Conditional deletion of the human ortholog gene Dicer1 in Pax2-Cre expression domain impairs orofacial development

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Introduction

Defects in palatogenesis are of complex etiology and represent some of the most common congenital malformations in humans, with an average prevalence of 1.2:1000 live births worldwide. The mechanisms involved in this process depend on a tightly regulated network of signaling molecules that control cell proliferation, apoptosis, and development. Recent studies indicate that microRNAs (miRNAs), a highly conserved class of small noncoding RNAs, regulate gene expression by repressing protein translation or inducing mRNA degradation. Mature miRNAs are single-stranded RNAs (ssRNAs) of approximately 22 nucleotides in length, generated by DICER1, an RNase III family member. DICER1 deletion disrupts numerous physiological processes that are dependent on miRNA-mediated gene regulation and is associated with early embryonic lethality. Human studies investigating the role of DICER1 during palatogenesis are limited. Previously, a genome-wide scan for nonsyndromic cleft lip and palate
in multigenerational Indian families suggested evidence of linkage at several chromosomes regions including 14q32, which comprises the genomic location of \( \text{DICER1} \).\(^{[31]} \) More recently, Li, et al.\(^{[32]} \) showed that rs7205289 in pre-miR-140, which had been previously implicated in zebrafish palatogenesis,\(^{[33]} \) may contribute to human nonsyndromic cleft palate susceptibility by influencing the processing of mature miR-140. Although groundbreaking, these studies highlight the need for additional analyses on the expression of \( \text{DICER1} \) and its dependent miRNAs, as well as their regulatory function during mammalian palatogenesis and orofacial development.

To investigate \( \text{Dicer1} \) function during palatal and orofacial morphogenesis, we utilized a \( \text{Dicer1} \) conditional knockout (CKO) mouse model in which the floxed \( \text{Dicer1} \) alleles are deleted through \( \text{Cre} \)-mediated recombination following \( \text{Pax2-Cre} \) expression.\(^{[26,30,34]} \) \( \text{Pax2} \) is the earliest transcription factor to be expressed in the prospective mid-hindbrain area, around embryonic (E) day 7.5 in mouse\(^{[34‑37]} \) and as such may affect cranial neural crest (CNC) migration, proliferation, and the differentiation of CNC-derived tissues, including the formation of skeletal structures associated with the craniofacial region.\(^{[38]} \)

**Materials and Methods**

**Animals**

All animal care and use was approved by the Creighton University Institutional Animal Care and Use Committee (IACUC). \( \text{Pax2-Cre/Dicer1}^{\text{loxP/loxP}} \) CKO mice were generated as previously described.\(^{[30]} \) Control animals consisted of \( \text{Dicer1}^{\text{loxP/loxP}} \) mice not carrying the \( \text{Pax2-Cre} \) transgene. To examine \( \text{Pax2-Cre} \) expression domains, \( \text{Dicer1}^{\text{loxP/loxP}} \) was mated with \( \text{Pax2-Cre/Rosa26R} \) females. Timed pregnancies were set up overnight. Noon of the next day was considered embryonic day 0.5, and pregnancies were counted forward from that point. Embryos were harvested by caesarean section at different stages of embryonic development.

**H and E staining and whole-mount skeletal staining**

Hematoxylin-Eosin (H and E) staining, whole-mount skeletal staining, and Von Kossa staining were performed as described elsewhere.\(^{[39,40]} \)

**In situ hybridization and quantitative real-time PCR**

\( \text{Dicer1} \) ISH was performed as previously described.\(^{[30]} \) Gene-specific RT-QPCR on total RNA isolated from palatal tissue was performed as described elsewhere.\(^{[41]} \) Detection of miR-101b, miR-140, and miR-145 (Exiqon, Inc. Woburn, MA, USA) was performed as described by Weston and colleagues.\(^{[42]} \) Student’s \( t \)-test was performed on normalized miRNA expression values to assess statistical significance (\( P \leq 0.01 \) was considered significant).

**Proliferation assays**

Labeling and detection of mitotically active cells by the thymidine analog 5-ethyl-2'-deoxyuridine (EdU) (50 mg/kg) in DMSO was performed as previously described.\(^{[41,43]} \) Negative controls consisted of saline-injected females. Five animals per genotype and time point were analyzed. The number of EdU-positive cells were counted. Student’s \( t \)-test was performed, \( P \leq 0.01 \) was considered significant.

**Apoptosis assay**

Coronal sections (10 µm) from WT and \( \text{Dicer1} \) CKO at different embryonic time points were processed using the ApopTag Plus \( \text{In situ} \) apoptosis fluorescein detection kit (Chemicon International, Inc. Temecula, CA, USA) and counterstained with DAPI. Control tissue sections were treated with DNase I (positive control) or DNase I buffer without the enzyme (negative control), before the ApopTag reaction. Counting of apoptotic nuclei was performed at 20µm intervals. Five cross-sections were analyzed per genotype. Four areas (120 µm × 120 µm) were selected within cross-sections. The ratio of fluorescein-positive nuclei to the total (DAPI-stained) nuclei was calculated per section. Student’s \( t \)-test was performed, \( P \leq 0.01 \) was considered significant.

**Results**

**Pax2-Cre-mediated Dicer1 deletion induces embryonic lethality and craniofacial abnormalities**

\( \text{Dicer1} \) ablation in the \( \text{Pax2-Cre} \) expression domain results in impaired growth of the mid-hindbrain and late embryonic lethality at E18.5.\(^{[30]} \) Gross morphological analyses of E17.5 \( \text{Dicer1} \) CKO mutants revealed...
Expression of Pax2-Cre and Dicer1 microRNAs during palatal shelf development

To determine the pattern of Pax2-Cre expression in the embryonic orofacial region, Pax2-Cre/Dicer1loxP/WT mice at E12.5 and E17.5 and Dicer1 CKO mice at E17.5, all of them carrying a Rosa26-LacZ reporter allele (Rosa26R), were examined by X-gal staining. At E12.5, X-gal staining, which highlights all progeny of the Pax2-Cre-expressing cells, shows positive cells throughout the mid-hindbrain and in the region of the first pharyngeal arch of the Pax2-Cre/Dicer1loxP/WT/Rosa26R, including the developing palatal shelves [Figures 2a and b]. At E17.5, Pax2-Cre-positive cells continued to be expressed in the mid-hindbrain and throughout the secondary palate in the Pax2-Cre/Dicer1loxP/WT/Rosa26R [Figure 2c], while Dicer1 CKO mice exhibited diminished X-gal staining and a complete cleft of the secondary palate [Figure 2d].

To confirm Pax2-Cre-mediated Dicer1 deletion in a spatially restricted pattern, we generated a Dicer1 riboprobe and examined its expression by ISH in WT and Dicer1 CKO mice at E12.5 [Figure 3]. Compared to the WT [Figures 3a, c and e], only residual Dicer1 expression was observed in the mid-hindbrain, brainstem, and palatal shelves [Figures 3b, d, and f], which corresponds to the Pax2-Cre expression domain.

Palatogenesis in the Dicer1 conditional knockout mouse

To assess the stage at which morphological differences first occur during the Dicer1 CKO palatal development, E11.5, E13.5, and E16.5 WT control and Dicer1 CKO mutant mouse heads were coronally sectioned and stained with H and E. At E11.5, coronal sections showed comparable orofacial and palatal morphology in both mutant and WT embryos, suggesting that, prior to E11.5, orofacial development is not impacted by Dicer1 loss [Figures 4a and b]. Although Pax2-Cre expression in mice has been shown to start as early as one somite stage\cite{97} morphological differences in palatal development did not become conspicuous until around E13.5. At this stage, the palatal shelves of Dicer1 CKO embryos are vertically positioned along the side of the tongue, in a pattern similar to the control embryos. Nevertheless, the palatal shelves in Dicer1 CKO embryos exhibit a slight reduction in size, suggesting developmental delay in bilateral palatal outgrowth of the maxillary process [Figures 4c and d]. At E16.5, control embryos showed completion of palatal development, which is hallmarked by fusion between the palatal shelves, disappearance of the medial epithelial seam, and fusion with the primary palate anteriorly [Figure 4e]. In addition, intramembranous ossification of the palatal bones is visible. In comparison, E16.5 Dicer1 CKO mice showed normal primary palatal development, but exhibited a complete secondary palatal cleft [Figure 2d]. Further histological analysis of the Dicer1 CKO shows that the palatal shelves exhibit arrested growth, remaining vertically oriented in position [Figure 4f], and are morphologically equivalent to an E13.5 WT embryo [Figures 4d and f]. Furthermore, intramembranous ossification is completely absent at the palatal shelf region, although the nasal septal cartilage, that develops from the Pax2-Cre-negative frontonasal region, is present [Figure 4f]. Collectively, the present results show that Dicer1 deletion by Pax2-Cre does not affect primary palatal development, nor does it impact the initial stage of palatal development; however, it disrupts the growth and fusion of the palatal shelves.

CNC-derived facial and anterior skull bone development in the Dicer1 CKO

To assess cartilage and bone development E17.5 Dicer1 CKO mice and WT littermates were stained with Alcian Blue and Alizarin Red, respectively. Compared to the WT, Dicer1 CKO exhibited complete loss or abrogated development of several CNC-derived bones in the viscerocranium, anterior cranial vault, and prechordal skull base [Figures 5a-e]. Specifically in the facial skeleton, the squamosal, jugal (zygoma), and palatine bones of the maxilla were absent, while the mandible, tympanic ring, vomer, and frontal process of the maxilla were reduced in size when compared to WT littermates [Figures 5c and d]. In the anterior part of the skull, the presphenoid, alisphenoid, and orbitosphenoid were absent...
from the cranial base, while the medial portion of the frontal bones in the calvaria and the basisphenoid of the cranial base exhibited impaired growth [Figure 6d]. No differences were observed between Dicer1 CKO and WT mice in mesodermally derived skeletal elements of the posterior skull, including the parietal, intraparietal, petrous temporal, basioccipital, exoccipital, and supraoccipital [Figures 5c-f]. Collectively, the observed pattern of skeletal abnormalities suggest that Pax2-Cre-mediated Dicer1 deletion disrupts intramembranous and endochondral bone ossification of specific CNC-derived skeletal elements in the region of the first pharyngeal arch and anterior skull.

To determine the pattern of mineralization during bone formation, tissue sections from the heads of E17.5 WT and Dicer1 CKO embryos were analyzed with Von Kossa stain. WT mice demonstrated normal intramembranous and endochondral ossification with mineral deposition in the secondary palate, mandible, and premaxilla [Figures 6a and c]. In contrast, in the region of the presumptive secondary palate, Dicer1 CKO showed only limited mesenchymal condensation and a complete absence of mineralization in the truncated palatal shelves [Figures 6b and d].
Cell proliferation and apoptosis in the Dicer1 CKO mouse

To investigate possible mechanism(s) associated with impaired palatal outgrowth in Dicer1 CKO, pregnant mice were injected with EdU at E11.5, E13.5, and E17.5 and analyzed for EdU incorporation by immunohistochemistry. At embryonic day 11.5, the total number of EdU-positive (EdU⁺) cells in the region of the developing brain and first pharyngeal arch in Dicer1 CKO mice were markedly decreased when compared to littermate controls [Figures 7a-d]. As expected, in regions outside the Pax2 expression domain, the density of EdU⁺ cells was comparable between mutant and WT littermates [Figures 7a, b, detail box]. Double staining of E11.5 Dicer1 CKO and WT sections with EdU and ApopTag was performed [Figures 7c and d]. Contrasting with WT littermates [Figure 7c], apoptotic (ApopTag) cells were detected throughout the orofacial region of Dicer1 CKO (data not shown) and were particularly concentrated in the developing palatal areas [Figure 7d]. At E13.5 (P = 0.0061) and E17.5 (P = 0.0032), the density of proliferating (EdU) cells was significantly lower in Dicer1 CKO [Figures 8a-h]. Moreover, the number of apoptotic cells increased significantly at E13.5 (P = 0.0083) [Figure 8d, detail]. ApopTag-positive cells were not observed in the palatal region of WT littermates at E13.5 and E17.5 nor at E17.5 in Dicer1 CKO (data not shown). Comparative RT Q-PCR analyses of dissected palatal tissue from WT and Dicer1 CKO mice at E11.5, E13.5, and E17.5 revealed a marked increase in the levels of apoptotic markers Caspase 3 (P = 0.0003) and p53 (P = 0.0003) in mutant mice, particularly at E13.5. Moreover, expression levels for senescence marker p21 were also higher in Dicer1 CKO at E11.5 (P = 0.0009) and E13.5 (P = 0.0008). At E17.5 WT control and Dicer1 CKO animals showed comparable expression levels with the exception of p21 (P = 0.013), [Figure 9].

miRNA expression in palatal tissue of Dicer1 CKO

To further assess the impact of Dicer1 deletion, we examined miRNA expression in dissected palatal tissue of Dicer1 CKO and WT control mice at E13.5 and E17.5. The choice of miRNAs was based on previous reports implicating their role in cell proliferation, craniofacial development, and predicted target genes. These miRNAs include miR-101b, miR-140, and miR-145. Among predicted targets, these three miRNAs have been associated with regulation of Sox9, Col10a1, Runx1, Zeb2, Pdgfc, Foxo1, Bmp3, and Eya3. The three miRNAs tested were found to be significantly downregulated at E13.5 (P = 0.0002, 0.0006, and 0.0002, respectively) and E17.5 (P = 0.0001, 0.0004, and 0.0001, respectively) in Dicer1 CKO [Figure 10].

Discussion

Palatogenesis and cranioskeletal development depends on proper migration, patterning, proliferation, and differentiation of CNC cells and involve a variety of genes that must be expressed at critical levels in specific spatiotemporal sequences. Dicer1-dependent miRNAs are important effectors of post-transcriptional gene regulation during embryonic development, and have been implicated in impacting neural crest survival. Recently, a genome-wide scan in multigenerational Indian families has suggested evidence of linkage for chromosome 14q32, the region encompassing the DICER1 gene, and nonsyndromic cleft lip and palate.

Early in embryogenesis, CNC cells populating the first pharyngeal arch and anterior cranial skeleton originate from an area of mid-hindbrain boundary, corresponding to the Pax2/Pax2-Cre expression domains. Here, we extend the results of previous studies showing that Pax2-Cre and Dicer1 expression overlaps in the lateral palatal shelves and demonstrate that Pax2-Cre-mediated Dicer1 ablation leads to a secondary palatal cleft and defects in CNC-derived skeletal elements associated with the first pharyngeal arch and anterior skull. Interestingly, formation of the primary palate, which differentiates from the frontonasal region, is not impacted.

Pax2 is the earliest transcription factor to be expressed in the prospective mid-hindbrain area, preceding the onset of several signaling molecules, including Wnt1. Likewise, the Pax2-Cre transgene is detected in the mid-hindbrain region at E7.5, with expression in the first pharyngeal arch mesenchyme around E9.5. Mutations in Pax2 result in loss of the midbrain and cerebellar regions, suggesting that Pax2 is required for maintenance of the signaling molecules associated with mid-hindbrain and CNC development.
cell development and, therefore, may be expressed in those migrating CNC cells populating the first pharyngeal arch. Hence, ectopic Pax2-Cre expression in the mesenchyme of the first pharyngeal arch and palatal regions of the Pax2-Cre/Dicer1CKO mice may be reflective of the endogenous Pax2 expression in the mid-hindbrain region from which CNC cells first originate. Expression of the endogenous Pax2 gene appears to be downregulated in normal developing palatal tissue, but not in the Pax2-Cre construct, where X-gal staining is still detected in CNC-derived structures of Pax2-Cre/Dicer1CKO [Figures 2a-c]. Further studies are needed to test this hypothesis.

Evidence for miRNA-mediated gene regulation during neural crest development has emerged from studies examining the Wnt1-Cre/Dicer1CKO mice, which exhibit defects in all neural crest-derived tissue. In contrast, defects in the Dicer1 CKO model are specifically limited to the secondary palates and the skeletal elements derived from CNC cells of the first pharyngeal arch and
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anterior skull, providing a unique model in which to study the impact of Dicer1-dependent miRNA depletion during secondary palatogenesis.

During craniofacial morphogenesis, cell cycle progression and exit are tightly coordinated to ensure that cellular proliferation, differentiation, and apoptosis occur in the appropriate spatiotemporal sequence. Dicer1 in turn regulates all these processes by mediating the biogenesis of small RNAs, including miRNAs. While all three miRNAs examined were found to be significantly downregulated, residual expression was still observed and may be reflective of differential cellular Dicer1 retention in both Pax2-Cre and non-Pax2-Cre expressing cells and/or delayed depletion of mature Dicer1 mRNA.

To date, a number of Dicer1 CKO models have been generated that confirm the importance of Dicer1 in different aspects of cell growth and death. Parallel to these studies, we show here that Pax2-Cre-mediated loss of Dicer1 early in craniofacial development impairs proliferation, differentiation, and survival of CNC-derived craniofacial structures associated with the first pharyngeal arch and anterior cranial base. While proliferating cells were still detectable in many regions of the face and developing brain of Dicer1 CKO at different time points, they were less abundant compared to the WT animals, particularly in the developing palatal shelves. Moreover, the number of apoptotic cells and the expression levels for the senescence marker p21 and the apoptotic markers Caspase 3 and p53 in the developing palatal shelves increased significantly as the embryos developed, supporting our earlier observation that Dicer1 ablation may not affect CNC cell migration and initial proliferation. Rather, we hypothesize that Dicer1 deletion in the Pax2-Cre expression domain contributes to premature cell cycle exit and cell death during palatogenesis. This hypothesis is consistent with previous studies showing that that progenitor cells are less dependent on miRNAs than their differentiated progeny. Therefore, lack of Dicer1 and, consequently mature miRNAs in the mid-hindbrain boundary would not impact CNC cell migration to the first pharyngeal arch, but rather impair the growth and differentiation of the resultant CNC-derived mesenchyme and the formation of skeletal elements associated with the craniofacial region.

Alterations in the mandible are known to cause abnormal displacement of the tongue, which mechanically prevents the elevation of the palatal shelves, and leads to palatal clefting. In the case of mechanical hindrance, mandibular hypoplasia is the notable defect and is not associated with additional craniofacial abnormalities. In contrast, palatal clefting associated with mandibular hypoplasia and abrogated growth of cranioskeletal components, such as those...
seen in Dicer1 CKO and other mouse models, is often attributed to impaired cell proliferation and/or cell survival. Therefore, we hypothesize palatal clefting in Dicer1 CKO is resultant of arrested palatal growth caused by decreased proliferation and increased apoptosis, rather than as a consequence of mandibular hypoplasia. This premise is supported by histological, proliferation and apoptosis assays, showing comparable palatal development in Dicer1 CKO and WT mice up to E11.5, followed by a significant increase in apoptosis in Dicer1 CKO palatal shelves at E13.5. Palatal growth arrest is more conspicuous at E17.5 in Dicer1 CKO. At this time point, palatal shelves are vertically oriented, similar in size to stage E13.5 WT, exhibiting limited mesenchymal condensation, and complete absence of mineralization. These data suggest that Pax2-mediated deletion of Dicer1 may prevent differentiation of CNC-derived mesenchymal cells into osteoblasts and account for the abrogated skeletal development observed in the Dicer1 CKO facial skeleton and anterior skull.

As a whole, this study highlights the developmental impact of inhibiting miRNA biogenesis following Dicer1 deletion in the Pax2-Cre expression domain and reinforces their importance for regulating the signaling pathways involved in the differentiation of CNC-derived skeleton of the first pharyngeal arch. Analyses of specific miRNA species in orofacial development, combined with targeted and controlled manipulation of those miRNA, are likely to enhance our ability to develop future therapies aiming to treat orofacial malformations.

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