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論　文　内　容　の　要　旨

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論文題名	Retinoic acid deficiency mediated by temporospatial loss of *Rdh10* underlies the etiology of midfacial defects (Rdh10の機能阻害によるレチノイン酸シグナルの低下は顔面正中裂の原因となる)

論文内容の要旨

Introduction During embryonic craniofacial development, the facial primordium is composed of seven prominences: the frontonasal process and two paired maxillary processes and mandibular processes. These primordia are populated by cranial neural crest cells and covered by the facial ectoderm. Adequate growth and fusion of each facial primordium are critical for normal craniofacial development and any disturbance in these processes could result in a wide range of congenital craniofacial deformities, including orofacial cleft. Orofacial cleft is a group of defects including isolated cleft lip and/or cleft palate and sometimes accompanied by symptoms such as missing or extra teeth. In humans, orofacial cleft would also be classified in different types according to where the clefts exist. Lateral cleft lip and palate are the most frequent forms of orofacial cleft and therefore the mechanisms are relatively well studied. In contrast, the etiology of midfacial cleft, which exhibits the cleft at the middle of the face, is largely elusive. It is well known retinoic acid(RA) signaling is involved in patterning and growth of the facial primordia by regulating cellular behaviors and gene expression. In our previous study, embryonic elimination of *Rdh10*, a rate limiting enzyme for synthesizing RA, resulted in severe midfacial defects, including midfacial cleft. However, the cellular and molecular mechanisms underlying this midfacial cleft caused by RA deficiency are still unknown. In the present study, we investigated the association of disturbed RA signaling and midfacial cleft using tamoxifen-induced conditional *Rdh10* knock-out mice (*Ert2Cre; Rdh10*$^{fl/fl}$) as a model.

Methods and Results We first confirmed the efficiency of elimination of *Rdh10* and compared spatiotemporal distribution of RA in the developing craniofacial region between control and *Ert2Cre; Rdh10*$^{fl/fl}$ mice. By administrating tamoxifen at E7.0, substantial reduction of *Rdh10* and *Rare-LacZ* expression was seen in the developing frontonasal process. These data indicate *Rdh10* plays a dominant function in activating RA in the developing frontonasal process. The heads of the embryos were dissected at different developmental stages followed by morphological assessment with whole-mount DAPI staining. As a result, we detected midfacial cleft as early as E11.5. From histological analysis of the same samples, arrested upper incisor development and ectopic nodules or chondrogenesis could also be observed. Interestingly, most of the affected tissues were restricted to the derivatives of frontonasal process, whereas structures in the maxillary processes, such as the upper molars, were relatively intact. In order to assess the behaviors of cranial neural crest cells, expression of *Sox9* and *Tfap2a* were analyzed by *in situ* hybridization in E9.5 embryos. The expression of both genes showed no noticeable difference in the frontonasal process between mutant and control embryos at E9.5, which indicates the production and migration of cranial neural crest cells are normal in the mutant mice. We further assessed apoptosis as well as proliferation in the developing frontonasal process at E10.5 via TUNEL staining and immunohistochemistry of pHH3. As a result, significant elevation of apoptosis in the mesenchyme of developing frontonasal process could be seen in mutant embryos, while the number of

proliferating cells was comparable between control and mutant mice. These results indicate RA signaling is essential for the survival of post-migratory cranial neural crest cells. Previous reports showed that loss of *Alx3* and *Alx4* in mice would result in elevated apoptosis in the developing frontonasal process and midfacial cleft. Furthermore, mutation of *Alx1* and *Alx3* is associated with human frontonasal dysplasia, of which symptoms include midfacial cleft. Interestingly, we detected substantial reduction of *Alx1* and *Alx3* in the developing frontonasal processes of E10.5 mutant embryos. These results strongly indicate that RA signaling is required to activate craniofacial *Alx1* and *Alx3* expression, and its disturbance results in elevated apoptosis and midfacial cleft. We further investigated the expression profiles of *Fgf8* and β-catenin in the developing frontonasal process, which are known as crucial signaling molecules for craniofacial development. Ectopic *Fgf8* could result in ectopic nodules and cartilage in the chick craniofacial region. Continuous activation of β-catenin could result in midfacial cleft together with ectopic cartilage in the craniofacial region. Consistent with previous reports, *Fgf8* and β-catenin showed noticeable upregulation in the mutant frontonasal process at E10.5. Hedgehog(Hh) signaling is another well-known signaling pathway regulating craniofacial development. Antagonism or enhancement of Hh signaling in ventral forebrain or frontonasal process could correspondingly result in holoprosencephaly and hypertelorism, which could both be associated with midfacial cleft and upper incisor patterning. Interestingly, *Shh* expression was reduced in the oral ectoderm at E10.5 but showed persistently expanded expression in the ventral forebrain in E11.5 mutant mice. Furthermore, *Gli1* and *Ptch1*, readout genes of Hh signaling, were ectopically expressed at the middle of the frontonasal process and also elevated in the ventral forebrain at E11.5. Importantly, the severity and penetrance of midfacial cleft in the mutant was significantly reduced by administrating cyclopamine, an inhibitor of Hh signaling. These results strongly suggest that disturbed Hh signaling by reduced RA signaling underlies the etiology of midfacial cleft in the mutant embryos.

We also discovered upper incisor defects in the mutant embryos, such as missing and split tooth germ. In order to identify which developmental stage is specifically influenced by RA during incisor development, we assessed the expression of P21 and SOX2 to indicate dental lamina and dental placode. As a result, we detected lack of invagination with reduced cell proliferation in SOX2-expressing dental lamina at E11.5 and noticeable reduction of P21 expression in dental placode at E13.5 in the mutant embryos. These results clearly indicate upper incisor defects in the mutant embryos begin at the initial stage. Next, we assessed a series of inductive signals which are essential for initiation of tooth development. Combined with RNAseq data, we assessed *Pax9* and *Pitx2*, of which mutations are associated with missing teeth and arrestment of dental lamina. Additionally, downregulation of *Bmp4* in dental mesenchyme is suggested to result in splitting the Shh expression domain and incisor placode. The results showed downregulation of all the above inductive signals in either dental epithelium or mesenchyme, indicating that RA orchestrates the critical molecular interactions during odontogenesis. Finally, these results demonstrate that RA signaling is also required for the initiation and patterning of the upper incisor development.

Conclusions Altogether, these data indicate that spatiotemporal *Rdh10* and RA signaling in the embryonic frontonasal process are important for regulating genes which govern midface development. These insights would shed light on the etiology of human midfacial cleft with novel molecular and cellular mechanisms.

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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

本研究の目的は胎児発生に重要なレチノイン酸シグナルが前顔面の発生において果たす役割を詳細に解析する事である。レチノイン酸合成に必要な Rdh10 遺伝子を胎生時期特異的に除去したマウスを解析する事によってレチノイン酸シグナルが頭部神経提細胞の生存に必要である事、顔面正中裂を引き起こす様々な遺伝子発現に影響する事を証明した。

以上のことより、本研究はレチノイン酸シグナルの低下が顔面正中裂に至るメカニズムの一端を明らかにしたことから、博士（歯学）の学位論文として価値のあるものと認める。