Choanal atresia and stenosis: Development and diseases of the nasal cavity

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Abstract

Proper craniofacial development in vertebrates depends on growth and fusion of the facial processes during embryogenesis. Failure of any step in this process could lead to craniofacial anomalies such as facial clefting, which has been well studied with regard to its molecular etiology and cellular pathogenesis. Nasal cavity invagination is also a critical event in proper craniofacial development, and is required for the formation of a functional nasal cavity and airway. The nasal cavity must connect the nasopharynx with the primitive choanae to complete an airway from the nostril to the nasopharynx. In contrast to orofacial clefts, defects in nasal cavity and airway formation, such as choanal atresia (CA), in which the connection between the nasal airway and nasopharynx is physically blocked, have largely been understudied. This is also true for a narrowed connection between the nasal cavity and the nasopharynx, which is known as choanal stenosis (CS). CA occurs in approximately 1 in 5000 live births, and can present in isolation but typically arises as part of a syndrome. Despite the fact that CA and CS usually require immediate intervention, and substantially affect the quality of life of affected individuals, the etiology and pathogenesis of CA and CS have remained elusive. In this review I focus on the process of nasal cavity development with respect to forming a functional airway and discuss the cellular behavior and molecular networks governing

this process. Additionally, the etiology of human CA is discussed using examples of disorders which involve CA or CS.

Introduction

The craniofacial complex in vertebrates comprises multiple sensory organs that are essential for life. Proper head and facial development requires the co-ordinated growth and fusion of multiple facial primordia, nasal cavity invagination, and connection of the nasal cavity to the nasopharynx to form a continuous airway. Each step in this process plays a critical role in producing functional structures, and the perturbation of any step in the process could result in a wide range of craniofacial abnormalities. The complexity of head and facial development helps to account for the high incidence of craniofacial anomalies, which comprise more than 30% of all congenital defects [1]. Choanal atresia (CA), characterized by complete blockage between the nasal cavity and nasopharynx, is a relatively rare craniofacial defect, occurring in 1/5000 live births. Patients exhibiting bilateral CA need immediate surgical correction to avoid complications such as cyanosis caused by respiratory distress [2]. Compared to cleft lip and/or palate, which are well-studied craniofacial anomalies known to result from perturbed embryonic facial prominence growth and fusion, the cytological and molecular etiologies of CA remain

elusive and thus require further investigation. Accepted theories for explaining the developmental pathogenesis of CA include persistence of the buccopharyngeal membrane from the foregut, abnormal persistence or location of mesoderm forming adhesions in the nasochoanal region, abnormal persistence of the nasobuccal membrane of Hochstetter, and misdirection of neural crest cell migration [3]. However, there is little direct evidence supporting these biological mechanisms due to a lack of relevant models for studying CA [4]. The lack of relevant models may be due in part to simply overlooking the condition, since the phenotype occurs inside the head and is not immediately visible. Indeed, even in humans, some CA patients are not diagnosed until adulthood [2,5]. For these reasons, it remains important to clarify the pathology of CA in order to further our understanding of the etiology and pathogenesis of craniofacial malformations. We also know from clinical experience that CA frequently presents in different syndromes and disorders as part of a complex phenotype[6-8]. Interestingly, genes that are mutated in different syndromes which also exhibit CA or CS can be categorized according to their association with certain signaling pathways which are important for cellular activities such as *FGF* signaling and neural crest development, respectively. These facts suggest the possibility of common molecular or cellular mechanisms underpinning CA during embryonic development. Prior reviews have focused on the clinical aspects of CA, but

very few literature reviews have discussed the etiology and pathogenesis of CA and CS. In this review, I introduce the anatomical structures which are essential for forming a functional airway and focus on the specific cellular molecular mechanisms that govern their development. Additionally, congenital human diseases which exhibit CA are discussed together with their associated phenotypes in order to consider possible mechanisms leading to CA and CS phenotypes. By investigating and reviewing the etiology and pathogenesis of CA, I discuss the importance of understanding the biological mechanisms of nasal airway and choanae formation. This is critical not only for explaining the pathogenesis of craniofacial anomalies but also for understanding the basic mechanisms that govern morphogenesis during embryogenesis.

Development of nasal cavity and continuous airway

The nasal placode develops within the epithelium of the frontonasal process beginning around embryonic day (E) 9.5 of mouse embryo development, and represents the first step in nasal cavity formation [9,10]. Subsequently, the medial nasal, lateral nasal and maxillary processes grow and fuse [11]. Simultaneously, the nasal placode invaginates toward the inside of the head to produce the nasal cavity [12]. When the medial and lateral nasal processes fuse at the anterior part of the lambdoidal region, an epithelial structure

called the nasal fin remains at the posterior end of the lambdoidal region. The nasal fin gradually thins and becomes the oronasal membrane. Later, the oronasal membrane breaks down, resulting in connection of the nasal airway and nasopharynx with the primitive choanae[13] (Figure 1). The epithelial seam between the medial and lateral nasal processes is removed by mechanisms including cell death and/or epithelial mesenchymal transformation [14,15]. However, the nasal fin has to persist so that primitive choanae can form properly [4]. The development of the frontonasal region during this period is highly conserved among various species, as shown by principal component analysis[16]. Yet, the way that primitive choanae develop appears to vary among different species. Three-dimensional reconstruction using optical projection tomography has revealed that chicken and some reptiles maintain the connection between the nasal and oral cavities. In contrast, mammals such as mice temporarily separate the nasal and oral cavity and form the choanae later to produce a functional airway extending from the nostril[17].

After the development of primitive choanae, the nasal cavity continuously grows and multiple types of neurons differentiate in the nasal epithelium, enabling the animal to sense odors[12]. As the nasal septum grows vertically, it meets the secondary palatal shelf and fuses in the middle of the head (Figure 2)[18]. As a result, the nasal cavity becomes completely separated from the oral cavity, which enables an animal to avoid the

impaction of food in the nasal cavity, which could cause sinus infection during eating, or the inhalation of food and interference with breathing. It is well known that multiple cell populations are involved in these processes of nasal cavity formation, and some of their possible roles are discussed in this review. Especially, neural crest cells have been suggested to play important roles in CA and CS from the phenotype of neurocristopathies. Exactly how neural crest cells control nasal cavity and choanae development is still largely elusive. However, considering the importance of cranial neural crest cells for proper patterning of craniofacial structures, one explanation of CA and/or CS is neurocristopathies related to mispatterned craniofacial structure.

Congenital human anomalies which can be associated with CA

In order to consider possible mechanisms by which CA can occur, it is helpful to discuss human syndromes which exhibit CA as part of their condition. Human disorders in which CA is a feature are listed with their characteristic phenotypes and their affected genes in Tables 1 to 6. For some of these disorders, we can speculate that the CA phenotype correlates with certain cellular or developmental mechanisms, as discussed below.

Neurocristopathy

Aberrant cranial neural crest cell development has been proposed as one of the cellular mechanisms underpinning the pathogenesis of CA [3]. Therefore, it is reasonable to expect that other syndromes categorized as neurocristopathies (disorders of neural crest cell development) would also exhibit problems in connecting the nasal cavity to the pharynx (Table.1). Several neurocristopathies which also exhibit CA are described in this section in order to show their phenotypic correlations and the possible relationship between defects in neural crest cell development and CA.

CHARGE SYNDROME

CHARGE syndrome is characterized by craniofacial defects such as orofacial clefting, cranial nerve anomalies and ear deformities [19,20]. Most of these phenotypes are known to be associated with defects in cranial neural crest cell development [21,22]. Mutations in *CHD7* are present in 90-95% of CHARGE syndrome patients [19]. CHARGE syndrome frequently includes CA, with the occurrence of CA being as high as 65% in patients with this syndrome [19,20], and a mouse model in which *Chd7* is mutated also exhibits malformed primitive choanae [23]. Interestingly, various animal model investigations have revealed indispensable roles for *Chd7* in multiple aspects of neural crest cell development [24-27]. These findings suggest that mutations in *Chd7* perturb cranial neural

crest development in the etiology of CA in CHARGE syndrome. Moreover, tissue-specific deletion experiments have revealed that epithelial expression of *Chd7* is essential for olfactory neuroectoderm development [28]. These findings support the idea that *CHD7* plays diverse roles in proper airway formation. This is consistent with the broad expression of *Chd7* in craniofacial tissues during embryogenesis [23,29].

Interestingly, *CHD7* is known to interact with the retinoid signaling pathway during the development of certain organs, such as the inner ear and the brain [24]. Moreover, retinoid signaling is well known to play critical roles during craniofacial development, and loss of retinoid signaling results in a broad array of developmental defects, including CA [4,30]. It is intriguing to note that many craniofacial defects, such as cleft palate and CA, have been commonly observed in both *Chd7* mutant and in retinoid signaling defective animals. In fact, modulating retinoid signaling in a *Chd7* mutant mouse partially rescued the phenotype [24,31]. These facts together with the overlapping domains of *Chd7* expression and retinoid signaling reporter (*Rare-LacZ*) activity during craniofacial development in mice suggest a possible correlation between these signaling pathways in proper nasal cavity and primitive choanae development.

It is important, however, to note the possibility of numerous direct and indirect mechanisms by which CA and choanal malformation could be associated with mutation

of *CHD7*. Further extensive research including analyses of genotype-phenotype correlation in CHARGE syndrome, and conditional (tissue and stage specific) *Chd7* knock out experiments will be required to interrogate these mechanisms.

TREACHER COLLINS SYNDROME

Treacher Collins syndrome (TCS) presents with a wide spectrum of craniofacial defects, often including CA [8,21,32]. Mutations in *TCOF1*, *POLR1C* and *POLR1D* are responsible for causing TCS, and all three genes play roles in ribosomal RNA production and subsequently ribosome biogenesis [33,34]. The fact that other similar disorders caused by gene mutations that also affect ribosome biogenesis, such as Diamond-Blackfan anemia [35] or Acrofacial dysotosis[36], can also present with CA suggests that the process of ribosomal biogenesis is critical in the progenitor cells of craniofacial tissues for connecting the nasal airway to the nasopharynx. A mouse model of Treacher Collins syndrome which lacks *Tcof1* function displays elevated neuroepithelial cell death, which results in a reduced number of migrating cranial neural crest cells during embryogenesis [37,38]. At a later stage, *Tcof1* mutant mice exhibit multiple craniofacial defects, including cleft palate or high arch palate, and notably, CA [39]. Zebrafish models of Treacher Collins syndrome in which either *Polr1c* or *Polr1d* was disrupted also displayed elevated cell

death in association with severe craniofacial defects [40]. Interestingly, the elevated cell death is caused by activated *P53* signaling. The Treacher Collins syndrome phenotype can be prevented by suppressing apoptosis via chemical (pifithrin-α) or genetic inhibition of *P53* [37,40]. Further investigation revealed a critical role for *Tcof1* in preventing oxidative stress-induced DNA damage, which can also lead to p53-dependent cell and tissue apoptosis [41]. Remarkably, dietary supplementation with N-acetylcysteine, a strong antioxidant, can reduce p53 activity and neuroepithelial apoptosis and dramatically improve the craniofacial phenotype of *Tcof1* mutant mice [41]. These results also strongly suggest that CA in Treacher Collins syndrome is associated with defects in cranial neural crest cell development. These findings also suggest that it may be possible to prevent the Treacher Collins syndrome phenotype, including CA, by promoting the survival of progenitor neural crest cells. Furthermore, a recent study noted that some commonly used drugs such as statins can titrate the level of P53 in cancer cells [42], suggesting that such drugs might be another tool to ameliorate the phenotype of Treacher Collins syndrome and the pathogenesis of CA.

DIGEORGE SYNDROME

Hemizygous deletion of chromosome 22q11.2 in humans results in DiGeorge syndrome,

which is another neurocristopathy associated with a broad range of craniofacial abnormalities, including CA [43]. Mutations in *TBX1,* which lies within the deletion region, has been shown to be a major contributor to the overall phenotype of DiGeorge syndrome [44-48]. Furthermore *Tbx1* plays a critical extrinsic role in neural crest cell development, especially migration, which is essential for proper craniofacial and cardiovascular development [49,50]. TBX1 can activate *Cxcr4*, which encodes a chemotactic guidance molecule for neural crest cells [51]. These results suggest that disruption of neural crest cell migration may underlie the pathogenesis of CA in DiGeorge syndrome. Interactions between *Tbx1* and retinoid signaling during neural crest development have also been reported and may contribute to the pathogenesis of CA in DiGeorge syndrome [52,53]. Consistent with this idea, deletion of *ALDH1A2*, which plays a critical role in synthesizing retinoic acid, produces a phenotype similar to that observed in DiGeorge syndrome [54]. Considering the fact that retinoid signaling plays indispensable roles in primitive choanae development, particularly in connecting the nasal cavity to the nasopharynx [4,30], it is likely that disruption of retinoid signaling in association with mutations in *TBX1*, contributes to the CA phenotype in DiGeorge syndrome. However, the pathogenesis of CA in DiGeorge syndrome model organisms remains to be properly anatomically described, and clarifying this issue in the future could provide novel

insights into the etiology of CA.

AXENFELD-RIEGER SYNDROME, TYPE 1

Mutations in the *PITX2* gene result in Axenfeld-Rieger syndrome, Type 1, which is associated with a wide spectrum of craniofacial defects, including aberrant tooth development, high arch palate and CA [55]. Furthermore, *Pitx2* has been shown to interact with RA signaling to govern early cranial neural crest cell survival and migration in zebrafish [56]. It has also been reported that neural crest specific deletion of *Pitx2* results in disrupted craniofacial structures, including eyes [57]. Each of these phenotypes suggests there may be a correlation between cranial neural crest cell development and proper connection of the nasal pharynx to the airway. However, it is also important to note the varied penetrance of CA in different neurocristopathies, which suggests that perturbation of neural crest cell development may be one of many single or multifactorial causes of CA. Many questions therefore still remain. In the future it will be necessary to revisit animal models which exhibit neural crest cell defects and abnormal airway and choanal morphology in order to reveal the mechanistic relationship between neural crest cells during choanae development and the pathogenesis of CA and CS.

CROUZON SYNDROME, PFEIFFER SYNDROME, APERT SYNDROME, BEARE-STEVENSON CUTIS GYRATA SYNDROME and ANTLEY-BIXLER SYNDROME (Diseases associated with mutations in FGFRs)

There is a spectrum of syndromes which are caused by mutations in FGFRs (Fibroblast Growth Factor Receptors) (Table 2). These syndromes include Crouzon syndrome, Pfeiffer syndrome, Apert syndrome, Beare-Stevenson Cutis Gyrata syndrome and Antley-Bixler syndrome [6,7,58,59]. These syndromes frequently exhibit craniosynostosis, which is defined as the premature fusion of cranial sutures as a result of accelerated bone formation [60]. Biochemical assays have demonstrated enhanced dimerization of FGFRs in human craniosynostoses associated with mutations in *FGFRs,* which results in exaggerated FGF signaling [61]. Also, a number of animal models with mutations equivalent to these human mutations have been established, and they phenocopy human craniosynostosis [62-65]. Interestingly, CA is frequently observed in individuals with craniosynostosis. Considering that thickening of the posterior vomer could result in narrowing of the choanae, accelerated bone formation may therefore obstruct the nasal airway in the pathogenesis of CA or CS in humans [66,67]. This implies that the CA phenotype observed in disorders associated with enhanced FGF signaling may share some common mechanistic features with craniosynostosis. A similar biological

mechanism may therefore also underpin Shprintzen-Goldberg syndrome, since this syndrome exhibits craniosynostosis together with CA [68,69]. Paradoxically, a CA phenotype has not yet been reported in animal models of these FGF signaling-related diseases [62,65]. The incidence of CA or CS is considerably lower in mice than in humans and this may be due in part to differences in the anatomical structures of the maxillary complex between mice and humans and distinct mechanisms for establishing the airway connection. The bones which surround the secondary choanae (choanal openings at the posterior end of the palate) in the human skull (vomer, sphenoid body, medial pterygoid plate and palatine bones) are anatomically located differently in the mouse craniofacial skeleton [66]. It is important to note that various facial dismorphologies caused by premature fusion of sutures in the growing head have the potential to affect the position and/or size of the choanae, which in turn can result in CA or CS [66,70]. In fact, it has been reported that in representative craniosynostoses, such as Apert syndrome and Pfeiffer syndrome, affected patients exhibit a considerably smaller upper airway lumen compared to normal individuals, and this maybe a consequence of facial dismorphology [66].

CRANIODIAPHYSEAL DYSPLASIA, MARSHALL-SMITH SYNDROME, MARSHALL SYNDROME, RAINE SYNDROME (Diseases with accelerated bone

formation)

Similar in principle to craniosynostosis, there are many other syndromes that exhibit CA together with accelerated bone formation in the face (Table 3). They include Craniodiaphyseal dysplasia, which can present with CA or CS, and that results from mutation of the *SOST* gene [71,72]. Marshall-Smith syndrome, which is caused by mutations in the *NFIX* gene, is another condition that presents with CA, together with accelerated cranial skeletal maturation [73,74]. Similarly, Marshall syndrome, which is caused by mutations in *COL11A1,* also exhibits CA together with accelerated osseous maturation, including the skull [75]. Furthermore, Raine syndrome, which is caused by *FAM20C* gene mutations, also shows increased skull ossification and CA [76]. These disorders suggest there may be a correlation or association between accelerated bone formation in embryonic and postnatal development and the pathogenesis of CA and/or CS.

FRASER SYNDROME, ANIRIDIA 1, BOSMA ARHINIA MICROPHTHALMIA SYNDROME (Deficiency of mid-facial external apparatus)

There is a spectrum of syndromes in which CA is a feature, together with defects in mid-facial organs, such as the eyes and nose (Table 4). Fraser syndrome, for example,

presents with multiple phenotypes, including CA and cryptophthalmos [48,77]. Aniridia 1 patients can exhibit CA in addition to developmental eye defects in association with mutations in *PAX6* [78]. Recently, a mutation in *SMCHD1* was identified in familial Bosma arhinia microphthalmia syndrome patients, who exhibit CA with severe facial hypoplasia, including agenesis of the nose and eyes [79-81]. There are several mechanisms which could explain the development of CA in this set of disorders. The first is general disruption of embryonic mid-facial development, which could result in hypoplasticity or absence of fundamental structures of the nasal cavity. Another is disruption of placode development, which is critical for initiating both optic vesicle and nasal epithelial morphogenesis. Interestingly, many of the syndromes listed in Tables 1-6 display eye and/or midfacial defects together with CA, which suggests that proper midfacial development is important for normal primitive choanae formation.

HOLOPROSENCEPHALY 1, SOLITARY MEDIAN MAXILLARY CENTRAL INCISOR, PALLISTER-HALL SYNDROME (Diseases caused by disrupted SONIC HEDGEHOG signaling pathway)

The Sonic hedgehog (SHH) signaling pathway plays critical roles in midfacial development [9,14]. In particular, the strength and extent of the SHH gradient governs the

width of the midface [82]. Consistent with this idea, reduced SHH signaling induces holoprosencephaly which is characterized by varying degrees of hypoterolism. The most severe forms of holoproencephaly are sometimes associated with CA [83]. However, even a mild form of holoprosencephaly such as cebocephaly, which is characterized by a single nostril or solitary median maxillary central incisor, can also exhibit CA [84]. Furthermore, Pallister-Hall syndrome, which is caused by mutation of *GLI3*, a negative regulator of the SHH signaling pathway, can also present with CA [85,86]. These findings indicate that proper midfacial development which is regulated by SHH signaling is critical for ensuring that the nasal airway connects to the nasopharynx (Table 5).

AMNIOTIC BAND CONSTRICTION

Amniotic band constriction is a disorder of development which can affect multiple tissues such as the face and limbs, resulting in clefts and amputations, respectively [48]. Some reports have also noted CA in association with Amniotic band constriction[87,88]. The cause of the Amniotic band constriction phenotype is posited to be the physical constriction of tissues by amniotic bands resulting from a ruptured amniotic membrane during embryogenesis [89]. However, there are some phenotypes such as internal organ defects which can not be explained by this theory, suggesting that amniotic band

constriction may be a secondary consequence of a yet to be identified primary defect [48]. For these reasons, the mechanistic connection between Amniotic band constriction and CA requires further investigation.

Animal models which show developmental defects in airway formation

Animal model studies have revealed that signaling pathways involving retinoic acid and *Tbx22* are critical for proper nasal airway development. As noted earlier, retinoid signaling plays important roles during multiple aspects of craniofacial development[90-92]. Retinoic acid is the active metabolic derivative of vitamin A and binds to retinoic acid receptors (RARs) which then act as transcription factors that regulate target genes [90,91,93]. It is well known that two oxidation reactions are required to convert vitamin A into retinoic acid. In the first oxidation step, RDH10 converts vitamin A into retinal. This is followed by the second oxidation step, in which ALDH1A1, 2 or 3 converts retinal into retinoic acid [90,91,93]. Expression of a retinoid signaling reporter (*Rare-LacZ*) in mice revealed that retinoid signaling is continuously active during multiple steps of craniofacial development [4,90]. Importantly, strong retinoid signaling activity appears after E12.0 specifically in the area of the primitive choanae, and then continues in the position where the nasal cavity connects with the oral cavity (Figure 3). Additionally,

Rdh10, *Aldh1a2* and *3* are expressed in similar regions around the primitive choanae (Figure 3). These results suggest that vitamin A is converted into retinoic acid during craniofacial development in and around the primitive choanae and the invaginating nasal cavity. More importantly, *Rdh10* loss-of-function in mouse embryos results in a substantial reduction of retinoid signaling and severe craniofacial defects, including a midfacial cleft and CA [4,92]. Epithelial rather than cranial neural crest cell expression of *Rdh10* from E7.5 to E9.5 has been shown to be indispensable for connecting the nasal airway to the nasopharynx [4]. *Rdh10* mutant mice also exhibit abnormal nasal cavity epithelial differentiation [4]. The etiology of CA in *Rdh10* loss-of-function mouse embryos was shown to be associated in part with elevated *Fgf8* expression and increased cell death, as well as reduced proliferation in the nasal fin. Enhanced *Fgf8* expression in the nasal fin was also observed in *Aldh1a3* knock-out mice, which exhibited diminished retinoid signaling and CA [30]. Considering that CA can be prevented in both *Rdh10* and *Aldh1a3* mutants by supplementation with either retinal or retinoic acid, respectively, this implies that deficient retinoid signaling causes elevation of FGF signaling, which in turn disrupts the behavior of nasal fin cells, resulting in CA [4,30]. Since the shape of the nasal cavity is severely distorted in *Rdh10* mutants, the failure to connect the nasal cavity with the nasopharynx may be caused by retarded invagination of the nasal

epithelium. There is some evidence indicating a role for FGF signaling in the cellular and molecular mechanisms of epithelial folding or invagination, specifically during lung budding, which is accomplished by airway epithelial invagination [94]. Interestingly, epithelial invagination during *Drosophila* trachea development is driven by FGF signaling-controlled epithelial cell rounding during proliferation [95,96]. In addition, inhibition of retinoid signaling in mice causes reduced cell proliferation in the developing nasal fin [4]. These findings are indicative of the spatiotemporal importance of proper FGF signaling in the nasal fin for invagination. FGF signaling is also known to be important for neuroectodermal differentiation in the nasal cavity, indicating that it may play multiple roles [12]. These results suggest that FGF signaling is important for proper nasal epithelium morphogenesis and maturation.

Neural tube closure is another example where perturbation of epithelial folding can result in craniofacial abnormalities such as anencephaly [97]. Polarized constriction of actin bundles at the apical side of the epithelial cells, which is governed by the planar cell polarity (PCP) signaling pathway, is critical for this process [98]. Furthermore, it has been shown that mice lacking *Rho-GTPase* family genes, which are important for the PCP signaling pathway, develop severe craniofacial defects, including facial clefting [99,100]. From these phenotypic similarities, one can speculate that a link between retinoid and

Rho-GTPases signaling pathways may underlie the etiology of CA. Although a universal mechanism for epithelial invagination remains elusive, these findings support a synergistic role for these mechanisms in proper nasal cavity development and primitive choanae formation.

The family of genes encoding retinoic acid receptors (RARs) is critical for retinoid signaling. Mutation of members of this set of genes has been shown to result in a variety of craniofacial defects, such as severe maxillary hypoplasia or facial clefting, which resembles *Rdh10* mutant phenotypes [101-104]. CA has not been described in these mutants; however, it remains possible that the phenotype has simply been overlooked. For future investigations, in order to specifically discover detailed airway phenotypes including CA or CS in animal models, histology, micro-CT, and or magnetic resonance imaging microscopy would be very powerful and should provide us further knowledge about the etiology of various airway defects [66].

Tbx22 is another gene whose mutation is known to cause CA together with submucosal cleft in mice [105]. Interestingly, ossification of the palate region in *Tbx22* mutant mice is retarded, which suggests that the mechanism underlying CA in these mice is different to that in models of retinoid deficiency, in which accelerated bone formation blocks the airway [4]. In humans, mutations in *TBX22* result in X-linked cleft palate and

ankyloglossia. Considering the fact that ankyloglossia can result from a persistent frenum, which is part of the oral epithelum, a similar mechanism that causes the oronasal membrane to persist might also underpin the pathogenesis of CA.

Environmental factors related to the etiology of CA

It is well known that many craniofacial anomalies are multifactorial in origin, which means that they occur as a result of the sum of genetic and environmental factors [106]. This is especially true for cleft lip and/or palate, which are the most frequent congenital craniofacial malformations. Cohort and case control studies have identified certain environmental factors as risk factors, including alcohol, smoking and excessive intake of vitamin A [107-109]. One cohort study revealed that smoking during pregnancy is a risk factor for isolated CA [110]. It is important to note that smoking can inhibit the synthesis of vitamin A [111]. These findings together with the phenotypes associated with retinoid deficiency in mice indicate a possible synergistic risk of smoking and retinoid deficiency in the pathogenesis of CA. Excessive alcohol consumption has also been shown to inhibit the retinoid signaling pathway [112,113]. Furthermore, in zebrafish, alcohol exposure phenotypes can be rescued by supplementation with retinoic acid [114]. Ethanol exposure has also been shown to interfere with SHH signaling [115] and ethanol exposure worsens

the craniofacial phenotypes in mice which carry mutations in SHH signaling pathway genes [116]. Although there is no clear evidence to date that connects ethanol consumption to human CA, it will be important to consider the biological impact of excessive ethanol consumption as a risk factor for CA in future studies. Additionally, there are reports that prenatal use of anti-thyroid medication such as carbimazole can cause CA in offspring [110,117]. Furthermore, warfarin intake has also been reported to have a teratogenic effect inducing a variety of embryonic defects, including CA [118]. Taken together with the high frequency of other craniofacial defects, including craniosynostosis, in the offspring of anti-thyroid medication-treated pregnant women, this implies that environment-gene disruption of cranial neural crest cell development can underlie the etiology and pathogenesis of CA. Collectively, these findings illustrate the importance of environmental factors in the pathogenesis of CA and indicate that further investigation of environmental influences in the pathogenesis of CA should be a focus for the future.

Concluding remarks

Analyses of human disorders and genetically modified animal models which exhibit CA, have revealed perturbation of epithelial invagination, altered neural crest cell migration and premature bone formation as critical factors in the pathogenesis of CA. Bidirectional

oral ectoderm and nasal epithelium invagination is critical for primitive choanae development and for connecting the nasal cavity to the nasopharynx. Signaling via retinoic acid and SHH appears to underpin much of the etiology of CA. Retinoid signaling in particular seems to play a pivotal role by bridging many signaling pathways, including FGF, TBX1 and CHD7 signaling. Moreover, reduced retinoid signaling during primitive choanae development in mice impacts epithelial cell proliferation and leads to cell death via disruption of FGF signaling, followed by CA. Further investigation of retinoid signaling and the morphogenesis of progenitor tissues that form the choanae will be necessary for a complete understanding of the etiology and pathogenesis of CA and CS, and may uncover elusive mechanisms that could lead to their prevention.

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Figure Legends

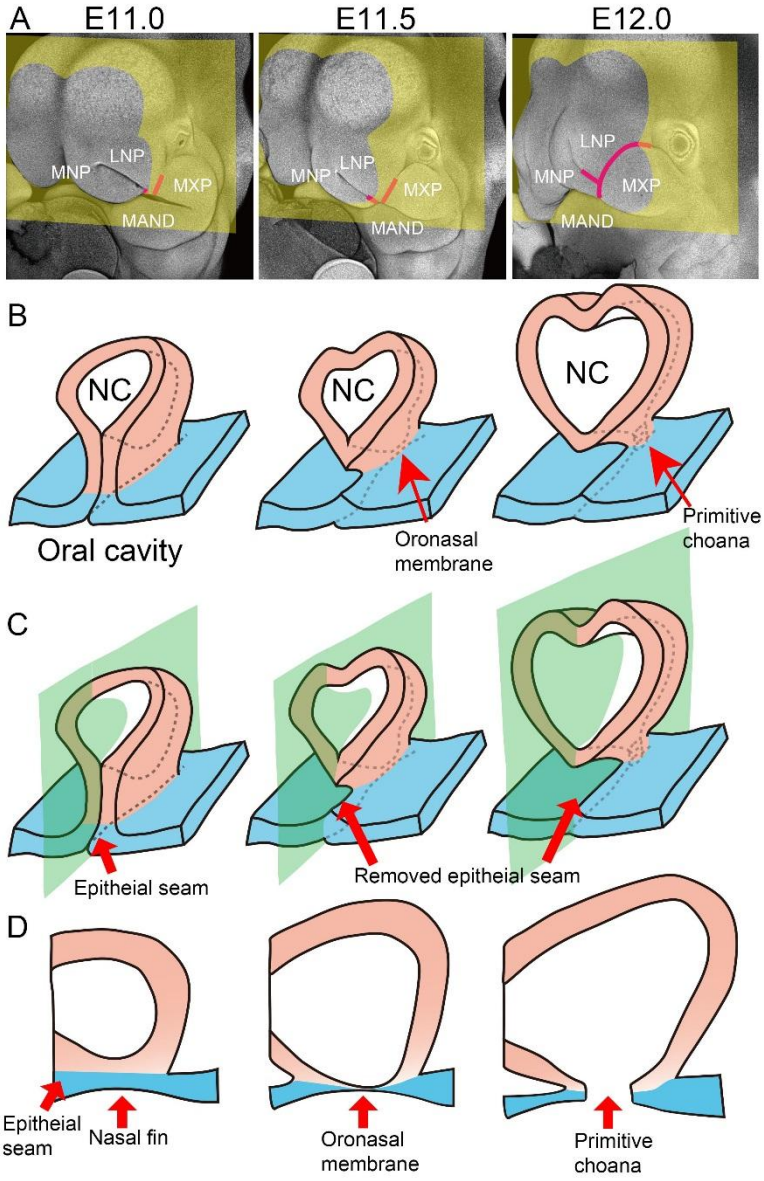

Figure.1

Figure 1. Invagination of nasal and oral epithelium takes place during embryonic craniofacial development. (A) Oblique view of developing mouse head with whole mount nuclear fluorescent imaging at E11.0, E11.5 and E12.0, which was modified from reference 9. Yellow color indicates the level of frontal section shown in B. Red lines indicate the position where the facial processes fuse. (B-D) Schematic drawing of nasal (pink) and oral epithelium (light blue) during the process of nasal fin, oronasal

membrane and primitive choana formation. Green color in (C) indicates the level of section which is shown in (D). (D) Sagittal view of the process of primitive choana development. The drawings in B-D represent one-half of the nasal area. MNP: medial nasal process, LNP: lateral nasal process, MXP: maxillary process, MAN: mandible, NC: nasal cavity.

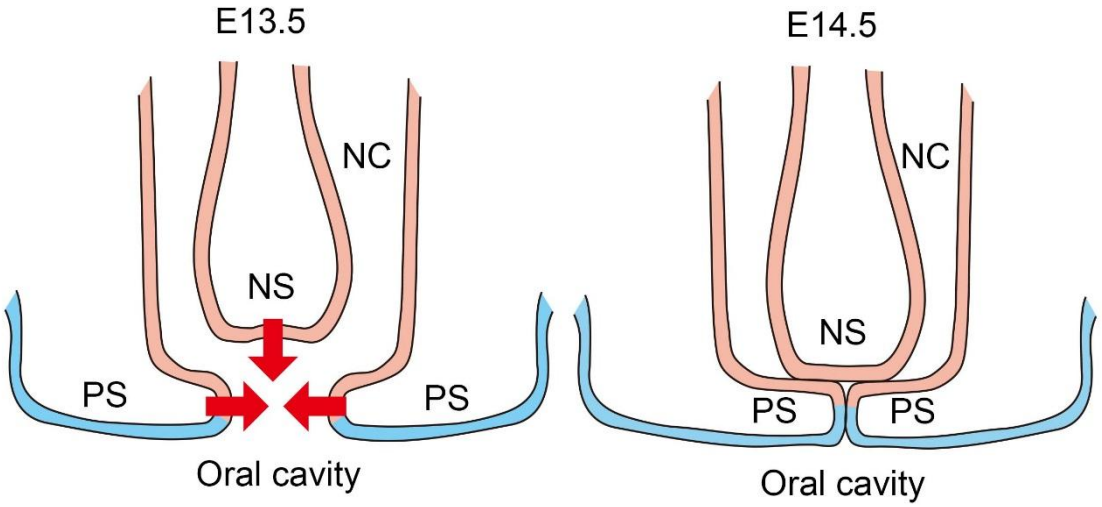

Figure.2

Figure 2. Schematic drawing of the process of nasal septum and secondary palate fusion. The two figures represent the frontal plane of the maxilla from E13.5 to 14.5. Pink and light blue color indicate nasal epithelium and oral epithelium, respectively. Red arrows indicate the direction of growth of nasal septum (NS) and secondary palatal shelf (PS). By the fusion of palatal shelves and nasal septum, the nasal cavity (NC) becomes completely separate from the oral cavity. NC: nasal cavity, NS: nasal septum, PS: palatal shelf.

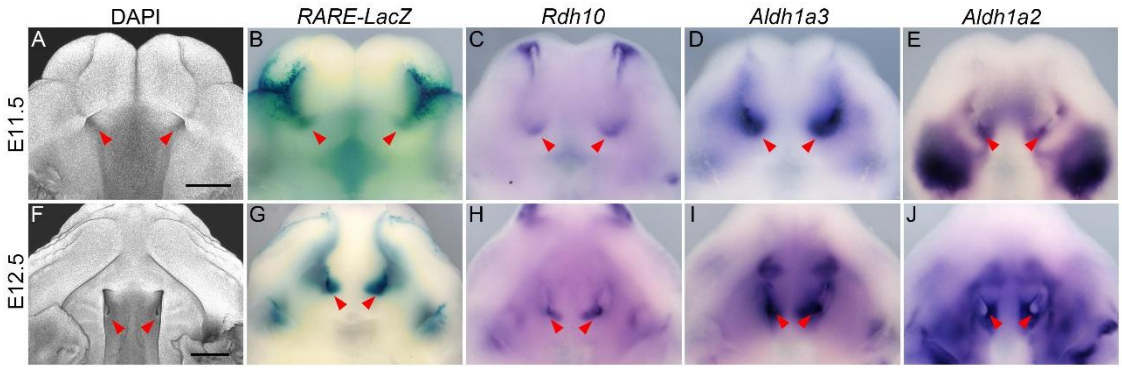

Figure.3.

Figure 3. Expression pattern of *RARE-LacZ* reporter and retinoic acid signaling-related genes during choana development. Intra-oral view of maxillary complex at E11.5 (A) and E12.5 (F) using whole-mount nuclear fluorescent imaging. (B and G) *LacZ*-stained *RARE-LacZ* embryo's maxilla. (C-E and H-J) Whole-mount *in situ* hybridization of genes indicated at the top. Scale bar: 500 μm.

Table 1 List of human neurocristopathies associated with CA

Disease	Other representative phenotypes	Reported gene or locus of mutation
ACROFACIAL DYSOSTOSIS, CINCINNATI TYPE (OMIM # 616462)	micrognathia, downslanting palpebral fissures, lower eyelid clefts, inferiorly displaced orbits, midfacial hypoplasia	POLR1A
AXENFELD-RIEGER SYNDROME, TYPE 1 (OMIM # 180500)	abnormal development of the anterior segment of the eye, dental hypoplasia, failure of involution of periumbilical skin, maxillary hypoplasia	PITX2
CHARGE SYNDROME (OMIM #214800)	coloboma of the eye, heart anomaly, retardation of mental and somatic development, microphallus, ear abnormalities and/or deafness, cleft palate	CHD7
DIAMOND-BLACKFAN ANEMIA 10 (OMIM # 613309)	micrognathia, cleft palate, low-set and posteriorly rotated ears, mandibulofacial dysostosis	RPS26
DIGEORGE SYNDROME (OMIM # 188400)	parathyroid hypoplasia, thymic hypoplasia, and outflow tract defects of the heart	TBX1
TREACHER COLLINS SYNDROME 1 (OMIM # 154500)	downslanting eyes, coloboma of the lid, micrognathia, ear deformities, hypoplastic zygomatic arches, hearing loss, cleft palate	TCOF1

Table 2 List of human diseases with FGFR mutation associated with CA

Disease	Other representative phenotypes	Reported gene or locus of mutation
ANTLEY-BIXLER SYNDROME WITHOUT GENITAL ANOMALIES OR DISORDERED STEROIDOGENESIS (OMIM # 207410)	craniosynostosis, midface hypoplasia, joint contractures	FGFR2
APERT SYNDROME (OMIM # 101200)	craniosynostosis, midface hypoplasia, syndactyly	FGFR2
BEARE-STEVENSON CUTIS GYRATA SYNDROME (OMIM # 123790)	craniosynostosis, and ear defects, cutis gyrata, acanthosis nigricans, anogenital anomalies, skin tags, prominent umbilical stump	FGFR2
CROUZON SYNDROME (OMIM # 123500)	hypertelorism, exophthalmos and external strabismus, parrot-beaked nose, short upper lip, hypoplastic maxilla, and a relative mandibular prognathism	FGFR2
CROUZON SYNDROME WITH ACANTHOSIS NIGRICANS (OMIM #612247)	downslanting palpebral fissures, exophthalmos, ocular hypertelorism, midface hypoplasia, convex nose, posteriorly rotated ears, acanthosis nigricans	FGFR3

HYPOGONADOTROPIC HYPOGONADISM 2 WITH OR WITHOUT ANOSMIA (OMIM # 147950)	absent or incomplete sexual maturation, anosmia, cleft palate, hearing loss	FGFR1
PFEIFFER SYNDROME (OMIM # 101600)	craniosynostosis, midface deficiency, broad thumbs, broad great toes, brachydactyly, syndactyly	FGFR1 or FGFR2

Table 3 List of human diseases with accelerated bone formation associated with CA

Disease	Other representative phenotypes	Reported gene or locus of mutation
CRANIODIAPHYSEAL DYSPLASIA, AUTOSOMAL DOMINANT (OMIM # 122860)	hyperostosis, sclerosis, macrocephaly, hypertelorism, a broad flat nasal bridge with saddle nose, and prominent mandibles	SOST
MARSHALL SYNDROME (OMIM # 154780)	accelarated osseous maturation including skull, prominent forehead, small face, prominent eyes, micrognathia	COL11A1
MARSHALL-SMITH SYNDROME (OMIM #602535)	accelerated skeletal maturation, failure to thrive, dysmorphic facial features	NFIX
RAINE SYNDROME (OMIM #259775)	increased skull ossification, narrow prominent forehead, proptosis, depressed nasal bridge, midface hypoplasia	FAM20C

Table 4 List of human diseases with mid-facial external apparatus defect associated with CA

Disease	Other representative phenotypes	Reported gene or locus of mutation
BOSMA ARHINIA MICROPHTHALMIA SYNDROME (OMIM #603457)	severe hypoplasia of the nose and eyes, palatal abnormalities, deficient taste and smell, inguinal hernias, hypogonadotropic hypogonadism with cryptorchidism	SMCHD1
FRASER SYNDROME (OMIM # 219000)	cryptophthalmos, syndactyly, ambiguous genitalia, laryngeal and genitourinary malformations, oral clefting, and mental retardation	FRAS1or GRIP1 or FREM2

Table 5 List of human diseases with mutation in SHH signaling associated with CA

Disease	Other representative phenotypes	Reported gene or locus of mutation
HOLOPROSENCEPHALY 1 (OMIM % 236100)	retinal coloboma, solitary upper incisor, microcephaly, short stature, mental retardation	21q22.3
PALLISTER-HALL SYNDROME (OMIM # 146510)	digital abnormalities, hypothalamic hamartoma, pituitary dysfunction, visceral malformations	GLI3
SOLITARY MEDIAN MAXILLARY CENTRAL INCISOR (OMIM # 147250)	short stature, midnasal stenosis, solitary upper incisor, holoprosencephaly	SHH

Table 6 List of human diseases associated with CA

Disease	Other representative phenotypes	Reported gene or locus of mutation
ANIRIDIA 1 (OMIM # 106210)	absence of the iris	PAX6
ANKYLOBLEPHARON-ECTODERMAL DEFECTS-CLEFT LIP/PALATE (OMIM # 106260)	ectodermal dysplasia, ankyloblepharon, cleft lip and palate	TP63
ANTLEY-BIXLER SYNDROME WITH GENITAL ANOMALIES AND DISORDERED STEROIDOGENESIS (OMIM # 201750)	radiohumeral synostosis, midface hypoplasia, joint contractures,	POR
APLASIA CUTIS CONGENITA, NONSYNDROMIC (OMIM # 107600)	thin skin, syndactyly, imperforate anus, pulmonary hypoplasia	BMS1
BREASTS AND/OR NIPPLES, APLASIA OR HYPOPLASIA (OMIM % 113700)	absence of breasts and/or nipple, absence of the mammary gland	Unknown
BURN-MCKEOWN SYNDROME (OMIM #608572)	sensorineural deafness, cardiac defects, narrow palpebral fissures, coloboma of the lower eyelids, prominent nose with high nasal bridge, short philtrum, cleft lip and/or palate, protruding ears	TXNL4A
CAT EYE SYNDROME (OMIM # 115470)	coloboma of the iris, anal atresia, downslanting palpebral fissures, preauricular tags and/or pits, heart and renal malformations	22q11
CHROMOSOME 9p DELETION SYNDROME (OMIM # 158170)	delayed motor development, trigonocephaly, wide nasal bridge, large upper lip, high-arched palate, long fingers and	9p

	toes, flat feet, and dermatoglyphic peculiarities	
COFFIN-SIRIS SYNDROME 1 (OMIM # 135900)	mental retardation, hypertrichosis, sparse scalp hair, and hypoplastic or absent fifth fingernails or toenails	ARID1B
CONSTRICTING BANDS, CONGENITAL (OMIM % 217100)	amputated fingers, facial cleft	Unknown
CORPUS CALLOSUM, AGENESIS OF, WITH MENTAL RETARDATION, OCULAR COLOBOMA, AND MICROGNATHIA (OMIM # 300472)	coloboma of the iris, high forehead, retrognathia, mental retardation	IGBP1
DIABETES MELLITUS, NEONATAL, WITH CONGENITAL HYPOTHYROIDISM (OMIM # 610199)	neonatal diabetes, hypothyroidism	GLIS3
DIARRHEA 3, SECRETORY SODIUM, CONGENITAL, WITH OR WITHOUT OTHER CONGENITAL ANOMALIES (OMIM #270420)	congenital secretory sodium diarrhea, corneal erosions, punctate keratitis	SPINT2
ECTRODACTYLY, ECTODERMAL DYSPLASIA, AND CLEFT LIP/PALATE SYNDROME 1 (OMIM % 129900)	Ectrodactyly, ectodermal dysplasia, clefting, anomalies of lacrimal ducts, urogenital defects, hearing loss	7q11.2-q21.3
FEINGOLD SYNDROME 1 (OMIM # 164280)	microcephaly, limb malformations, esophageal and duodenal atresias, mental retardation	MYCN

FEINGOLD SYNDROME 1 (OMIM # 164280)	normochromic macrocytic anemia, reticulocytopenia, absent erythroid progenitors in the bone marrow, craniofacial defects, upper limb malformation, heart defect, urinary defect	RPS26
FRYNS MICROPHTHALMIA SYNDROME (OMIM 600776)	microphthalmia, glaucoma, oblique facial cleft, lid colobomas	Unknown
FRYNS SYNDROME (OMIM % 229850)	gastroschisis, central nervous system defects, midline cleft of the upper lip and alveolar ridge, cleft nose	Unknown
HYPOTHYROIDISM, THYROIDAL OR ATHYROIDAL, WITH SPIKY HAIR AND CLEFT PALATE (OMIM #241850)	athyroidal hypothyroidism, spiky hair, cleft palate, bifid epiglottis	FOXE1
LENZ-MAJEWSKI HYPEROSTOTIC DWARFISM (OMIM # 151050)	bone dysplasia, enamel hypoplasia, syndactyly, loose skin	PTDSS1
MANDIBULOFACIAL DYSOSTOSIS, GUION-ALMEIDA TYPE (OMIM #610536)	microcephaly, midface and malar hypoplasia, micrognathia, microtia, dysplastic ears, preauricular skin tags, significant developmental delay, speech delay	EFTUD2
MCKUSICK-KAUFMAN SYNDROME (OMIM # 236700)	hydrometrocolpos, polydactyly	MKKS
MENTAL RETARDATION, AUTOSOMAL DOMINANT 6 (OMIM # 613970)	mental retardation	GRIN2B
MENTAL RETARDATION, X-LINKED 99, SYNDROMIC,	mild mental retardation, short stature, scoliosis, hip dysplasia,	USP9X

FEMALE-RESTRICTED (OMIM # 300968)	postaxial polydactyly, pes cavus, anal atresia, hearing loss	
MICROCEPHALY 10, PRIMARY, AUTOSOMAL RECESSIVE (OMIM # 615095)	small head size, low sloping forehead, micrognathia, prominent helices, prominent nasal bridge, cataracts	ZNF335
RADIAL RAY HYPOPLASIA WITH CHOANAL ATRESIA (OMIM 179270)	flattened nasal bridge, esotropia, finger abnormalities	Unknown
SHPRINTZEN-GOLDBERG CRANIOSYNOSTOSIS SYNDROME (OMIM # 182212)	craniosynostosis, hypertelorism, downslanting palpebral fissures, high-arched palate, micrognathia, low-set posteriorly rotated ears	SKI
VAN MALDERGEM SYNDROME 1 (OMIM # 601390)	mental retardation, osteopenia, thickened skull base and frontal bones, narrow thorax, short clavicles, subluxation of the radial heads, and hand and feet abnormalities, abnormal teeth, high-arched palate, thick gums, hypospadias	DCHS1

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