Title
2D Geometric Morphometric Analysis of the Relationship Between Sonic Hedgehog Expression Domains and the Embryonic Face Shape

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2D Geometric Morphometric Analysis of the Relationship Between Sonic Hedgehog Expression Domains and the Embryonic Face Shape

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2D Geometric Morphometric Analysis of the Relationship Between Sonic Hedgehog Expression Domains and the Embryonic Face shape

Janice J. Hwang

Abstract

Objectives: Craniofacial malformations are among the most common birth defects, affecting ~1 in every 700 live births. However, our understanding of the mechanisms that result in these diseases is limited. Many cases have been associated with genetic predispositions that have allowed us to attempt to investigate the underlying mechanisms for these diseases. Sonic Hedgehog (SHH) signaling pathway has undergone significant investigation due to the involvement of *Shh* in the development of the midface. In humans, deletion of a single copy of *Shh* is associated with a spectrum of phenotypes comprising Holoprosencephaly (HPE), ranging from mild hypotelorism and midfacial hypoplasia to cyclopia indicates that diseases like HPE are multifactorial. Although our long-term goal is to discover the underlying mechanisms that cause these diseases, we must first understand the normal progression of development before trying to understand the abnormal. Therefore, the goal of this study was to determine how *Shh* expression and face shape relate with each other during normal development.

Methods: We collected wild-type chicken embryos at 72 hrs, 96 hrs, and 120 hours of incubation. Chickens infected with RCAS-*wnt3a* at 72 hrs of incubation were also included in our sample. We used *in situ* hybridization to identify *Shh* expression domains and 2D geometric morphometrics to quantify changes in shape in *Shh* expression domains and face shape. We performed Principal Components Analysis (PCA) as well as multivariable

regression analyses of *Shh* expression shape and face shape on somite number and centroid size. We used Partial Least Squares (PLS) to evaluate covariation in shape between Shh expression domains and facial shape.

Results: Changes in *Shh* expression shape and face shape are dependent on developmental time. As the embryos progressed in development, there were significant changes in both *Shh* expression shape and face shape. While the overall size of the embryo grew, both the *Shh* expression shape and face shape constricted. More specifically, *Shh* expression shape tapered into a narrow V-shaped band in the ectoderm of the stomodeum while face shape constricted as a result of the nasal pits growing closer together while the mouth became smaller. Our PLS regression identified that the changes in *Shh* expression and face shape are correlative in which *Shh* expression shape is associated with face shape at specific timepoints during development.

Conclusion: Changes in *Shh* expression shape correlate with changes in face shape. This suggests that *Shh* expression shape may serve as a predictor for face shape during embryonic growth. Although we are unable to determine if *Shh* is directly responsible for the observed changes in face shape, this potential predictive relationship could be valuable for future studies to identify when and how disease progression initiates.

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1. INTRODUCTION

1.1 Complexity of Craniofacial Anomalies

Craniofacial birth defects are one of the leading causes of infant death in the United States. Although these malformations are understandably among the most common serious birth defects, affecting ~1 in 700 live births (Heron et al. 2009), our understanding of the mechanisms that contribute to these diseases is limited. Many of these malformations have been found to occur unpredictably. But generally, many cases have been associated with an underlying genetic factor. In fact, many of these defects demonstrate a high rate of heritability (Grosen et al., 2010, 2011) suggesting that genetic factors are clearly involved (Taniguchi et al., 2012). However, the wide range of phenotypic expression of these malformations indicates that these defects are multifaceted and the underlying mechanisms for many of these diseases are complex and remain unknown.

Several studies have identified additional factors that may be associated with craniofacial malformations. Some of these factors include maternal exposure to tobacco smoke (Little, 2004), alcohol use (Bell, 2014), hypoxia (Smith, 2013), folic acid deficiency (Little, 2008), obesity (Stott-Miller, 2010), and diabetes (Correa, 2008). Although the environmental effect may have a smaller role when compared with genetic mutation, interactions between these two factors may play a significant role in improper facial development. This may even explain how individuals affected by the same craniofacial disease may share the same genotype while expressing different levels of phenotypic severity (Nanni et al., 1999). More specifically, people who have the same genetic predisposition may

experience varying levels of exposure to environmental teratogens that results in a wide range of phenotypic expression. These diseases may even represent extremes of normal facial morphology (Young et al., 2010), leaving some cases undiagnosed. Thus, defining the progression of normal morphological development is a crucial first step to understanding the etiology of these complex diseases.

1.2 Facial development

Normal facial development is an intricately timed process that occurs in the early stages of embryonic growth. In humans, the facial region develops during the fourth and eighth embryonic weeks during which a complex series of biochemical mechanisms result in corresponding anatomical changes. This process begins shortly after the anterior neuropore closes. At this point in normal development, the brain and face begin to develop congruently with each other. The brain essentially acts as a platform for the face. If the brain is smaller, then the platform is smaller; thus, the face may develop prognathism when fully developed (Diewert and Lozanoff, 1993; Diewert et la., 1993). But ultimately, little is known about how the brain and face communicate with one another during development and whether or not neurological mechanisms ultimately drive the outgrowth and development of the face.

In normal situations, as the forebrain enlarges, it eventually creates the frontonasal process as the overlying ectoderm is pushed forward and laterally. Soon after, there is mesenchymal growth in the first branchial arch that develops the maxillary process and the mandibular process. The medial and lateral nasal processes develop from the nasal placodes as these placodes slowly sink down forming a downward facing “horseshoe”

before the two processes meet and fuse together (Som, 2013). During normal craniofacial development, these facial prominences must properly grow and fuse together at the appropriate times and this process is highly dependent on complex mechanisms (Jiang, 2006). The medial nasal process fuses with the maxillary process to begin the formation of the upper lip. The upper lip completes development as the two medial nasal processes fuse together and create the philtrum and columella. Meanwhile, as the maxillary process migrates medially, it also contributes to the development of the upper cheek regions, resulting in the connection between the upper jaw and lip. Likewise, the lateral nasal process merges with the maxillary process to form the lateral nose and the lateral border of the nostril which establishes a transition from the nose to the cheek (Som, 2013).

1.3 Signaling Pathways

These prominences grow and merge together in a specific way in order to properly develop the face. Surrounding tissues including the ectoderm, endoderm, and neural tube transmit complex molecular mechanisms and patterning information in order to drive cell proliferation and tissue growth that allow these different prominences to fuse together correctly (Adameyko and Fried, 2016; Chai and Maxson, 2006; Singh and Groves, 2016). Likewise, the mesenchyme also exchanges signals to the ectoderm to regulate growth and to help continue facial formation and growth (Van Otterloo et al., 2016). These signals are intricate and require the integration of multiple signals between the ectoderm and mesenchyme. Some of these signaling pathways include Fibroblast Growth Factor (FGF), Bone Morphogenetic Protein (BMP), Wingless-Integrin (Wnt), Sonic Hedgehog (SHH), Platelet Derived Growth Factors (PDGF), and Retinoic Acid (RA) (Geetha-Loganathan et al., 2014, Hu et al., 2015, Song et al. 2004, Scarano et al., 2016). These pathways are

only a few that are known, and many other pathways remain to be identified. In fact, surprisingly little is known about the genetic programs and how they influence morphological changes in the ectoderm and mesenchyme and conversely, how the ectoderm and mesenchyme influence the genetic pattern of nearby tissues. This process of facial development is incredibly complex and countless factors contribute to and can influence the outcome. The main concern arises from the fact that any interruption in this complex interplay between signals and tissues may ultimately lead to major structural changes ultimately developing into craniofacial malformations (Roessler et al., 2009). Here we focus on one pathway—Sonic hedgehog (Shh), because it is both highly conserved among animals (Lemos et al., 2004) and plays a critical role in craniofacial development (Hu and Marcucio, 2009).

1.4 Sonic Hedgehog Signaling Pathway

Sonic Hedgehog is one of three members of the Hedgehog signaling pathway. Together, Shh, Indian hedgehog, and Desert hedgehog play an important role in vertebrate development and growth (Lee et al., 2016, Briscoe and Therond, 2013, Ingham and McMahon, 2001). Of these three, Shh is highly involved in craniofacial morphogenesis and its proper function contributes to the separation of the two halves of the forebrain, ultimately establishing the facial midline (Chiang et al., 1996). In the embryonic head, *Shh* is initially expressed in the mesendoderm, including the prechordal plate and prosencephalon. Development continues as the prosencephalon is divided into the diencephalon and the telencephalon. Meanwhile, the original *shh* expression domain observed in the prosencephalon is carried into the diencephalon while a new expression domain is initiated in the telencephalon (Marcucio et al., 2005). Later in development, a

new expression domain of *Shh* is found in the frontonasal ectodermal zone (FEZ), a distinct region located in the stomodeal ectoderm. *Shh* lines the roof of the mouth and works in conjunction with fibroblast growth factors to regulate proximodistal growth and dorsoventral patterning within the frontonasal process (Hu et al., 2002). *Shh* is also observed in the medial nasal processes and maxillary process as they fuse to form the upper lip (Guilherme et al., 2016). This suggests that Shh signaling influences the outgrowth and patterning of the upper midface (Chong et al., 2012) and that proper signaling activity may allow for a predictive relationship for shape variation within the upper jaw and other structures within the midline.

The facial processes and domains with Shh signaling activity coordinate together in order to establish a temporo-spatial relationship as the craniofacial region develops. This is a very intricate and complex process that involves interactions with numerous signaling pathways. It begins with the release of Shh from the surface of signaling cells as a result of the combined activity of Dispatched (Disp), a sterol-sensing domain protein (Ma et al., 2002) and Scube2, a secreted glycoprotein (Creagna et al., 2012). Once it has been released, Shh can work in both short and long-ranges to signal embryonic tissues (Gritli-Linde et al., 2001). These tissues receive Shh via a number of receptor proteins including Patched1 (Ptch1) (Goodrich et al., 1996), Lrp2 (Saito et al., 1994), Growth arrest-specific1 (Gas1) (Martinelli and Fan, 2007), and Cdon and Boc (Kang et al., 2002, 1997). Once Shh is received, the receptor proteins, such as Ptch1, initiate many downstream intracellular signaling pathways that contribute to development (Goodrich et al., 1996).

This process requires proper coordination with other signaling pathways. If one step in this process deviates from normal, there are bound to be detrimental downstream effects

that will negatively affect craniofacial development. For instance, because *Shh* is eventually expressed in the palatal shelves and secondary palate (Cobourne and Green, 2012), inhibiting Shh signaling has been associated with improper lip and palate formation ultimately leading to clefting of the lip or palate (Heyne et al., 2015). Disruptions in Shh signaling can result in a wide range of craniofacial malformations and is most commonly associated with Holoprosencephaly (HPE).

1.5 Holoprosencephaly

HPE is the most common developmental defect of the forebrain and may be one of the leading causes of infant death with an incidence as high as 1 in every 250 conceptions and in ~1 in every 16,000 live births (Roach et al., 1975). These numbers suggest the high rate of intra-uterine fetal fatalities that occur among those affected by HPE, indicating the severity of this disease. This condition results from the failure of the early forebrain, or the prosencephalon, to divide into two distinct hemispheres (Geng and Oliver, 2009). This failure to properly develop the two lobes of the brain has a detrimental effect on subsequent formation of the midline structures of the face. The clinical representation of HPE is variable and manifests in a wide range of craniofacial abnormalities that is categorized into three subtypes: alobar, semilobar, and lobar. A milder subtype called middle interhemispheric fusion variant (syntelencephaly) also exists (Rajalakshmi et al, 1993). Alobar is the most severe form of HPE, resulting from the complete failure of the prosencephalon to divide. The forebrain remains a single vesicle and is accompanied by cyclopia in which a single midline eye is located below a proboscis (Figure 1.1). Midline

clefts are also commonly observed in addition to cyclopia. This form of HPE has a very low survival rate with those affected by it dying within days of birth (Bullen et al., 2001). Semilobar HPE is characterized by partial division of the forebrain where the posterior forebrain separates while leaving the anterior forebrain intact. Around 50% with semilobar HPE have been found to survive beyond one year of age (Bullen at al., 2001). Lobar HPE has a more favorable prognosis and occurs when the forebrain is almost but not completely separated into two distinct hemispheres. Both semilunar and lobar HPE can present with a range of facial anomalies such as nasal anomalies, iris coloboma, and cleft lip and palate. Syntelencephaly is the mildest subtype of HPE and results from incomplete separation of the posterior frontal and parietal lobes. Those affected by syntelencephaly often present with milder craniofacial features such as ocular hypotelorism, pre-maxillary agenesis, and a single median maxillary central incisor (Ming and Muenke, 2002). Some patients may not present with any obvious craniofacial malformations (Cohen and Sulik, 1992), leaving many affected people undiagnosed. The clinical presentation of HPE is incredibly broad as described. In addition to its contribution to these facial deformities, HPE is associated with neurological impairment in the more severe cases due to improper brain development. Milder cases present with normal cognitive ability (Muenke et al., 1994).

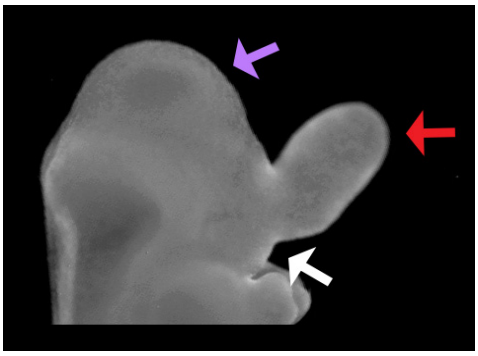

Figure 1.1. Loss of Shh function causes alobar HPE. The forebrain remains a single vesicle (purple arrow). A large midline proboscis (red arrow) is located above a cyclopic eye (white arrow).

1.6 Etiology of HPE: Genetic and Environmental

The etiology of HPE has been studied and several factors have been elucidated. Genetically, *Shh* was one of the first identified genes to cause HPE (Roessler et al., 1996). This coincides with our understanding of the Shh signaling pathway's involvement in the development of the facial midline. So if the signaling pathway deviates from normal, then facial development must also deviate from normal. However, this is an oversimplification of this genetic component. It is, in fact, a very complicated and multifaceted process that contributes to the development of a wide range of phenotypes that comprise HPE.

As previously described, Shh regulates the development of the FEZ, a signaling center that is crucial for regulating the patterning and growth of the middle and upper face (Hu and Marcucio, 2003). Interestingly, ectopic Shh signaling can cause hypertelorism (increased space between the orbits) and can also potentially cause facial duplication in severe cases (Hu and Helms, 1999). But this phenotypic expression is not seen in HPE. In HPE, we commonly see hypotelorism (decreased space between the orbits). Perturbing the Shh signaling pathway during brain development alters *Shh* expression in the FEZ and disrupts proper face development by preventing the proper expansion and growth of the frontonasal process which causes a narrowing of the face, resulting in phenotypes such as hypotelorism (Marcucio et al., 2005, Hu and Marcucio, 2009). Deletion of both copies of the *Shh* gene results in severe HPE-like phenotypes in mice that resemble the alobar form of HPE in humans with a single forebrain vesicle and midline proboscis. However, deletion of one copy of the *Shh* gene in mice does not produce obvious facial malformations (Chiang et al., 1996). Contrarily, humans who are heterozygous for the *Shh* gene display a range of phenotypes that comprise HPE from

hypoteolorism and facial hypoplasia to complete cyclopia (Roessler et al., 2009; Muenke and Cohen, 2000). The fact that a single mutation in the Shh signaling pathway can induce a variety of phenotypes suggests that there are multiple factors that contribute to the spectrum of craniofacial malformations found in HPE patients.

Maternal environmental factors have also been identified that may contribute to the development of HPE. Only a few factors have been formally recognized including insulin-dependent diabetes mellitus (1% risk of HPE) (Barr et al., 1083) and maternal alcoholism which worsens with smoking (RR 1.4) (Croen et al., 2000). Other factors have been identified in association with HPE such as prenatal exposure to drugs (Repetto et al., 1990) and infections (cytomegalovirus, toxoplasma, and rubella) (Frenkel et al., 1990; Lison et al., 1967; Castel et al., 1976). However, our understanding of the cause for the phenotypic variation within HPE affected individuals remains unclear. While genetics remains a significant factor in HPE, the variability in phenotype implies that haploinsufficiency for a specific gene is not generally sufficient to cause HPE and that other factors are likely involved. While *Shh* remains a major contributing factor involved in HPE, affecting approximately 22.3% of reported HPE cases (both point mutations and overall large deletions (Bendavid et al., 2006)), additional factors must be taken into consideration to account for the variability of disease (Nanni et al. 1999). For instance, a family with the same *Shh* mutation may be present in individuals with HPE, individuals with microforms of HPE, and also in individuals who are asymptomatic (Roessler et al., 1996). Therefore, a phenotype associated with a *Shh* mutation is incredibly variable and may be a cumulative result of multiple genetic and environmental influences.

1.7 Summary and Significance

Our understanding of facial development is limited due to its complexity. Therefore, when craniofacial abnormalities form, it is generally unknown which stages of facial development are affected. We understand that there exists a complex network of pathways that work together to regulate the growth and development of the face and that disrupting any point during this cascade of events causes a downstream effect that ultimately affects the morphology of the face. For instance, when *Shh* expression is inhibited in the FEZ, the resultant face is narrow and truncated (Marcucio et al., 2005). However, when Shh signaling is activated, we observe ectopic *Shh* expression that expands dorsally and the face becomes abnormally wide (Hu and Marcucio, 2009). This suggests that specific gene expression patterns can potentially characterize developmental processes and phenotypic variation. Given the voids in our understanding of the mechanisms underlying the severity and variation of disease, we seek to quanitfy the pattern of expression of Sonic Hedgehog (SHH) during different time points of normal embryonic development. Our goal is to understand how the face shape and *Shh* gene expression are coordinated. Previous studies have shown that *Shh* expression migrates as facial development progresses (Marcucio et al., 2005). Thus, understanding how *Shh* expression and morphology relate may broaden our understanding of phenotypic variation and, more importantly, elucidate how things deviate from normal in individuals affected by craniofacial malformations.

In this study, we used in situ hybridization to identify *Shh* domains in chicken embryos at different timepoints of embryonic development. Geometric morphometrics was then

performed on 2D photos to quantify both gene expression and face shape to assess three specific aims:

1) to evaluate changes in shape of the *Shh* expression during early stages of development;

2) to evaluate changes in facial shape morphogenesis during early stages of development;

3) to compare how changes in gene expression relate to facial morphogenesis during these stages of development in both wildtype and experimental samples.

We hypothesize that changes in *Shh* expression and facial morphogenesis follow trends that parallel each other during development. More specifically, as *Shh* expression changes, we anticipate seeing a correlative change in facial morphogenesis.

2. MATERIALS AND METHODS

2.1 Wild-Type Chicken Embryos

Fertile White Leghorn chicken eggs were incubated in a humidified chamber at 37.5◦C. Day 0 was identified as the day when the eggs were placed into the incubator. Embryos were sacrificed on days three through five. On the day of collection, the shell was opened to directly access the embryos which were collected from extraembryonic membranes. Fifty-eight embryos were collected and then washed in 1x PBS and fixed overnight in 4% paraformaldehyde in 1x PBS at 4◦C. A total of 58 embryos were included in this study, ranging from 72hrs to 120hrs of development (HH stages 19-27). To further objectively quantify developmental time, for each embryo we counted the number of somites caudal

to the hindlimb bud (tail somites). In total, our sample over this time period ranged from 11-21 tail somites.

2.2 In Situ *Hybridization*

SHH expression was analyzed by whole mount *in situ* hybridization. Tissues were hybridized using 0.5-1µg/ml digoxygenin-labeled Shh cRNA probes. Tissues were then washed and incubated with an alkaline phosphatase-conjugated anti-digoxygenin antibody (Boehringer). NBT.BCIP substrate (Roche) was used for color detection. Expression domains were evaluated using 2D geometric morphometric analysis.

2.3 RCAS-wnt3a Chicken Embryos

Ten embryos infected with RCAS-*wnt3a* and RCAS-AP (control) were added from a previous study completed by the Marcucio lab for further experimental analysis. *Wnt3a* is an agonist of the Wnt-signaling pathway that previous experiments show expands the *Shh* expression domains and alters facial shape. We include this data to test whether mechanistic changes to *Shh* expression are predictive of facial shape changes.

2.4 2D Geometric Morphometrics

To quantify gene expression and face shape, we used 2-dimensional (2D) geometric morphometrics. Embryos were imaged by conventional photography using a Leica MZFLIII dissecting microscope with a Leica LUI-750 camera. We identified thirty-eight facial and twenty *Shh* landmarks on these 2D embryo images in ImageJ and recorded the x,y,z, coordinates (Figure 2.1). Raw coordinate data was imported into the software

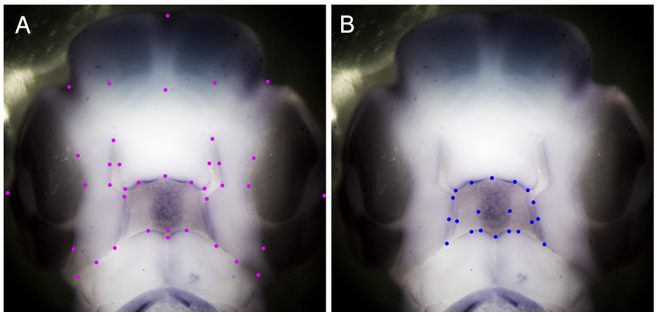

Figure 2.1 Landmarks A) 38 face shape landmarks and **B)** 20 *Shh* expression shape landmarks.

MorphoJ (Klingenberg, 2008)**.** We performed a Procrustes superimposition to remove differences in location and scale, leaving shape alone. To evaluate shape variation, on the resulting Procrustes data, we performed Principal Components Analysis (PCA), a multivariate regression of shape on somite number, and a multivariate regression of shape on centroid size. Finally, we performed a Partial Least Squares (PLS) analysis to evaluate covariation between the observed changes in face shape with *Shh* expression shape in both normal and experimental samples. For the remainder of this study, the term "face shape" is used to describe the midface, consisting of the nasal pits and mouth.

3. RESULTS

3.1 Shh expression

Many studies highlight the importance of Shh signaling in the development of the midface (Chiang et al., 1996, Chong et al., 2012) and other studies emphasize that disturbances within the pathway may lead to improper facial morphogenesis (Ming and Muenke, 2002). Our goal was to better understand how *Shh* expression changes during normal development of the face. To do so, we collected chicken embryos from a series of consecutive time points during a critical time period in facial morphogenesis in which *Shh* expression is known to play a major role. We began by examining 2D *Shh* expression.

Embryos with 11 tail somites had an *Shh* expression shape that was restricted to the roof of the stomodeum. The expression domain at this stage is broad and slightly constricts in the middle. As development progresses, *Shh* expression appears to consolidate into a straight but tapered band. By 21 tail somites, the ventral aspect of *Shh* expression appears to have extended toward the globular process of the frontonasal process. The zone of expression on the roof of the stomodeum is narrowed to a V-shaped band that tapers as it extends through the roof of the mouth (Figure 3.1A).

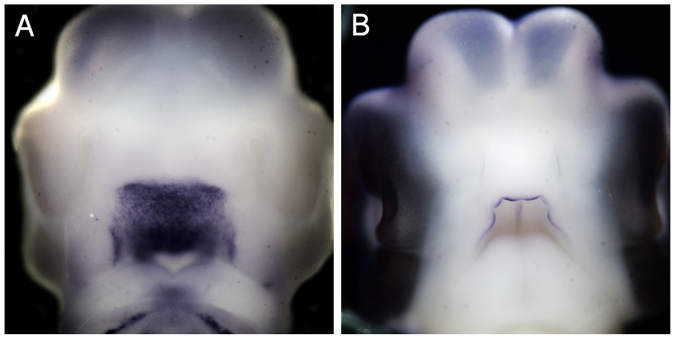

Figure 3.1 Observable changes
A) *Shh* expression shape is broad in early embryos. B) As embryos progress in development, *Shh* expression shape constricts into a narrow V-shaped band on the roof of the stomodeum while the expression domains on the medial nasal processes extend to the globular process to meet with the maxillary prominences.

3.2 Facial morphogenesis

We also evaluated 2D face shape changes during normal morphogenesis. The overall facial complex increases in size with the mid-brain and forebrain expanding laterally and superiorly. Likewise, the developing eyes move distally. While these structures grow outwards, the nasal pits become narrower and move closer in proximation due to the growth of surrounding tissues. The globular processes of the FNP move closer to the distal tip of the MxP in preparation for subsequent fusion of the prominences (Figure 3.1B). “Face shape” refers to midline structures such as nasal pits and the mouth.

3.3. Changes observed in Shh expression shape and face shape are coordinated with developmental time

The goal of our work was to assess how *Shh* expression and face shape changes relate with each other during normal embryonic development. We have previously identified that changes in *Shh* expression may play a role in variability in midfacial malformations observed in craniofacial defects such as HPE (Young et al., 2010), suggesting the importance of normal Shh signaling in development. Therefore, we wanted to assess how *Shh* expression and facial shape relate in normal facial morphogenesis.

Changes in face shape and *Shh* expression shape were evaluated using PCA of the Procrustes-transformed shape data and multivariate regressions of face and gene shape on both the number of somites and centroid size. Our PCA indicated several principal components that are involved in the variation in shape that we observed in the embryos. PC1, PC2, and PC3 demonstrated 57%, 19% and 5% involvement in variation, respectively (Figure 3.2A). PC2 was excluded from further evaluation due to the fact that it was influenced by the orientation of the face in the 2D image. Evaluation of PC1 and PC3 suggested that the observed variation was a result of development. PC1 followed a consistent pattern with a growing head and narrowing *Shh* expression shape. We also noted that although the head was growing larger, the face shape became narrow as the nasal pits grew closer in proximity while the mouth constricted. When plotted together, PC1 and PC3 indicated a general trend between PC1 and shape (Figure 3.2B). As PC1 increased, the face shape and *Shh* expression domain shape changed significantly together (Figure 3.3A). As the overall head expanded with growth, both the face shape and *Shh* expression shape constricted (Fig 3.2B, Fig 3.3A). More specifically, as *Shh*

expression shape tapered into a narrow triangle, the nasal pits appeared to have grown closer together as the mouth became smaller in size.

Next, a multivariate regression of shape on the number of somites was completed. The number of somites present is an indicator of how far development has progressed (Gilbert, 2000). Therefore, an increasing number of somites indicates that the embryo is farther along in development. This corresponds with our expectation that as growth and development proceeds, the embryo head will continue to grow until maturation. Our results are consistent with this understanding. As the number of somites increased, there was an observable and significant change in face shape and *Shh* expression ($P<0.001$). Generally, the embryo was clearly growing as a result of developmental progress. However, like we observed with PC1, as the overall face expanded, both the midface and *Shh* expression domain narrowed (Fig 3.3C).

Likewise, a multivariate regression of shape on centroid size indicated a significant relationship that closely resembled the relationship between shape and the number of somites ($P<0.001$). As centroid size increased, face shape narrowed while *Shh* expression shape constricted. Similarly to somites, centroid size is also arguably an indicator of growth and development. More specifically, centroid size is determined by the square root of the sum of the squared distances from the center of the configuration of landmark points (Bookstein, 1991). So this value increases as the evaluated objects (embryo heads) increase in size. Therefore, we anticipate seeing that as centroid size increases, the embryos will grow in size due to progression in development. As expected, our analysis revealed this correlation between centroid size and face shape as well as

with *Shh* expression shape (P<0.001). As centroid size increased, face shape increased while both the midface and *Shh* expression domain narrowed in size (Fig 3.3B).

Together, these data suggest that *Shh* expression shape and face shape are both significantly influenced by development. As time progresses, the embryos grow. As growth proceeds, *Shh* expression shape becomes narrow as face shape constricts.

3.4 Shh expression shape predicts face shape

Finally, PLS was used to evaluate the relationship between changes observed in face shape and *Shh* expression shape. Figure 3.4 shows a significant correlative relationship between the development of face shape and *Shh* expression (P<0.001). As the size of the overall head grew, the face shape constricted while the gene expression domain tapered into a triangular shape with the base of the triangle at the most ventral edge of the FNP where the medial nasal processes and stomodeum meet. Figure 3.5 includes RCAS-wnt3a embryos that present with widened domains of *Shh* expression. Although these embryos were collected at 72hrs like our youngest WT embryo samples, they presented with wider *Shh* expression shapes and as a result, the face shapes were also wider than our WT samples. The fact that these samples were plotted on the lower end of the PLS regression plot indicates that their shapes represent the developmental progression of younger embryos. Therefore, we conclude that *Shh* expression shape is predictive of face shape in developing embryos.

4. Discussion

Facial morphogenesis is an complex process that relies on properly orchestrated mechanisms. It is well understood that the brain is a key contributor in regulating facial morphogenesis (Adameyko and Fried, 2016; Chai and Maxson, 2006; Singh and Groves, 2016). Previous studies have shown that disturbances to the Shh signaling pathway lead to a variety of craniofacial dysmorphologies, suggesting that the *Shh* gene is an important contributor to proper facial development (Lemos et al., 2004, Hu and Marcucio, 2009, Chiang et al., 1996). In this study, we focused on elucidating the relationship between face shape and *Shh* expression shape during development. We hypothesized that changes in *Shh* expression shape correspond with changes in face shape.

4.1 Face shape changes correlate with changes in Shh expression shape

From our three methods of evaluating changes in *Shh* expression shape and face shape, we found evidence that as the embryos progressed in development, there was a significant change in the two evaluated shapes. We noted that as the embryos proceeded in their developmental growth, *Shh* expression shape narrowed in the stomodeum while the face shape also narrowed as a result of the nasal pits growing medially and the mouth constricting in size.

Furthermore, we incorporated an experimental group of RCAS-*Wnt3a* embryos to evaluate how a change in *Shh* expression shape may relate with our findings. RACS-*Wnt3a* embryos present with a larger zone of expression for *Shh*. If our finding, suggesting that there is a direct relationship between *Shh* expression and face shape is true, then we would see that the face shapes of these experimental embryos are predictable based

on the *Shh* expression shape. When incorporated into our PLS regression, we found that the RCAS-*Wnt3a* embryos followed the anticipated relationship. Because these embryos presented with a larger *Shh* expression shape, we anticipated that these embryos would fall on the left side of linear prediction. This turned out to be true as these embryos presented with wider faces as a result of farther set nasal pits and a larger mouth.

Therefore, we identified that the changes observed in *Shh* expression shape and face shape significantly correlate with each other suggesting a predictive linear relationship between the two shapes.

4.2 Shh expression shape may serve as a new method for developmental staging

Harvesting age is routinely used for staging documentation due to simplicity. But because there are so many differences in developmental progression among embryos from the same liter, unexpected variation may influence any statistical analyses that are conducted. For instance, our study collected samples from three different time points (72 hrs, 96 hrs, 120 hrs). However, when staged according to the number of somites, each harvesting time point presented with embryos at different developmental stages. Thus, it is important to implement methods that allow for correct identification of precise developmental timing.

The results of this study indicate a potential new method of developmental staging. We have identified that *Shh* expression shape corresponds with facial development. This suggests that *Shh* expression shape could potentially serve as a way to evaluate developmental progression. However, this is only applicable when staging normal

development. In situations where alterations in *Shh* expression is anticipated, expression shape can ultimately serve as a predictor for face shape.

4.3 Clinical implications

We have described a predictive relationship between *Shh* expression shape and face shape. As *Shh* expression shape tapered in shape, the embryonic face shape changed as a result of the nasal pits growing closer together. This may occur as a result of several morphological changes. For instance, the tissues between the nasal pits and eyes grow, allowing the eyes to move distally while the nasal pits grow medially. Also, the fused medial nasal prominences become narrow as they extend down as a result of growth, which also constricts the mouth. This may occur as a result of *Shh*'s involvement in the growth and development of the FNP via the FEZ. Previous work completely by the Marcucio laboratory (Hu, 2003) indicated the FEZ as an important molecular boundary that is responsible for activating a cascade of events that ultimately contributes to the development of the upper jaw. *Shh* is one of the gene expression domains in the FEZ that works with *fgf8*-expressing cells to regulate the formation of the primary palate. Several different signaling pathways are involved with this process and disturbances to any of these pathways can have detrimental downstream effects. For instance, SHH and BMP signaling work together to regulate the expansion of the *Shh* domain into the nasal pits, maxillary processes and globular process which, in turn, could affect the fusion of these prominences (Hu, 2015). This is an important consideration due to the fact that disruptions in *Shh* expression in this region influence midface development (Chiang et al., 1996). When there is a defect in *Shh* expression, many midface malformations that comprise HPE may appear ranging from the most cases with cyclopia and the mildest

cases with a narrow midface (Bullen et al., 2001, Ming and Muenke, 2002). However, when *Shh* is overly expressed, the midface appears wider with the eyes set farther apart (Hu and Helms, 1999). So decreased *Shh* expression leads to varying degrees of midface constriction while increased *Shh* expression leads to varying degrees of midface expansion. Therefore, understanding how *Shh* expression progresses in normal development is crucial in understanding the mechanistic basis for variation during facial morphogenesis. Our results demonstrate how *Shh* expression relates to facial development and more importantly, we identified that *Shh* may potentially serve as a predictor for face shape. As developmental time progressed, we observed that the face shape narrowed as a result of *Shh* expression shape constriction. Conversely, RCAS-*wnt3a* presented with wider face shapes, which resulted from wider *Shh* expression shapes regardless of developmental stage.

Although we were able to demonstrate that *Shh* expression shape relates with face shape during embryonic development, we did not investigate the mechanistic interactions. In other words, we do not know if *Shh* is directly responsible for the resultant face shape. While it is noteworthy to delineate a relationship between *Shh* expression shape and face shape, further work needs to be conducted to evaluate how the different signaling pathways interact to drive craniofacial morphogenesis.

5. Conclusions

In summary, we have determined that *Shh* expression shape may potentially serve as a predictor of face shape during early embryogenesis. As the embryo develops, *Shh* expression shape in the ectoderm of the stomodeum tapers into a narrow V-shape.

Meanwhile, face shape constricts as the nasal pits grow closure together and the mouth becomes smaller. These shape changes appear to follow a linear pattern in which face shape might be dependent on *Shh* expression shape. The fact that this correlation exists between these two shapes suggests that there is a potential relationship in which *Shh* is responsible for variation in midline structures of the face. Further research is required to understand the mechanisms that drive facial morphogenesis.

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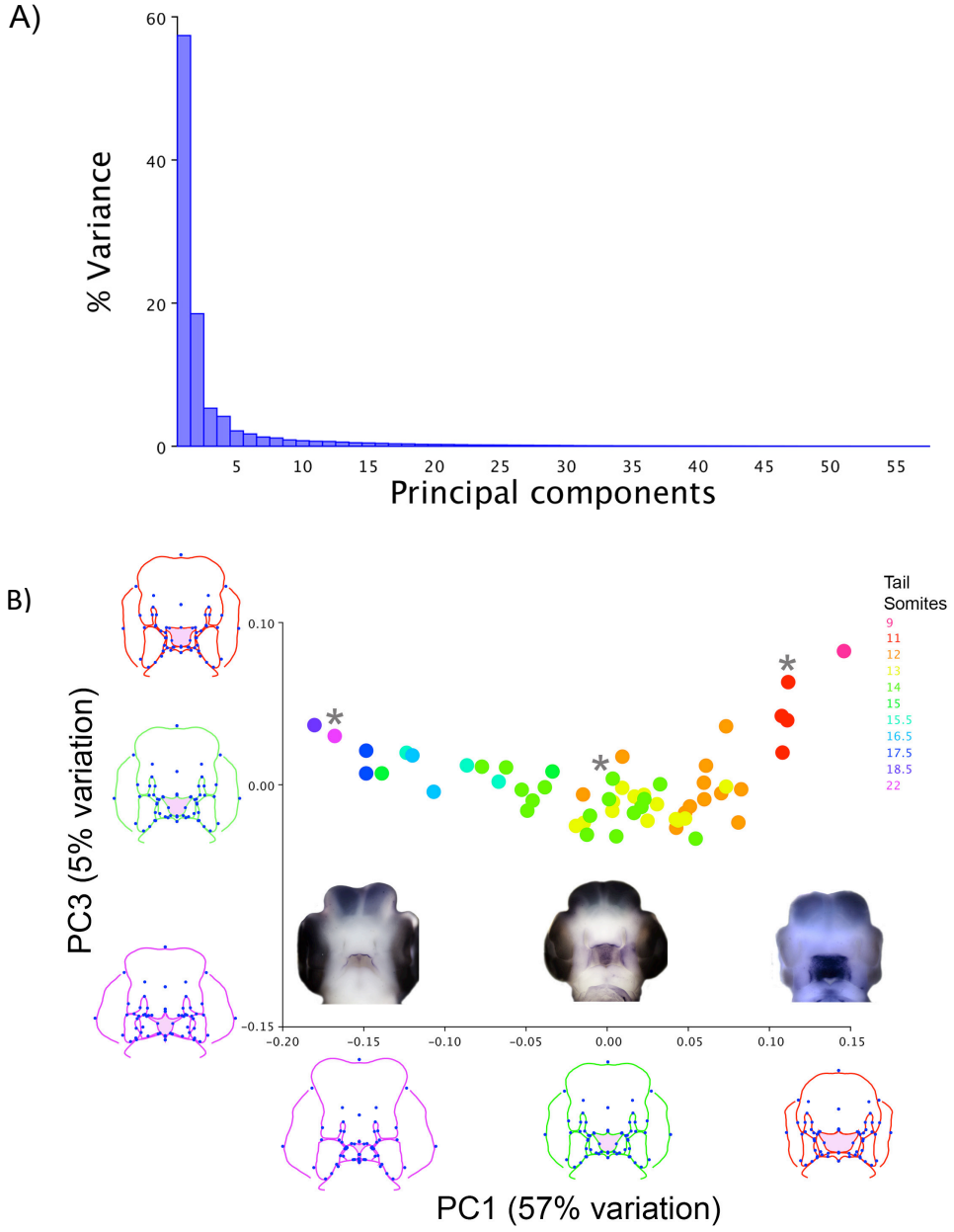

Figure 3.2. Principal Component Analysis
A) PC1 explains 57% of variation observed in *Shh* expression domain and face shape. PC2 explains 19%, while PC3 explains 5% of variation. PC2 is excluded from further evaluation due to the fact that it explains the orientation of the face in the 2D image.
B) Changes in *Shh* expression and face shape follow a trend that appears to be dependent on developmental progression. As the embryo ages, there is a noticeable change in both *Shh* expression domain and face shape. *Shh* expression domain becomes narrower while the face shape also narrows as a result of the nasal pits growing closer together as well as the mouth constricting in size. However, we are unable to determine whether *Shh* is directly responsible for these observed shape changes.

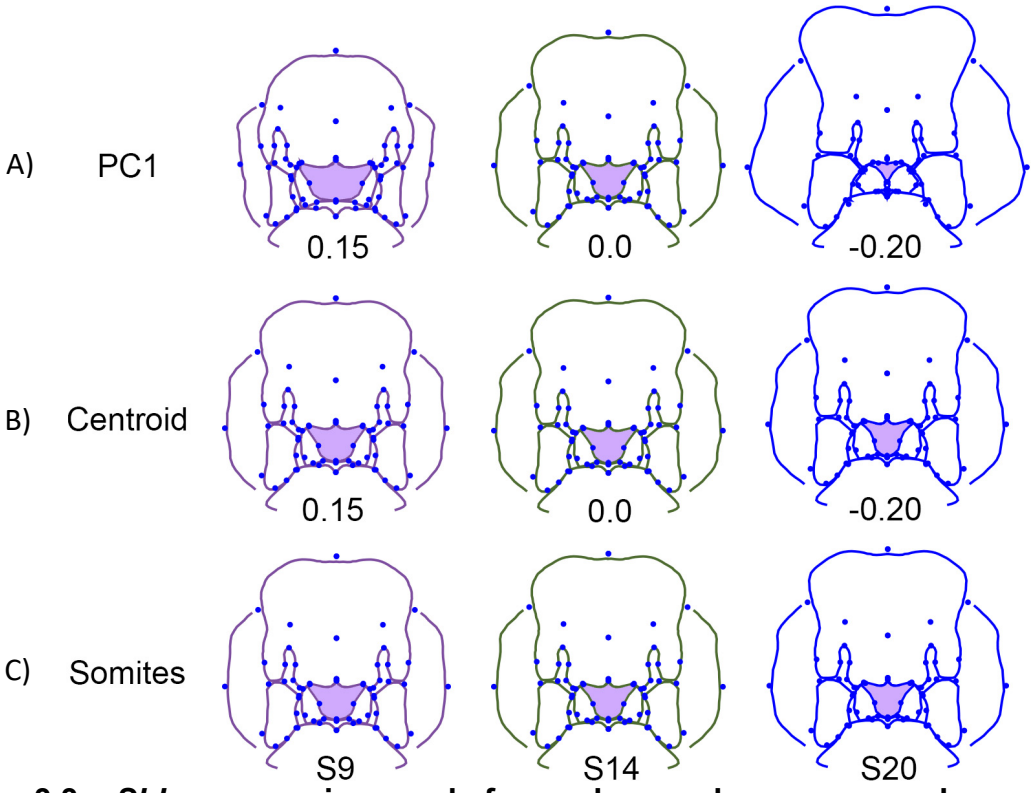

Figure 3.3. *Shh* expression and face shape changes are dependent on developmental timepoint.

Changes in *Shh* expression and face shape were evaluated based on PC1, number of somites, and centroid size at three different timepoints. A) PC1 explained 57% of changes observed among the embryos (Fig 1a). At -0.2, the embryo face shape appears immature with a wide *Shh* expression domain. As PC1 increases, the face shape begins to mature and enlarge while *Shh* expression begins to constrict and narrow. B) As centroid size increased, face shape increased while the *Shh* expression domain narrowed in size. Because an increase in face shape is dependent of growth, an increase in centroid size is also dependent of growth. All observed changes are significant with $P<0.001$. C) As somite number increased, there was a gradual change in face shape and *Shh* expression. The face expanded while the *Shh* expression domain narrowed. Because the number of somites is an indicator of embryonic development, these observed changes may be associated with developmental stage.

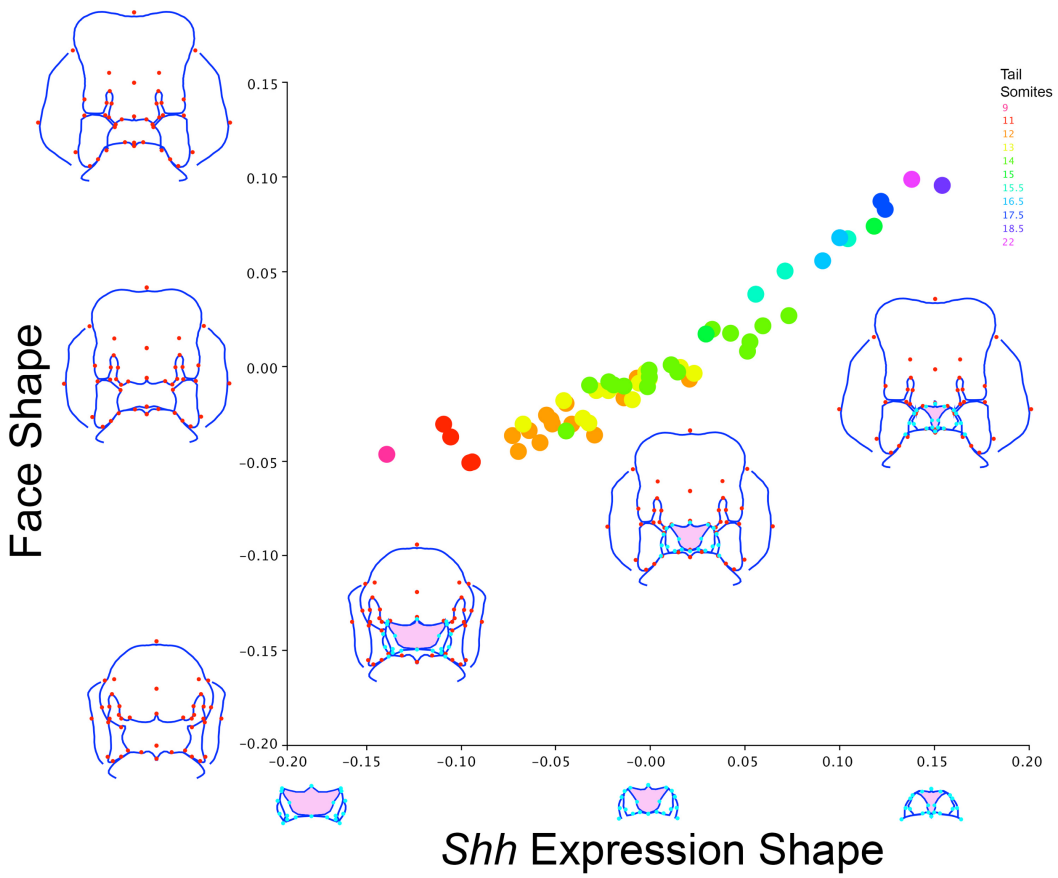

Figure 3.4. Changes in face shape and *Shh* expression shape are related.
A significant correlative relationship is noted between *Shh* expression shape and face shape (P<0.001). As *Shh* expression shape narrows, the embryo head expands as it grows outwards while the midface constricts. This suggests that *Shh* expression shape may be a predictor for face shape in developing embryos.

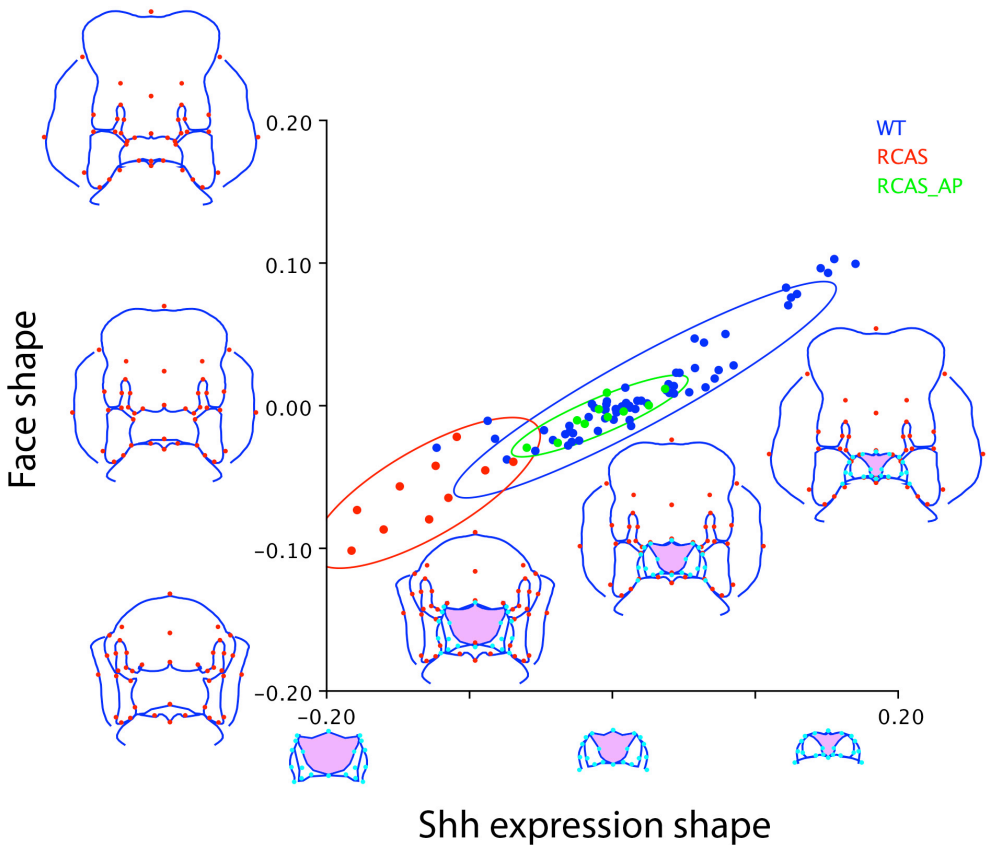

Figure 3.5. SHH expression shape predicts face shape during early development. RCAS-*wnt3a* embryos present with an expanded domain of *Shh* expression. The observed relationship between face shape and *Shh* expression shape in RCAS-*wnt3a* embryos is consistent with that of our WT embryos. As development progresses, *Shh* expression shape tapers into a narrow triangular zone while the overall head grows and the midface constricts. The mouth becomes smaller while the nasal prominences grow closer together.

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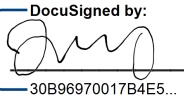
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