The Association Between Runx Signaling and Craniofacial Development and Disease

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Abstract

Purpose of Review The Runx family genes (*Runx1, Runx2, Runx3*, and *Cbfb*) are important transcriptional regulators in the development of various tissues. We herein highlight the roles of the Runx family genes in morphogenesis in the craniofacial regions and in the pathogenesis of congenital morphological problems in these regions.
Recent Findings A recent analysis using conditional *Runx* mutant animals and a human genetic study identified the novel roles of *Runx* genes in the development of the tooth, salivary glands, and the palate. In an animal study, Runx1/Cbfb signaling was found to regulate the *Lgr5* expression and maintain the stem cells in the dental epithelium in the growing incisors. Aberrant Runx1/Cbfb signaling induced male-specific involution of the convoluted granular cell differentiation of the submandibular gland. In palatogenesis, Runx1/Cbfb signaling regulated the *Tgfb3* expression in the fusing palatal epithelium through Stat3 activation.
Summary The combination of a human genetic study and a phenotype analysis of mutant animals revealed the various roles of Runx genes in the development of the tooth, palate, and salivary glands. Runx genes have functional redundancy in various tissues, which still hinder the roles of Runx genes in morphogenesis. Future studies may reveal the novel roles of Runx signaling.

Keywords Runx1 · Runx2 · Cleft palate · stem cells · incisors

Introduction

Runx2 is a master gene for bone development [1] and haploinsufficiency of Runx2 causes cleidocranial dysplasia [2]. As suggested by the function of Runx2, this disease is characterized by problems in skeletogenesis, such as missing clavicles and opened anterior fontanelle. A recent study further showed that osteoporosis and osteopenia are detected in this syndrome [3•]. Supernumerary teeth are also among the characteristic features of this syndrome [4]. Interestingly, *Runx2* deletion disturbed tooth development in mice [5], whereas haploinsufficiency induces extra tooth generation in humans [4]. The tooth is a mineralized tissue, like bone; however, unlike bone, teeth are classified as skin appendages and their morphogenesis is regulated by epithelial-mesenchymal interaction. Indeed, tooth development arrest in *Runx2* deletion is due to disturbed Runx2-regulated Fgf signaling in the process of epithelial-mesenchymal interaction [6]. A recent study further demonstrated that other Runx family genes are also involved in the morphogenesis, differentiation, and maintenance of stem cells in various organs. In the present review, we introduce novel roles of Runx signaling in the craniofacial region.

Runx genes encode transcription factors sharing a conserved region of 128 amino acids termed the runt domain (RD) [7]. There are three members of RUNX gene family in mammals: RUNX1, RUNX2, and RUNX3. These Runx family transcription factors form a heterodimeric transcription complex with Core binding factor β (Cbfb), a cofactor of the Runx family, which enhances the binding affinity of the complex for DNA.

Runx1, Runx3, and Cbfb, a cofactor for the Runx genes, are also involved in skeletal development. *Runx3* is expressed in osteoblasts and *Runx3* deletion results in congenital osteopenia in mice [8]. *Runx1* is coexpressed in early stages of intramembranous ossification in craniofacial bones [9]. The conditional deletion of *Runx1* demonstrates a delay in sternal development and further double null mutation of *Runx1* and

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Runx2 results in complete lack of a sternum [10]. Furthermore, as is observed in *Runx1* mutants, *Cbfb* null mutation has been demonstrated to be associated with skeletal defects [11]. Taken together, Runx2 is dominant in bone development, but other Runx genes also have redundant and regulatory roles.

The null mutant phenotypes in each Runx gene display distinct tissue-specific roles in mice; RUNX1 is required for hematopoiesis; RUNX2 is essential for osteogenesis; and RUNX3 is crucial for the development of dorsal root ganglia neurons and T-lymphocytes (reviewed in [12]). Both *Runx1* and *Runx2* null mutants are lethal in early embryogenesis [1, 13], and *Runx3* mutants cannot survive long after birth. A recent phenotype analysis of their conditional mutants or conditionally rescued mutants further revealed the additional and various roles of the Runx family genes in various biological events (e.g., cancer development, organogenesis, and morphogenesis).

The aim of this review is to summarize the findings from current studies that showed the importance of the Runx genes in the development of tissues and organs in the craniofacial regions and Runx-related human disease.

CCD and Supernumerary Teeth

The etiology of the supernumerary teeth in *Runx2* mutant mice remains to be elucidated at the molecular and cellular levels. In rodents, *Runx2* null mutant demonstrated extra buds at the lingual side of the upper tooth buds [14]. This evidence directly suggests that Runx2 inhibits the initiation of extra tooth formation in early tooth development. However, the tooth development does not proceed beyond the cap stage in *Runx2* null mutants and supernumerary teeth are not evident in these mutants [5]. Furthermore, the Runx2 mouse CCD model is not ideal for the study of supernumerary teeth since mice are monophydont. Humans are diphydont and the supernumerary teeth in man have been shown to be supernumerary to the second dentition [15]. Unlike humans, rodents do not show supernumerary teeth with *Runx2* haploinsufficiency.

The budding of the tooth germs is regulated by various molecules. Activation of canonical Wnt signaling in the dental and oral epithelium induces extra tooth buds as well as supernumerary teeth in rodents [16, 17]. Interestingly, treatment with BIO, a small-molecule GSK3 inhibitor and, therefore, activator of canonical Wnt signaling, prevents the induction of supernumerary teeth in epithelial Wnt-activated mutants ex vivo [18•]. Furthermore, BIO also inhibits the successive formation of M2 molars in WT mice molar in vitro [18•]. Taken together, mesenchymal Wnt signaling prevents the formation of successive or extra tooth, unlike the stimulatory roles of epithelial Wnt signaling in the induction of tooth budding. In this study, it was demonstrated that Wnt inhibitors, such as *Axin2* or *Drapc1*, were activated in *Runx2* null

mutants. Hence, it is likely that antagonistic interactions between Runx signaling and Wnt inhibitor at the dental mesenchyme surrounding the tooth buds may—at least in part—explain the molecular etiology of the supernumerary teeth in CCD patients [18•].

The Involvement of Runx Genes in Amelogenesis and the Maintenance of the Dental Epithelium

Some CCD patients demonstrate enamel hypoplasia [19]. *Runx2* is expressed in ameloblasts, which is derived from the dental epithelium, during the late secretory and maturation stages [20], and the epithelial-specific ablation of *Runx2* results in hypomaturation of the enamel matrix with *Klk4* downregulation in mice. In these mutants, the tooth morphology was not affected; however, the enamel matrix was remarkably abraded in aging with significant downregulation of the *AMELX*, *AMBN*, and *ENAM* expression [21•].
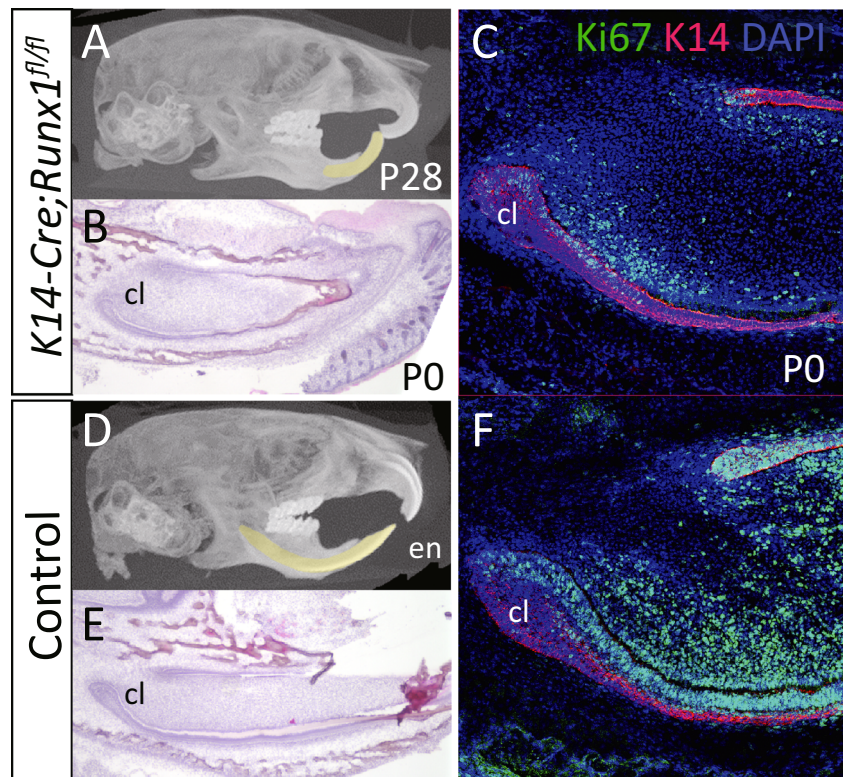
Runx1 is also expressed in ameloblasts and the dental epithelium [22]. Humans with Braddock-Carey syndrome, in which A 21q22 contiguous gene syndrome encompasses *RUNX1*, show enamel hypoplasia [23]. In mice, epithelial deletion of *Runx1* results in the absence of enamel formation in the incisors and enamel hypoplasia with tipping in the molars [24••]. Similar defective phenotypes in the enamel matrix were also demonstrated in *Cbfb* mutants [25].

On the other hand, *Runx3* null mutants did not show phenotypes associated with tooth development [22]. Collectively, Runx1 and Runx2 with Cbfb, cofactor, are involved in enamel formation in amelogenesis. Since the expression of *Runx1* and *Runx2* partly overlap in ameloblast differentiation, their function could be redundant. Hence, the deletion of both *Runx1* and *Runx2* may possibly cause more severely defective amelogenesis, which would further elucidate the distinct roles of Runx signaling in enamel formation.

The Involvement of Runx Genes in the Maintenance of Stem Cells

The incisors of rodents continuously elongate throughout their lifetime and specifically contain an epithelial stem cell compartment at the cervical loop. In conditional null mutants of each *Runx1* and *Cbfb*, the incisors show remarkable shortening with shrinkage of the cervical loop and with a significant reduction in the proliferative activity of the ameloblast precursors [24••, 25]. In the cervical loop of mutant incisors, the expression of *Lgr5*, a stem cell marker, is remarkably disturbed, indicating that Runx1 is involved in the maintenance of the epithelial stem cells in the growing incisors [24••] (Fig. 1).

Fig. 1 *K14-Cre*; *Runx1fl/fl* mice showed extremely short incisors and enamel defects (A) in comparison to control mice (C). On the other hand, there was no significant difference in the molar root length or morphology. Double immunostaining for Ki67 and K14 revealed that the epithelium in the cervical loop of the *Runx1* mutant (B) was significantly less proliferative than that in the control (D). Nuclei were counterstained with DAPI

This regulatory role of Runx1 in stem cell maintenance is also evident in hair follicles [26]. In the growing incisors, *Runx2* is expressed in the cervical loop and *Runx3* was observed in ameloblast precursors [24••]; however, neither *Runx2* nor *Runx3* null mutants showed shortening of the incisors [21•]. Taken together, among the Runx genes, *Runx1* is dominantly involved in the maintenance of stem cells.

The molecular mechanisms underlying the regulation of the *Lgr5* expression by Runx remain to be elucidated. *Lgr5* is known to be a Wnt target gene; however, an analysis using the canonical Wnt signaling reporter mouse demonstrated that *Lgr5* is not directly regulated by Wnt signaling in the mouse incisors [27]. In our study, *Runx1* abrasion disturbed Stat3 phosphorylation specifically at the cervical loop regions. In vitro, Stat3 inhibitors (e.g., AG490 or S3I-201) disturbed the *Lgr5* expression at the WT cervical loop and Stat3 activation using ZnCl2 rescued the disturbed *Lgr5* expression in the *Runx1* mutant incisors [24••]. Interestingly, a ChIP analysis demonstrated that both Runx and Stat transcriptional factors bind to the promoter region of *Lgr5*, suggesting that Runx1-Stat3 regulatory network could directly—at least in part—regulate the expression of *Lgr5*.

As stated above, Runx1 affects ameloblast differentiation; however, it was curious that the enamel phenotypes were remarkably more severe in incisors than in molars. Such a difference could be due to the disturbance of the supply of the ameloblast precursors in the *Runx1* mutant incisors.

The Involvement of Runx in Exocrine Gland Development

Salivary glands are regulated by epithelial-mesenchymal interaction, as well as teeth. In development of the salivary gland, *Runx1* is specifically expressed in the ductal epithelium of the submandibular glands. Interestingly, in male mice, *Runx1* conditional null mutants exhibited involution of the granular convoluted tubules (GCT). GCT is a secretory canal lying between the intercalated and striated ducts and is specifically induced in the postnatal stage under the control of androgens [28]. Such androgen-specific characteristics are also supported by its involution by castration in male mice [29]. In these *Runx1* mutants, serum androgen levels remained unchanged and testis development was not affected, however, the expression of *Crisp3*, a biomarker gene of the AR pathways, is significantly downregulated, and it was assumed that Runx1 directly regulates the sensitivity of GCTs cells to androgen and contributes to the induction of the GCTs in male mice [30•]. Epithelial *Cbfb* null mutant mice demonstrate similar ductal phenotypes with the involution of GCT, indicating that Cbfb is also an indispensable cofactor in Runx signaling in postnatal development of the salivary glands [31]. Taken together, Runx1/Cbfb signaling is involved in sexual dimorphism in the induction of GCTs in the presence of androgen. To date, no reports have demonstrated the possible association between genetic *Runx1* or *Cbfb* mutations and salivary gland disease.

Runx3 is also expressed in both ductal and serous or mucous acinous cells of the parotid, submandibular, and sublingual salivary glands and in adenoid cystic carcinoma cells [32]. *Runx3* is a tumor suppressor gene in gastric cancer [33] and the expression of *Runx3* was downregulated in carcinoma cells, which could be associated with tumor progression [32]. A CpG island analysis demonstrated that *Runx3* is methylated in these carcinoma cells.

Methylation of the runt-related transcription factor-3 CpG island spreads the most from the 5′ region to the transcription start site in adenoid cystic carcinoma tissues, and the transcription start site may be a critical region for runt-related transcription factor-3 methylation. The spreading pattern of the runt-related transcription factor-3 methylation may play a role in the progression of adenoid cystic carcinoma [34].

The Involvement of Runx in Cleft Palate

The expression of *Runx2* is evident in the palatal mesenchyme and *Runx2* deletion results in complete cleft palate in mice [35]. In humans, association analyses of four populations identified single nucleotide polymorphisms (SNPs) that showed significant excess maternal transmission and suggested that *RUNX2* may influence the risk of CL/P [36]. On the other hand, there is no evident association between CCD and cleft palate; however, a clinical report described that CCD patients with mutation of p.R225Q, which is located closely to the Runt binding region, had a median pseudo-cleft palate [37]. The deletion of *Hoxa2*, a homeobox gene, results in cleft palate with the upregulation of Runx2 in mice, suggesting that palatal *Runx2* is regulated by *Hoxa2*, presumably by regulating the osteogenic differentiation of the palatal mesenchyme [38].

Runx1 is specifically expressed at the fusing palatal epithelium [22, 39••]. In humans, microdeletion encompassing *RUNX1* causes Braddock-Carey syndrome and, as well as thrombocytopenia, cleft palate is among the characteristic features of this disease [40]. In mice, epithelial abrasion of *Runx1* results in anterior-specific cleft palate due to disintegration of the fusing epithelium; however, cleft palate is not induced in the secondary palate of the mutants [39••]. Previous studies have also demonstrated that the molecular and the cellular mechanism in the palatal fusion do not differ along the anterior-posterior axis and some molecules are specific to the either anterior or posterior palate [41]. Anterior cleft is a rare cleft phenotype in mutant mice and some signaling molecules and transcription factors, such as *Bmp4*, *Msx1*, *Shh*, and *Shox2* are specific to the anterior regions. *Shox2* null mutants and *Bmp4*-rescuing *Msx1* null mutants also showed anterior cleft [41]. In *Runx1* mutants, the expression of previously known anterior-specific molecules, such as *Bmp4*, *Msx1*, or *Shox2*, was not deviated; however, *Tgfb3* was significantly

downregulated at the primary palate. *Tgfb3* is strongly expressed in the fusing palatal epithelium and null mutation of *Tgfb3* results in complete cleft palate in mice (Fig. 2) [42]. On the other hand, the epithelial-specific ablation of *Tgfb3* results in anterior cleft [43], which is similar to the phenotypes in *Runx1* mutants. TGFB3 protein rescues *Runx1* mutants from anterior cleft, which suggests that *Tgfb3* could be a critical target molecule for Runx signaling in anterior palatal development [39••] and the Runx-Tgfb3 signaling axis is independent from molecular networks that have been previously reported to regulate palatogenesis.

Noteworthy, Runx1 regulates the phosphorylation of Stat3, which further regulates the expression of *Tgfb3* in the fusing epithelium. The application of Stat3 inhibitor remarkably downregulates the expression of *Tgfb3* and also disturbs the fusion between the primary and secondary palate. Interestingly, the inhibition of Stat3 also downregulates the expression of *Runx1*, indicating that Runx1 and Stat3 phosphorylation reciprocally regulate their activity. On the other hand, *Tgfb3* and *Runx1* are both expressed in all regions of the palatal fusion and the reason why *Runx1* deficiency causes anterior-specific cleft palate remains to be elucidated. One possible explanation is the involvement of the *Socs3* expression in the anterior palate [39••]. *Socs3* is a direct target molecule for Runx transcription factors and a negative regulator of Stat3 phosphorylation. Interestingly, Socs3 is localized at the anterior palate regions and *Runx1* deficiency results in the remarkable upregulation of *Socs3*, which may subsequently attenuate the phosphorylation of Stat3 [44••]. Another explanation is the functional redundancy between Runx1 and Runx2. Indeed, *Runx2* is expressed at the fusing palatal epithelium as well as the palatal mesenchyme; however, its expression at the palatal epithelium of the primary palate is not obvious [45]. To evaluate this hypothesis, a further analysis using double mutants is needed to confirm the roles of Runx signaling in palatal fusion.

Like Runx1, Cbfb is essential to mouse anterior palatogenesis. Epithelial *Cbfb* deletion results in anterior cleft palate, specifically due to the failed disintegration of the fusing epithelium, as is observed in *Runx1* mutants. *CBFb* haploinsufficiency due to interstitial deletion was shown to cause cleft palate and congenital heart anomalies in humans [46, 47]. In these mutants, the expression of *Tgfb3* is also disrupted in the area of failed palatal fusion, in which Stat3 phosphorylation is also affected. Stat3 signaling is activated in a number of cancers [48], in the immune response, and also in response to various environmental signals [49]. Hyper-IgE syndrome is caused by heterozygous mutations of *STAT3* and demonstrates primary immune deficiency and cleft palate is often observed in this syndrome [50]. However, the possible mechanisms underlying the involvement of Stat3 in the pathogenesis of the cleft palate remain be elucidated. In in vitro cultures of isolated primary palate tissue, a Stat3 inhibitor
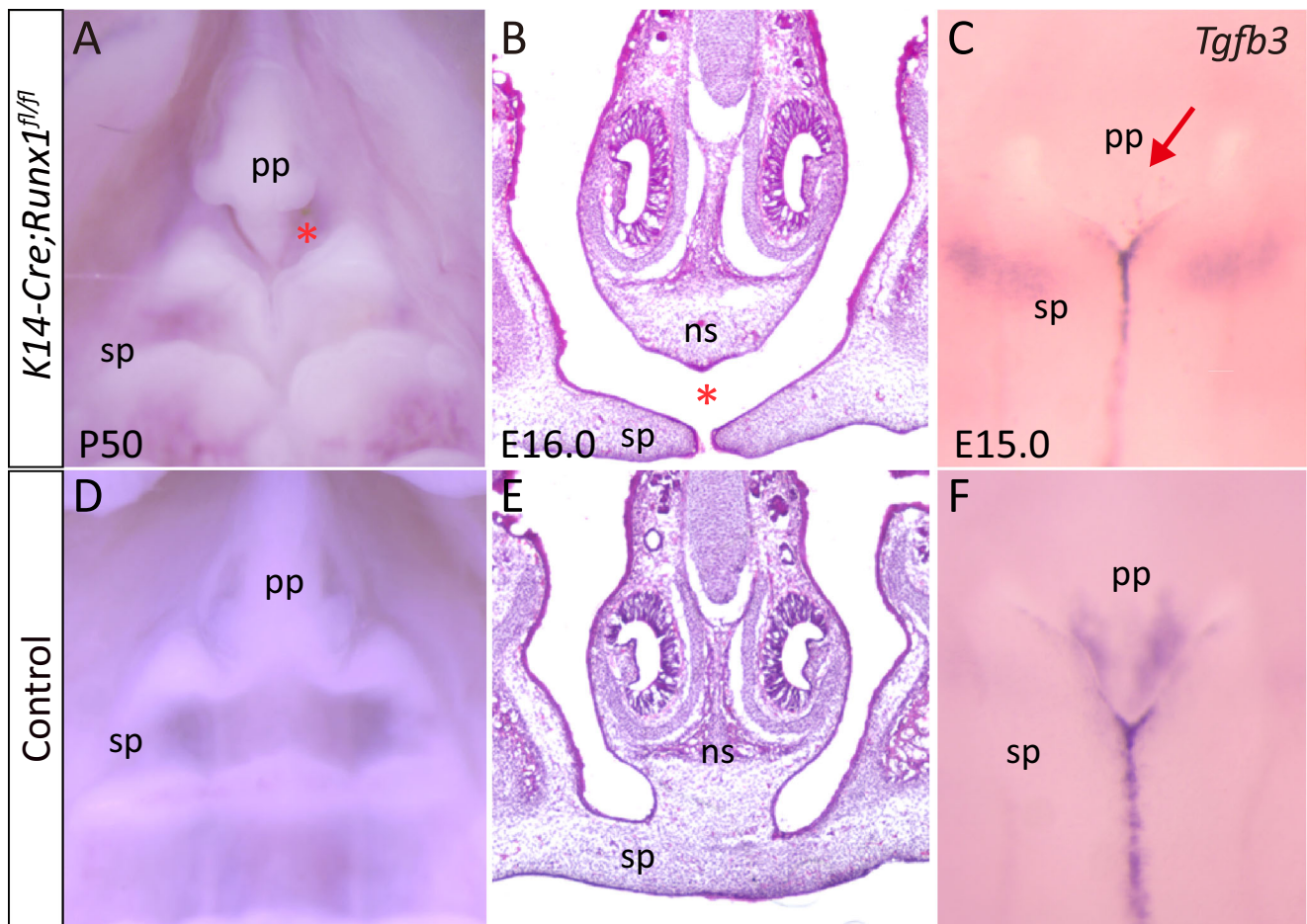
Fig. 2 Occlusal views of *K14-Cre*; *Runx1fl/fl* and control mouse palate demonstrate anterior cleft palate at the boundary between the primary and secondary palate at P50 (A, D). Frontal histological sections at 16.0 reveal failed palatal fusion in the *Runx1* mutant shelves (B, E). Whole-mount in situ hybridization analyses show that the expression of *Tgfb3* is markedly disturbed at the primary palate regions in *Runx1* mutant mice (C, F)

disturbed epithelial fusion with the remarkable downregulation of the expression of *Tgfb3*. Taken together, Stat3 signaling could mediate the functional roles of Runx1/Cbfb signaling in the induction of *Tgfb3* at the fusing palatal epithelium in the anterior palate [39••, 44••].

Prevention is the ultimate objective with regard to cleft palate. Non-syndromic cleft palate is a multifactorial genetic disease and the combination of genetic susceptibility factors and environmental risk factors can trigger pathologic palatogenesis. In our study, we identified Stat3 signaling as a downstream pathway of Runx1/Cbfb signaling. It is also known that Stat3 is activated by various molecular signals, such as EGF signaling and IL-6 signaling, and further activated by various environmental modifications and the application of various pharmaceutical agents. Indeed, maternal folic acid application attenuates the risk of cleft palate and also activates Stat3 phosphorylation [44••]. Actually, we demonstrated that folic acid rescues the cleft palate phenotype of the *Runx1* mutants with the activation of Tgfb3, suggesting that pharmaceutical modulation of Stat3 signaling could be used for preventing CP in parents with genetic mutations that affect Stat3 signaling [44••].

Summary and Future Directions

Regarding craniofacial development, the analysis of mutant phenotypes clarified various roles of Runx family genes in tooth, craniofacial, and salivary gland development. Furthermore, recent findings on the involvement of Runx signaling in cancer cells have provided a key understanding of the roles of Runx signaling in cellular differentiation, stem cell maintenance, and signal transduction in morphogenesis. Each gene of the Runx family often has the functional redundancy, which potentially complicates the evaluation of the roles of redundant genes and which also hinders the elucidation of the unknown roles of Runx genes. Future studies using tissue-specific multiple null mutant animals might reveal novel roles of Runx signaling.

Genetic studies have demonstrated that *Runx1* SNPs are associated with methacholine responsiveness, which is further

modified by a history of IUS exposure [51]. *Runx2* SNPs also influence susceptibility to CL/P through interaction with environmental tobacco smoke [52]. As discussed above, Runx1/Cbfb signaling affects Stat3 activity, and Runx1 might have some regulatory roles in the pathogenesis of disease. In the future, an understanding of the etiology and the mechanisms of Runx gene family–related diseases in combination with genetic and environmental factors might be useful for molecular diagnostics and pharmaceutical modulation for the prevention of such diseases.

Declarations

Conflict of Interest Takashi Yamashiro, Hiroshi Kurosaka, and Toshihiro Inubush declare no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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•• Of major importance

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