ASYMMETRY PATHWAY GENES IN FACIAL ASYMMETRY

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ABSTRACT

Symmetry is considered an attractive facial characteristic, though it is very rare as most humans have some degree of asymmetry. Though most cases are subtle enough to go undetected, more severe cases of asymmetry can result in marked facial deformity. It has been found that facial asymmetry presents as a comorbid condition with other severe malocclusions. While many causes of facial asymmetry have been thoroughly investigated, such as cleft lip/palate, unilateral craniosynostosis and hemifacial microsomia, the genetic cause of asymmetries associated with dentofacial malocclusions has yet to be investigated.

Studies have shown that muscle strength, size, and fiber type can influence bone size and density. The masseter muscle is frequently involved in development of sagittal malocclusion, and left/right fiber-type differences have been previously found in subjects with facial asymmetry. Still, the contribution of muscle function to the development of facial asymmetry is unclear. Symmetry is a genetically controlled human characteristic, as is the careful breaking of symmetry during development to allow for appropriate organ formation necessary for life. Many genes have been identified to play a role in this initial breaking of symmetry, as well as the maintenance of left-right differential expression. Among these genes, the Nodal Pathway plays a particularly important role. Expression of Nodal Signaling Pathway (NSP) genes, which provide positional and patterning information during embryogenesis, vary significantly in facial asymmetry subjects. *PITX2*, a downstream NSP effector, patterns changes in gene expression on left-right sides through gradient density signaling. *PITX2* is reportedly expressed in adult human skeletal muscle satellite cells suggesting NSP gene activity is a key factor in both

development and maintenance of facial asymmetry in adulthood. A human transcript microarray was used to evaluate whether these genes involved with normal coding of symmetry during development were differentially expressed in masseter muscle from patients with or without facial asymmetry.

This study evaluated orthognathic surgery patients with varying skeletal malocclusions. The patients were categorized based on vertical, transverse, and sagittal discrepancies, as well as the presence or absence of facial asymmetry (n= 93). The subjects were appropriately diagnosed as open or deep bite, class I, II, or III, and treatments planned for the appropriate surgical correction. All of the patients required bimaxillary sagittal split osteotomy, at which point masseter muscle samples were collected. Eleven patients were included in this study. Of the eleven, two of the patients were classified with facial asymmetry. Samples were disrupted in QIAzol Lysis Reagent, RNA was isolated using a Qiagen miRNeasy Mini Kit according to the manufacturer's instructions, and quality of the total RNA was tested by Agilent Bioanalyzer and Nanodrop spectrophotometry. Samples were used for quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and protocols for microarray analysis were conducted as described in the Ambion WT Expression Manual and the Affymetrix GeneChip Expression Analysis Technical Manual.

The eleven samples were analyzed by Principal Components Analysis (PCA) to detect fold-changes for each transcript. From this, differences in global gene expression between the two asymmetric and eight remaining subjects were evident, as the two asymmetric patients clustered separately from the malocclusion patients without asymmetry. To find differentially expressed transcripts step-up t-tests were performed to correct for false discovery rate (FDR) comparing the two asymmetric samples to the eight symmetric samples. Differences were considered significant if step-up p-values were <0.05 and fold differences were >±2 between groups.

Differential gene expression was confirmed with ten of the 22 Nodal Pathway genes in asymmetric patients. Extracellular mediators *Nodal* and *Lefty* expression was 1.39 and 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 asymmetric subjects. Two of the three Nodal Pathway mediators decreased significantly with negative fold differences (-7.02, -2.47; $P < 0.003$). *PPM1a*, one of the two negative intracellular mediators, had a significantly decreased fold difference of -3.79 with asymmetry (P , 4.59×10^{-6}). *PITX2*, an asymmetry factor known to influence the size of type II skeletal muscle fibers in adults was also decreased in facial asymmetry ($P < 0.05$).

The down regulation of Nodal Pathway genes suggests its involvement in development of asymmetry. *PITX2* expression differences also contribute to skeletal and muscle development in this condition. The overall data trend was for moderate to large decreases in gene expression for approximately 60% of the Nodal pathway genes. Further histological 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 methods used in this study should be conducted with a larger sample population, including a wider variety of different types of asymmetry.

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CHAPTER 1

INTRODUCTION

Bilateral asymmetry is an anthropometric trait commonly found in humans. For example, preference for right hand usage has been documented for over fifty centuries and its resultant functional bias promotes asymmetry in arm bone length and diaphyseal breadth (Coren and Porec, 1977; Auerbach and Ruff, 2005). Differences in lower limb length of up to 10mm exist in 95% of people, but are generally considered subclinical normal variations that may become more apparent later in life (O'Brien et al 2010). Asymmetry is also a common characteristic of the human face, but recognized subconsciously as a normal appearance (Farkas and Cheung 1981). Composites of left and right side mirror images of the human face, first studied by Hallervorden, each give a different appearance and are recognized as abnormal masked versions of a normally appearing asymmetric face (Hallervorden 1902; Lundstrom 1961). Like the upper limbs, the right facial side is usually larger, but the chin is more often deviated to the left side (Haraguchi and Takada 2008). The mandible, along with constituent dental alveolar bones are typically the most symmetric given the ability of the dentition to adapt to differences by eruption and occlusal positioning (Vig and Hewitt 1975). 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 centerline by a few degrees (Lundstrom 1961).

Craniofacial asymmetry requiring corrective surgery often results from specific pathological processes that ranges from childhood trauma, arthritis, condylar aplasia, and ocular hypertelorism to craniofacial anomalies such as unilateral craniosynostosis and hemifacial microsomia (Proffit et al. 1980; Kejliberj 1998; Farronato et al. 2011; Leonardi and Barbato 2007; Miamoto et al. 2011; Pelo et al. 2011; Yanez-Vico et al. 2013). 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 (Chew 2006). 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 (Severt and Proffit 1997). 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 (Lundstrom 1961).

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 (Person 1973). 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 (Wolff et al. 1993; El-Gheriani et al. 2003). 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 (Bailey et al 1999). Asymmetry detected by this approach may result from imbalanced growth and function of both the jaw and associated muscles (Kaban 2009; Kwon et al. 2007; Yu et al. 2004). Obwegeser proposed that imbalances of mandibular growth occur in two different forms of prognathism; hemimandibular hyperplasia and hemimandibular elongation (1986). 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 contributes to development of asymmetry are less clear (Kwon et al. 2007). 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 (Burr 1997).

Bite force is associated with muscle cross sectional area and mechanical advantage, but muscle size is not always associated with facial skeletal form (Throckmorton et al. 1980; Hannam and Wood 1989; Kiliaridis and Kalebo 1991; Raaadsheer et al. 1996; Van Spronsen et al. 1997). 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 (Mew 2004). 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 deviation and the vertical facial dimension is decreased (Raoul et al. 2011; Rowlerson et al. 2005; Sciote et al. 2012).

PITX2 is a gene active in the Nodal Pathway that determines left-right asymmetry during embryogenesis (Yoshioka et al. 1998; Shen 2007). Recent reports show *PITX2* to be expressed in satellite cells of adult human skeletal muscle (Knopp 2013). *PITX1*, another member of the *PITX* gene family, produces fiber atrophy when overexpressed in masseter muscle (Padey et al. 2012). 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.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 Asymmetry in Normal Human Anatomy

Organisms throughout all kingdoms display various body structures, from the simplest lack of any organization, to beautifully constructed ordered growth, to very complicated genetically dictated organ systems. Organization of body structures has become generally more organized through evolution. Humans generally display bilateral asymmetry while internally displaying a complicated lack of asymmetry to facilitate the demands of Homo Sapien life.

2.1.1 Symmetry and Asymmetry as a Human Norm

From a very early age, children develop the skills to identify symmetry versus asymmetry. Recognition of vertical symmetry, represented by a shape with equal right-left sides reflected along a vertical axis, has been found to be innate in newborns (Bornstein and Krinsky 1985). Furthermore, infants develop a preference for symmetry by 12 months (Griffey and Little, 2014). Using eye-tracking studies, it has been found that infants between 12 and 24 months seem to prefer symmetrically computerized faces versus asymmetric faces. The studies show babies will gaze at the symmetric images a significant amount of time longer than non-symmetric images. This preference persists in to adulthood. Perrett et al. found that adults judge perfectly bilateral symmetric faces to be more attractive than asymmetric faces (1999). Facial attractiveness is influenced by multiple factors. Averageness, the effect of blended features to create a composite single image, has been found to play a key role in attractiveness and preference (Cellerino, 2003). In addition to averageness, gender-specific features and age can influence facial attractiveness. However, symmetry seems to play one of the most influential roles in human facial preference.

Humans prefer to look at symmetric images and faces. Having an attractive and symmetric face can play a significant factor in a person's life. Attractive faces are perceived as being more trustworthy. Because of this, these people tend to have higher self-esteem and a higher quality of life. These factors can have further impacts on factors such as BMI, income, and job security. It also has an effect on mate quality, especially considering the role of symmetry in facial attractiveness increases as humans go through puberty (Vingilig-Jaremko, L. 2014). Symmetry is just one of the factors influencing facial harmony. Facial harmony encompasses more characteristics than symmetry, including facial form and feature archetype (Claes, 2013). These features can vary to a certain extent and still be considered "normal" and accepted. Dysmorphometrics is the measurement of morphological abnormality. Using twin studies and computer-generated images, dysmorphometrics can be studied to identify to what degree certain features can vary before faces become perceived as being discordant. From these studies, it has been found that a certain degree of asymmetry is unnoticeable and considered normal in appearance.

The human body as a whole is classified as being "bilaterally symmetric" with the sagittal plane separating the body in to two mirror images (Valentine, J.W., 2013). This characteristic is shared with more than 99% of the Animal kingdom. Multiple advantages to having a bilateral body plan include, but are not limited to, the capability to have a central nervous system, a distinct caudal and dorsal end, and the ability to streamline (Finnerty 2005).

Still, while the human body displays bilateral symmetry, a certain degree of asymmetry exists. VanValen suggests subclassifying these structural asymmetries based on the degree of genetic versus environmental influence (Graber 149). The three classifications are directional asymmetry, antisymmetry, and fluctuation asymmetry. Directional asymmetry occurs as a result of normal development with a significant genetic influence. This type of asymmetry results in one side developing differently than the other. Genetically determined asymmetries, differential development of lung lobes for example, are a feature of normal human anatomy and physiology. Without these asymmetries, the human body simply could not function. While much of what is considered normal human anatomy is symmetric, particularly the musculo-skeletal system, life depends on certain structures to develop unilaterally or asymmetrically. A human heart cannot pump blood, a stomach cannot digest food, and a liver cannot filter blood as perfectly symmetric organs.

Antisymmetry is also under genetic influence and considered developmentally normal. In contrast with directional asymmetry, the side that develops differently cannot be predicted before development. Antisymmetry is also much less common than directional asymmetry.

The third type of asymmetry VanValen defines is fluctuating asymmetry. Fluctuating asymmetry has less of a genetic influence, with the affected side being random. Most bilateral facial features are generally under the same genetic influence. The same set of genes encodes the development of the left lateral incisor as the right lateral incisor. Any variance among these normally bilateral structures would theoretically be more likely due to environmental factors. In this way, fluctuating asymmetry is a reflection of the body's inability to develop a perfectly bilateral, homologous structure. The degree of a person's fluctuating asymmetry may indicate how well the genome can react to certain environmental circumstances and still develop the ideal bilaterally symmetric phenotype.

While the affected side of fluctuating asymmetry is genetically random, population studies reflect a trend towards preferential sidedness throughout time. For over 50 centuries, humans have consistently displayed a preference toward right-handedness with 92.6% of the population being right-handed, regardless of geography or culture (Coren and Porac 1977). This preference may be an effect of or could be the cause of a skeletal length discrepancy, as humans also historically display a discrepancy in right versus left humeral bone length and diaphyseal breadth (Blackburn 2011). Minor right-sided skeletal asymmetry first appears in infants, suggesting a genetic influence. This asymmetry is amplified throughout growth and development as right-sidedness becomes more pronounced in adolescents and adults. These findings suggest biomechanical forces playing a larger role in bilateral asymmetry. Furthermore, the bias toward right-sidedness is more evident in upper limbs (Auerbach and Ruff 2006). Auerback and Ruff found that asymmetries in lower limbs were closer to zero with a trend toward longer femurs on the left side. They proposed that this difference was due to a functional "crossed symmetry". Evidence that behavioral asymmetry influences a morphologic asymmetry is also supported by studies observing increased skeletal asymmetries in arm lengths of racquet athletes.

Similarly, there is a trend toward sidedness of the face as well. For example, 70% of unilateral cleft lips are found on the left side. It has also been well documented that hypodontia is 1.5x more common unilaterally than bilaterally (Trakiniene et al., 2013). When a tooth is unilaterally congenitally missing, it is more commonly missing on the left side only (Hedayati and Dashlibrun, 2013). These variations from the bilateral "norm" are actually common, universal features of the human anatomy.

Facial sidedness is also characteristic of asymmetries. Studies evaluating dental and mandibular asymmetries suggest a dominance for the left side over the right side (Biagi et al. 2012). Maxillary length show significantly significant differences with the right side more commonly longer than the left. Because of this, like upper limbs the right facial side is usually longer (Lundstrom, A. 1961). The longer right maxilla normally results in a deviation of the chin to the left side (Haraguchi et al. 2008).

2.2 Genetic Role in Asymmetry

2.2.1 Breaking of Asymmetry During Development: The Nodal Pathway

The asymmetric organ placement and morphology is a highly conserved feature of almost all vertebrates. For centuries, scientists have sought to understand how the breaking of symmetry and formation of chirality is established during embryogenesis. It is an important question to answer, as a deviation from the normal asymmetric development can result in sever birth defects. Often, errors in development lead to the inability of vasculature to connect properly to the lungs, heart, or other organs, resulting in significant impacts on patient health, perhaps resulting in death.

Three embryonic germ cell layers are formed during the third week of development in a process known as gastrulation. This process is initiated by a primitive streak along the central axis of the epiblast. The cells of this invagination differentiate in to the endoderm and mesoderm with the cephalic end becoming the primitive pit. Invading cells at the cephalic end become the notochord, which forms the midline axis for the development of the axial skeleton. On either side of the notochord, ectoderm cells begin to differentiate and thicken forming into neural folds and grooves. From these ectodermally derived tissues, the spinal cord and neural tissues of the branchial arches are formed. These later stages of gastrulation are the beginnings of symmetric cellular patterns along each axis (Sadler 1990). Next in normal development, breaking of symmetry must occur to establish right and left sides relative to the notochord.

While the process of the establishment of left-right asymmetry during development is still unclear, it is generally agreed that it occurs by three major phases. First, the left-right axis is established relative to the dorsal-ventral and anterior-posterior axes. Next, the differences established in the first step cause differential expression of genes on each side of the left-right midline axis. The differential gene expression during the second stage results in the third stage: left-right differences in cell behavior on each side, resulting in asymmetric cell migration rates, organ morphology, and organ position.

Various models of left-right asymmetry have been proposed outlining the origin of asymmetry, the mechanism for aligning the left-right axis, and the differential cell action on each side of the midline. The most prominent and likely models all involve the intracellular cytoskeletal element of the centriole as the source of the asymmetry. To date, the three most popular and likely models are the ion flux model, the chromatid segregation model, and the planar cell polarity model (Levin 2013). In mammals, dynein molecular motors develop, rotating monocilia on the extracellular surfaces of the cells of the primitive node. This creates a flow of morphogenic vesicles laterally to the left side of the neural fold.

Regardless of how the breaking of asymmetry is initially established, differential gene expression, cellular activity gradient, and varied organ development is essential during gastrulation. The Nodal pathway is a series of genes that provides positional and patterning information to cell fields during embryogenesis. The signals are essential for mesoderm and ectoderm induction and patterning (Schier 2009). Nodal itself is a transcription factor that initiates the pathway, inducing chirality in the endoderm and mesoderm cell layers. It is the origin of the initial of the three stages involved in the breaking of symmetry.

The Nodal Pathway is very complex involving extracellular processing of *Nodal* precursors, overlapping but non-redundant ligands, and extracellular inhibitors. Of note is the inhibitor *Lefty,* which blocks *Nodal* signaling by binding to the *Nodal* protein itself, as well as extracellular *Nodal* receptors. This complex pathway is requires for asymmetric organogenesis and in its absence, organ asymmetry is lost or randomized. The establishment of *Nodal* differentially in the left lateral plate mesoderm is initiated by its expression in the cells of the embryonic midline. Differential expression of these proteins auto-regulate and have long-range and short-range effects. Nodal signals also display concentration-dependent effects. For these reasons and more, it is easy to see that the *Nodal* pathway is both robust and intricate. Many questions remain regarding the signaling mechanisms, pathway kinetics, diffusion, processing, trafficking, and degradation of the genes and proteins involved in this precise pathway.

2.2.2 Maintenance of Asymmetry: *PITX2* and Other Downstream Regulators

While *Nodal* is involved in the initiation of chirality and asymmetry in the endoderm and mesoderm germ layers, a downstream effector known as paired-like homeodomain transcription factor 2, PITX2, establishes the third stage of asymmetric organogenesis. *PITX2* is a highly conserved transcription factor that patterns the changes in gene expression on left-right sides through gradient density signaling and differential cellular action. It is responsible for correct organ positioning, gut and body rotations, and other lateral plate mesoderm derivatives (Ryan et al. 1998). It is fundamental to proper development of the brain, pituitary, heart, and facial structures (Kioussi et al. 2002).

PITX2 is important in the development of mesoderm-derived first brachial arch structures and is necessary to cellular differentiation of pre-myoblast cells. It is necessary for the development of masticatory muscles. Additionally, its presence is necessary for the induction of the ectoderm to form tooth buds in the first brachial arch and normal skeletal development. These findings suggest that normal *PITX2* expression is necessary or the development of the human jaw complex. While abnormal *PITX2* expression and signaling is establish prior to the migration of neural crest cells in to the first brachial arch, its expression or lack of expression persist in many of the muscle cells of adult human head and neck structures. Clearly, *PITX2* is essential for normal facial muscle development. In particular, *PITX2* initiate the myogenesis of extra-ocular muscles (Shih et al. 2007a.).

Skeletal muscle is very capable of regeneration and repair. Populations of resident stem cells, termed satellite cells, have been found in adult muscle tissues located on the surface of the myofiber and are in art responsible for this maintenance. Though generally mitotically inactive, studies have found that activation of these cells produces myoblast progeny that allows for homeostasis, hypertrophy, and maintenance of mature muscle. This is possible through the redeployment of developmental myogenic regulatory networks, including *PITX2*. While much is known regarding the role of *PITX* genes in embryonic and fetal muscle development, little is known about the role of these genes in controlling adult myogenesis. Still, it is well regarded that *PITX2* is upregulated and redeployed in adult muscle satellite cells to increase myogenic differentiation and allow for maintenance and repair of skeletal muscle (Knopp et al. 2013).

2.3 A Review of Facial Asymmetry

As previously discussed, human faces are characteristically bilateral, with a mirror image across the sagittal plane. Still, slight facial asymmetries are found in all individuals, even the most aesthetically attractive faces. These asymmetries are minor and indiscernible, generally unnoticed in everyday life. Early orthodontists recognized asymmetry as a normal human condition. In 1954 Fischer stated, "Facial asymmetry is a natural phenomenon and there is nothing abnormal about it." Thompson further distinguished asymmetries, recognizing that "normal asymmetry is not very evident, whereas the abnormal asymmetry is quite obvious." (1943). Still, the difference between "normal asymmetry" versus "abnormal" is not easily defined. The distinction between the two could be the effect of a single large deviation or the additive effect of multiple minor deviations (Claes 2013).

2.3.1 Evaluation of Facial Asymmetry

A thorough evaluation of each individual patient, both clinically and radiographically, is necessary to determine an accurate orthodontic diagnosis. Only from a proper diagnosis can the orthodontist then formulate an appropriate treatment plan. This is even more essential in patients presenting with facial asymmetries.

Diagnosis of an asymmetry from the clinical exam includes assessment of the soft tissue feature, the skeletal base, and the dentition. Commonly this assessment is made from the frontal view, but additional analysis can be performed from the basal view or face-down. Lateral asymmetry can exist from a muscular etiology, a skeletal etiology, or a functional etiology, existing only during movement (Sutton, 1968). The first step in clinically appraising the bilateral symmetry of the face is defining facial points and the median line. Multiple facial points can be defined and identified. Examples include the pupils, suborbits, zygomatic arches, canthus of the eyes, lip commissures, and points along the inferior border of the nose and chin. Using these points, variance from normal can be assessed and measured based on their location in reference to the median line. The median line is an extension of the sagittal plane and is not easily distinguished. Sutton proposes using three central facial features, though often all three do not actually lie on the same line. The three points he uses are: subnasale (the junction of the lower border of the nasal septum and the surface of the upper lip), prosthion ("the lowest point of the intermaxillary suture, upon the alveolar margin, between the two first incisors"), and pogonion ("the most prominent point in the middle line of the anterior surface of the chin"). Other structures that may be used to divide the face medially include but are not limited to glabella and soft tissue nasion.

Quantifying and qualifying structures of the face in markedly asymmetric patients is even more difficult than in "normal" patients. Often it is difficult to determine which structures to use as reference and which structures are actually causing the asymmetry. Proffitt suggests dividing the face in to subunits and evaluating each independently, and then in relation to each other. In this way, the source of the asymmetry can more accurately be determined.

After the presence or lack of an asymmetry is determined, it is prudent for the orthodontist to preform a thorough clinical exam. This would include documentation quantifying and qualifying several facial characteristics. Previously discussed was the evaluation of facial features. Dental features may also be the cause or effect of an asymmetry, and should therefore be evaluated thoroughly as well. This would include evaluating the jaws, alveolar process, and dentition at rest, smiling, and in function. At each of the positions, the clinician should document various features. Included are: gingival show, occlusal cant, anterior teeth inclination, lip position relative to incisal edge and smile arch. The maxillary dental midline should be recorded, as well as the mandibular dental midline with an open mouth, at initial contact, and in centric occlusion. Dental arch asymmetries may also be present. These asymmetries could be severe and include the whole dental arch and skeletal base, or as simple as a molar asymmetry due to early loss of deciduous teeth.

Radiographic analysis is another essential aspect of evaluation and diagnosing facial asymmetry. Most orthodontic exams include a panoramic radiograph. This image can be used to identify gross anomalies, supernumerary or missing teeth, and to evaluate the shape and height of the mandibular ramus and condyle. While panoramic radiographs can be useful to evaluate posterior mandibular asymmetry, evidence suggests there will be some under diagnosis (Kambylafkas et al. 2006). Further studies suggest that the use of panoramic images to assess mandibular asymmetry should be done with caution and with the understanding of the limitations of the image (VanElslande et al. 2008). While vertical measurements are more accurate than horizontal or angular measurements, they are still not true representations of the accurate object, as false magnification values may skew the values.

Success in evaluating panoramic radiographs has been found using the Levandoski Panoramic Analysis (1993). This method, detailed in 1993, is a simple system to evaluate the temporomandibular joints (Levandoski 1993). This method has further been expanded by Piedra to analyze facial and dental asymmetries (1995). These methods have been found to be useful in the analysis and diagnosis of dental and facial asymmetries and its relation to facial photograph findings. A vertical line is drawn through the nasal septum as a skeletal midline reference. A perpendicular is drawn to the right and left maxillary tuberosities. From this horizontal, three vertical, perpendicular lines are drawn per side: through the sigmoid notch, the tip of the mandibular condyles, and the tip of the coronoid processes. This is a quick and efficient method to identify and diagnose the presence of facial asymmetry with a panoramic radiograph (Padron and Protillo 2009). While these lines may provide quantitative measurements, due to the effect of magnification, panoramic evaluation is mostly qualitative.

While panoramic radiographs are the most common qualitative diagnostic aid in orthodontics, the lateral cephalogram is the most commonly used quantitative evaluation (Pereira and Ravi). These two-dimensional images are used to measure the shape, position, size, and orientation of different structures of the face. All of these structures are projected onto a single sagittal plane image and can therefore be skewed by superimposition. The information gathered from these images is further limited to the vertical and anteroposterior dimension. While useful in measuring facial structures of symmetric patients, these images are further skewed in asymmetric patients (Gateno et al. 2011). Recent studies have shown that for measurements of shape, roll and yaw asymmetries distort two-dimensional cephalometric measurements. For measurements of size, two-dimensional images are distorted by yaw asymmetries, and for measurements of orientation, linear and angular measurements are distorted in patients with facial asymmetry (Gaterno 2011).

Due to the limitations of two-dimensional analyses, a shift has occurred toward three-dimensional analysis with computed tomography. These images can be used to diagnose various facial asymmetries. Measurements used to diagnose and quantify asymmetry with CT scans include upper midline deviation, maxillary canting and maxillary arch form asymmetry, as well as menton deviation, the relationship of gonion to midsagittal plane, rams height, and frontal ramus inclination.

2.4 Congenital Disorders (Prenatal)

Congenital disorders are conditions that are present at birth and generally arise during the first months of development. These disorders can range in severity and causation. Often the disorders have abnormalities beyond dental deformities. Because of this, treatment of these conditions trend towards a team approach, with the orthodontist playing an integral part of that team. In this way, the clinician plays an essential part in determining the timing and sequence of orthodontic treatment in conjunction with the patients other health care needs.

2.4.1 Cleft Lip and Palate

One of the most commonly encountered craniofacial congenital disorders an orthodontist will encounter is cleft lip and palate. Occurring in approximately 1:700 live births in the United States, Clefts of the lip and palate are the fourth most common craniofacial birth defect (Vig 2011 Graber pg 966). Cleft lip and palate has a multifactorial etiology with a range of manifestations and abnormalities. Typical facial disturbances include a retrognathic maxilla, flattening of the cranial base, hyperdivergence, an increased anterior face height and decreased posterior face height (Abuhijeleh et al. 2014). Cleft lip and palate are due to the disruption of the formation of the facial organ systems, particularly the fusion of the primary and/or secondary palate. This general happens between days 28-55 of development (Profitt 115).

2.4.2 Hemifacial Microsomia

Also known as craniofacial microsomia, this developmental disorder is generalized as lack of development of lateral facial areas. The disturbance of normal development occurs between days 19 and 28 f embryonic development due to loss of neural crest cell migration. The reason for the loss of migration is unknown. The result of this disruption of development is a deficient or missing mandible, ramus, and associated muscle and fascia, as well as deformation of the external ear. The syndrome affects the lateral and lower areas of the face most drastically due to the longest migration path of the neural crest cells of these structures. While midline defects are rare, as the affected cells seem to always complete their aberrant migration, facial asymmetry is always observed (Proffit 117).

2.5 Selection of Genes of Interest

It is clear that dentofacial malocclusions can be highly influence genetics and muscle phenotypes. Particularly, the development of facial asymmetry can be directly or indirectly caused by complex genetic interactions involving the Nodal Pathway. Therefore, three specific genes involved in this pathway were selected as a focus of this study. As previously discussed, *PITX2*, a transcription factor that patterns the changes in gene expression on left-right sides, was chosen as the main focus. *NOMO1,2,3* (Nodal Modulator 1, 2, and 3) three highly similar pathway antagonists, and *SMAD4* (Mothers Against Decapentaplegic Homolog 4), a gene that encodes signaling transcription proteins, were selected due to their involvement in the NSP (Gene Cards 2014).

CHAPTER 3

AIMS OF THE INVESTIGATION

The overall aim of this investigation is to evaluate the potential relationship between the development of facial asymmetry, associated malocclusions, subject phenotypes, and masseter muscle fiber type composition and the expression levels of Nodal Pathway Genes. The study focused on three genes of interest: *NOMO1,2,3, SMAD4,* and *PITX2.*

This study has three specific areas of focus. First, a microarray analysis was done to compare global gene expression of isolated RNA from masseter muscle between subjects with facial malocclusion with or without facial asymmetry. The microarray data was then plotted to separate subjects according to differences using a Principal Components Analysis.

Second, expression data for sets of genes associated with development of laterality and symmetry in normal development will be collected, sorted and summarized according to their significance and fold difference between subjects with and without facial asymmetry.

Three specific genes (*NOMO1,2,3, SMAD4,* and *PITX2*) were further investigated with RT-PCR experiments. These genes were chosen based on a literature review, identifying them as important genetic loci involved in the breaking and maintenance of symmetry. The expression of these genes was characterized based on vertical and sagittal dentofacial diagnoses of the subjects, as well as gender and presence or absence of TMD symptoms.

Thirdly, sections of masseter muscle samples were stained for fiber type composition. The expression of the genes of interest was analyzed to correlate a relationship between expression levels, patient characteristics, and fiber type analysis. After initial significance was found with *PITX2* expression and malocclusions, the study focused primarily on this gene.

CHAPTER 4

MATERIALS & METHODS

4.1 Muscle Samples

Subjects for this study were recruited from the Universite de Lille Department of Oral and Maxillofacial Surgery. The subjects were identified from the dentofacial deformities population and were treatment-planned to undergo maxillofacial surgery for correction of malocclusion with concurrent marked jaw discrepancies. All subjects read and signed an informed consent, and the research protocol was validated by the French Independent Ethical Committee (named CCPP) and Temple University Review Board of Temple University. All subjects had a non-contributory medical history. The French surgical team before orthognathic surgery did diagnosis of skeletal classification, based on the surgical repositioning needed for each patient. Orthodontic diagnoses were made before surgery using the Delaire analysis (Delaire, et al., 1981) and diagnosis was used to determine the type of surgical repositioning needed to correct jaw deformation and malocclusion.

Maxillary and cranial symmetry was identified on the posterior-anterior cephalometric radiographs by drawing a vertical reference line through the crista galli and the superior aspect of the nasal septum. This vertical line was drawn perpendicular to a line between the intersections of the greater wings of the sphenoid and the lateral margins of the orbits. Asymmetry was identified on the submentovertex cephalometric radiograph by evaluating for imbalances between the midpalatal raphe line (representing the median sagittal plane), which was drawn perpendicular to the intercondylar axis reference line. Patients were identified as asymmetric if the vomer bone, which represented the sagittal axis, deviated relative to the sagittal reference line on the submentovertx radiograph.

Comparing the dental mandibular midline relative to the vertical reference line on the posterior-anterior cephalogram identified mandibular asymmetry. Any deviation of this midline was acknowledged.

Malocclusions were diagnosed in the vertical dimension (open bite and deep bite malocclusions), the sagittal dimension (Class II and III), and in the transverse dimension (posterior facial asymmetry and mandibular asymmetry). All patients received the Jaw Pain and Function (JPF) questionnaire, translated into French, with a self-rating scale for evidence of TMD. Based upon the several diagnoses and evaluations, patients were sorted by gender, malocclusion type, facial symmetry, and TMD status.

All subjects had, at minimum, a mandibular bilateral sagittal split osteotomy using Epker's technique. This osteotomy separates the ascending branch of the mandible from the dental arch to permit repositioning of the mandible in to a better occlusal position after adaptive movement. Depending on the type and amplitude of correction needed, it is sometimes necessary to cut the pterygo-masseteric sling to reposition and realign bones (JOMS reference 41). A Tessier's distractor was used to completely separate the two bony pieces by more than one inch. During the 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, these lacerated, exposed masseter muscle fibers are removed as clinical waste to avoid being interpositioned between the bony pieces or being introduced in the suction drain. During this procedure, approximately $0.5cm^3$ of masseter muscle tissue was excised on left and right sides from a consistent site in the middle of the deep layer 1.5 cm from the lowest point of the mandible's angle as described previously by Rowlerson et al. (2005) (**Figure 1**).

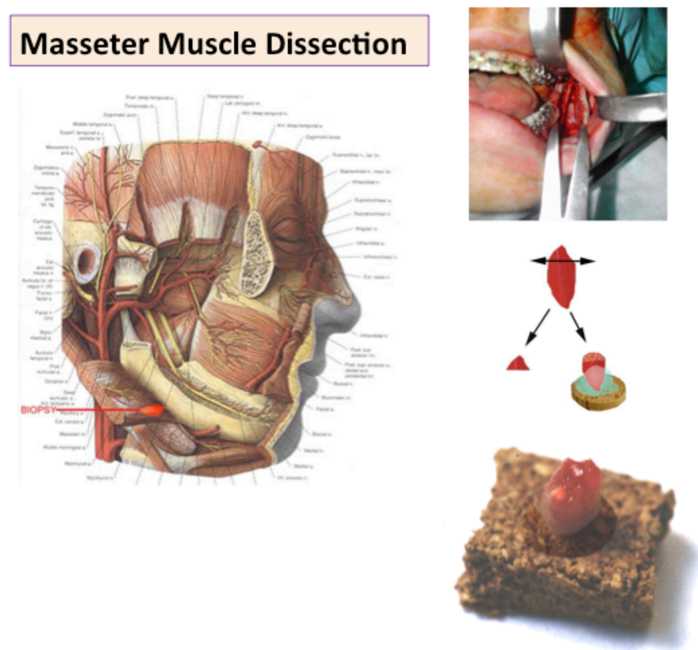

Figure 1: Masseter muscle biopsy site, surgical procedure, and isolated sample
Illustrates the anatomical area of masseter muscle biopsy (right) and the surgical procedure, removal, and mounting of the masseter sample for cryosectioning (left).

Immediately after resectioning in the operating room, muscle samples from left and right sides were mounted for sectioning, snap frozen and stored at -80°C. The samples were then transported on dry ice to the Biological Resources Centre, Universite de Lille, where they were managed and stored using procedures compliant with ISO9001:2008 and the French Norm NFS 96 900. Muscle was shipped on dry ice in lots of 60 specimens to Dr. Sciote's laboratory at the Kornberg School of Dentistry at Temple

University. Upon arrival, muscle was stored at -80°C until sectioning and isolation of total RNA for microarray analysis and RT-PCR.

Either a left or right masseter muscle from eleven of the 93 patient masseter samples were selected for microarray analysis based on operator analysis of subjects with the greatest mandibular growth rotation as describe in Skieller et al (1984). The eleven samples (mean subject age 28) included four Class II deep bite subjects, four Class II open bite, and three Class III open bite. Two of the eleven patients (Subject 1 and 2) were classified with posterior facial asymmetry of the ptyergiod plates, vomer bone, and septal cartilage from evaluation of submental vertex and view radiographs and posterior-anterior cephalograms. Both asymmetrical patients had masseter muscle samples taken from the long side (i.e. the side opposite of the chin deviation) for genetic analysis and none reported a history of facial trauma. Patient 1 also had evidence of condylar resorption as determined by evaluation of panoramic radiography with resulting mandibular asymmetry to the left. The radiographic analysis revealed evidence of flattening of both condylar heads, greater on the left side.

The sequela of this finding was greater, progressive mandibular retrusion with clockwise rotation of the mandible on the left due to decreased ramus height, which contributed to the diagnosis of mandibular midline left of facial midline. Patient 1 was classified as a skeletal Class II anterior open bite with a mandibular asymmetry (<3 mm) with significant posterior facial asymmetry. Patient 2 was diagnosed as a skeletal Class III anterior open bite with mandibular asymmetry (<3 mm) and significant posterior facial asymmetry. Patient 3, a 41 year old female with a Class II open bite, was confirmed to have obstructive sleep apnea. Although genetic analysis was conducted on this subject the patient was removed a priori from the comparisons in this study. None of the subjects had systemic conditions, facial trauma, tumor, condylar hypertrophy, arthritis, or developmental conditions that might influence craniofacial growth.

Masseter biopsies from eleven of the study subjects were used to generate microarray data. The dentofacial classification of these patients is listed in **Table 1**.

Table 1. Orthognathic Surgery Subjects Included in the Microarray Analysis of Masseter Muscle Gene Expression

			Skeletal Classification		
Subject	**Gender**	**Age**	**Sagittal**	**Vertical**	**TMD**
1	F	15	Class II	Open	Yes
2	F	17	Class II	Open	No
3	F	41	Class II	Open	Yes
4	F	41	Class II	Open	No
5	F	24	Class II	Deep	No
6	F	47	Class II	Deep	Yes
7	M	17	Class II	Deep	Yes
8	F	34	Class II	Deep	No
9	F	16	Class III	Open	No
10	F	53	Class III	Open	No
11	F	18	Class III	Deep	No

Samples from these subjects, as well as 45 additional subjects were included in the RT-PCR experiments.

4.2 Jaw Pain and Function Analysis

All subjects preformed a tempomandibular pain survey pre-surgery. Developed by Gerstner, Clark, and Goulet (1994), the questionnaire asked eight questions related to jaw pain and five questions related to joint function. This enabled subjects to self-rate their pre-surgical pain and function levels. The purpose of the survey was to classify patients as those with tempomandibular disorder (TMD), those without tempomandibular disorder, and subjects with tension-type headaches. Gerstner et al. (1994) showed that the questionnaire could not distinguish TMD from those with muscular related headaches. The questionnaire would reliably identify TMD subjects from the control with 90.3-97.7% sensitivity and 95.7-100% specificity. The questionnaire numerically quantified TMD symptoms. This high reliability identified TMD patients at a rating of 5 to 9. For the purposes of identifying patients with TMD in our investigation, we used a cutoff value of 6 or higher. A copy of this questionnaire is included (Appendix A). In addition to the JPF questionnaire, subjects were tested for signs and symptoms of temporomandibular disorder at the time of the pre-surgical examination**.** Of the eleven surgical subjects, four were diagnosed as having signs and symptoms of TMD (patients 1,3,6, and 7).

4.3 Microarray Analysis

4.3.1 RNA Isolation and Reverse Transcription

Single masseter samples from either the left or right side of each of the selected subjects were transported on dry ice to the University of Pennsylvania Molecular Profiling Facility and University of Pennsylvania Center for Musculoskeletal Disorders where procedures for microarray target preparation and hybridization were conducted. Total RNA was isolated using Qiagen miRNeasy® procedures (Qiagen Inc., Valencia, CA) according to the manufacturer's specifications. Tissue was disrupted in QIAzol Lysis® Reagent (700 µl) with a generator probe, and RNA was isolated using a miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quality of the total RNA was tested by Bioanalyzer and Nanodrop spectrophotometry (Agilent, anta Clara, CA) and the Affymatrix GeneChip Expression Analysis Technical Manual (affymetrix Inc, Santa Clara, CA). A 250-ng alquot of total RNA was converted to first-strand cDNA by reverse transcriptase with pol(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.

4.3.2 Microarray Hybridization and Analysis

Labeled cDNA (5.5ug) was added to an Affymatrix hybridization cocktail, heated at 99ºC for five minutes and hybridized for sixteen hours at 45ºC to Human Transcriptome 2.0® (HTA-2.0) GeneChips (Affymetrix Inc., Santa Clara CA). The microarrays were then washed at low stringency with 0.9M sodium chloride, 60mM sodium phosphate, 6mM ethylenediaminetetraacetic acid (EDTA), pH 7.4 (6X SSPE) and high stringency with 100mM 2-(N-morpholino) ethanesulfonic acid (MES), 0.1M sodium chloride buffer (NaCl) and stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycoerythrin stain. A GeneChip 3000 7G® (Affymetrix Inc., Santa Clara CA) scanner was used to collect fluorescence signal. Fluorescence data from the 70,534 transcripts of the array were normalized and converted to Log2-transformed values by Affymetrix Command Console with Partek Genomics Suite, v6.6 software.

4.4 Principal Component Analysis

A Principle Component Analysis (PCA) distinguished greatest gene expression differences between the eleven subjects included in the microarray analysis. Differences were illustrated by representing the linear combination of all gene transcripts on each axis. The result was a clustering of subject points based on similarity of RNA expression levels of each of the transcripts. Closer subject points signifies more similar RNA expression values with clustering of subject point implying even greater similarity of gene transcripts. Dr. John Tobias at The University of Pennsylvania performed the analysis using Partek software.

4.5 RT-PCR

4.5.1 Total RNA Isolation for RT-PCR

Total RNA was isolated with TRIzol as described previously (Horton et al, 2008). First, muscle was disrupted by TRIzol® reagent (Invitrogen, Carlsbad, CA). The RNA extracts were then digested with DNase I, and RNA was re-isolated with RNAqueous® (Ambion, Austin, TX). The presence and concentration of isolated RNA was verified and quantified by absorbance at A_{260}.

4.5.2 RNA Quantification

RT-PCR was performed to validate microarray expression values for three target genes selected for analysis from the microarray data. The genes of interest were a set of highly related *Nodal* modulator genes (*NOMO1,2,3*), a gene involved in positive intracellular signal transduction of the transforming growth factor-beta (*SMAD4*), and an upstream regulator of *Nodal* (*PITX2*). Reverse transcription polymerase chain reaction

(RT-PCR) assays were performed in triplicate for each of the three target genes. The TaqMan® (Applied Biosystems, Foster City, CA) RT-PCR protocol utilized commercial gene specific primer probe sets and an endogenous control gene hypoxanthine phosphribosyltransferase 1 (*HPRT1)* in a RNA-to-C_T 1-Step® reagent in an Applied Biosystems Step One Plus® instrument.

The *HPRT1* gene functions in the synthesis of purine nucleotides. It is expressed in all human tissues so it is considered a housekeeping gene. Running comparison assays using a *HPRT1* ensures that expression levels can be quantified for the three target genes by the comparative threshold cycle ($\Delta\Delta C_T$) method (Livak & Schmittgen, 2001). This method compares the fold differences in RNA expression between the subjects and a standard calibrator sample as related to the internal control gene.

Here, a commercial preparation of purified human skeletal muscle RNA (Ambion) was used as the standard control or calibrator. Standard plots for amplification comparisons were made using 1, 10 and 100ng of skeletal muscle RNA prepared by serial dilution. Then amplification plots for the internal control gene, *HPRT1* (**Figure 2**) and the three genes of interest, *NOMO1,2,3*, *SMAD4*, and *PITX2* (**Figures 3, 4, 5**) were determined. The standard curves for *HPRT1* (internal control) and the target genes showed that amplification efficiencies were >90% and within 10% of one another. From this, the reference calibrator standard was determined to be 15 ng of skeletal muscle RNA.

HPRT1

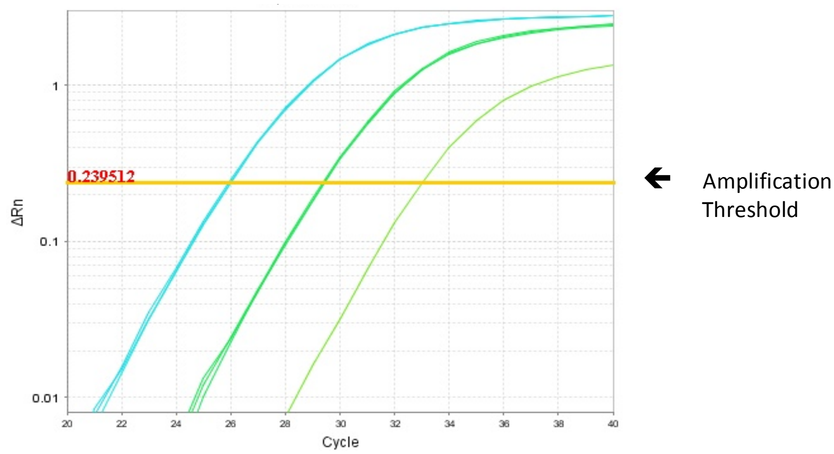

Figure 2: RT-PCR Standards Amplification Plot of Internal Control Gene *HPRT1*
Amplification thresholds are used to determine the fluorescence level above base, but below exponential amplification phase. Standard human skeletal muscle RNA in the amounts of 100ng (Blue), 10ng (Green), and 1ng (Light Green) were used.

NOMO1,2,

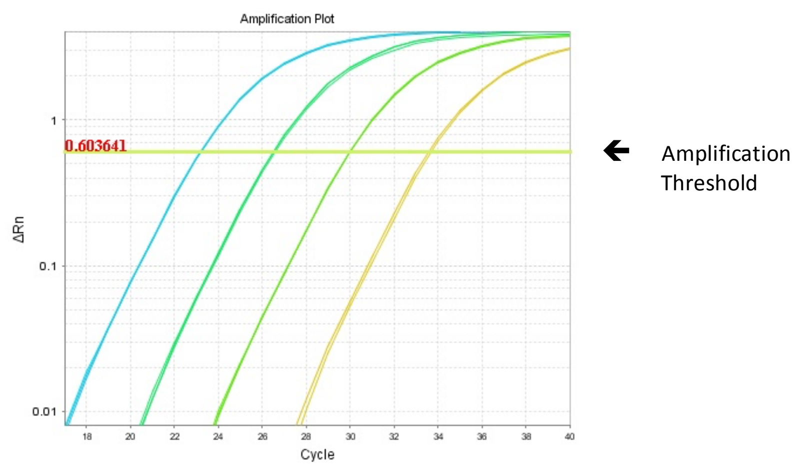

Figure 3: RT-PCR Standards Amplification Plot for *NOMO1,2,3*
Amplification thresholds are used to determine the fluorescence level above base, but below exponential amplification phase. Standard human skeletal muscle RNA in the amounts of 100ng (Blue), 10ng (Green), and 1ng (Light Green) were used.

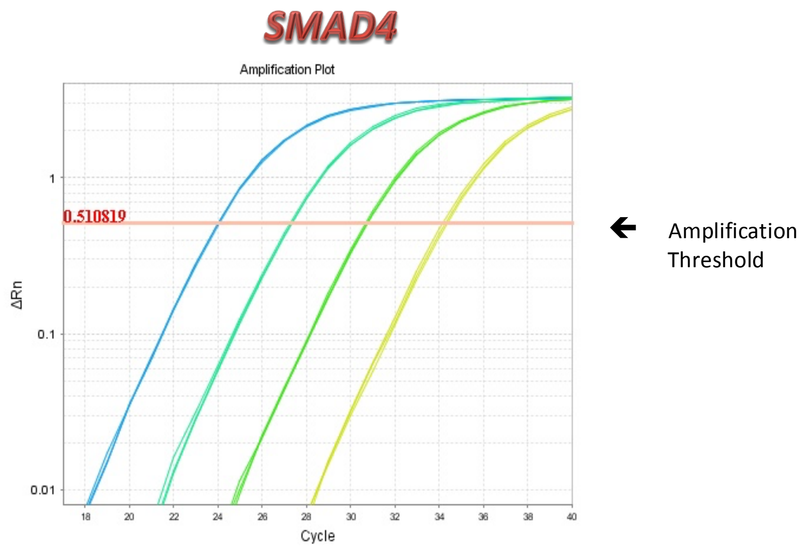

Figure 4: RT-PCR Standards Amplification Plot for *SMAD4*
Amplification thresholds are used to determine the fluorescence level above base, but below exponential amplification phase. Standard human skeletal muscle RNA in the amounts of 100ng (Blue), 10ng (Green), and 1ng (Light Green) were used.

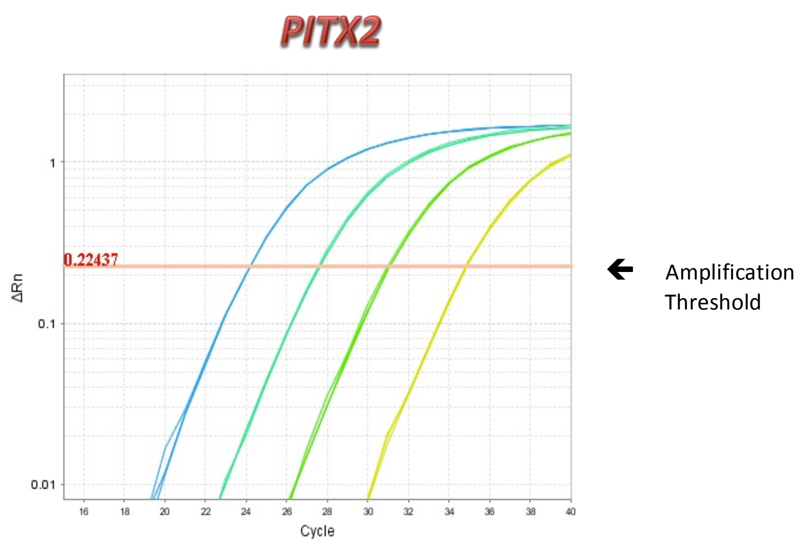

Figure 5: RT-PCR Standards Amplification Plot for *PITX2*
Amplification thresholds are used to determine the fluorescence level above base, but below exponential amplification phase. Standard human skeletal muscle RNA in the amounts of 100ng (Blue), 10ng (Green), and 1ng (Light Green) were used.

The standard amplification plots of the target genes and RNA from *HPRT1* should show parallel slopes and the amplification efficiency of the three genes of interest should be greater than 90%. Our values met these conditions. Two standard curves were generated to compare the data: one for *NOMO1,2,*3 and *SMAD4*, and a separate curve for *PITX2*. The slope for *HPRT1* in the first data set was -3.41 and the slope for *NOMO1,2,3* was -3.48, and for *SMAD4* was -3.42. The efficiency percent for *HPRT1* was 96.46% and the efficiency percent for *NOMO1,2,3,*and *SMAD4* were 93.96% and 96.12% respectively (**Figure 6, Table 2**). For the second data set, the slope for *HPRT1* was -3.38 and the slope for *PITX2* was -3.43. The efficiency percent for *HPRT1* was 97.72% and for *PITX2* was 95.79% (**Figure 7, Table 2**). The genes were amplified greater than 90% and the comparative C_T (concentration threshold) could be utilized to quantify RNA expression of the genes of interest.

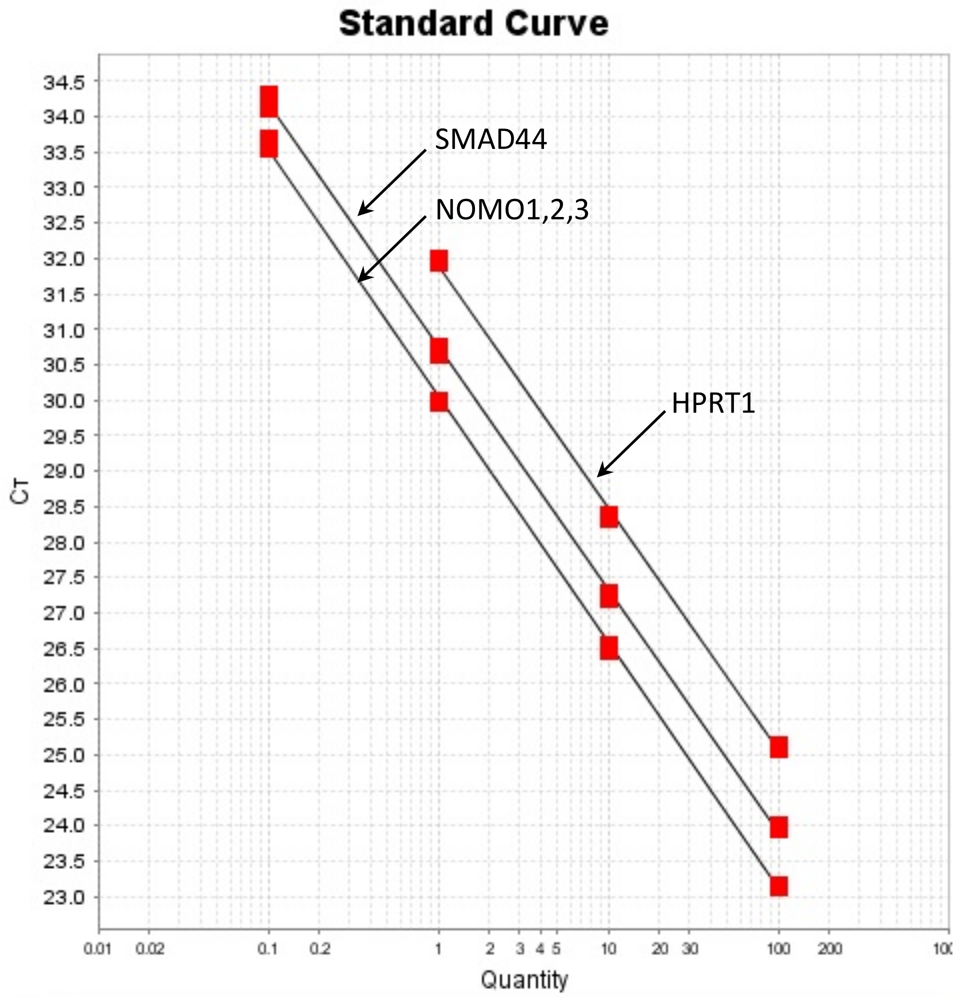

Figure 6: Standard Curves for *NOMO1,2,3, SMAD4,* and *HPRT1*
Illustrates generally parallel slopes between the RT-PCR standard curves for the genes of interest in relation to a calibrator gene (*HPRT1)*. The Y-axis (C_T) represents the fluorescence level at threshold amplification and is plotted against the X-axis (Quantity) which represents the amount of RNA expression in nanograms.

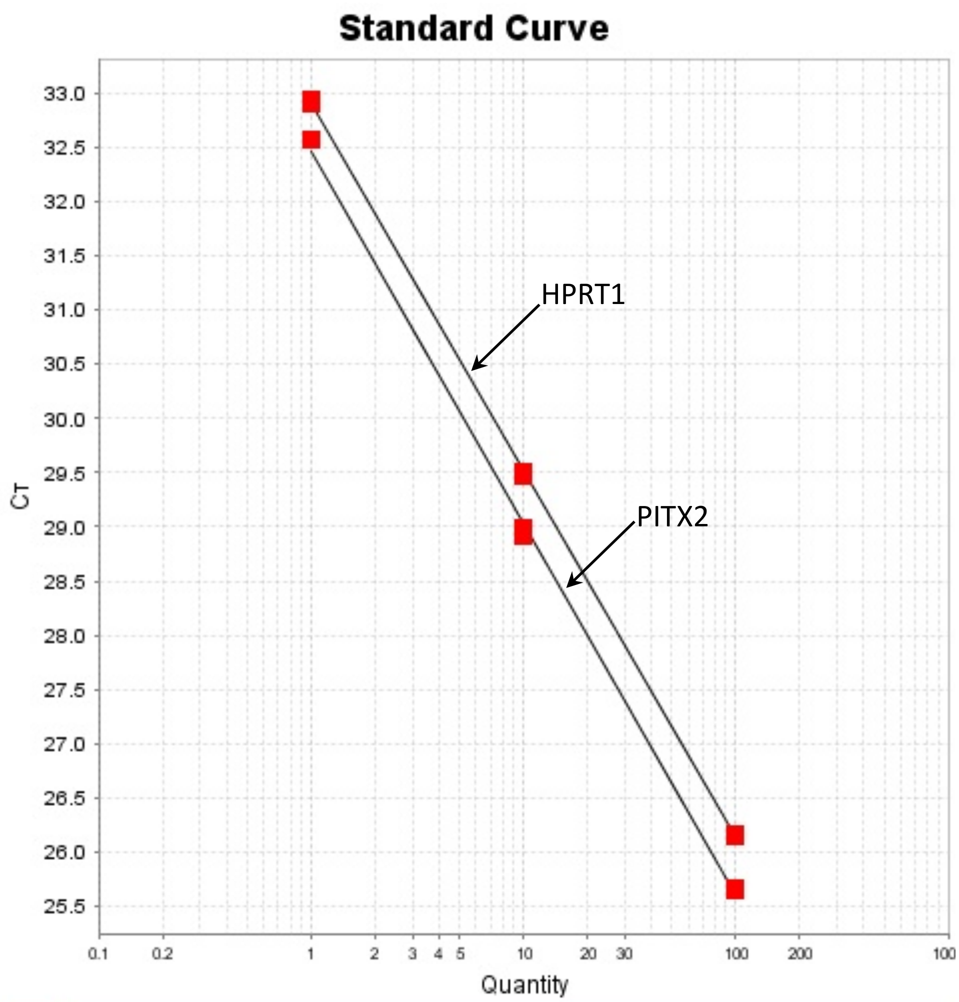

Figure 7: Standard Curves for *PITX2* and *HPRT1*: Illustrates generally parallel slopes between the RT-PCR standard curves for the genes of interest in relation to a calibrator gene (*HPRT1)*. The Y-axis (C_T) represents the fluorescence level at threshold amplification and is plotted against the X-axis (Quantity) which represents the amount of RNA expression in nanograms.

Table 2. Amplification characteristics of internal control gene *HPRT1* and experimental genes of interest *NOMO1,2,3*, *SMAD4,* and *PITX2* by RT-PCR

Gene	Slope	Y Intercept	R^2	Efficiency %
Gene Assay Set #1				
HPRT1	-3.41	31.88	0.99	96.46
NOMO1,2,3	-3.48	30.04	0.99	93.96
SMAD4	-3.42	30.74	1.00	96.12
Gene Assay Set #2				
HPRT1	-3.38	32.89	1.00	97.72
PITX2	-3.43	32.46	0.999	95.79

The comparative threshold cycle (CT) method ($\Delta\Delta C_T$) using the following calculation determined relative quantities:

Fold Change = $2^{-\Delta\Delta CT}$ where:

ΔC_T (Masseter Muscle Sample) = C_T (*TNF*, Masseter RNA) – C_T (*HPRT1*, Masseter RNA)

ΔC_T (Calibrator Skeletal Muscle Sample) = C_T (*TNF*, Skeletal Muscle RNA) – C_T (*HPRT1*, Skeletal Muscle RNA)

$\Delta\Delta C_T = \Delta C_T$ (Masseter Muscle Sample) – ΔC_T (Commercial Skeletal Muscle Sample)

$$2^{-\Delta\Delta CT} = 2^{-[\Delta CT \text{ (Masseter Muscle Sample)} - \Delta CT \text{ (Commercial Skeletal Muscle Sample)}]}$$

Assays were performed three times for the three genes of interest in 56 subjects. The expression level data was averaged with each assay. Results were then reported as fold differences between target genes and the standard commercial skeletal muscle RNA expression levels.

4.6 Masseter Muscle Sample Fiber Type Analysis

During the bilateral sagittal split osteotomy procedure in France, masseter muscle samples were collected from the discarded muscle. The samples were from the deep anterior portion of the muscle. Next, the samples were snap frozen and stored at -80° C. The samples were then analyzed for gene expression and histologic analysis after being shipped to Dr. Sciote's lab at Temple University Dental School (Sciote, 2012; 2013).

In the lab, the samples were cryosectioned at 10 μm thickness to obtain serial cross sectional slices, and sections were mounted on glass microscope slides for immunostaining with five antibodies specific for myosin heavy chain (MyHC) isoforms: anti-type I, anti-type IIX, anti-type IIA, anti-type neonatal and anti-α-cardiac (atrial) as described previously (Sciote, 1994; 1998). Any tissue section series that did not have consistent antibody reactions for all stains or acceptable morphology of muscle fibers were discarded from the data. Image J image-analysis software, available from the National Institutes of Health, was used to measure fiber type cross-sectional areas. Measurement error was tested using intra-rater reliability in determination of fiber area (by repeating morphometric tracing of all fiber areas in one biopsy by one examiner), This resulted in an R^2 value of 0.94.

The purpose of determining the differential composition of muscle fiber type in the samples was due to the fact that different fiber types have different characteristics and purposes. Type I fibers are used to maintain posture, particularly freeway space, as they are fatigue resistant. In contrast, Type II fibers can either be fatigue resistant (type IIA) or fatigueable (type IIX). Type I fibers are slow contracting while both Type IIA and Type IIX fibers are fast contracting. The hybrid fibers combine both slow and fast contractile properties (Sciote, 1994). These hybrid fibers are rare in skeletal muscle (more commonly found in skeletal muscle pathology). Masticatory muscles are distinct in that hybrid muscle is always found.

4.7 Statistical Evaluation

Affymetrix Transcriptome Analysis Console Software was used to analyze the data of the differentially expressed genes. Eleven CEL files were imported to Partek Genomics Suite (v6.6; Partek Inc, St. Louis *P* MO). RNA normalization was applied, resulting in log2-transformed intensities for 70,534 transcript IDs. The IDs corresponded to 24,953 unique gene symbols, their transcriptional variants, and additional content provided on the platform.

A *t*-test was preformed to find differentially expressed transcripts comparing the two asymmetric samples (Subjects 1 and 2) to the eight symmetric samples. Because relatively small sample sizes are used to compare thousands of differences in gene expression levels, a statistical approach to correct any false discovery rate was necessary. Therefor, resulting values were corrected for false discovery rate (step up P value) using the method of Benjamini and Hochberg as implemented in Partek Genomics Suite. Differences were considered significant if step-up $P < 0.02$ and fold differences were greater than +/- 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 because accurate power analysis cannot be calculated without making too many unfound assumptions. Raw CEL files for each of the eleven subjects were uploaded into the GEO database with the following ID link: GSE57775. Included in GEO is a Matrix file summarizing the experimental protocol and an Affymetrix matrix table listing transcript expression levels for each subject.

CHAPTER 5

RESULTS

5.1 Statistical Differences between Groups in Microarray Analyses

5.1.1 Principal Components Analysis

Intersample differences in global gene expression between subjects in the microarray analysis were visualized according to variations in three dimensions by a Principal Components Analysis (PCA). In a summary plot (**Figure 8**), each axis is a linear combination of all 70,534 transcripts that has its own coefficients selected to best show sample variation. The number of each color-coded dot represents one subject corresponding to the same numbered patient shown in Table 1. Malocclusion classification is depicted by the color of the subject dot in which red signifies a skeletal Class II deep bite subject, blue signifies a skeletal Class II open bite subject, and green signifies a skeletal Class III open bite subject. The PCA plot shows that the overall expression data of patients with posterior midfacial asymmetry (Subjects 4 and 10) sorted separately from subjects of other malocclusion/facial groups. All malocclusion subjects without facial asymmetry, regardless of sagittal and vertical dimensions, were clustered into one group. The patient identified as having obstructive sleep apnea (Subject 3) also sorted separately. While genetic differences exist within the symmetric malocclusion group, the greatest degree of genetic difference is found between malocclusion patients on the basis of presence or absence of facial asymmetry.

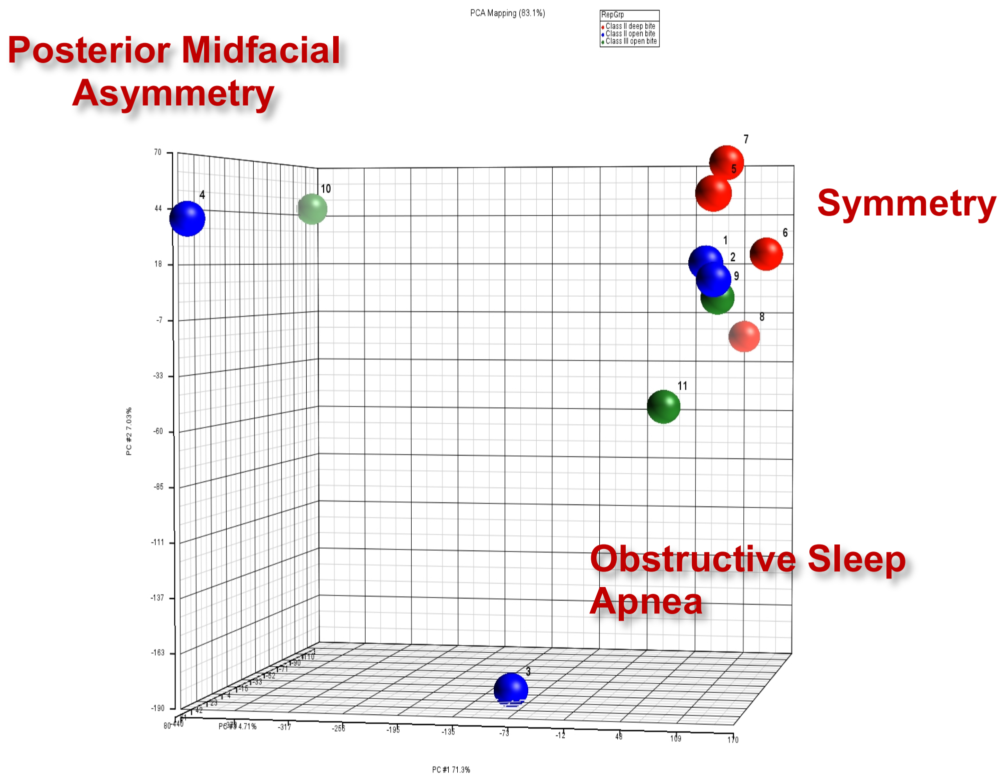

Figure 8. Principal Component Analysis. The PCA plots subjects according to genotype and clusters subjects with highest similarity of gene expression.

All transcripts were evaluated for evidence of differential expression between symmetry and asymmetry groups. Differential expression of transcripts was considered significant if there was a greater than +/-2 fold difference between the groups and a step-up *P* value <0.05. Using these values, approximately one-third of the array, approximating 22,000 gene transcripts, was determined to be significant. These results are not related to the results from the PCA comparison plots, but rather a reflection of the statistical cutoffs for significantly differentially expressed transcripts.

In order to determine the biological significance for these samples clustering separately, we investigated a subset of genes that are responsible for regulating normal formation of symmetry during development. Included in these gene sets are the Nodal Pathway genes and *PITX2.*

5.1.2 Differences in Nodal Pathway Gene Expression in Microarray Analysis

The Nodal Pathway dictates normal formation of laterality at specific time points during development. These genes pattern asymmetry in the heart, lungs, and other organs. Because the facial asymmetry patients clustered independently from the symmetric patients, the Nodal Pathway genes were selected to further investigate whether gene expression differed between these two groups. These genes were chosen a priori due to their biological effect on formation of laterality, as well as their documented effects on first branchial arch structures.

Expression levels between asymmetry Subjects 1 and 2 and the eight malocclusion subjects of the symmetry group differed by several fold with significant step-up *P* values identified for ten of the twenty-two Nodal pathway genes of interest (**Table 3**).

Table 3: Differences in Nodal Pathway Gene Expression in Masseter Muscle from Subjects with Malocclusion and Facial Asymmetry Compared to Symmetric Subjects by Microarray Analysis.

Transcript ID	Symbol	Gene	Gene Description	Fold Difference	Step-Up P-Value
TC05001797.hg.1	PITX1	Pituitary Homeobox 1	Downstream Organogenesis	1.13	0.02
TC04001471.hg.1	PITX2	Pituitary Homeobox 2	Downstream Organogenesis	-1.10	0.06
TC08001753.hg.1	FOXH1	Forhead Box H1	Nuclear Transcription Factor	1.29	0.0002
TC01006387.hg.1	LEFTY1	Lefty 1	Negative *Nodal* Regulation	1.54	4E-06
TC01003895.hg.1	LEFTY2	Lefty 2	Negative *Nodal* Regulation	1.38	0.00003
TC10001365.hg.1	NODAL	Nodal	Cell differentiation signaling	1.84	9E-06
TC16000180.hg.1	NOMO1	Nodal Modulator 1	Protein Modulator	-5.63	0.0003
TC16000912.hg.1	NOMO2	Nodal Modulator 2	Protein Modulator	-5.81	0.0003
TC16000194.hg.1	NOMO3	Nodal Modulator 3	Protein Modulator	-5.65	0.0003
TC09000919.hg.1	CER1	Cerberus 1	Negative *Nodal* Regulation	1.90	0.0009
TC02002344.hg.1	CFC1B	Cryptic Family Protein 1 B	*Lefty* Mediator	1.31	0.0005
TC02000813.hg.1	CFC1B	Cryptic Family Protein 1 B	*Lefty* Mediator	1.27	0.0001
TC10001623.hg.1	ACTR1A	Alpha-centracin 1A	Surface Receptor	-4.64	1E-06
TC02002121.hg.1	ACTR1B	Alpha-centracin 1B	Surface Recetpor	-1.05	0.29
TC02000378.hg.1	ACTR2	Alpha-centracin 2	Surface Receptor	-2.47	0.00005
TC04000711.hg.1	SMAD1	Mothers against decapentaplegic homolog 1	Positive Intracellular Mediator	-1.22	0.094
TC18000496.hg.1	SMAD2	Mothers against decapentaplegic homolog 2	Positive Intracellular Mediator	-2.47	0.00002
TC15000622.hg.1	SMAD3	Mothers against decapentaplegic homolog 3	Positive Intracellular Mediator	-3.14	0.0001
TC18000998.hg.1	SMAD4	Mothers against decapentaplegic homolog 4	Positive Intracellular Mediator	-5.91	1E-06
TC0X000195.hg.1	USP9X	Probable ubiquitin carboxyl-terminal hydrolase FAF-x	Positive Intracellular Mediator	-7.03	4E-07
TC14000362.hg.1	PPM1A	Protein phophatase 1A	Negative Intracellular Mediator	-3.80	5E-06
TC19001087.hg.1	RANBP3	Ran-binding protein 3	Negative Intracellular Mediator	-1.20	0.089

Expression of the genes for *Nodal* and *Lefty,* which are extracellular mediators of differentiation, were 1.84 to 1.39 fold greater ($P < 3.41 \times 10^{-5}$), whereas the integral membrane modulators of *NODAL*, *NOMO 1,2,3*, were -5.63 to -5.81 ($P < 3.05 \times 10^{-4}$) less in asymmetry subjects. Expression values of two of the three surface activin receptors were also significantly decreased: *ACTR2* was -2.47 ($P < 5.06 \times 10^{-5}$) and *ACTR1A* was -4.64 ($P < 9.65 \times 10^{-7}$). Also in the array data, microRNAs *MiR-15B/16,* negative regulators of post-transcriptional gene expression that target the Nodal type II receptor *Acvr2a* gene (aka *ACTR2*) in frog development (Martello et al., 2007), had fold increases of +1.5. Fold differences among four of the five positive intracellular mediators exceeded the negative threshold (<-2.0) in the range of -7.02 to -2.47 ($P < 0.003$). *SMAD4*, which in addition to its critical role with co-partner *SMAD2* in the NODAL pathway also mediates signal transduction of Transforming Growth Factor (*TGF-β*), was expressed -5.91 fold less in asymmetry subjects with a P value of $1x10^{-6}$. *USP9x* gene, which encodes a deubiquitinating enzyme that maintains Smad4 activity (Dupont et al., 2009), was likewise significantly down-regulated with asymmetry (-7.03 fold; P $4x10^{-7}$).

In contrast, *PITX2*, a transcription factor whose expression is controlled by Nodal and, in turn, acts as an upstream effector of Nodal, showed no significant fold difference (-1.10; $P < 0.1$) between asymmetric and symmetric subjects. However, because left–right expression of *PITX2* determines asymmetrical development of left sided visceral organs, participates in myogenic development and maintenance of extraocular muscles and influences the size of type II skeletal muscle fibers, it was considered an important gene of interest in this study.

5.2 Overview of RT-PCR Expression Analysis

Three genes of interest (*NOMO1,2,3, SMAD4,* and *PITX2)* were selected for analysis in a larger population to verify the microarray data and to further characterize differential expression levels in masseter muscle samples between asymmetry and symmetry subjects with malocclusions. RT-PCR was performed on RNA from masseter muscle samples of the orthognathic surgery patients to quantify the three genes.. Quantitative data were analyzed for gene expression differences between right and left masseter muscles and bilateral average values when comparing subject groups.

5.2.1 RT-PCR Subject Characteristics

NOMO1,2,3, SMAD4, and *PITX2* expression levels from both the right and left side masseter muscle biopsies were determined for 47 subjects (**Table 4**) Of the 47 subjects, we obtained expression results for *NOMO1,2,3* in 19 subjects, for *SMAD4* in 15 subjects, and for *PITX2* in 13 subjects. Of these subjects, 30 were female (64%) and the mean age was 23.8 years. Of the total subjects, 31 were diagnosed as having a Class II malocclusion, and 16 were Class III. Vertical classifications broke down in to 11 normal, 21 open, and 15 deep bites. Asymmetry was identified in 53% of patients (22 symmetric and 25 asymmetric) and signs and symptoms of temporomandibular disorder were present in 19% (9 Subjects). All facial asymmetries, including those as a result of imbalanced posterior midfacial growth, were considered significant if they caused a facial midline deviation of four millimeters or greater.

Table 4: Summary of patient phenotypes for RT-PCR masseter muscle samples.

	Sagittal	**Vertical**	**Presence of Symmetry**
Classification	Class II	Normal	Symmetric
Subject Number	31	11	22
Classification	Class III	Open	Asymmetric
Subject Number	16	21	25
Classification		Deep	
Subject Number		15	

5.2.2 RT-PCR Expression Data

Right and left side masseter muscle samples were analyzed for expression levels of the three genes of interest. Any subject that did not have data for both sides was not included in analyses because the value collected from a single side might not accurately represent both sides. It is likely that a discrepancy, though slight, exists between expression on contralateral sides.

First, expression levels of the three genes of interest were compared between right and left side masseter muscles from patients without regard to symmetry or malocclusion classification (**Figure 9**). Subjects that did not have data for both sides were excluded in this analysis because the value collected from a single side might not accurately represent both sides. It is likely that a discrepancy, though slight, exists between expression on contralateral sides. *NOMO1,2,3* included 19 subjects and had similar expression values on the left and right sides (0.332 and 0.312 respectively) that were not significant, p=0.6396. The sample for *SMAD4* included 16 subjects, and, like *NOMO1,2,3,* did not have a significant difference between expression on each side (0.463 on the left and 0.420 on the right), p=0.3326. *PITX2* expression values included 13 subjects and also did have a significant increase p=0.9721, with the left side producing RNA values of 0.918 and the right side yielding 0.913.

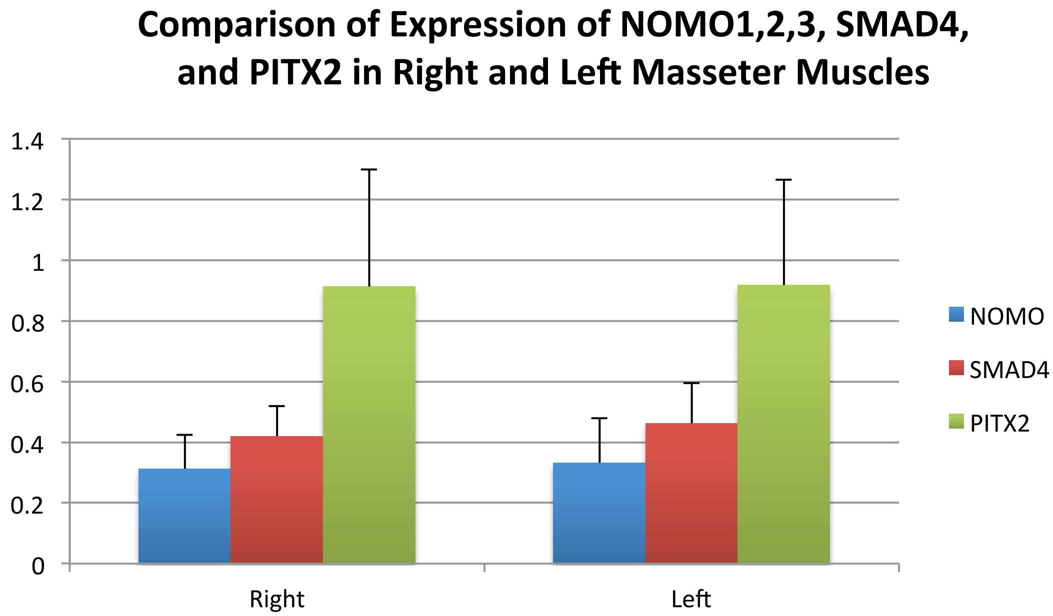

Figure 9: Expression values for the three genes of interest comparing left and right masseter muscle samples in all patients.

Next, gene expression values were compared for left and right side masseter muscles of subjects with skeletal symmetry (**Figure 10**). Values were obtained for eight subjects for *NOMO1,2,3,* seven subjects for *SMAD4,* and seven subjects for *PITX2.* Expression values for *NOMO1,2,3* were right - 0.324 and left – 0.301 and were not significantly different, $p = 0.7241$. Expression values for *SMAD4* were right - 0.420 and left - 0.460 and were not significantly different, $p = 0.5092$. Expression values for *PITX2* were right – 0.928 and left – 0.828 and were not significantly different, $p = 0.5912$.

Comparison of Expression of NOMO1,2,3, SMAD4, and PITX2 in Right and Left Masseter Muscles of Patients with Facial Symmetry

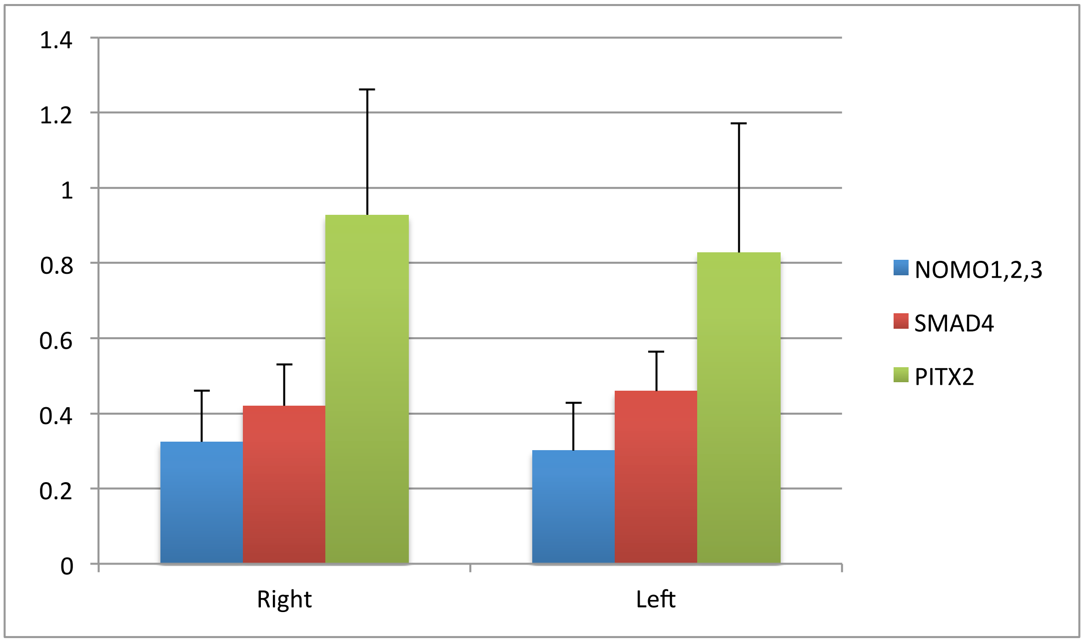

Figure 10: Expression values for the three genes of interest comparing left and right masseter muscle samples in symmetric patients.

An analysis of muscle from asymmetry patients was done (**Figure 11**) in the same way. Once again, values were obtained for 8 subjects for *NOMO1,2,3,* 7 subjects for *SMAD4,* and 7 subjects for *PITX2.* The 11 *NOMO1,2,3* subjects had values of 0.355 on the left and 0.301 on the right with a difference of 0.114. This difference was not significant, $p = 0.3335$. The 8 *SMAD4* subjects yielded a difference of 0.132 with 0.466 expression on the left and 0.419 expression on the right. This differences was also not significant, $p = 0.4677$. Finally, the 6 asymmteric *PITX2* samples had 1.024 expression on the left and only 0.896 expression on the right. This resulted in a right versus left lateral difference of 0.338, which sot significant at $p = 0.586$.

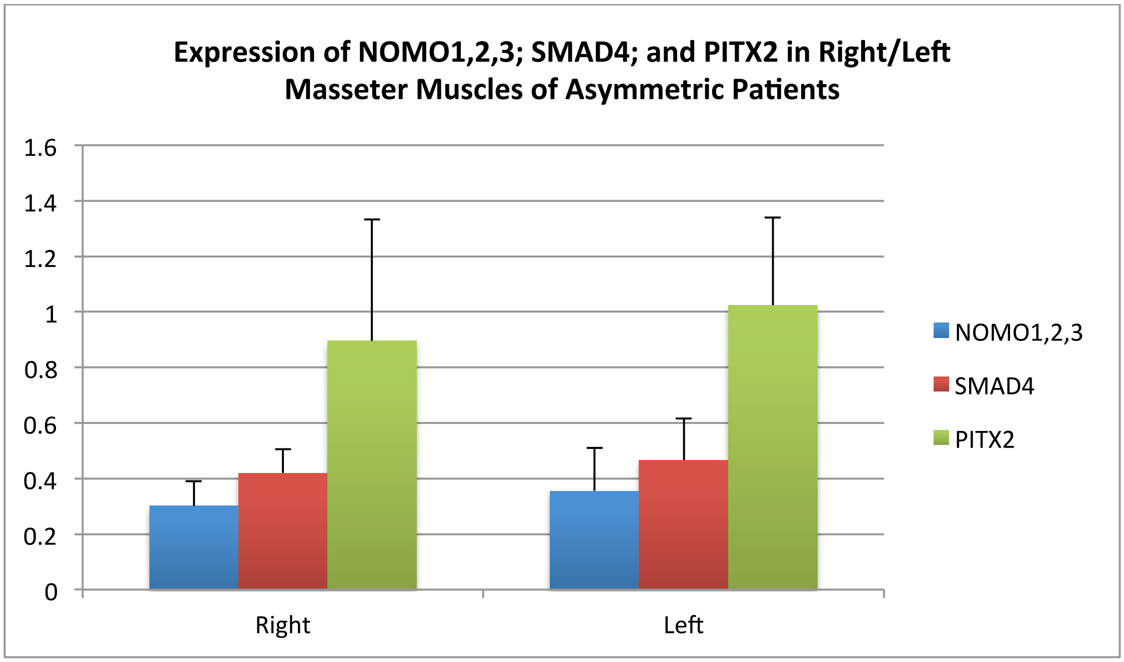

Figure 11: Expression values for the three genes of interest comparing left and right masseter muscle samples in asymmetric patients.

Having compared gene expression differences between right and left sides in the three groups, treated as independent variables, the results were not significant. We then assembled **Table 5** to compare lateral difference values (LED) for the entire group relative to each other. Based on this comparison it became evident that the LED between groups was highest in the asymmetric subjects compared to symmetric subjects in all genes of interest, and especially for PITX2.

Table 5. Lateral Expression Differences (LED) in *NOMO1,2,3, SMAD4,* and *PITX2* in Masseter Muscle of Orthognathic Surgery Subjects With and Without Facial Asymmetry

	n	LED *NOMO1,2,3*	LED *SMAD4*	LED *PITX2*
All Subjects	47	0.022	0.047	0.006
Symmetric	22	0.023	0.040	0.099
Asymmetric	25	0.114	0.132	0.338

Seeing these data trends we compared LED between symmetric and asymmetric groups for the three genes using ANOVA (**Figure 12;Table 6**). Overall LED gene expression was significantly different between groups, $p = 0.029$. For individual gene comparisons there was no significant difference for NOMO, ($p = 0.386$) of SMAD4, ($p = 0.246$). For PITX2 there as a difference approaching significance at $p = 0.06$.

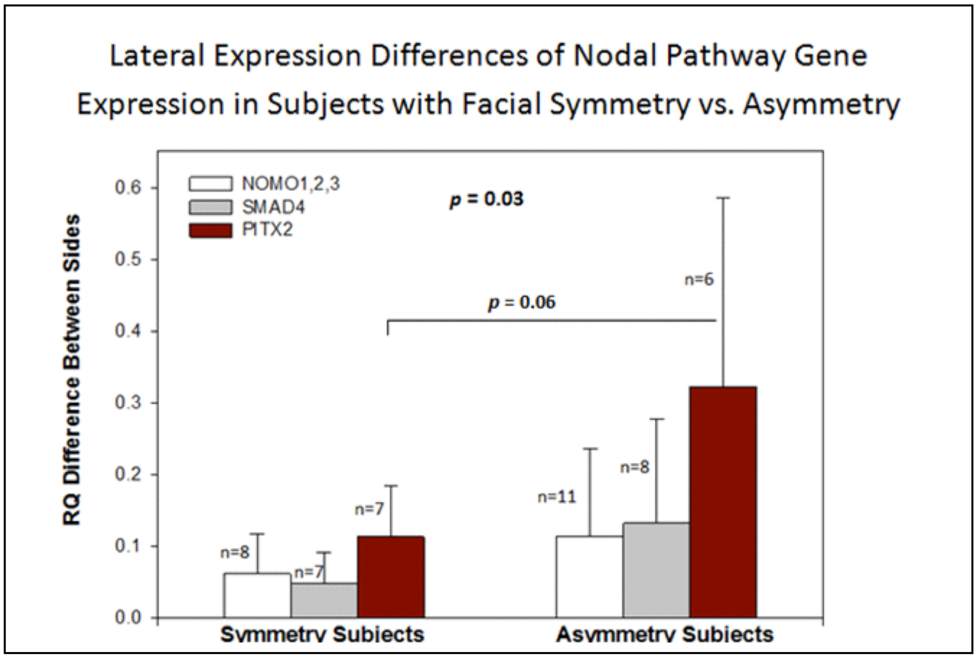

Figure 12: Lateral differences in right versus left masseter muscle gene expression varied significantly in patients with facial symmetry versus asymmetry

Table 6: Comparative Alayses of Expression Levels for Symmetry Versus

Table 6. Comparative Analyses of Expression Levels for Symmetry Versus Asymmetry			
Gene	**Statistical Analysis**	**Subject Number**	**p-value**
NOMO1,2,3, SMAD4, PITX2	ANOVA	47	0.029*
NOMO1,2,3	Unpaired t test	17	0.3865
SMAD4	Unpaired t test	15	0.2459
PITX2	Unpaired t test	13	0.0665*

5.2.3 Gene Expression Values vs. Malocclusion

After establishing gene expression values in comparison to symmetry versus asymmetry, further statistical analyses were performed on the basis of patient characteristics. Lateral expression differences (LED) were calculated as the difference between expression in the left muscle sample and expression in the right muscle sample. Average values between the two samples were also calculated.

Using ANOVA analyses, both LED and average expression values were compared for *NOMO1,2,3, SMAD4,* and *PITX2* individually, as well as the three genes in total. These values were then independently analyzed on the basis of: Asymmetric vs. symmetric, sagittal classification (Class I, II, or III), vertical classification (normal, open, or deep), gender, and the presence or absence of TMD. The significant results are reported in **Table 7**.

Table 7: ANOVA Analyses for Gene Expression Versus Malocclusion Classification

Gene	Value	Classification	P-value (*significant)	Subject Number
PITX2, NOMO1,2,3, SMAD4	LED	Symmetric	0.0006*	23
PITX2	LED	Asymmetric	0.015*	16
PITX2	LED	Asymmetric, no TMD	0.120	13
PITX2	Average	Symmetric	0.015*	5
PITX2	LED	Class II and Class III, Asymmetric	0.041*	8
PITX2	LED	Class II, Asymmetric	0.002*	6

As shown above, significance was found (p= 0.0006) for lateral expression difference of *NOMO1,2,3, SMAD4,* and *PITX2* collectively in the 23 patients in the patients with skeletal symmetry. Outside of this value, all significant correlations could be attributed to expression levels of *PITX2*. For average expression values of *PITX2* a correlation was found with symmetric patients with a p-value of 0.015. For lateral expression differences, correlations of *PITX2* was found in all asymmetric patients (p=0.015), Class II asymmetric patients (p=0.002), and Class II and III asymmetric patients (p=0.041).

5.3 Muscle Fiber Type Correlation

Correlation statistical tests were performed to determine the relationship between *NOMO1,2,3, SMAD4,* and *PITX2* expression and masseter muscle fiber type. Staining and fiber typing was preformed as described in the Materials and Methods and both mean area and percent occupancy were calculated for each fiber type.

Kendall Tau correlations were preformed comparing the expression of each gene and the pecent occupancy of each fiber type. These tests were further investigated on the bases of: Symmetry versus asymmetry, symmetry versus asymmetry in Class II subjects, symmetry versus asymmetry in Class III subjects, symmetry versus asymmetry in normal vertical subjects, symmetry versus asymmetry in open bite subjects, and symmetry versus asymmetry in deep bite subjects.

PITX2 was chosen as a focus for the final portion of our investigation. It was selected based on the previous results, showing it in particular had highly significant correlation between expression and maloclusion in various different classifications (**Table 8**).

The Kendall tau statistical tests resulted in no significance between any of the gene expression values and muscle fiber type for any of the classifications, with the exception of average *PITX2* expression and Type I fibers in all patients. This relationship resulted in a Kendall tau rank of -0.467 and a p-value of 0.01.

Table 8: Correlation Analyses for *PITX2* Expression Levels, Muscle Fiber Percent Occupancy, and Symmetry Classification

Table 8. Correlation Analyses for *PITX2* Expression Levels, Muscle Fiber Percent Occupancy, and Symmetry Classification			
	Statistical Analysis	**Subject Number**	**p-value**
Average *PITX2* Expression	**Kendall tau Rank**		
Type I	-0.467	18	0.01
Type IIA	-0.067	18	0.56
Type IIX	-0.333	12	0.64
	Pearson Correlation		
Type I	-0.670	18	0.002*
Average *PITX2* Expression, Asymmetric	**Kendall tau Rank**		
Type I	0.600	14	0.22
Type IIA	0.056	14	0.32
Type IIX	0.308	8	
RL Difference *PITX2* Expression	**Kendall tau Rank**		
Type I	-0.214	18	0.536
Type IIA	0.286	18	0.386
Type IIX	0	12	1
RL Difference *PITX2* Expression, Asymmetric	**Kendall tau Rank**		
Type I	-0.429	14	0.230
Type IIA	-0.048	14	0.220
Type IIX	-0.157	8	0.393

A Pearson Correlation was also performed for these subjects, once again resulting in significant results (p=0.002) (**Figure 13**).

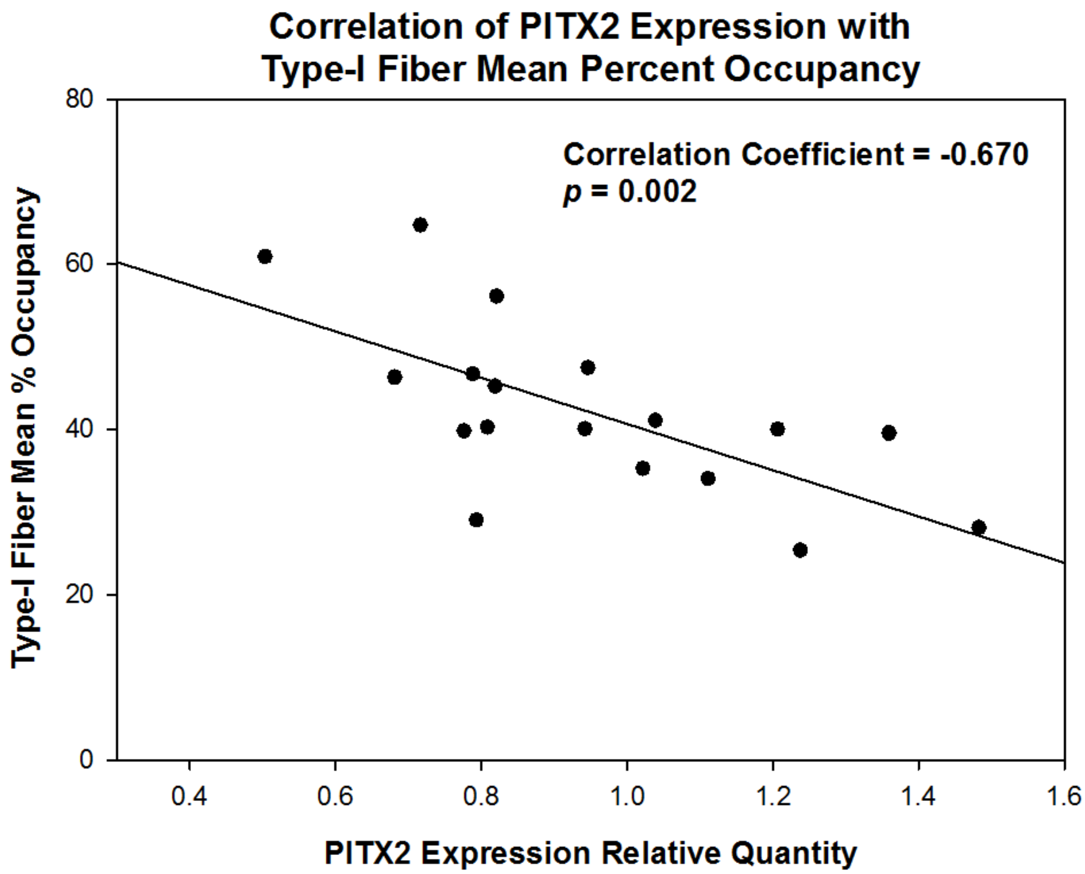

Figure 13: Pearson correlations confirm the influence of *PITX2* on skeletal muscle in patients with and without facial asymmetry. Decreased *PITX2* expression was found to result in decreased percentage of Type I myosin in the collected masseter muscle samples with significance (P<0.006).

CHAPTER 6

DISCUSSION

6.1 Asymmetry in Human Anatomy

Left right asymmetry is established very early in embryonic development and can be defined as the distinction of left versus right sides in the human body. The distinction is not only between the anatomy of left and right body halves (based on organ position, how they are connected, and body form) but also on how the asymmetric halves are established morphometrically. Regular left/right asymmetry is necessary for life, but deviation from the norm can have effects that range from mild skeletal imbalances (such as facial asymmetry) to severe serious conditions (Sutherland and Ware 2009). These conditions include apparently random organization of organs, to irregular intestinal looping, to congenital heart defects. Many require immediate surgical attention, while others are tragically incompatible with life (Kennedy et al. 2007).

Bilateral asymmetry was established relatively late in overall biologic evolution, though it is not distinct to humans or even mammals (Tena 2015). Asymmetry in the roundworm is established as early as the 6 cell stage and develops in to asymmetric substructure patterning (Wood et al. 1991). A left/right axis is also observed in the fruit fly with bilaterally asymmetric organ, brain, and male genital development (Adam et al. 2003). Each of these is induced by genetic expression influences (Coutelis et al. 2013).

A more elaborate and general left right asymmetry has been established in vertebrates, in contrast to the previous invertebrate examples where the asymmetry is restricted to certain substructures. Currently, two main hypotheses exist regarding how and when the left right axis is first established in vertebrates (Vandenberg and Levin 2013). One model suggests the establishment of the axis late in embryogenesis due to directional fluid flow as a result of the movements of motile cilia during late gastrulation and early neurulation. Another popular hypothesis supports the establishment of asymmetry much earlier in development, as soon as first cell cleavage after fertilization. This results in biophysical, molecular, and bioelectrical asymmetries. These early asymmetries are a result of chiral cytoskeleton and asymmetric segregation of chromatids. Further research suggests that the development of a bilateral axis is not mutually exclusive, but could be a result of both of these processes. Regardless of the mechanism of initial disruption of symmetry, the resultant effect is the same: asymmetric gene expression (particularly *Nodal, Lefty,* and *PITX2*) (Vandenberg 2012).

6.2 Craniofacial Asymmetry

Lateralization of humans occurs on multiple levels: from brain development, to organ positioning, to behavioral differences (Francks 2015). Right-left differences of the human brain can result in functional differences and preference. Studies have shown that 85% of human fetuses at 10 weeks gestational age move their right arm more than their left arm (Hepper et al. 2013). This percentage is strikingly similar to the adult rate of right-handedness and suggests an early embryonic establishment of laterality as a precursor for adult asymmetry.

Craniofacial asymmetry is present in almost the entire population to some degree. In general, the asymmetry is so mild that it is not obviously detected and considered within normal limits. More severe and noticeable facial asymmetries can be the result of a range of processes. These include but are not limited to facial trauma, congenital abnormalities, and environmental factors. As each craniofacial asymmetry variation can have a distinct etiology, they each result in distinct and importantly separate characteristics.

6.2.1 Nodal Pathway Genes During Development

During embryonic development, the breaking of symmetry during pattern formation and differentiation is essential. The Nodal Pathway is an integral part of this process, which occurs during gastrulation (Shen 2007). In particular, it is necessary for the establishment of the primary body axes, as well as the proper development of ectoderm and mesoderm layers. It has found to be conserved functionally over various species, including humans.

The Nodal Pathway is a complex network of ligands that are members of the tranforming growth factor-beta superfamily. The initiate extracellularly, binding to a membrane kinase receptor, and signal through the cytoplasm with the *SMAD* family of proteins, finally forming transcriptional complexes in the nucleus (Scheir 2003). Additionally, this pathway can be antagonized by *Lefty*, a highly diverged subclass of transforming growth factor-beta, by blocking receptor complexes (Chen and Shen 2004). The *Cerberus* family of cysteine-rich extracellular proteins are also *Nodal* inhibitors, but rather than blocking receptors, these proteins have a direct interaction with Nodal ligands (Piccolo et al. 1999).

The Nodal pathway is transduced downstream by a *SMAD* family of receptor-associated proteins (Massague et al. 2005). The pathway is also coupled with the transcription factor *FOXH1* and *LHX1*. Most of these studied proteins of the Nodal pathway are activated by gene transcription, while a few are transcriptionally repressed (Dickmeis et al 2001).

The initiation of the Nodal Pathway results in the generation of a signaling gradient that is essential for embryonic tissue patterning. This is due to a dose-dependent response in the field of developing cells (Ashe and Briscoe 2006). The ligands have a long range of action, with both *Nodal* and *Lefty* being shown to travel long distances of up to 500μm in chick embryos (Sakuma et al 2002). In mouse studies, *Nodal* is found primarily on the left side of the developing body axis, with its activation being blocked by the presence of *Lefty* on the right side of the axis. The ability of these ligands to have a long range of action is due to two factors: stability and efficiency of protein processing (LeGood et al. 2005).

Body development and patterning are specifically dose-dependent. This has been shown in *Nodal*-mediated mesoderm cellular response and loss of vegetal axis patterning in mutant knockout studies (Dougan et al. 2003). Additionally, more progressively severe defects are observed in mesoderm development with increasing doses of Nodal pathway mutants, including mutations of *SMAD* family proteins. This dependence could be due to differing levels of pathway activity, differing durations of exposure, or a combination of the two (Gritsman et al. 2000).

The Nodal pathway early in embryogenesis has been found to have numerous functions, most notably the signaling and antagonism in endoderm and mesoderm induction as well as neural left-right patterning. The pathway is essential for mesoderm formation, being dose and time-dependent for proper ventral-dorsal specification (Lee et al. 2001). Variable defects in mesoderm and endoderm development are observed in mutants with altered *Nodal* expression levels (Chen et al. 2006).

Similarly, dose-dependent responses to the Nodal Pathway are essential for the formation of the endoderm. These levels are higher than those necessary for mesoderm formation (Vincent et al. 2003). Mouse models show that knockout mutants display a reduced definitive endoderm while over-expressions of *Nodal* results in excess axial mesoderm (Hart et al. 2002).

Proper left-right patterning is dependent on appropriate Nodal Pathway activity. During axis specification, the pathway regulates left-sided positional information from the primitive node to the left lateral plate mesoderm. *Nodal* activity is then up regulated on the left side of the node resulting n asymmetric expression which leads to soft-tissue specificity in lateral development (Raya and Belmonte 2006).

6.2.2 *PITX* Genes During Development

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 (Shih et al. 2008). Masticatory muscles are absent in *PITX2* mutant mice, while muscles in the other branchial arches develop, but have some significant deformations. In the first arch *PITX2* is necessary to establish premyoblast specification of mesoderm and induction of ectoderm for tooth bud formation (Shih et al. 2007). 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 (Shih et al. 2007). After organogenesis is completed *PITX2* continues to be expressed in most muscles of the head and trunk, and persists into adulthood (Shih et al. 2007). 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 (Tessari et al. 2008). 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 genetic variations close the gene locus in humans is associated with susceptibility to atrial fibrillation (Kirchof et al. 2011). Similar experiments demonstrate that *PITX2* expression is required for normal functioning of adult extraocular muscles (Hebert et al. 2013).

6.2.3 Genetic Influence in Congenital Versus Post-Natal Asymmetry

Congenital defects are defined as those that are present at birth, regardless of the cause. Congenital craniofacial anomalies can be the result of genetic, environmental, or unknown disturbances in the developmental process (Young et al. 2014). These anomalies can occur during development when facial prominences and the pharyngeal arch derivatives are unable or fail to grow, merge, or form correctly according to the facial plan of that species (Feng et al 2009). Examples of congenital craniofacial anomalies include, but are not limited to, Cleft lip and/or cleft palate, craniosynostosis, hemifacial microsomia, vascular malformation, hemangioma, or positional plagiocephaly.

Cleft lip with or without cleft palate occurs in 7 in 1,000 births and is the most common congenital cause of malocclusion (Stuppia et al. 2001). About 5-7% of cases are syndromic and are associated with concurrent medical conditions. These conditions are the result of different genetic diseases or chromosomal disorders. The remaining cases are non-syndromic and are usually the cause of a complex interaction of genetic influence, environmental factors, and the interaction between the two during embryonic development and morphogenesis. In either case, multiple genetic loci could contribute to the development of a cleft (Ward et al. 2002). Bilateral cleft Lip and Palate is associated with other malocclusions and often show a relatively protruded premaxilla, retrognathc maxilla, increased maxillary width, increased nasal width, reduced posterior maxillary height, a short and retrognathic mandible, and reduced upper airway (Starbuck et al. 2014). This disorder and the associated craniofacial features are present at birth and can worsen during growth.

Hemifacial microsomia is the second most common congenital craniofacial defect with an occurrence of one in 5,600 births. It results due to the asymmetric and variable malformation of the first and second pharyngeal arches and affects the maxilla, mandible, orbit, ear, and facial soft tissues (Fan et al. 2005). It can occur bilaterally and in concurrence with other pharyngeal arch disorder, including Treacher Collins and Nager syndromes. Studies suggest the disturbance in neural crest cell migration is due to the overlapping pathogenesis of genetic and environmental factors. Once again, this malocclusion is presents at birth.

In contrast to congenital craniofacial anomalies, malocclusions can develop post-natally. In these cases, the facial forms are harmonious at birth, but become disproportionate or malformed through growth. Such is the case with many severe orthodontic cases with vertical and sagittal malocclusions. The etiology of developmental skeletal malocclusion is multifactorial (Huh et al. 2013). It includes genetic, environmental, and behavioral influences. Genetic influences on the development of dentofacial deformity include the heritable effects of maxillary and mandibular jaw morphology, as well as the masticatory muscles. The muscles of mastication can lead to the development of malocclusion by exerting variable forces of stretching and compression during both chewing and postural activity. Studies have previously shown an association between masticatory muscle fiber type size, type, and number with the development of skeletal malocclusions (Sciote et al. 2012).

The importance of genetic variations through gene polymorphisms is continually being explored, particularly epigenetic factors (Beunen et al. 2003). Epigenetic factors are mechanisms that change gene expression beyond the primary DNA structure. These influences contribute to the growth and development of facial phenotypes.

6.2.4 Previous Findings of Genetic Influences on Malocclusion

Malocclusion is a multifactorial, heterogeneous trait that results in impaired aesthetics, function, and decreased quality of life. It is a worldwide affliction and is not limited to a certain population or culture (Claudino and Traebert 2013). To date, most genome linkage studies have focused on Class II malocclusions, particularly in Asian and Hispanic families. One genome wide association study has identified class III gene loci and various point gene mutations in association with mandibular prognathism (Li et al. 2010). A heterozygous missense mutation in the *DUSP6* gene has also been recently discovered to have a likely effect on maxillary hypoplasia (Nikopensius et al. 2012). Fewer widespread genetic studies exist linking genetic associations with Class I and Class II malocclusions. Gutierrez et al. detected an association between *NOGGIN* and mandibular hypoplasia, while Pillas et al. found associations between dental crowding and irregularities with various genes including *HOXB, EDA, XEDAR,* and *BMP2* (2010; 2010).

While informative, these earlier studies were limited. More recently, genetic studies utilizing comprehensive phenotyping are being pursued to reduce heterogeneity and increase power for detecting genetic associations. This is accomplished via quantitative and categorical phenotype utilizing two-dimensional lateral cephalometric approaches and geometric morphometrics. This enables greater resolution in determining shape variation in complex structures, thereby more accurately classifying specific malocclusion phenotypes (Zelditch et al 2004).

Recently, da Fontoura et al. (2015) performed a more comprehensive study to link genotype and phenotype in a more specific and thorough manner. These studies used RT-PCR to determine genotype and Principle Components Analysis (PCA) to determine phenotypic variability. They were able to identify four Principle Components (PCs 1-4) to explain about 69% of total phenotypic variation. PC1 accounted for 32.7% of variance and depicted vertical discrepancies ranging from skeletal open to skeletal deep bites. Sagittal variation (PC2) accounted for 21.7% of variation for Class II to Class III patients. PC3 captured shapes ranging from a large ramus height, a small mandibular body, increased mandibular body, and steep cranial base explained 8.2% of variation. Finally, 6.6% of variation from PC4 was explained by posterior condylar head inclination variability and reduced anterior-posterior chin projection.

Genotypic associations with the phenotypic variability resulted in significant findings and specific linkages with gene loci and mutations. Two genes, *SNAI3* and *TWIST1* were particularly suggestive of the development of skeletal malocclusion. Specifically, *SNAI3* was found to be associated with variation in severely convex to concave profiles, while *TWIST1* is related to mandibular body length These studies further validate that genes and loci are strongly correlated with vertical and horizontal shape variation of the jaws, as well as mandibular rotation and condylar angulation. It is becoming increasingly clear that specific genetic loci play a significant role in the development of malocclusions.

6.3 Analysis of Results

6.3.1 Unique Features of Facial Asymmetry in Our Subjects

There are various kinds of craniofacial asymmetry and numerous methods of measuring the craniofacial skeleton (Vig and Hewitt 1975). Our study focuses on subjects with 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 (Cendekiawan et al. 2010). Many authors have described the correlation between the cranial base angle and sagittal jaw dimesnions (Klocke et al. 2002; Hopkin et al. 1968; Scott 1967). 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 naris area 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 of microarray data. 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, in conjunction with differences in jaw muscle function, given the fact that jaw muscle phenotype is related to facial asymmetry (Raoul et al. 2004). This work helps us understand how sub classifications of facial asymmetry arise and how symmetry regulatory genes contribute to development of malocclusion.

6.3.2 Nodal Pathway Gene Expression in Adult Masseter Muscle

In our study, gene expression in masseter muscle was compared between 11 subjects with skeletal malocclusion to determine the principle component differences in gene expression between groups. The two subjects with facial asymmetry (Subjects 4 and 10) clustered separately from the other malocclusion subjects. These subjects showed differential expression regardless of sagittal malocclusion, as one had a class II and the other a class III malocclusion. Seeing this trend, we chose to investigate the possibility that molecular pathways which pattern asymmetry in development could be active and regulated differentially in masseter muscle. After a review of literature, genes involved in and associated with the Nodal Pathway were chosen to further investigate. 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 (Raoul et al. 2011).

Almost all of the molecular signals described in the Nodal Pathway, were expressed at significantly higher or lower levels in the asymmetric subjects in comparison to the symmetric subjects (Padey et al. 2012). 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 Pathway 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.

Masseter muscle samples from the side opposite of the chin deviation had increased expression of *PITX1* compared to muscle from symmetric subjects. 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. The fold differences for *PITX1* and *PITX2* gene expression fold values 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.

6.3.3 Differential Gene Expression in Facial Asymmetry

Using masseter muscle samples, we were able to quantify gene expression in 47 subjects for the three genes of interest: *NOMO1,2,3, SMAD4,* and *PITX2. NOMO1,2,3* and *SMAD4* were chosen to further investigate as the initial microarray resulted in significant fold differences in gene expression in symmetric versus asymmetric patients. Both genes are known to encode for proteins involved in the Nodal Pathway. *PITX2* was chosen as a gene of interest due to the previously reported intimacy between gene expression differences and development of right/left symmetry.

As expected, expression values varied by particular gene. When comparing lateral expression differences between sides, all three genes showed similar values on each side for symmetric patients, as well as the total sample (symmetric and asymmetric patients). Lateral expression differences were greatest in patients with facial asymmetry. This is to be expected, and confirms our hypothesis, that the genes involved in the Nodal Pathway are differentially expressed in right and left sides in patients with facial asymmetry. This further confirms their involvement with left/right patterning and the development of transverse malocclusion. While increased, the LED for *NOMO1,2,3* and *SMAD4* were not statistically significant. However, the LED for *PITX2* did show statistical significance with a P value of 0.03. Once, again, this confirms the involvement of specific gene transcripts in the patterning of left and right sides.

6.3.4 Gene Expression and Muscle Fiber Type

Multiple statistical evaluations were performed to determine any significant correlations between muscle fiber type and the presence of asymmetry. We hypothesized that lateral gene expression differences could possibly produce differential skeletal muscle morphology with one side being stronger than the other. This could potential cause an asymmetry as the forces on the bone would not be the same on either side, thereby resulting in a skeletal disharmony.

After through statistical correlations, however, only one was found to be significant: average *PITX2* expression and Type 1 fibers in all patients. Both a Kendall Tau and Pearson Correlation confirmed this relationship. We found that as expression of *PITX2* decreased, the percentage of Type 1 myosin fibers also decreased. This relationship is understandable and agrees with previous findings that *PITX2* is essential for non-limb myogenesis and muscle maintenance (Sadie et al. 2013). Decreased expression of this gene results in a decreased ability of the organ to build and maintain muscle, particularly Type 1 fibers.

Understanding how *PITX2* influences first brachial arch functions may provide new insights into the development of facial asymmetry and masticatory fiber type properties in TMD. It is already known that *PITX2* is especially important in maintenance of adult heart and extraocluar muscle functions. In adult mouse and human heart, *PITX2* is expressed at much higher levels in the right atrium, and altered expression produces atrial fibrillation (Kirchof et al. 2011). In extraocular muscle, *PITX2* is abundantly expressed, and inactivation leads to decreased expression of I, IIX and α-cardiac myosin heavy chain (MHC), but not IIA or neonatal MHC (Porter et al. 2001; Zhou et al. 2009). Given the unusual presence of α-cardiac MHC in human masseter, it is likely that *PITX2* influences its expression. Our microarray data indicates that *PITX2* is abundantly expressed in masseter muscle (Rowlerson et al. 2005). Therefore our goal is to determine how differences in masseter muscle *PITX2* expression in facial asymmetry might affect fiber type properties and development of TMD.

6.4 Future Studies

This work helps us understand how sub-classifications of facial asymmetry arise and how symmetry regulatory genes contribute to the development of malocclusion. Further studies could strengthen the validity of our results by utilizing the most advanced technology available. Three-dimensional CBCT images, as well as three-dimensional photography could be used to more accurately diagnose facial classifications. In particular, these tactics would be useful in determining the precise location and degree of any malocclusion and asymmetry.

Furthermore, exploring a larger subject population that includes a variety of different types of facial asymmetry should strengthen the results obtained by RT-PCR. This would strengthen the power of the study, and could be used to expand results by additional molecular studies. Immunohistochemical staining of masseter samples using protein antibodies produced by the genes of interest would be helpful in determining levels of RNA expression and protein expression for the genes of interest. The data could also be quantified by tagging the antibody with fluorophore. Western Blot could also be effective in identifying the presence of the proteins of the genes of interest in the muscle samples from our subjects.

Though tedious, the study could be expanded extensively by exploring other genes involved in the Nodal Pathway. Additional studies would also include determining the effect of differential expression of the genes of interest and the correlation with the side to which the asymmetry occurs. The long-term goals of these studies would be to explore and verify the effect of each of these genes by using knock-out mice. These would enable us to confirm the effect of the genotype on the phenotype.

CHAPTER 7

CONCLUSIONS

Variation of Nodal Pathway gene expression effect muscle and skeletal development and directly influence facial structures and skeletal malocclusion.

1. *PITX2* is differentially expressed in masseter muscle of adults with facial asymmetry.
2. Significant expression differences occur bilaterally with facial asymmetry when TMD or C-II malocclusion is present
3. PITX2 expression actively controls fiber type occupancy, which can influence the development of asymmetry, TMD, and vertical or sagittal malocclusion

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.

7.1 Potential Health Significance

Orthodontics is an ever-changing science and art that demands the clinician to be constantly pushing the envelope and keeping up with advances in technology and information. With the completion of the mapping of the Human Genome, genetic studies on humans soared exponentially. As the affects of genes on musculoskeletal properties and facial development are further discovered and confirmed, diagnosis of malocclusion and facial deformity is becoming more thorough and accurate. In short, the more accurate the diagnosis, the more appropriate the treatment. By utilizing this knowledge, clinicians may have a more exact understanding of the growth of the patient, as well as the etiology and the further progression of deformity. With these characteristics in mind, proper treatment can be more accurately determined from an early age, perhaps decreasing the severity of the developing malocclusion.

While it is unlikely that a simple saliva sample could provide the entire genetic profile of the patient, it could potentially be utilized to identify specific markers for the genetic predilection of normal or abnormal growth. With concurrent advancement of pharmacology and genetics, perhaps treatment could include genetic therapy and medication. This could be extremely beneficial for the field of orthodontics, as it could decrease treatment time, predict outcome stability, and allow for less aggressive methods in the treatment of severe deformities.

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APPENDICES

APPENDIX

Jaw Pain and Function Questionnaire – English Version

Jaw symptom and oral habit questionnaire

First name : L | Date : 19/02/2013

Last name : VG

Identification : 004

Instructions: Please check the appropiate answer to the following questions | Examiner : R. Nicot

A	Jaw pain questions	Doesn't hurt at all	Hurts a little	Hurts a lot	Almost unbearable	Unbearable pain without relief
1	Does it hurt when you open wide or yawn ?	X				
2	Does it hurt when you chew or use the jaws ?	X				
3	Does it hurt when you are not chewing or using the jaws ?	X				
4	Is your pain worse on waking ?	X				
5	Do you have pain in front of the ears or earaches ?	X				
6	Do you have jaw muscle (cheek) pain?	X				
7	Do you have pain in the temples ?		X			
8	Do you have pain or soreness in the teeth ?	X				
B	Jaw function questions	No	Maybe a little	Quite a lot	Almost all the time	All the time without stopping
9	Do your jaw joints make noise so that it bothers you or others?	X				
10	Do you find it difficult to open your mouth wide ?	X				
11	Does your jaw ever lock closed so you cannot open it?		X			
12	Does your jaw ever lock open so you cannot close it?	X				
13	Do you have a problem with your bite being uncomfortable?	X				