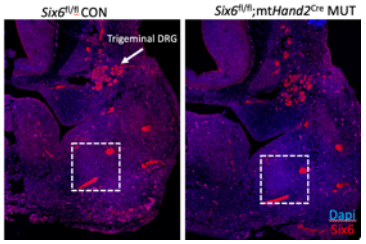


Figure 2: Immunofluoresence staining with antibody againt Six6 at E12.5 for control and mutants. High Six6 expression in the trigeminal dorsal root ganglion and trigeminal and hypoglossal nerve. Decreased expression in mandibular mesenchyme in the mutant.

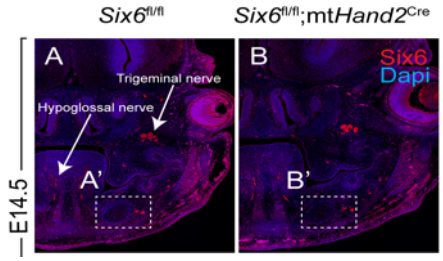


Figure 3: Immunofluoresence with antibody againt Six6 at E14.5 for control and mutants with decreased expression in Meckel's cartilage in mutants.

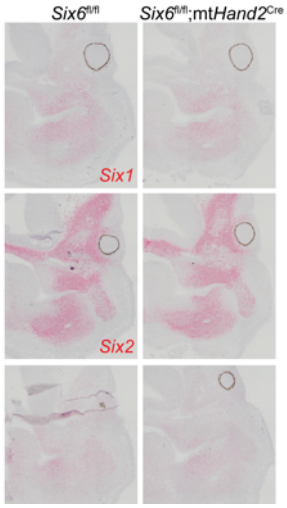


Figure 4: RNAscope showing expression of *Six1, Six2, and Six4* in control and *Six6*$^{fl/+}$mutant (N=1) at E12.5.

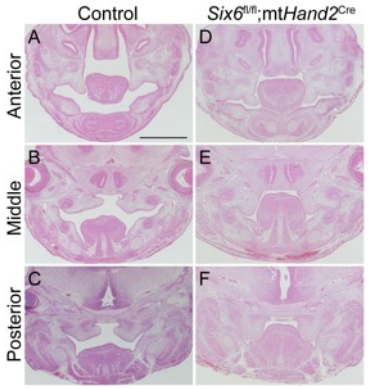


Figure 5: H&E staining of anterior, middle, and posterior sections from control and mutant (N=4) embyryos at E14.5.

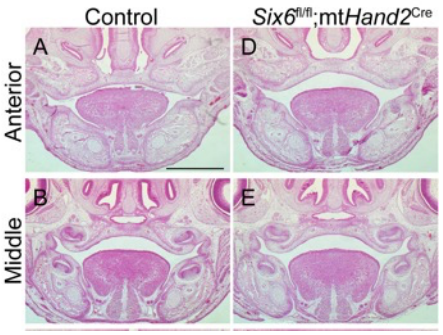


Figure 6: H&E staining of anterior, middle, and posterior sections from control and mutant (N=4) embyryos at E16.5. No cleft of the secondary palate observed.

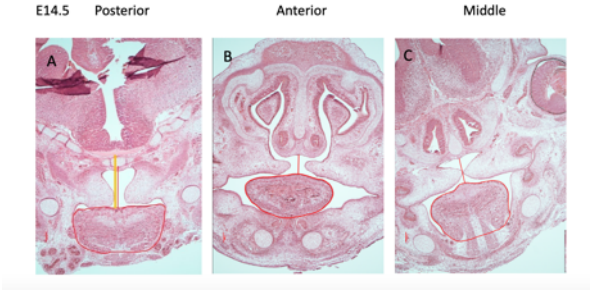


Figure 7: Tongue area and position at E14.5 in a sample. (A) dorsum of tongue to cranial base noted by the yellow line. (B) (C) dorsum of tongue to nasal cavity noted by the red line.

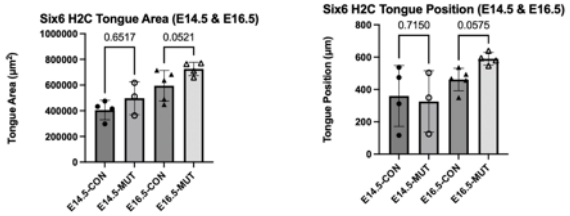


Figure 8: Comparison of tongue area and tongue position between controls and mutants at E14.5 and E16.5. Each dot represents a sample. Bars represent standard deviation.

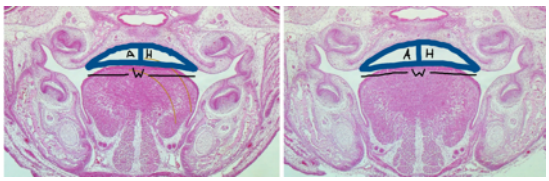
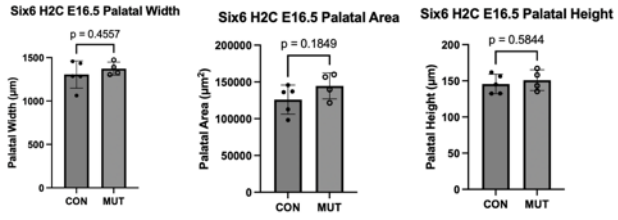


Figure 9: Palatal area, width, and height measured in a sample.

Figure 10: Comparison of palatal width, palatal area, and palatal heights between controls and mutants at E16.5. Each dot represents a sample. Bars represent stadard deviation.



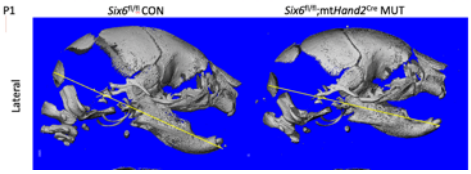


Figure 11: Line used to measure position of mandible relative to skull in control and mutant pups at P1. Landmarks to measure were adapted Richtsmeier Lab at Penn State (41).

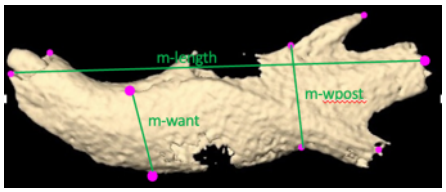


Figure 12: Measurements of length, anterior width, posterior width for hemi-mandibles in at P1. Landmarks adapted from Richtsmeier Lab at Penn State (41).

Measure #	P1 Mouse Skull
Six6fl/fl (Hand2Cre neg) CON 3577	9.399
	9.361
	9.365
	9.395
	9.36
average	9.376
Six6fl/fl;Hand2Cre/+MUT 3578	9.354
	9.363
	9.385
	9.353
	9.372
average	9.3654
Six6fl/fl (Hand2Cre neg) CON 3586	8.969
	8.962
	8.956
	8.954
	8.958
average	8.9598

Table 1: MicroCT for Skull Length for Control (N=2) and Mutant (N=1)

Table 2: MicroCT for Length and Width for Control (N=2) and Mutant (N=1)

	Left mandible	Left mandible	Left mandible	right mandible	right mandible	right mandible
Measure #	length	W1 (anterior)	W2 (posterior)	length	W1 (anterior)	W2 (posterior)
Six6fl/fl (Hand2Cre neg) CON 3546	5.498	0.939	1.24	5.445	0.954	1.237
	5.452	0.933	1.237	5.389	0.981	1.24
	5.449	0.926	1.218	5.414	0.96	1.242
	5.381	0.963	1.219	5.357	0.946	1.236
	5.456	0.97	1.223	5.335	0.952	1.245
average	5.4472	0.9462	1.2274	5.388	0.9586	1.24
Six6fl/fl;Hand2Cre/+MUT 3547	5.477	0.953	1.235	5.496	0.969	1.273
	5.488	0.994	1.276	5.451	1.005	1.272
	5.476	0.988	1.258	5.448	0.995	1.293
	5.448	0.98	1.269	5.457	1.01	1.268
	5.464	0.986	1.252	5.437	1.05	1.253
average	5.4706	0.9802	1.258	5.4578	1.0058	1.2718
Six6fl/fl (Hand2Cre neg) CON 3548	5.199	0.914	1.198	5.045	1.072	1.162
	5.153	0.921	1.211	5.137	1.097	1.177
	5.095	0.939	1.186	5.048	1.065	1.219
	5.14	0.927	1.176	5.095	1.35	1.221
	5.103	0.938	1.201	5.07	1.086	1.217
average	5.138	0.9278	1.1944	5.079	1.134	1.1992