Roles of GASP-1 and GDF-11 in Dental and Craniofacial Development

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Purpose: Growth and differentiation factor (GDF)-11 is a transforming growth factor-β family member that plays important regulatory roles in development of multiple tissues which include axial skeletal patterning, palatal closure, and tooth formation. Proteins that have been identified as GDF-11 inhibitors include GDF-associated serum protein (GASP)-1 and GASP-2. Recently, we found that mice genetically engineered to lack both Gasp1 and Gdf11 have an increased frequency of cleft palate. The goal of this study was to investigate the roles of GDF-11 and its inhibitors, GASP-1 and GASP-2, during dental and craniofacial development and growth.

Methods: Mouse genetic studies were used in this study. Homozygous knockout mice for Gasp1 (Gasp1−/−) and Gasp2 (Gasp2−/−) were viable and fertile, but Gdf11 homozygous knockout (Gdf11−/−) mice died within 24 hours after birth. The effect of either Gasp1 or Gasp2 deletion in Gdf11−/− mice during embryogenesis was evaluated in Gasp1−/−;Gdf11−/− and Gasp2−/−;Gdf11−/− mouse embryos at 18.5 days post-coitum (E18.5). For the analysis of adult tissues, we used Gasp1−/−;Gdf11+/− and Gasp2−/−;Gdf11+/− mice to evaluate the potential haploinsufficiency of Gdf11 in Gasp1−/− and Gasp2−/− mice.

Results: Although Gasp2 expression decreased after E10.5, Gasp1 expression was readily detected in various ectodermal tissues at E17.5, including hair follicles, epithelium in nasal cavity, retina, and developing tooth buds. Interestingly, Gasp1−/−;Gdf11−/− mice had abnormal formation of lower incisors: tooth buds for lower incisors were under-developed or missing. Although Gdf11+/− mice were viable and had mild transformations of the axial skeleton, no specific defects in the craniofacial development have been observed in Gdf11+/− mice. However, loss of Gasp1 in Gdf11+/− mice occasionally resulted in small and abnormally shaped auricles.

Conclusions: These findings suggest that both GASP-1 and GDF-11 play important roles in dental and craniofacial development both during embryogenesis and in adult tissues.

Key Words: Cleft palate; Craniofacial abnormalities; GASP-1; GDF-11; Microtia; Tooth abnormalities

INTRODUCTION

Growth and differentiation factor (GDF)-11 is a transforming growth factor (TGF)-β family member closely related to myostatin (MSTN); GDF-11 and myostatin share 89% amino acid sequence identity and 96% sequence positives within the mature C-terminal region. However, targeted mutations of each Mstn and Gdf11 gene in mice exhibit distinct phenotypes: Mstn mutant mice show a dramatic increase in muscle mass while Gdf11 mutant mice show anteriorly directed transformations of the axial skeleton and a range of palatal anomalies. Nevertheless, due to the high degree of homology between myostatin and GDF-11, binding molecules that inhibit myostatin activity may also act similarly on GDF-11.

Several myostatin and GDF-11 binding proteins have been identified as GDF-11 inhibitors include GDF-associated serum protein (GASP)-1 and GASP-2. Recently, we found that mice genetically engineered to lack both Gasp1 and Gdf11 have an increased frequency of cleft palate. The goal of this study was to investigate the roles of GDF-11 and its inhibitors, GASP-1 and GASP-2, during dental and craniofacial development and growth.
identified, including GDF-associated serum protein (GASP)-1 and GASP-2. GASP-1 shares 54% amino acid sequence identity and 69% sequence positives with GASP-2 and has the same domain organization as GASP-2. Previously, we reported that native forms of Gasp1 and Gasp2 proteins generated in mammalian cells inhibit both myostatin and GDF-11 by blocking binding of the ligand to its receptor. These binding proteins exhibit distinct tissue/time specific expression patterns, which may contribute to differential regulation of myostatin and GDF-11 activities in specific tissues, resulting in differential effects on their growth and development.

Biological roles of GASP-1 and GASP-2 have been suggested in mouse models. Mice lacking both Gasp1 and Gasp2 have reduced muscle weights, a shift toward more oxidative fiber types, and impaired muscle regeneration ability, the reverse of what is seen in Mstn-/- mice. Mice lacking Gasp2 have posteriorly directed transformations of the axial skeleton, in contrast with what is seen in Gdf11-/- mice. Although Gasp1-/- mice did not show any phenotype related to the axial skeletal patterning, loss of Gasp1 in Gdf11-/- mice dramatically increased the frequency of cleft palate. Interestingly, Gdf11 has also been reported as an important regulator in tooth development by inducing differentiation of pulp stem cells into odontoblasts for reparative dentin formation. However, dental phenotypes have not been evaluated in relation to GDF-11 inhibitors.

The goal of this study was to investigate the roles of GDF-11 and its binding molecules during dental and craniofacial development. Specifically, we evaluated genetically engineered mice that lack various combinations of genes, including Gdf11 and its binding molecules, Gasp1 and Gasp2.

**MATERIALS AND METHODS**

1. **Mice**

Gasp1, Gasp2, and Gdf11 knockout mice have been described previously. Gasp1 and Gasp2 homozygous knockout (Gasp1-/- and Gasp2-/-) mice were viable and fertile, but Gdf11 homozygous knockout (Gdf11-/-) mice died within 24 hours after birth. To analyze the effect of Gasp1 deletion in Gdf11-/- mice, Gasp1+/- mice were mated with Gdf11+/- mice. Gasp1+/-;Gdf11+/- mice from this cross were intercrossed to obtain Gasp1+/-;Gdf11+/- mice, and Gasp1+/-;Gdf11+/- mice were intercrossed again to obtain Gasp1+/-;Gdf11+/- mouse embryos. Similar strategies were employed to obtain Gasp2+/-;Gdf11+/- mouse embryos. All mice were handled and housed according to the protocols that were approved by the Institutional Animal Care and Use Committees at Johns Hopkins Medical Institutions.

2. **In Situ Hybridization**

For analysis of Gasp1 expression patterns, full-length cDNA of Gasp1 was used as a probe for in situ hybridization. Time-mated C57BL/6 female mice were harvested to collect embryos on 17.5 days post-coitum (E17.5). Embryo heads were embedded in optimal cutting temperature (OCT) compound and frozen rapidly using dry ice. Frozen tissues were coronally-sectioned at 10 µm and stained as described.

3. **Histopathologic Analysis**

To evaluate development of craniofacial tissues in Gasp1-/-; Gdf11-/- mice, female mice from the intercrosses between Gasp1-/-;Gdf11+/- mice were harvested to collect embryos at E18.5. For histologic analysis, embryo heads were embedded in OCT compound, and 10 µm frozen coronal-sections were taken and subjected to H&E. Embryo livers were used to extract DNA for genotyping.

**RESULTS**

We examined the expression patterns of Gasp1 to determine whether it is expressed in tissues that would be consistent with regulation of GDF-11 function. As we previously reported, the expression of Gasp1 in adult mice was detected in many different tissues, including skeletal muscle, which is the predominant site of myostatin expression. Here, we examined the expression patterns of Gasp1 during embryogenesis. Although we were unable to detect any expression of Gasp1 at E9.5 by whole-mount in situ hybridization (data not shown), Gasp1 expression was readily detected in various ectodermal tissues at E17.5, including hair follicles, epithelium in nasal cavity, retina, and developing tooth buds (Fig. 1). In contrast, Gasp2 expression decreased after E10.5, and in adult tissue, its expression was limited.
to testis. These distinct tissue expression patterns during development may contribute to differential regulation of myostatin and GDF-11 activities in specific tissues including muscle, tooth, palate, and ear.

Mice lacking Gdf11 (Gdf11<sup>-/-</sup>) have been shown to exhibit a range of palatal defects with approximately 60% penetrance of cleft palate (i.e., 12/20 Gdf11<sup>-/-</sup> mice analyzed have cleft palate). Interestingly, although nearly all mice lacking Gasp1 exhibited normal palate development, loss of Gasp1 had a dramatic effect on the frequency of palatal defects in Gdf11<sup>-/-</sup> mice, with 15/15 of Gasp1<sup>-/-</sup>;Gdf11<sup>-/-</sup> mice analyzed having cleft palate. Interestingly, GDF-11 has also been previously reported as an important regulator in tooth development by inducing differentiation of pulp stem cells into odontoblasts for reparative dentin formation. To evaluate possible dental phenotypes, we examined tooth development in Gasp1<sup>-/-</sup>;Gdf11<sup>-/-</sup> and Gasp1<sup>-/-</sup>;Gdf11<sup>+/+</sup> mice, and found abnormal formation of lower incisors in addition to cleft palate in Gasp1<sup>-/-</sup>;Gdf11<sup>-/-</sup> mice: tooth buds for lower incisors were under-developed or missing (Fig. 2).

Not all the tissues in the craniofacial region complete their developmental processes during embryogenesis. The external ear (also known as auricle) is one of them. Mice are born with closed ears, as the main body of the auricle folds over to cover the external auditory canal until birth. Ears appear as nubs until 2 to 3 days and open at 3 to 5 days as the auricles start to unfold and come away from the head. The auricles continue to grow throughout the lifetime. As Gdf11<sup>-/-</sup> mice died perinatally, we were unable to observe the postnatal growth patterns of the auricles in those mice. No specific defects in the craniofacial development have been observed in either Gdf11<sup>-/-</sup> or Gasp1<sup>-/-</sup> mice.

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**Fig. 1.** Expression patterns of Gasp1 by in situ hybridization of mouse embryos at 17.5 days post-coitum (E17.5). (A-E) Drawing represents a lateral view of the head of a mouse embryo with the planes of coronal sections. Although Gasp1 expression was not observed at E9.5, Gasp1 is highly expressed in most of ectodermal tissues at E17.5 which include hair follicles (h.f.), epithelial cells in nasal cavity (n.c.), retina (r.), and developing tooth buds (arrows).

**Fig. 2.** Craniofacial defects in Gasp1<sup>-/-</sup>; Gdf11<sup>-/-</sup> mouse embryos. H&E stained coronal sections of the mouse heads at 18.5 days post-coitum (E18.5). (A) Normal palate (p) and developing tooth buds (arrows, upper fours for molars and lower two for incisors) are found in Gasp1<sup>-/-</sup>;Gdf11<sup>-/-</sup> mouse. (B) The palatal shelves fail to fuse, and tooth buds for lower incisors are under-developed (left) or missing (right) in Gasp1<sup>-/-</sup>; Gdf11<sup>-/-</sup> mice.
Interestingly, however, loss of Gasp1 in Gdf11−/− mice occasionally resulted in small and abnormally shaped auricles (also known as microtia) (Fig. 3). Loss of Gasp2 in Gdf11−/− mice did not result in microtia.

**DISCUSSION**

GDF-11 has been reported as an important regulator in palatal formation during embryogenesis: mice lacking Gdf11 (Gdf11−/−) have been shown to exhibit a range of palatal defects. GDF-11 has also been reported to be expressed in terminally-differentiated odontoblasts and act as an important regulator in tooth development. Overexpression of Gdf11 in vivo stimulated the reparative dentin formation during pulpal wound healing by inducing differentiation of pulp stem cells into odontoblasts. 

Although myostatin is a TGF-β family member closely related to GDF-11, Mstn mutant mice did not exhibit phenotypes related to skeletal defects including palatal formation and dental malformation. However, mice lacking both Mstn and Gdf11 (Mstn−/−;Gdf11−/−) had more extensive transformations of the axial skeleton than mice lacking only Gdf11 and also had other skeletal defects, including cranial and forelimb digit defects, which were not seen in Gdf11−/− mutants. Mstn−/−;Gdf11−/− mice showed severe craniofacial microsomia and occasionally exhibited extra limbs as compared to Gdf11−/− mice. These findings suggest that myostatin may also play an important role in skeletal formation.

Due to the high degree of homology between myostatin and GDF-11, proteins that inhibit myostatin activity generally act similarly on GDF-11, and it has been reported that both Gasp1 and Gasp2 inhibit both myostatin and GDF-11 by blocking binding of the ligand to its receptor. By using mutant mice that lack various combinations of myostatin/GDF-11 inhibitors in a Gdf11 null background, we showed that loss of Gasp1 in Gdf11−/− mice dramatically increased the frequency of abnormal formation of lower incisors in addition to cleft palate, but Gasp2 deletion in Gdf11−/− mice did not increase the frequency of dental malformation and cleft palate. Because homozygous deletion of Gdf11 causes perinatal lethality in mice, we maintained Gasp1−/−;Gdf11−/+ and Gasp2−/−;Gdf11−/+ mice for the intercrosses to obtain Gasp1−/−;Gdf11−/+ and Gasp2−/−;Gdf11−/+ mouse embryos. Interestingly, we found that loss of Gasp1 occasionally developed microtia in Gdf11−/− mice while loss of Gasp2 did not develop microtia in Gdf11−/− mice. The mechanism how Gasp1 deletion in Gdf11−/− and Gdf11−/+ mice interrupts processing of dental and craniofacial development remains unknown. However, considering a possible role of myostatin in skeletal formation and the functional redundancy of myostatin and
GDF-11, disinhibition of myostatin activity by removing myostatin inhibitor Gasp1 may trigger disorganized development in Gdf11<sup>−/−</sup> and Gdf11<sup>+/−</sup> mice.

Microtia is a congenital anomaly of the external ear that ranges in severity from mild structural abnormalities to complete absence of the ear and a common feature of craniofacial microsomia. Although some candidate genes responsible for microtia have been identified, the etiology largely remains unknown. Interestingly, a few TGF-β family members such as bone morphogenetic protein-5<sup>12,13</sup> and GDF-6<sup>8,14</sup> were reported as candidate genes. Therefore, it was not surprising to find that GDF-11, also a TGF-β family member, may play an important role in ear formation.

It is important to understand the pathogenesis of dysmorphic tooth formation and craniofacial defects to improve dental management in patients with these symptoms. Our study suggests that GASP-1 and GDF-11 may play important roles in dental and craniofacial development. However, in the current study, the biological evaluation of homozygous deletion of Gdf11 gene had to be focused on embryonic stages due to perinatal lethality. Further studies using conditional knockout techniques in genetically engineered mice will be essential in evaluating phenotypes induced by tissue and cell specific deletion of Gdf11 gene at specific time points.

**CONFLICT OF INTEREST**

No potential conflict of interest relevant to this article was reported.

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**REFERENCES**