From shape to cells: mouse models reveal mechanisms altering palate development in Apert syndrome

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SUMMARY

Apert syndrome is a congenital disorder characterized by severe skull malformations and caused by one of two missense mutations, S252W and P253R, on fibroblast growth factor receptor 2 (FGFR2). The molecular bases underlying differential Apert syndrome phenotypes are still poorly understood and it is unclear why cleft palate is more frequent in patients carrying the S252W mutation. Taking advantage of Apert syndrome mouse models, we performed a novel combination of morphometric, histological and immunohistochemical analyses to precisely quantify distinct palatal phenotypes in Fgfr2+/S252W and Fgfr2+/P253R mice. We localized regions of differentially altered FGF signaling and assessed local cell patterns to establish a baseline for understanding the differential effects of these two Fgfr2 mutations. Palatal suture scoring and comparative 3D shape analysis from high resolution μCT images of 120 newborn mouse skulls showed that Fgfr2+/S252W mice display relatively more severe palate dysmorphologies, with contracted and more separated palatal shelves, a greater tendency to fuse the maxillary-palatine sutures and aberrant development of the inter-maxillary suture. These palatal defects are associated with suture-specific patterns of abnormal cellular proliferation, differentiation and apoptosis. The posterior region of the developing palate emerges as a potential target for therapeutic strategies in clinical management of cleft palate in Apert syndrome patients.

INTRODUCTION

Apert syndrome [OMIM 101200] is a rare congenital disorder with disease prevalence of 15–16 per million live births. Patients with Apert syndrome are characterized by premature fusion of the coronal suture(s) and severe craniofacial dysmorphology (Cohen and MacLean, 2000), but also exhibit many other developmental defects, including limb abnormalities, heart and lung defects, as well as neural malformations that could lead to cognitive impairment (Cohen and MacLean, 2000). In humans, there are at least 70 nucleotide substitutions that alter the amino acid sequence of fibroblast growth factor receptor (FGFR) genes (Hébert, 2011). Two of these, Ser252Trp (S252W) and Pro253Arg (P253R), occur on neighboring amino acids on the linker region between the second and third extracellular immunoglobulin domain on fibroblast growth factor receptor 2 (FGFR2) and together are responsible for almost 99% of reported cases of Apert syndrome. Approximately 67% of individuals with Apert syndrome have a FGFR2 S252W mutation, while the remaining 33% carry the FGFR2 P253R mutation (Park et al., 1995; Wilkie et al., 1995). The phenotypic outcome of Apert syndrome mutations is generally similar, such that genetic testing is required to identify the causative mutation. Comparative analyses suggest only highly localized phenotypic differences between the two FGFR2 mutations (Slaney et al., 1996). Cleft palate is 3.5 times more frequent in patients carrying the S252W mutation, whereas digit fusion (i.e. syndactyly) is more severe in patients with the P253R mutation (Slaney et al., 1996). These qualitative analyses are, however, based on relatively small samples of patients and details of differential effects of FGFR2 mutations on patients with Apert syndrome remain poorly understood.

Given the relatively low prevalence of Apert syndrome in humans, analysis of appropriate and representative mouse models for Apert syndrome, such as Fgfr2+/S252W and Fgfr2+/P253R mice (Wang et al., 2005; Wang et al., 2010), is crucial for an understanding of the developmental mechanisms underlying the phenotypic differences among patients carrying one FGFR2 mutation or the other. The correspondence between Apert syndrome mouse models and human patients with Apert syndrome has been demonstrated at the morphological, histological and molecular levels (Chen et al., 2003; Wang et al., 2005; Wang et al., 2010; Holmes et al., 2009; Aldridge et al., 2010; Du et al., 2010; Martinez-Abadias et al., 2010; Martinez-Abadias et al., 2011), validating the use of large experimentally controlled samples of mouse models to elucidate the complex etiology of Apert syndrome. Using a comparative sample of newborn Fgfr2+/S252W and Fgfr2+/P253R Apert syndrome mouse models and their unaffected littermates (n=73) we found that murine skull dysmorphologies parallel those described in Apert syndrome patients, and detected significant morphological differences between the two Apert syndrome mouse models (Martinez-Abadias et al., 2010). A particular highly localized difference at the posterior region of the palatine bone prompted us to perform the current detailed analysis of the palate of Apert syndrome mouse models. Here, we analyze with higher precision the palatal dysmorphologies of Fgfr2+/S252W mice.
**TRANSLATIONAL IMPACT**

**Clinical issue**
Apert syndrome is a rare congenital disorder characterized by skull malformations and facial abnormalities. Almost 100% of Apert syndrome cases are caused by one of two amino acid substitutions, S252W or P253R, in a protein involved in FGF/FGFR signaling, fibroblast growth factor receptor 2 (FGFR2). Though the causative FGFR2 mutations have been identified, we still have little understanding of how they contribute to abnormal craniofacial phenotypes. Despite overall similarity between patients carrying one FGFR2 mutation or the other, it has been proposed that patients carrying the S252W mutation present with more severe palatal dysmorphology than patients carrying the P253R mutation. In addition, cleft palate occurs 3.5 times more frequently in the former group. In humans, it is difficult to differentiate the specific effects of each mutation because of the relatively low incidence of the disease and high level of phenotypic variation. For this reason, mouse models have become a valuable tool for determining the molecular mechanisms that alter palate development and lead to cleft palate in Apert syndrome.

**Results**
To determine how the two major Apert syndrome mutations lead to different palatal dysmorphologies, this study utilizes Fgfr2+/S252W and Fgfr2+/P253R mouse models. The authors report anatomical differences between the two mutant mouse models in terms of size and shape of the palatine bones, and demonstrate that the most striking abnormalities are associated with the Fgfr2 S252W mutation. These include patency of the inter-premaxillary suture, a wider separation of the palatine shelves and fusion of the suture between the palatine bones and maxilla, bones that together define the secondary palate. Furthermore, the authors’ cellular analysis reveals suture-specific aberrant cellular proliferation, differentiation and apoptosis in newborn mice.

**Implications and future directions**
This work shows that the two Apert syndrome mutations affect the development of the mouse palate in different ways. The results provide further evidence that the S252W mutation causes more severe palatal phenotypes than the P253R mutation. However, in line with previous analyses of craniofacial phenotypes, these results also suggest that ‘severity’ may be a highly localized phenomenon and that the local effects of altered FGF/FGFR signaling should be considered cell- and tissue-specific. Among the localized anatomical sites demonstrating perturbed FGF/FGFR signaling as a result of the S252W mutation is the posterior region of the palate, revealing a potential target for new therapeutic strategies in the clinical management of Apert syndrome.

RESULTS
To comparatively assess the effects of FGFR2 mutations on palate morphology, we analyzed patterns of palatal shape variation using geometric morphometric (GM) methods (Dryden and Mardia, 1998; Lele and Richtsmeier, 2001). Three-dimensional (3D) coordinates of a set of ten landmarks located along the medial aspects of the horizontal plate of the right and left palatine bones were collected from the reconstructed isosurfaces of the μCT images of each specimen (Fig. 1, Table 1) using heads of newborn Fgfr2+/S252W (n=24) and Fgfr2+/P253R (n=41) Apert syndrome mice, as well as their unaffected littermates that do not carry the mutation and that we use as controls (Fgfr2+/− S252W, n=26; Fgfr2+/− P253R, n=29). We assessed the pattern of fusion of five palatal sutures (inter-premaxillary, inter-maxillary, inter-palatine, right and left maxillary-palatine), qualitatively scoring the sutures as patent, partially fused or completely fused as visualized on μCT (see Fig. 1) (see Materials and Methods for more details).
Palatal dysmorphology in Apert syndrome

**Differential palate dysmorphologies in Fgfr2<sup>+/S252W</sup> and Fgfr2<sup>+/P253R</sup> Apert syndrome mice**

Palate shape information was extracted from the landmark coordinates using a General Procrustes Analysis (GPA), a procedure that superimposes configurations of landmarks and adjusts for the effects of orientation and scale (Dryden and Mardia, 1998). Allometric shape variation related to size was accounted for statistically by computing a multivariate regression of shape on size (see Materials and Methods for details). We used the allometry-adjusted Procrustes coordinates as input data for canonical variates analysis (CVA), a discriminant analysis used to explore shape variation and to maximize separation between the four pre-defined groups of newborn mice, including: Fgfr2<sup>+/S252W</sup> and Fgfr2<sup>+/P253R</sup> Apert syndrome mice and their respective unaffected littermates.

The first two canonical variates (CV1 and CV2) explain almost all the morphological variation of the sample (95.92%) and distribute the specimens into three different clusters: a cluster of Fgfr2<sup>+/P253R</sup> Apert syndrome mice, a cluster of unaffected littermates from both models and a cluster of Fgfr2<sup>+/S252W</sup> Apert syndrome mice (Fig. 2A). The CVA showed that the main differences in palatal shape occur between Fgfr2<sup>+/S252W</sup> and Fgfr2<sup>+/P253R</sup> Apert syndrome mice, which are distributed at the positive and negative extremes of the first canonical axis, respectively (Fig. 2A). Unaffected littermates show an intermediation position along CV1 (Fig. 2A).

Comparison of the associated shape changes corresponding to the extreme negative and positive CV1 values (Fig. 2B) shows that the palatine bones are contracted along the antero-posterior and medio-lateral axes in Fgfr2<sup>+/S252W</sup> Apert syndrome mice (Fig. 2Bb). In comparison to the mean shape, the anterior landmarks located along the outline of the palatine bones of Fgfr2<sup>+/S252W</sup> mice are shifted to a more posterior position (landmarks 1-4), whereas the posterior landmarks are shifted to a more anterior position (landmarks 5-10), and all landmarks of the palatal shelves are displaced laterally (Fig. 2Bc).

Table 1. Anatomical definitions of collected palatal landmarks displayed in Fig. 1

| Landmark (right, left) | Anatomical definition |
|------------------------|-----------------------|
| (1) ralp, (2) lalp     | Most anterolateral point on the posterior palatine plate |
| (3) ramp, (4) lamp     | Most anteromedial point on the posterior palatine plate |
| (5) rpmp, (6) lpmp     | Most posteromedial point on the posterior palatine plate |
| (7) rmps, (8) lmps     | Most anterolateral indentation at the posterior edge of the palatine plate |
| (9) rplpp, (10) lplpp  | Most posterolateral point on the posterior palatine plate |

For more information visit [http://www.getahead.psu.edu/LandmarkNewVersion/P0_Mouse_Palate.html](http://www.getahead.psu.edu/LandmarkNewVersion/P0_Mouse_Palate.html).
GDMA confidence interval testing (Lele and Richtsmeier, 1995; Lele and Richtsmeier, 2001) reveals significant differences in the way that the horizontal plates of the palatine bones of Fgfr2+/S252W and Fgfr2+/P253R Apert syndrome mice differ from their respective unaffected littermates (α=0.10). These analyses support the results of Procrustes-based analyses and provide statistical evidence of the differences in the localized effects of these two neighboring FGFR2 mutations on palatal development. GDMA confidence intervals demonstrate that the distance between the most anterolateral aspect of the horizontal plate of the palatine bone (where the palatine plates meet and eventually fuse with the maxillary alveolus; between landmarks 1 and 2) is increased relative to unaffected littermates in the Fgfr2+/S252W mutant mice but reduced relative to unaffected littermates in Fgfr2+/P253R mice. Posteriorly (between landmarks 7 and 8), the palate of Fgfr2+/S252W Apert syndrome mice is wider, but there is no difference between Fgfr2+/P253R mice and unaffected littermates. The horizontal plates of the palatine bones are also more profoundly reduced along the anteroposterior axis in Fgfr2+/S252W Apert syndrome mice. Together these observations suggest that the positioning of the maxillary alveolus and deficiency in development of the palatine plate contribute to the differences in the effects of the two FGFR2 mutations.

**More severe palate dysmorphologies in Fgfr2+/S252W Apert syndrome mice**

To statistically test the degree of differentiation in palatal shape between groups, we computed the Mahalanobis distances between all possible pairs of groups (Klingenberg and Monteiro, 2005). Results indicate that based on palatine morphology, Fgfr2+/S252W and Fgfr2+/P253R Apert syndrome mice are significantly different from each other as well as from their unaffected littermates, even after correcting for multiple testing by Bonferroni (Table 2). Unaffected littermates from S252W and P253R models are not significantly different from each other (Table 2). If we consider the Mahalanobis distance from each mutant Apert mouse model to their respective unaffected littermates as a measure of severity of the palatine dysmorphology, our results confirm that the palates of Fgfr2+/S252W Apert syndrome mice are more severely affected than those of Fgfr2+/P253R Apert syndrome mice, because the Mahalanobis distance between mutant and unaffected littermates is 1.4 times greater in Fgfr2+/S252W mice (Table 2).

**Table 2. Pairwise Mahalanobis distances between Fgfr2+/S252W and Fgfr2+/P253R Apert syndrome mice and their unaffected littermate controls**

|        | Fgfr2+/S252W | Fgfr2+/S252W | Fgfr2+/P253R | Fgfr2+/P253R |
|--------|--------------|--------------|--------------|--------------|
| Fgfr2+/S252W | <0.0001      |<0.0001       |<0.0001       |              |
| Fgfr2+/S252W |                |              |<0.0001       | 0.29         |
| Fgfr2+/P253R | 4.07*        | 2.70*        |              |<0.0001       |
| Fgfr2+/P253R | 3.40*        | 1.31         | 2.06*        |              |

Lower left off-diagonal matrix: Mahalanobis distance values. Upper right off-diagonal matrix: associated P-values estimated after 10,000 permutation rounds. *Statistically significant values after Bonferroni’s correction (P>0.05/6=0.008).
Disease Models & Mechanisms

DMM

RESEARCH ARTICLE

Differential fusion patterns of palatal sutures in Apert syndrome mouse models

Comparison of the qualitative scoring of palatal suture patency showed that the inter-maxillary and the inter-palatine sutures are patent (~75-100% of cases) or just partially fused (~0-25%) in all four groups (Fig. 3). Although there are no significant differences between mutant and unaffected littermates in terms of the fusion pattern of these two midline sutures, Fgfr2+/S252W Apert syndrome mice show a greater degree of patency of the inter-palatine suture (Fig. 3).

Fgfr2+/S252W and Fgfr2+/P253R Apert syndrome mice show a higher tendency towards patency (~60%) or partial fusion (~20-40%) of the inter-premaxillary suture relative to their unaffected littermates, in which the inter-premaxillary suture is usually fused (~40-95%) or partially fused (~60-5%) at P0 (Fig. 3). Apparent differences in the pattern of fusion of the inter-premaxillary suture between the two groups of unaffected littermates are not relevant. Complete inter-premaxillary suture fusion (FFF) was observed in 22 out of 23 unaffected littermates of the S252W model, whereas 26 out of 30 unaffected littermates of the P253R model have the inter-premaxillary suture completely (FFF) or almost completely fused (PFF). The palates of unaffected littermates from both Apert syndrome mouse models are thus morphologically similar and comparable, as confirmed by the rest of the morphometric analyses (Fig. 2; Table 2).

Finally, we identified a difference in fusion patterns of the maxillary-palatine sutures between Fgfr2+/S252W and Fgfr2+/P253R Apert syndrome mice. In unaffected littermates of both models and in Fgfr2+/P253R Apert syndrome mice, the right and left maxillary-palatine sutures are patent (~70-95%) or just partially fused (~5-30%) (Fig. 3B-D), whereas these sutures are either partially (~35%) or completely fused (~25%) in Fgfr2+/S252W Apert syndrome mice at P0 (Fig. 3A).

Abnormal palate development in Fgfr2+/S252W Apert syndrome mice

Patterns of cell proliferation, differentiation and apoptosis were assessed to establish a baseline for understanding the effects of the FGFR2 mutations on localized cellular behaviors that underlie Apert syndrome. We focused our comparative histological analysis and immunohistochemical assays on the most significant differential effects detected on the palate morphology of Fgfr2+/S252W Apert syndrome mice at P0: the greater degree of fusion of the maxillary-palatine sutures, the greater separation of palatine bones at the midline and the higher tendency towards inter-premaxillary suture patency. Similar analyses of Fgfr2+/P253R Apert syndrome mice were not performed because the fusion pattern of the right/left maxillary-palatine in Fgfr2+/P253R mutant mice mimics the pattern of unaffected littermates (Fig. 3) and palatal shape changes in Fgfr2+/S252W mice are relatively mild, with morphologies that overlap with those of unaffected littermates (Fig. 2A).

Histological analyses confirmed that the maxillary-palatine sutures in Fgfr2+/S252W Apert syndrome mice commonly presented with localized partial premature fusion, showing variably fused osteogenic fronts with deposition of osteoid as compared with the distinct osteogenic fronts separated by undifferentiated mesenchymal cells in the unaffected littermates (Fig. 4A,B). In Fgfr2+/P253R mutant mice, the maxillary-palatine suture presents the overlap typical of the unaffected littermates, although the mesenchymal interface between the two bones is often less regular in mutants (Fig. 4A,B and not shown). Alkaline phosphatase (ALP) activity, an early marker of osteogenesis, is low or absent in normal suture mesenchyme (Fig. 4C). In the mutant suture, ALP is similarly absent from half the suture mesenchyme dorsal to the central locus of fusion, but appears in the half ventral to the point of fusion (Fig. 4D). Cell proliferation assessed by Ki67 staining showed similar numbers of positive cells in the unfused mutant suture mesenchyme and adjacent osteogenic faces as compared with sutures of unaffected littermates. Proliferation was maintained even as ALP activity rose within the mesenchyme of fusing sutures (Fig. 4E,F,O). Runx2 expression, an additional marker of osteoblast differentiation, was similar in mutants and unaffected littermates, being higher in osteoblasts closer to bone and lower in the suture mesenchyme (Fig. 4G,H). As revealed by TUNEL staining, there was almost no
apoptosis in the suture mesenchyme of unaffected littermates, but a low frequency of apoptosis was evident within the palatine and maxillary bones (Fig. 4I). In mutant sutures, the distribution and frequency of apoptotic cells was similar to sutures of unaffected specimens, even in the area of increased ALP activity. However, focal areas of intense apoptosis occurred in fusing sutures at the point where osteoid deposition bridges the overlapping bones in Fgfr2+/S252W mice (Fig. 4J). Antibody detection of the phosphorylated forms of the MAPK p38 and ERK1/2, major downstream effectors of FGFR signaling, showed no appreciable differences between mutant and unaffected littermates, although signals were decreased in the area of fusion dominated by apoptotic cells (Fig. 4K-N).

Histological analyses of the inter-palatine suture revealed palatine bones of Fgfr2+/S252W mice to be thicker and more robust relative to unaffected littermates (Fig. 5A,B). Both the osteogenic mesenchyme of the suture and the non-osteogenic mesenchyme ventral to the palatal bones were also thicker. Patency of the midline inter-palatine suture in both mutant and unaffected littermates at P0 (Fig. 5A,B), as well as the relatively wider separation of the palatine shelves in mutants detected by the CVA analysis was evident histologically (Fig. 5C,D). Ki67 staining showed no proliferation differences in either the osteogenic fronts of the palatine shelves or the intervening suture mesenchyme (Fig. 5E,F,O). Runx2 expression levels and distribution were similar in mutants and unaffected littermates, although the increased thickness of the mutant osteogenic mesenchyme is evident by the wider domain of Runx2 expression (Fig. 5G,H). Apoptotic cells, indicated by TUNEL staining, were infrequent among mesenchymal cells or within osteoblasts of both Fgfr2+/S252W Apert syndrome mice and unaffected littermates (Fig. 5I,J). Immunohistochemical staining for phosphorylated p38 and ERK1/2 showed no difference between mutants and unaffected littermates (Fig. 5K-N).

Histological analyses of the inter-premaxillary suture revealed striking differences between unaffected littermates and Fgfr2+/S252W mutants. The paired premaxillary bones in unaffected littermates are broad, consisting of trabecular bone ventrally, and are separated at the midline by a thick mesenchyme. Each premaxilla extends vertically as a thin compact sheet of bone curving around the overlying vomeronasal cartilage to end adjacent to the cartilage of the nasal septum. The mesenchyme separating these vertical sheets is sparse and flattened osteoblasts line their surface, separated from the vomeronasal cartilage on the opposite side by a mesenchyme a few cells in thickness (Fig. 6A). In contrast to unaffected littermates, the mutant premaxillary bones are thicker ventrally and extend vertically as broad trabecular wedges tapering to points adjacent to the cartilage of the nasal septum. The thin compact sheets of bone
seen in unaffected littermates seem to be retained in these wedges, forming the lateral side curving along the vomeronasal cartilages, from which trabecular bone extends medially. The mesenchyme of $Fgfr2^{+/S252W}$ mice separating these bones remains thick as it extends dorsally, but typically thins below the nasal septum. The septal cartilage is often misaligned with the intermaxillary suture mesenchyme in the mutant suture. Only a single cell layer typically separates the premaxillary bone from the vomeronasal cartilage. The vertical thickening of the intermaxillary bones and intervening mesenchyme greatly widens the distance between the paired vomeronasal organs in mutants compared with unaffected littermates (Fig. 6B).

Ki67 staining showed that the proportion of proliferating cells was significantly increased within the midline mesenchyme separating premaxillary bones in $Fgfr2^{+/S252W}$ mice compared with unaffected littermates. This difference was especially notable comparing the dorsal portion of the sutures (Fig. 6C,D,M). The expression levels of Runx2 were similar in $Fgfr2^{+/S252W}$ mice and unaffected littermates, with high expression in osteoblasts and low expression in the intervening mesenchyme, but this mesenchyme was more extensive in the mutant suture. The difference in the cellularity of the medial and lateral faces of the premaxillary bones is also readily appreciated with Runx2 imaging, with more Runx2-expressing osteoblasts lining the medial surfaces and fewer lining the lateral surfaces of mutant premaxillary bones compared with unaffected littermates (Fig. 6E,F). ALP activity levels were also similar in $Fgfr2^{+/S252W}$ mice and unaffected littermates, but decreased strongly in the sparse mesenchyme separating the vertical bone extensions in unaffected littermates (Fig. 6G,H). Apoptotic cells detected by TUNEL staining were infrequent in the suture mesenchyme but were common in the adjacent trabecular bone of both mutants and unaffected littermates and occurred with significantly increased frequency in the $Fgfr2^{+/S252W}$ mice (Fig. 6G,H,N). The levels of phosphorylated p38 (Fig. 6I,J) and phosphorylated ERK1/2 (Fig. 6K,L) were similar in mutant sutures and unaffected littermates.

**DISCUSSION**

Our results demonstrate the differential effects of the $S252W$ and P253R FGFR2 mutations on palatal morphogenesis and reveal the aberrant cellular behaviors associated with the phenotypic differences between $Fgfr2^{+/S252W}$ and $Fgfr2^{+/P253R}$ Apert syndrome mice. The morphometric analyses and the detailed scoring of palatal suture patency detected significant palatal shape differences between $Fgfr2^{+/S252W}$ and $Fgfr2^{+/P253R}$ Apert syndrome mice and underscored what has been reported in humans: individuals carrying the $S252W$ mutation show more severe palatal dysmorphologies. The histological and immunohistochemical analyses highlighted that these palatal defects are highly localized and suture-specific and can be associated with dynamic combinations of abnormal patterns of cellular proliferation, differentiation and apoptosis. The extent of these changes at P0 suggests their beginnings earlier in embryogenesis.
Fig. 6. Inter-premaxillary suture at P0. (A,B) H&E staining shows that mutant sutures present with aberrant trabecular overgrowth of the vertical extensions of the premaxillary bones (pm), thickening of the intervening suture mesenchyme and misalignment of the suture with the cartilage of the nasal septum (s). Widening of the mutant suture is evident in the greater separation of the cartilages (c) encapsulating the vomeronasal organs. (C,D,M) Immunofluorescent staining for Ki67 (gray channel shown) shows increased proliferation in mutant suture mesenchyme compared with unaffected littersmates. (E,F) Immunofluorescent staining for Runx2 (gray channel shown) shows similar expression levels between unaffected littermates and mutant mouse, although mutant suture mesenchyme is more abundant and highly Runx2-positive cells are more numerous along the bone-mesenchyme interface. (G,H,N) TUNEL staining (green) shows minimal apoptosis within the suture mesenchyme of unaffected littermates and mutant mice but a high frequency in the adjacent osteogenic domains (stained for ALP activity; red). In these regions, apoptosis is significantly higher in mutants compared with unaffected littersmates. (I-L) Phospho-p38 and phospho-ERK 1/2 levels are similar in mutant suture mesenchyme relative to unaffected littermates; *P<0.035. (M) Proliferation is significantly increased in mutant suture mesenchyme compared with unaffected littermates; *P=0.026. Images from A-L are from near-adjacent sections from the same littermate pair. Scale bar: 100 μm.

From bone palatal defects to cellular and molecular alterations in Apert syndrome mouse models

Differential palatal defects in Fgfr2+/S252W and Fgfr2+/P253R mutant mice involve gross shape changes of the palatine bones (Fig. 2) and differences in fusion patterns of palatal sutures (Fig. 3). The most striking palatal defects of Fgfr2+/S252W mice include palatal shelves that were contracted and reduced in length with an enlarged interpalatine distance (Fig. 2B), a greater tendency to fuse the right and left maxillary-palatine sutures and a tendency for the inter-premaxillary suture to remain patent (Fig. 3). Analysis of several litters and specimens per genotype was crucial in revealing highly localized differences in dynamic and variable developmental processes such as suture closure.

Our data confirm that premature fusion of the maxillary-palatine suture is a feature of palatal dysplasia in Apert syndrome (Purushothaman et al., 2011; Holmes and Basilico, 2012) and provide a snapshot of the process of closure of this suture (Fig. 4B,D). Apoptosis in the mutant maxillary-palatine suture is specifically increased in the areas of bone deposition that bridge the two bones, but not in areas of the suture with increased ALP activity that presumably precede fusion, or along bone surfaces in other regions of the maxillary and palatine bones (Fig. 4I,J). Increased apoptosis appears to be a later event in maxillary-palatine suture fusion, similar to its late occurrence in the fusing coronal suture in this Apert mouse model (Holmes et al., 2009).

Regarding the inter-palatine suture, no significant changes in cell proliferation (Fig. 5E,F), differentiation (Fig. 5G,H) or death (Fig. 5I,J) were found. However, the histological analysis revealed that the suture mesenchyme and the palatal bones were thicker in Fgfr2+/S252W mice relative to unaffected littermates. The greater midline separation of the right and left palatine bones observed in Fgfr2+/S252W mice (Fig. 2B; Fig. 5A,B) might be the result of changes occurring earlier in embryogenesis and undetectable at P0 and/or of other cellular processes and mechanisms not analyzed here. Considering that Fgfr2 is expressed in the outer layer of immature osteoblasts of the palatine bones during development (Rice et al., 2003), a possible explanation that requires further testing is that altered growth kinetics bestowed by a mutant Fgfr2 might affect bone shape throughout development, causing the localized thickening of palatine bones and the wider separation in the midline suture.

Our results reveal significantly abnormal cell patterns of proliferation, differentiation and apoptosis local to the inter-premaxillary suture, causing a lack of fusion and maldevelopment of the anterior palate in Fgfr2+/S252W mice. In contrast to what was observed at the maxillary-palatine suture, a high incidence of apoptosis was common to both the unaffected and mutant inter-premaxillary suture, revealing a distribution largely restricted to the bone-forming surfaces adjacent to the suture mesenchyme (Fig. 6G,H,L), occurring in the absence of suture fusion. Although the incidence was significantly higher in the mutant suture, this would appear to be a normal developmental process at this location,
accentuated by aberrant FGF/FGFR signaling, and perhaps related to the differentiation of osteoblasts from mesenchyme with the function of limiting osteoblast numbers. Coupled with the increased proliferation seen in mutant mesenchyme, the inter-premaxillary suture could provide an in vivo example of the finding in vitro that chronic FGF signaling or the expression of FGFR2 craniosynostosis mutant proteins induces proliferation in immature osteoblasts but apoptosis in mature osteoblasts (Mansukhani et al., 2000). The little-known roles of apoptosis in normal and pathological facial suture formation appears to be suture-specific and requires further study.

Finally, immunohistochemical staining suggested that altered FGF/FGFR signaling in palatal sutures did not trigger significant differences in activation of MAPK, p38 and ERK1/2 in any of the sutures analyzed, at least at P0. However, p38 and ERK1/2 are major effectors of the FGF signaling pathway and it has been shown that inhibition of ERK1/2 in mice carrying the S252W mutation significantly improves facial development, suggesting the importance at least of ERK1/2 activity in the Apert facial phenotype (Shukla et al., 2007). Although we did not see significant differences in FGFR2 signaling within sutures as measured by detection of phosphorylated p38 and phosphorylated ERK1/2, such changes might be subtle in vivo, generating aberrant phenotypes incrementally over time.

**Palate is a preferential target of the FGFR2 S252W Apert syndrome mutation**

Our results suggest that the S252W mutation leads to more severe palate dysmorphologies than the P253R mutation, confirming the pattern already described in patients with Apert syndrome (Slaney et al., 1996). Larger Mahalanobis distances (Table 2) and greater ranges of palatal shape variation (Fig. 2A) show that palates of Fgfr2+/S252W mutant mice are more severely affected. The palatal phenotype of Fgfr2+/P253R mice was not unaffected, however. Rather, the palates of the two Apert syndrome mouse models were affected differently (Fig. 2), resulting in varying palatal dysmorphologies that were more intense in Fgfr2+/S252W mutant mice. Fgfr2+/+ mice might show more severe malformations at other anatomical locations or have other tissues or developmental systems more severely affected. In fact, patients carrying the P253R mutation present with more severe syndactyly (Slaney et al., 1996).

**Perturbed FGF/FGFR signaling leads to palate dysmorphology in Apert syndrome**

Compelling evidence suggests that FGF/FGFR signaling plays an essential role in palatal development mediating cell communication and interaction (Wilke et al., 1997). Misregulation of FGF/FGFR signaling can result in severe dysmorphogenesis and disease, including malformations of the palate that occur as a part of a syndrome or contribute to the occurrence of isolated cleft palate (Dixon et al., 2011). For instance, 3-5% of human cases of nonsyndromic cleft lip and palate are associated with mutations and single nucleotide polymorphisms affecting genes directly involved in the FGF/FGFR pathway (Riley et al., 2007). Mouse models carrying mutations on Fgfr10, Fgfr18, Fgfr8 and Fgfr1, Fgfr2 display abnormal or cleft palate providing further support of the involvement of FGF/FGFR signaling in the pathogenesis of cleft palate (De Moerlooze et al., 2000; Abu-Issa et al., 2002; Liu et al., 2002; Ohbayashi et al., 2002; Trokovic et al., 2003; Rice et al., 2004; Wang et al., 2005; Martínez-Abadias et al., 2010; Snyder-Warwick et al., 2010; Wang et al., 2010; Purushothaman et al., 2011).

Several mechanisms involving alternative splicing of the FGFR2 molecules and abnormal interaction of mutant receptors with inappropriate ligands have been proposed to explain the pathogenesis of cleft palate in Apert syndrome. Ibrahimi and colleagues suggested that the differential increase in FGFR2-IIIc affinity for a specific ligand such as FGF2, which is expressed in both facial ectoderm and mesenchyme, could explain the higher frequency of cleft palate in Apert syndrome patients with the FGFR2 S252W mutation (Ibrahimi et al., 2001). Britto and colleagues proposed that the incomplete expressivity of cleft palate in humans with Apert syndrome is consistent with a dose-dependent effect of FGFR2 mutations on palatal shelf fusion via the FGFR2-IIIb isoform (Britto et al., 2002). In this case, the Apert mutations might confer a dominant-negative functional effect on FGFR2-IIIb in human palatal epithelia, resulting in failure of fusion of the palatal shelves (Britto et al., 2002). Hajihosseini and colleagues hypothesized that cleft palate in Apert syndrome is the result of loss-of-FGFR2-IIIb function in epithelial cells, secondary to alleles that cause a gain-of-FGFR2 function in mesenchymal cells (Hajihosseini et al., 2009).

These varying lines of evidence highlight altered cross-talk between epithelial and mesenchymal cells, mediated by FGF/FGFR signaling and causing palate malformations. Our findings in Apert syndrome mouse models suggest that cellular miscommunication, evidenced by local variation in patterns of cell proliferation, differentiation and apoptosis at P0, is associated with highly localized palate dysmorphologies that might be associated with aberrant signaling specific to early undifferentiated cell populations, formative sutures and/or bony precursors.

**Insights into altered palate development in Apert syndrome**

Patterns of palatal suture fusion scored in unaffected littermates suggest that in normal palate development the fusion progresses from anterior to posterior, with anterior palatal sutures fusing first before birth (i.e. inter-premaxillary suture) and posterior palatal sutures fusing postnatally (i.e. inter-maxillary, inter-palatine and maxillary-palatine sutures) (Fig. 3). This gradient is reminiscent of that described for normal development of the palate and tongue during elevation of the palatal shelves (Yu and Ornitz, 2011), suggesting a continuation of gradients of morphogenetic processes during early and late palatal development. Here we show that palate development in Fgfr2+/S252W Apert syndrome mice is altered by aberrant cellular behaviors that cause a premature fusion of anterior transverse palatal sutures (e.g. right and left maxillary-palatine sutures) (Fig. 4) as well as failure of closure of palatal midline sutures, such as the inter-palatine (Fig. 5) and the inter-premaxillary sutures (Fig. 6), which are patent or show osteogenic fronts that are more separated in Fgfr2+/S252W mutant mice. Interestingly, Apert syndrome FGFR2 mutations also cause premature fusion of other non-midline sutures of the craniofacial complex, such as the coronal and the premaxillary-maxillary sutures (Wang et al., 2005; Wang et al., 2010; Martínez-Abadias et al., 2010). This abnormal pattern of suture patency and closure is associated with the typical wide and short head shape (brachycephaly) and midfacial retrusion of Apert syndrome patients.
Disease Models & Mechanisms

Differences in fusion patterns of palatal sutures could stem from structural and/or suture-specific differences in localized FGF/FGFR signaling environments. Overall, the three sutures examined histologically at P0 have strikingly different fates and structural characteristics. The maxillary-palatine suture contains broadly overlapping bone edges separated by a reasonably narrow mesenchyme, similar to the coronal suture, where the degree of overlap is much shorter but which consistently fuses in Apert syndrome mice (Holmes et al., 2009; Wang et al., 2005). The inter-palatine suture, on the other hand, has a butted structure similar to the sagittal suture, which fuses infrequently if at all in Apert syndrome mice. The inter-premaxillary suture is unique, having a butted structure but also an extensive region of proximity between the two premaxillary bones, and it is the suture that in unaffected littermates more readily fuses. Although the basis for suture-specific differences in FGF signaling are unknown, in the mutant inter-premaxillary suture the result is a more extensive and proliferative suture mesenchyme relative to unaffected littermates, which might prevent close contact and fusion between the paired premaxillary bones at P0.

Considering the potential differential sensitivity of mesenchymal cells occupying specific sutures to the presence of mutant receptors and the selective distribution or concentration of FGF ligands (Hajihosseini et al., 2004), it is not surprising that the same FGFR2 mutation appears to have different or even opposite effects on the patency of varying cranial sutures. FGF/FGFR signaling is known to be complex, and tissue-specific responses are widespread and variable (Wilke et al., 1997; Ornitz and Marie, 2002; Dorey and Amaya, 2010; Hébert, 2011; Martínez-Abadías et al., 2011; Martínez-Abadías et al., 2013). The identity of FGFs expressed during early craniofacial morphogenesis and palatal formation have been extensively characterized (Britto et al., 2002; Bachler and Neubüser, 2001; Welsh et al., 2007), but the complement of FGFs expressed in the facial sutures during late embryological development is undefined. A broad range of FGFs is identifiable in the coronal suture during late gestation (Hajihosseini and Heath, 2002), so the repertoire of FGF signaling within the various facial sutures, modified by their particular affinity for the FGFR2 S252W and P253R mutant receptors, could be similarly complex and lead to such diverse phenotypic outcomes.

Our results add to the accumulating evidence that although FGF/FGFR signaling contributes to the development of many cranial tissues, the effects can be highly localized, varying according to the tissue considered and the time of development. Our analysis reveals several localized sites of altered palatal development and confirms that the posterior palate is one of the palatal regions most affected by perturbed FGF/FGFR signaling caused by the FGFR2 S252W mutation. These anatomical sites might serve as a potential target for therapeutic strategies in clinical management of cleft palate in Apert syndrome patients.

Materials and Methods

Mouse models

Fgfr2+/S252W and Fgfr2+/P253R Apert syndrome mouse models were bred on C57BL/6j genetic background for 20 generations to minimize phenotypic variation and were generated, euthanized, fixed and imaged in compliance with animal welfare guidelines approved by the Johns Hopkins University, the Mount Sinai School of Medicine and the Pennsylvania State University Animal Care and Use Committees. Further details on generation of targeting construct can be found elsewhere (Wang et al., 2005; Wang et al., 2010).

Scoring patterns of suture fusion

We assessed the pattern of fusion of five palatal sutures (inter-premaxillary, inter-maxillary, inter-palatine, right and left maxillary-palatine), qualitatively scoring the sutures as patent, partially fused or completely fused (Fig. 1). For the inter-premaxillary suture, we divided the whole length of the suture into three different segments (anterior, middle, posterior) and scored the degree of fusion at each segment as open (O), partial (P) or fused (F) independently. Once a code was obtained for each of the three sections of the inter-premaxillary suture, the three codes were assembled into a pattern of suture fusion. For example, individuals presenting with a completely open inter-premaxillary suture were coded as ‘OOO’ and were included in the analysis as ‘patent’, whereas individuals with a totally fused inter-premaxillary suture were coded as ‘FFF’ and were included in the analysis as ‘completely fused’. All those cases in which at least one segment was partially fused (P) were pooled into the ‘partially fused’ category.

Geometric morphometric analyses

General Procrustes Analysis (GPA), a procedure that superimposes configurations of landmarks by shifting them to a common position, rotating and scaling them to a standard size until a best fit of corresponding landmarks is achieved, was used to extract shape information (Dryden and Mardia, 1998). GPA, however, does not eliminate the allometric shape variation that is related to size. We adjusted for the effect of allometry (Drake and Klingenberg, 2008) by computing a multivariate regression of shape on centroid size, measured as the square root of the summed distances between each landmark coordinate and the centroid of the landmark configuration (Dryden and Mardia, 1998).

Canonical variates analysis (CVA) was performed to explore shape variation and to maximize discrimination between Apert syndrome mouse models and their unaffected littermates. CVA derives discriminant functions called canonical variates (CVs) that...
result from an optimal combination of variables (the Procrustes coordinates), so that the first one provides the most overall discrimination between groups; the second provides the second-most, and so on (Manly, 2004). The discriminant functions correspond to shape features that are independent or orthogonal, that is, their contributions to the discrimination between groups does not overlap.

Mahalanobis distances, a distance measure used in cluster analysis and classification techniques, were computed from the pooled within-group covariance matrix for all groups after adjusting for allometry to assess the degree of differentiation between all possible pairs of groups of Apert syndrome mouse models and their unaffected littermates (Klingenberg and Monteiro, 2005). Statistical significance for all pairwise comparisons was tested using permutation tests based on 10,000 iterations under the null hypothesis for complete dissimilarity. The $P$ values obtained were adjusted for the lack of independence using the Bonferroni method: the total comparisons were $c = k(k−1)/2$ (where $k$ is the number of populations) and the significance level 0.05 was divided by $c$ to guarantee real significance. All geometric morphometric methods based on Procrustes analysis were performed using MorphoJ (Klingenberg, 2011).

To statistically determine differences in the way the two mutant phenotypes differed from their respective unaffected littersates, we used a confidence interval procedure developed within EDMA, Euclidean Distance Matrix Analysis (Lele and Richtsmeier, 1995; Lele and Richtsmeier, 2001). EDMA converts 3D landmark data into a matrix of all possible linear distances between unique landmark pairs and tests for statistical significance of differences between shapes using non-parametric confidence intervals (Lele and Richtsmeier, 1995; Lele and Richtsmeier, 2001). An average form is estimated using the linear distance data and differences in 3D size and shape are statistically compared as a matrix of ratios of all like linear distances in the two samples. We tested for morphological differences in each mutant group as compared with its unaffected littersmates, and for differences in the mutant/unaffected contrasts between the two mouse models for Apert syndrome. The null hypothesis for each comparison is that there is no difference in shape between groups (or no difference in shape contrasts). Statistical tests for differences in specific linear distances were evaluated by a non-parametric bootstrapping procedure (Lele and Richtsmeier, 1995). For each linear distance, a ratio between the average values of that distance for each group was computed and confidence intervals for the null hypothesis were estimated. Differences with a $P$ value below 0.05 were considered significant.

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COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS
N.M.-A., J.T.R., Y.W. and E.W.J. conceived and designed the experiments. N.M.-A., G.H., T.P., Y.Z. and Y.W. collected data and performed the experiments. N.M.-A. and J.T.R. analyzed the data. G.H., T.P. and Y.W. contributed reagents and materials. N.M.-A. and J.T.R. wrote the paper. T.P., G.H., Y.W., E.W.J. and J.T.R. provided critical revision and final approval of the manuscript.

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Mesodermal expression of Fgfr2S252W is a key pathological mechanism in Apert syndrome. This mutation leads to an increase in FGFR2 signaling, which is thought to drive craniosynostosis and other phenotypic features of this pathology. Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome, both of which are caused by gain-of-function mutations in FGFR2 that activate receptor signaling. These mutations lead to hyperactivity of the MAPK pathway in the skull abnormalities of Apert syndrome Fgfr2(+P253R) mice.

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