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NODAL PATHWAY GENES ARE DOWNREGULATED IN FACIAL ASYMMETRY

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Abstract

Purpose—Facial asymmetry is a common comorbid condition in patients with jaw deformation malocclusion. Heritability of malocclusion is advancing rapidly, but very little is known regarding genetic contributions to asymmetry. This study identifies differences in expression of key asymmetry-producing genes which are down regulated in facial asymmetry patients.

Material and Methods—Masseter muscle samples were collected during BSSO orthognathic surgery to correct skeletal-based malocclusion. Patients were classified as Class II or III and open or deep bite malocclusion with or without facial asymmetry. Muscle samples were analyzed for gene expression differences on Affymetrix HT2.0 microarray global expression chips.

Results—Overall gene expression was different for asymmetric patients compared to other malocclusion classifications by principal component analysis ($P < 0.05$). We identified differences in the nodal signaling pathway (NSP) which promotes development of mesoderm and endoderm

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and left-right patterning during embryogenesis. *Nodal* and *Lefty* expression was 1.39–1.84 fold greater ($P < 3.41 \times 10^{-5}$) whereas integral membrane *Nodal*-modulators *Nomo1,2,3* were –5.63 to –5.81 ($P < 3.05 \times 10^{-4}$) less in asymmetry subjects. Fold differences among intracellular pathway members were negative in the range of –7.02 to –2.47 ($P < 0.003$). Finally *Pitx2*, a upstream effector of *Nodal* known to influence the size of type II skeletal muscle fibers was also significantly decreased in facial asymmetry ($P < 0.05$).

Conclusions—When facial asymmetry is part of skeletal malocclusion there are decreases of NSP genes in masseter muscle. This data suggests that the NSP is down regulated to help promote development of asymmetry. *Pitx2* expression differences also contributed to both skeletal and muscle development in this condition.

Keywords

ASYMMETRY; NODAL; PITX2; MALOCCLUSION

Introduction

Bilateral asymmetry is an anthropometric trait commonly found in humans. For example, preference for right hand usage has been documented for over fifty centuries¹ and its resultant functional bias promotes asymmetry in arm bone length and diaphyseal breadth.² Differences in lower limb length of up to 10 mm exist in 95% of people, but are generally considered subclinical normal variations that may become more apparent later in life.³ Asymmetry is also a common characteristic of the human face, but recognized subconsciously as a normal appearance.⁴ Composites of left and right side mirror images of the human face, first studied by Hallervorden⁵, each give a different appearance and are recognized as abnormal masked versions of a normally appearing asymmetric face.⁶ Like the upper limbs, the right facial side is usually larger^{6,7}, but the chin is more often deviated to the left side.⁷ The mandible along with constituent dental alveolar bone are typically the most symmetric given the ability of the dentition to adapt to differences by eruption and occlusal positioning.⁸ Because of this adaptation, locations of the dental arches in the head and of the teeth within the dental arches are often slightly asymmetric, but not recognized clinically. Finally, the midpalatal raphe line of the palate is typically used as a reliable axis of symmetry, yet it usually differs with the maxillary arch center line by a few degrees.⁶

Craniofacial asymmetry requiring corrective surgery often results from specific pathological processes that ranges from childhood trauma⁹, arthritis^{10,11}, condylar aplasia¹², and ocular hypertelorism¹³ to craniofacial anomalies such as unilateral craniosynostosis¹⁴ and hemifacial microsomia.¹⁵ More commonly, however, asymmetry is found in dentofacial deformity populations that have skeletal malocclusions. The National Dental Centre reports that among patients having orthodontic and surgical correction of malocclusion in the multiracial population of the Republic of Singapore, asymmetry occurs in 8% of skeletal class II and in 50% of skeletal class III individuals.¹⁶ In contrast, the prevalence of asymmetry in skeletal class II is 28% and 40% for skeletal class III in a dentofacial deformities population undergoing surgical corrections at the University of North Carolina.¹⁷ These data suggest that because asymmetry occurs through genetic and functional differences during growth and development, the proportion of sagittal, vertical

and transverse dysplasia present in a local population reflects their heritable background.⁶ In a study of asymmetry that occurred with mandibular prognathism in two families for up to four generations, one family had a chin deviation to the left throughout the pedigree while the other had deviation almost always to the right.¹⁸ This observation, along with more recent genetic findings, corroborates evidence that mandibular prognathism is a major transmissible Mendelian effect with dominant inheritance that has variable expressivity.^{19,20} Nevertheless, the genetic influences that produce similarities of laterality in subsequent generations are yet to be fully appreciated.

Consistent measurement of laterality in subjects is fundamental to the study of facial asymmetry. One approach is to determine facial height differences that produce discordance between anatomic landmarks on both sides of the median sagittal plane.²¹ Asymmetry detected by this approach may result from imbalanced growth and function of both the jaw²² and associated muscles.^{23,24} Obwegeser proposed that imbalances of mandibular growth occur in two different forms of prognathism; hemimandibular hyperplasia and hemimandibular elongation.²⁵ Hyperplasia produces a generalized enlargement to the affected side of the mandible and development of open bite, whereas elongation produces horizontal displacement of the mandible and chin towards the unaffected side with development of cross bite, rather than open bite. The way in which discrepancies of muscle function contribute to development of asymmetry are less clear.²⁶ It is known that muscle disuse produces decreased bone size and density, while increased physical activity have opposite effects, but the etiology of these relationships is not yet fully understood.²⁷ Bite force is associated with muscle cross sectional area²⁸ and mechanical advantage,²⁹ but muscle size is not always associated with facial skeletal form.^{30–32} By contrast, despite the generation of small forces that are difficult to quantify, postural muscle tone to maintain the freeway space between the teeth and protect the airway appears to be more important.³³ Our approach has been to determine differences between fiber-type properties of the masseter muscle, which maintains mandibular posture in the conscious state. This work has demonstrated that variations in area and size of fiber types contribute to differences in the vertical dimension of facial growth. Bilateral comparisons of masseter muscle fiber types made in subjects with or without facial asymmetry show no differences with symmetry, but significant differences are found between sides when asymmetry is present. Irrespective of the type of skeletal malocclusion, type II fast-contracting fibers are increased in masseter muscle on the same side as the deviation³⁴ and the vertical facial dimension is decreased.^{35,36}

PITX2, a gene active in the Nodal Pathway that determines left-right asymmetry during embryogenesis,^{37,38} has recently been reported to be expressed in satellite cells of adult human skeletal muscle.³⁹ *PITX1*, another member of the *PITX* gene family, produces fiber atrophy when overexpressed in masseter muscle.⁴⁰ Collectively, these findings suggest that Nodal Pathway genes are active in adult skeletal muscle and may be key factors in development, growth and maintenance of facial asymmetry. In order to determine if symmetry pathways are active in adult humans we compared gene expression in masseter muscle between dentofacial deformity subjects with and without facial asymmetry.

Materials and Methods

SUBJECTS

Subjects were recruited from the dentofacial deformities population at the Université de Lille Department of Oral and Maxillofacial Surgery, undergoing orthodontic and maxillofacial surgery treatment for correction of malocclusion with marked jaw discrepancies. The subjects were recruited after they had signed an informed consent, and the research protocol was validated by the French Independent Ethical Committee (named CPP) and the Temple University IRB Committee. All subjects had at least a mandibular bilateral sagittal split osteotomy using Epker's technique. This osteotomy separates the ascending branch of the mandible from the dental arch, permitting repositioning in good occlusal position after adapted movement. Depending on the movement type and amplitude, it is sometimes necessary to cut the pterygo-masseteric sling to realign bones.⁴¹ Finally a Tessier's distractor is used to completely separate the two bony pieces by more than one inch. During this procedure, the deep portion of the masseter muscle is exposed and muscle fibers are lacerated in the middle of the split. Before closing the surgical approach, to avoid muscle interposition between the bony pieces or being introduced in the suction drain, the lacerated muscle fibers are removed as clinical waste. These samples from left and right masseter are snap frozen in the operating room immediately after resection and then transported on dry ice to the Biological Resources Centre (BRC), Université de Lille, where they were they were managed and stored using procedures compliant with ISO 9001:2008 and the French Norm NFS 96 900 before express shipped to Temple University. Malocclusion classification is determined according to the type of surgical repositioning done to produce correction. Selected for study was either a left or right masseter muscle from 4 Class II deep bite malocclusion subjects, 4 Class II open bite and 3 Class III open bite. Facial asymmetry was present in one of the Class II open bites and one Class III open bite. In both asymmetry subjects masseter muscle from the side opposite of the chin deviation was used for genetic analysis. Finally one of the Class II open bite subjects had mandibular advancement surgery for correction of sleep apnea. This subject did not have facial asymmetry. Although genetic analysis was conducted on the sleep apnea subject, this patient was removed a priori from the comparisons in this study, and will be used to formulate a case report in a separate study. None of the subjects had systemic conditions, facial trauma, tumor, condylar hypertrophy, arthritis, or developmental conditions that might influence craniofacial growth.

GENETIC ANALYSIS

Procedures for microarray target preparation and hybridization were done at the Molecular Profiling Facility of the University of Pennsylvania. Muscle samples were disrupted in QIAzol Lysis Reagent and RNA was isolated using a Qiagen miRNeasy Mini Kit according to the manufacturer's instructions. Quality of the total RNA was tested by Agilent Bioanalyzer and Nanodrop spectrophotometry. Protocols for microarray analysis were conducted as described in the Ambion WT Expression Manual and the Affymetrix GeneChip Expression Analysis Technical Manual. A 250 ng aliquot of total RNA was converted to first-strand cDNA by reverse transcriptase with poly(T) and random oligomer primers that incorporated the T7 promoter sequence. Second-strand cDNA synthesis was

followed by in vitro transcription with T7 RNA polymerase for linear amplification of each transcript. Resulting cRNA was converted to cDNA, fragmented, assessed by Bioanalyzer, and biotinylated by terminal transferase end labeling. Labeled cDNA (5.5µg) was added to an Affymetrix hybridization cocktail, heated at 99°C for 5 min and hybridized for 16 h at 45°C to Human Transcriptome 2.0 GeneChips (Affymetrix Inc., Santa Clara CA). After low (6X SSPE) and high (100 mM MES, 0.1M NaCl) stringency washes, the microarrays were stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycoerythrin stain. A GeneChip 3000 7G scanner was used to collect fluorescence signal. Cel files containing individual probe intensities for each probe on each array were generated using Affymetrix Command Console software.

STATISTICAL EVALUATION

Data Analysis of differentially expressed genes was analyzed with affymetrix® Transcriptome Analysis Console (TAC) Software. 11 cel files were imported to Partek Genomics Suite (v6.6, Partek Inc., St. Louis, MO) where RNA normalization was applied resulting in log2-transformed intensities for 70,534 transcript IDs corresponding to 24,953 unique gene symbols, their transcriptional variants and additional content provided on the platform. The one sleep apnea subject was removed prior to Principal Components Analysis (PCA) which determined the largest difference in global gene expression between the 10 remaining subjects. To find differentially expressed transcripts a t-test was performed comparing the 2 asymmetric samples (subjects number 1 and 2) to the 8 non-asymmetric samples. Resulting p-values were corrected for false discovery rate (FDR, step-up p-value) using the method of Benjamini and Hochberg as implemented in Partek Genomics Suite. This statistical approach is necessary to correct FDR (false discovery rate) since relatively small sample sizes are used to compare thousands of differences in gene expression levels. Fold-change was also calculated for each transcript. Differences were considered significant if step-up p-values were < .02 and fold differences were $\geq \pm 2$ between groups. The present study utilizes the latest gene chip technology and analytical computations on a small subject population. No power analysis was included in the tests since accurate power analysis cannot be calculated without making too many unfounded assumptions.

Raw cel files for each of the 11 subjects were uploaded into the GEO database with the following ID link: _____ (to be added when manuscript is accepted for publication.)

Results

STATISTICAL DIFFERENCES BETWEEN GROUPS

Overall expression data for asymmetric patients sorted separate from subjects with other malocclusion classifications by PCA. The two subjects with facial asymmetry grouped as one cluster and all other malocclusion subjects, regardless of sagittal and vertical dimensions, grouped as a second cluster (Figure 1). Although there are genetic differences between the various malocclusion groups with symmetry, by far the largest genetic differences are when facial asymmetry is present or absent in skeletal malocclusions. Figure 1 shows a PCA plot summarizing the global inter-sample differences by visualizing high

dimensional variation in three dimensions. Each axis is a linear combination of all 70,534 transcripts, each with its own coefficient chosen in such a way to best show sample variations.

Transcripts were evaluated for differential expression and considered significant if they showed greater than ± 2 fold difference in gene expression and a step-up p-value < 0.05 . Applying this reasonable cut off value between groups, approximately 22,000 gene transcripts were found to be significantly different, representing about 1/3 of the entire array. This reflects statistical cutoffs for significantly differentially expressed transcripts and is not related to results found from the PCA comparisons.

In order to better understand the biological relevance of the results, we evaluated a subset of genes which contribute to the normal formation of asymmetry. This gene set included Nodal morphogens which pattern the left – right axis in vertebrate development,³⁸ and Pitx2 which patterns muscle and craniofacial development.⁴²

CLINICAL PRESENTATION OF ASYMMETRY AND SURGICAL CORRECTION

Diagnosis was performed by clinical examination, evaluation of dental study models, panoramic radiographs and radiographic analysis of lateral, posterior-anterior, and submentovertex cephalograms. Surgical treatment planning was conducted using a computer-assisted cephalometric analysis from Delaire (Tridim and Orqal ceph, Orthalis).

On the posterior-anterior cephalometric radiograph, asymmetry was identified using as vertical reference, the line passing through the crista galli and the superior part of the nasal septum, drawn perpendicular to the line between the intersections of the greater wings of the sphenoid bone and the lateral margins of the orbits. On the submentovertex cephalogram, asymmetry was evaluated for imbalances between the midpalatal raphe line (which represented the median sagittal plane) drawn perpendicular to the intercondylar axis reference line. Patients were included in the asymmetric group if the sagittal axis of the vomer bone was deviated relative to the median sagittal plane on the submentovertex radiograph. Mandibular asymmetry was identified by measuring the deviation of mandibular dental midline relative to the vertical reference line on the posterior-anterior cephalogram.

Subject number 1 presented a Class III open bite malocclusion, whereas subject 2 belonged to the Class II open bite group (Figures 2 and 3). From a facial asymmetry point of view, these two subjects had a marked posterior facial asymmetry. It included a significant deviation of the vomer bone and asymmetry of the pterygoid process of the sphenoid bone which could be clearly identified on the submentovertex and lateral cephalograms. The piriform apertures were also asymmetric and a tilt of the maxillary and palatine planes was also evident on the posterior-anterior radiographs. In addition to posterior facial asymmetry, subjects 1 and 2 presented a mild form of total facial asymmetry. Mandibular asymmetry included subject 1 with chin deviation to the right and subject 2 with chin deviation to the left.

The surgical correction for patient 1 combined a Le Fort I procedure to advance the maxilla with bilateral sagittal split osteotomy to retract the mandible and close the anterior bite, and

a reduction genioplasty. For patient 2, orthognathic surgery consisted of a Le Fort I osteotomy with maxillary impaction to correct the occlusal plane and anterior vertical excess, and a bilateral sagittal split osteotomy to advance the mandible. In both asymmetric subjects, bilateral sagittal split osteotomy performed a correction of mandibular asymmetry.

DIFFERENCES IN NODAL PATHWAY GENE EXPRESSION

The normal formation of laterality in the heart and other organs is controlled by nodal pathway gene expression at specific embryonic developmental periods. Since the effects of asymmetric nodal pathway gene expression are well documented for first branchial arch structures, we investigated differences in nodal pathway gene expression *a priori* as potential candidate genes for facial asymmetry. There were very significant differences in gene expression (fold differences) for many of the Nodal pathway genes (Table 1). Table 1 reports both the individual gene expression values and averages for asymmetric subjects 1 and 2. For statistical comparisons, their average values were compared to average gene expression values for the 8 malocclusion subjects without facial asymmetry. Individualized data for subjects 1 and 2 is presented in Table 1 to demonstrate their close similarity in gene expression. 10 of the 22 Nodal pathway genes had significant expression levels for both fold differences and Step-up p-values (marked with an * in Table 1). This represented approximately 45% of the Nodal pathway genes of interest. Extracellular mediators *Nodal* and *Lefty* expression were 1.39–1.84 fold greater ($P < 3.41 \times 10^{-5}$) whereas integral membrane *Nodal*-modulators *Nom1,2,3* were -5.63 to -5.81 ($P < 3.05 \times 10^{-4}$) less in asymmetry subjects. For the pathway's three surface Activin receptor molecules, two of the three were -2.47 (*ACTR2*) and -4.64 (*ACTRIA*) less in asymmetry subjects ($P < 5.06 \times 10^{-5}$ and $P < 9.65 \times 10^{-7}$). Four of the five positive intracellular pathway mediators had significantly negative fold differences in the range of -7.02 to -2.47 ($P < 0.003$). *PPM1A*, one of the two negative intracellular pathway mediators, had a significantly decreased fold difference of -3.79 with asymmetry ($P < 4.59 \times 10^{-6}$). *Pitx2*, an asymmetry factor known to influence the size of type II skeletal muscle fibers in adults was also decreased in facial asymmetry ($P < 0.06$), but with a fold difference less than 2.

Including the nodal pathway genes identified in Table 1, we were able to identify a total of 39 candidate genes of interest which have been reported to either participate in the nodal pathway or contribute individually to the development of asymmetry. In this larger grouping of genes only 28% reached our criteria for statistical significance, so caution must be used in interpreting our findings regarding nodal pathway genes in Table 1. However, the overall data trend for the genes listed in Table 1 was for moderate to large decreases in gene expression for 60% of them in the two subjects with facial asymmetry.

Discussion

DEVELOPMENT OF LEFT-RIGHT AXIS OF ASYMMETRY

During the third embryonic week formation of the three embryonic germ layers occurs through a process termed gastrulation. Gastrulation is initiated by formation of a primitive streak along the central axis of the epiblast through which invagination of epiblast cells form endoderm and mesoderm. At the cephalic end of the primitive streak the primitive node

forms an elevated area of cells which surrounds the primitive pit. Invading cells at the primitive pit migrate to the cephalic end of the embryo, forming the notochord. The notochord forms the midline axis of the embryo for development of the axial skeleton. The ectoderm overlying the notochord is induced to differentiate into the neural plate which initiates neurulation. Neurulation are the embryologic events that occur in the development of the nervous system. Neural folds develop as a thickening of cells to the left and right of the underlying notochord. A neural groove develops in the center of the folds that eventually unites at the upper aspects of the folds, forming the neural tube as the presumptive spinal cord. In the area where the neural folds are fusing, cells in the lateral crest area separate from the neuroectoderm and begin a migration passage into the developing branchial arches. These processes in late gastrulation and early neurulation periods begin as symmetric cellular patterns along the cephalic-caudal, dorsal-ventral and medial-lateral axes.^{43,44}

In order for normal development to occur, this symmetry must be broken to develop clear differences in the left – right axis overtop the notochord. Symmetry breaking may occur through various intracellular changes in different species, but in mouse and other mammals there is conserved development of dynein molecular motors which rotate monocilia above the extracellular surface of the primitive node.⁴⁵ This introduces a lateral flow of morphogenic vesicles to the left side of the neural fold. Asymmetric cascades of gene expression from these morphogens leads to nodal expression in the left lateral plate mesoderm. Nodal is a transcription factor that initiates the nodal molecular pathway to induce chirality and asymmetry in endoderm and mesoderm germ layers during late gastrulation and neurulation.⁴⁶ There are three stages in development of the embryonic left – right axis: 1) A break in symmetry by directional flow from monocilia above the primitive node; 2) Formation of asymmetric gene expression first induced by the effects of nodal expression in left lateral plate mesoderm; and 3) changes in gene expression, through gradient density signaling, to pattern asymmetry into developing tissues and organs.⁴⁷ This third stage of organogenesis is established by the nodal downstream effector, *Pitx2*. This highly conserved transcription factor patterns asymmetry for correct *situs* positioning, gut and body rotations, and other lateral plate mesoderm derivatives.⁴⁸

Pitx2 is especially important to development of mesoderm-derived first branchial arch structures. In addition to lateral plate mesoderm, *Pitx2* is also expressed in cephalic and first branchial arch mesoderm during gastrulation.⁴⁹ In the first arch *Pitx2* is necessary to establish premyoblast specification of mesoderm and induction of ectoderm for tooth bud formation.⁵⁰ Masticatory muscles are absent in *Pitx2* mutant mice, while muscles in the other branchial arches develop, but have some significant deformations. Lack of skeletal structure development also occurs, but is limited to the first arch. This suggests that normal *Pitx2* expression is essential for induction of jaw development. Abnormal *Pitx2* signaling is well established before migration of neural crest cells into the arch, which disrupts normal organogenesis of the jaws.⁴² After organogenesis is completed *Pitx2* continues to be expressed in most muscles of the head and trunk, and persists into adulthood.⁵⁰ It is especially important in maintenance of adult heart and extraocular muscle functions. In heart formation, abnormal *Pitx2* function may result in aortic and septal defects.⁵¹ In adult mouse and human heart *Pitx2* is expressed at much higher levels in the right atrium, in comparison

to the left atrium and ventricles. Mice with altered expression levels may develop atrial fibrillation. In humans, genetic variations close the *Pitx2* gene locus are associated with susceptibility to atrial fibrillation.⁵² Similar experiments demonstrate that *Pitx2* expression is required for normal functioning of adult extraocular muscles.⁵³

NODAL PATHWAY GENE EXPRESSION IN ADULT MASSETER MUSCLE

We compared gene expression in masseter muscle between 10 subjects with jaw deformation malocclusion to determine the principle component differences in gene expression between groups (Figure 1). The two subjects with facial asymmetry clustered separately from the other malocclusion subjects even though one had a class II and the other a class III malocclusion. This allowed us to investigate the possibility that molecular pathways which pattern asymmetry in development could be active and regulated differentially in masseter muscle. Almost all of the molecular signals described in the Nodal Pathway,³⁸ were expressed at significantly higher or lower levels in the asymmetric subjects in comparison to the symmetric subjects (Table 1). Pathway genes were organized by gene ontology to determine how differential expression might contribute to development of facial asymmetry. *Nodal* was almost 2 fold increased in masseter muscle in the asymmetric subjects and *Nodal* modulators were almost 6 fold decreased indicating that *Nodal* overexpression was present with asymmetry. Nodal negative regulators *Lefty 1 & 2* and *Cerberus* were elevated 1.5 to 2 fold with asymmetry which might indicate pathway activity to modulate Nodal overexpression. Most importantly however, *Pitx2* was down regulated, indicating that facial laterality might not have been adequately patterned. All of the positive and negative intracellular molecular mediators of the nodal pathway were down regulated, which might indicate that the intracellular pathway is not very active in masseter muscle from asymmetric subjects. The gene expression differences from microarray analysis present an exciting first insight into the relevance of Nodal Pathway genes in development of facial asymmetry, but must be interpreted with caution since 30% of total gene expression was different in subject comparisons. Therefore nodal pathway effects remain to be more fully explored in subsequent studies. Our results must be confirmed in additional RT-PCR experiments for individual gene expression levels, between left and right masseter muscle sides in symmetric and asymmetric faces, and for different types of facial asymmetry. Given our results, further exploration regarding gene expression differences between subjects with hemimandibular hyperplasia and hemimandibular elongation need further exploration.²⁵

There is likely to be different expression levels for Nodal Pathway genes between left and right side masticatory muscles when asymmetry is present, since we know that fiber type distributions are significantly different between facial sides.³⁴ The *Pitx* family of genes may be particularly important influences of differences in masseter muscle fibers. *Pitx2* is required for initiation and progression of myogenic lineage of masticatory muscles,⁴² and overexpression of *Pitx1* may cause skeletal muscle fiber atrophy.⁴⁰ Masseter muscle samples from the side opposite of the chin deviation had increased expression of *Pitx1* compared to muscle from symmetric subjects (Table 1). Fast-contracting type II fibers of masseter muscle on this facial side have decreased cell number and cell size, which could be an effect from increased *Pitx1* expression. This relationship must be more fully explored in future studies. The fold differences for *Pitx1* and 2 gene expression were less than two

between groups, and therefore not significant, but small variations in their gene levels may have biologically important effects given their key roles in development of first branchial arch structures.

Overall, nodal pathway gene expression may represent two distinct biological phenomena, remnants of an individual's developmental history and active differences in gene expression to maintain functional laterality. Differences in expression levels for the *nodal modulators* (Table 1) most likely represent remnants of symmetry breaking from an earlier period. An intriguing possibility however, is that *Pitx1* and *Pitx2* are actively expressed in masticatory muscle to maintain functional laterality when facial asymmetry is present. Future investigations will need to investigate differences in gene expression for these and similar candidate genes between left and right facial sides in relation to the pattern of skeletal asymmetry and masticatory muscle fiber type properties.

UNIQUE FEATURES OF FACIAL ASYMMETRY IN OUR SUBJECTS

There are various kinds of craniofacial asymmetry and numerous methods of measuring the craniofacial skeleton.⁸ Our study focuses on subjects with total facial asymmetry which included a significant posterior facial asymmetry. Among subjects from the facially symmetric group, some had a mild form of mandibular asymmetry but none had vertical asymmetry of the posterior face. So, the very clear distinction between the two groups suggests that genes known to contribute to asymmetry may lead to facial asymmetry, principally in the posterior areas of the face.

These findings are particularly encouraging given the role this posterior anatomic area plays in midfacial development, principally through growth of cranial base synchondroses. Indeed, cranial base synchondroses act as important growth centers for the craniofacial skeleton. Among midline chondral structures, the spheno-occipital synchondrosis seems to have the most prominent role in growth of the human skull.⁵⁴ Many authors have described the correlation between the cranial base angle and sagittal jaw dimensions.^{55–57} For example, an increased cranial base angle has been demonstrated to contribute to Class II malocclusion, whereas Class III deformity is associated with smaller linear and angular cranial base dimensions. Considering these factors, a bilateral asymmetry of posterior facial anatomy, including cranial base synchondroses and other posterior nasofacial structures, could lead to the development of mandibular asymmetry.

Moreover, the existence of a mild form of mandibular asymmetry in the group of symmetric patients suggests that posterior facial asymmetry with mild mandibular asymmetry and mandibular asymmetry which is isolated to the jaws are genetically distinct. Our subjects with posterior facial asymmetry clustered together, but subjects with isolated mandibular asymmetry did not cluster together from Principle Component Analysis. This highlights the multifactorial etiologies that may underlie different forms of mandibular asymmetry. To go further, we may be able to interpret the increase in the expression of *Pitx1* and the decrease in the expression of *Pitx2* in masseter muscle as a mandibular compensatory mechanism to the asymmetry of the middle third of the face in subjects with posterior asymmetry. This compensatory mechanism could explain the mild form of mandibular asymmetry in these patients. Cranial base growth drives the face in a direction eventually compensated by the

growth of the jaw bones themselves in conjunction with differences in jaw muscle function, given the fact that jaw muscle phenotype is related to facial asymmetry.³⁴

In conclusion, this work helps us understand how sub classifications of facial asymmetry arise and how symmetry regulatory genes contribute to development of malocclusion. Further histologic analysis of masticatory muscle fiber types will be necessary to determine how variations in bilateral masticatory muscle phenotype contribute to jaw asymmetry in subjects with posterior facial asymmetry. Finally, the experimental approach used in this study should be explored in a larger subject population which includes a variety of different types of facial asymmetry.

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Figure 1.

Plot of differences in total gene expression in masseter muscle for 10 malocclusion subjects identified by Principal Components Analysis. Subjects 1 and 2 labeled in blue were a Class III open bite and Class II open bite respectively; both had significant posterior facial asymmetry with a mild form of total facial asymmetry. The remaining 8 malocclusion subjects included 2 Class II open bites, 2 Class III open bites and 4 Class II deep bites. These subjects had facial symmetry and regardless of sagittal and vertical differences in malocclusion classification clustered as one group. Differences in expression of approximately 22,000 gene transcripts were highly significant when compared between the asymmetry pair and the remaining 8 malocclusion subjects.

Figure 2.

A. Posterior-anterior cephalometric radiograph of subject 1 with total facial asymmetry. There was significant asymmetry of the middle third of the face, an asymmetry of the piriform apertures and tilt of palatal plane associated with a mandibular deviation on the opposite side of mandibular midline. B. Submentovertex cephalogram demonstrating significant deviation in the sagittal plane of the vomer bone relative to the median sagittal plane. C. Lateral cephalogram highlighting the duplication (asymmetry) of the left and right pterygoid processes of sphenoid bone.

Figure 3.

Triptych illustration representing the posterior facial asymmetry found the vomer bone and the pterygoid process of the sphenoid bone in subject 2. A. Posterior-anterior cephalometric radiograph of subject 2 with total facial asymmetry. Asymmetry of the middle third of the face, asymmetry of the piriform apertures and tilt of palatal plane associated with a mandibular deviation on the opposite side of the mandibular midline were represented. B. Submentovertex cephalogram including midpalatal raphe line and intercondylar axis. Vomer bone is highlighted in orange whereas sphenoid bone is represented in yellow. C. Anatomical diagram representing the inferior view of cranial base.

DIFFERENCES IN NODAL PATHWAY GENE EXPRESSION IN MASSETER MUSCLE FROM SUBJECTS WITH MALOCCLUSION AND FACIAL ASYMMETRY

Table 1

Genes	Transcript ID	Expression Values ^a				Asym vs Sym	
		Asym (n = 2)		Sym (n = 8)			
		Subject 1	Subject 2	mean	mean		
NODAL and NODAL Modulators:							
NODAL	TC10001365.hg.1	65.23	73.86	69.55	37.83	1.84	9E-06
NOMO1**	TC16000180.hg.1	131.72	112.63	122.18	717.65	-5.63	0.0003
NOMO2**	TC16000912.hg.1	127.44	108.63	118.04	715.56	-5.81	0.0003
NOMO3**	TC16000194.hg.1	130.96	111.98	121.47	716.18	-5.65	0.0003
LEFTY Negative Regulation of NODAL:							
LEFTY1	TC01006387.hg.1	170.94	168.60	169.77	109.97	1.54	4E-06
LEFTY2	TC01003895.hg.1	224.07	196.47	210.27	151.76	1.38	0.00003
Cerberus Negative Regulation of NODAL:							
CERI	TC09000919.hg.1	50.74	85.77	68.26	34.72	1.90	0.0009
Downstream activator of NODAL Organogenesis Signaling:							
PITX1	TC05001797.hg.1	189.97	176.86	183.42	162.14	1.13	0.02
PITX2	TC04001471.hg.1	103.67	98.04	100.85	111.53	-1.10	0.06
Nodal Cell Surface Receptors:							
ACTRIA**	TC10001623.hg.1	55.38	52.97	54.18	252.63	-4.64	1E-06
ACTR1B	TC02002121.hg.1	201.56	209.44	205.50	215.37	-1.05	0.29
ACTR2**	TC02000378.hg.1	70.65	79.21	74.93	186.34	-2.47	0.00005
Mediates Lefty Cell Surface Signaling:							
CFC1B	TC02002344.hg.1	57.15	47.01	52.08	39.44	1.31	0.0005
CFC1B	TC02000813.hg.1	52.27	45.91	49.09	38.57	1.27	0.0001
Positive Intracellular Mediators of the NODAL Pathway:							
SMAD1	TC04000711.hg.1	117.73	109.43	113.58	139.64	-1.22	0.094

Genes	Transcript ID	Expression Values*				Asym vs Sym	
		Asym (n = 2)		Sym (n = 8)			
		Subject 1	Subject 2	mean	mean		
SMAD2**	TC18000496.hg.1	121.14	136.63	128.88	319.62	-2.47	0.00002
SMAD3**	TC15000622.hg.1	99.33	82.58	90.96	289.32	-3.14	0.0001
SMAD4**	TC18000998.hg.1	111.184	109.18	110.18	656.66	-5.91	1E-06
USP9X**	TC0X000195.hg.1	56.44	61.56	58.60	416.22	-7.03	4.00E-07
Negative Intracellular Mediators of the NODAL Pathway:							
PPM1A**	TC14000362.hg.1	84.86	88.31	86.59	331.38	-3.80	5E-06
RANBP3	TC19001087.hg.1	151.15	133.07	142.11	170.92	-1.20	0.089
Positive Nuclear Transcription Factors:							
FOXH1	TC08001753.hg.1	45.84	44.50	45.42	35.34	1.29	0.0002

Abbreviations: Asym, asymmetric (Subject 1, Class III Open bite; Subject 2, Class II Open bite); Sym, symmetric (8 Subjects with skeletal malocclusion without asymmetry); Fold diff, fold difference between average expression in Asym vs. Sym.

* Expression values are fluorescence signals detected for probes on the microarray, corrected for non-specific binding.

** Significant for step-up P-value with fold differences >±2 between groups.