Organized cell movement is a major mechanism underlying facial morphogenesis

by

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Abstract

The mechanisms of embryonic facial morphogenesis are poorly understood because direct visualization of the growing embryo is challenging. We refined an organ culture method to visualize the individual cells within the frontonasal mass at high resolution. The fate of the frontonasal mass is to form the premaxilla, nasal septum and facial midline. During normal development the frontonasal mass begins as wide prominence but then the nasal pits relocate to the midline, causing dramatic medio-lateral narrowing. We confirmed that in vitro, the frontonasal mass narrowed over a 48h period, similar to in vivo. Removing the eyes, brain and most of the surrounding face did not impede facial narrowing suggesting intrinsic rather than extrinsic mechanisms were involved. Indeed, blocking the cytoskeletal rearrangements with the Rho GTPase inhibitor, Y27632 (ROCKi) completely inhibited narrowing. Organ cultures were stained with Hoechst dye and imaged using confocal microscopy. Nuclei were imaged for 4-6 hours at 10 minute intervals. Manual cell tracking was carried out across the frontonasal mass. At 10X magnification, striking patterns of order, disorder and then order in vectors of movement were observed. In ROCKi treated cultures there was disorder for the entire culture period. Clustering the vectors according to similarity of the angle revealed large groups of cells were moving in a similar manner in the controls but in the ROCKi treated cultures, clusters were poorly defined and smaller in size. In order to assess symmetry and patterns of divergence and convergence the vector data was interpolated over an evenly spaced grid. The controls had a high degree of right-left symmetry whereas the ROCKi-treated cultures lacked symmetry. We also examined the data to look for sources or sinks in the cell movements. Divergence and convergence bands were located in the mediolateral axis with branches at the lateral edges. The change in direction from convergence to divergence and then back to convergence was rapid, often switching within 20 minutes. This rapid cycling is on the same scale as GTPase switching. Our data suggest that orchestrated mesenchymal cell behaviors, mediated by Rho GTPases are involved in convergent-extension in the face.
Lay summary

Deficient face formation in an embryo leads to serious birth defects such as cleft lip and palate. However, the cellular processes that are required for facial development are poorly understood because direct visualization of the growing embryo is challenging. New methods of observing face development in real time were pioneered. The chicken embryo was studied since embryos can be incubated to the exact stages when facial development is beginning. Conditions supporting the growth of facial processes outside of the egg were refined and then live imaging was carried out. Cells were marked with a fluorescent dye, that allowed tracking of movements over several hours. These experiments revealed that coordinated cell movements are driving facial shape. Cells appear to communicate with neighboring cells over significant distances. The orchestrated movements are characteristic of all cultures. This work will one day lead to methods of preventing congenital facial abnormalities.
Preface

All of the work presented hereafter was conducted in the Life Sciences Institute at the University of British Columbia, Point Grey campus.

I performed all of the work in Chapter 3, developing the methods for organ culture and live imaging. The lipid nanoparticles were generously provided by the Cullis Lab (Peter Cullis).

I was the lead investigator for all of the work in Chapter 4 where I was responsible for the majority areas of concept formation, data collection and analysis. Johnathan Woo, MSc Ped Dent 2014, carried out the Optical projection tomography scans in Figure 4.1. He did the original segmentation and volume calculations in Figure 4.1. He placed landmarks on the face and carried out geometric morphometrics analysis using MorphoJ. The linear measurements in Figure 4.1 were carried out by me. All other experiments were performed and analyzed by me.

I was the lead investigator for the work in Chapter 5. I performed the live imaging and did the analyses in Figures 5.1, 5.2. I was assisted with the cell tracking by Jaspreet Rekhi an undergraduate student and Takashi Akazawa, Visiting PhD student from Kagawa University, Japan. I consulted with Professor Leah Keshet-Edelstein and her post-doc Dr. Elisabeth Rens from the Department of Mathematical Sciences, UBC. I worked with my supervisor to generate the research questions that needed to be addressed with advanced mathematical analyses such as right-left symmetry, clustering and divergence-convergence. Dr. Rens ran the XY data for all of the movies through MatLab to generate the data in Figures 5.3 to 5.14. I worked with my supervisor to interpret the data.

I will be sole first author on two manuscripts based on this work. The first will be derived from Chapters 4 and 5. The second manuscript will be based on Chapter 3, development of live imaging techniques to study facial morphogenesis. Dr. Joy Richman was the supervisory author on both of these projects and was involved throughout in concept formation and thesis edits.
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List of abbreviations

ANOVA Analysis of variance
AP Anterior-posterior
BMP Bone morphogenetic protein
BrdU Bromodeoxyuridine
BSA Bovine serum albumin
cDNA Complementary DNA
CL/P Cleft lip and palate
CNCC Cranial neural crest cells
DAPI 4′,6-diamidino-2-phenylindole
DAX DIX Axin domain
DEP DVL, EGL-10, Pleckstrin
DIX DVL/Axin
DMEM: F12 Dulbecco’s modified eagle medium/Ham’s F-12
Dsh Dishevelled
DV Dorsal-ventral
DVL Dishevelled
ECM Extracellular matrix
EDTA Ethylenediaminetetraacetic acid
FGFR FGF receptor
FNM Frontonasal mass
GFP Green fluorescent protein
GM130 Golgi bodies
GS Goat serum
GTPase Guanosine triphosphatase
HH Hamburger Hamilton
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HOX Homeobox
HPE Holoproencephaly
JNK Jun N-terminal kinase
n Sample size
NC Neural crest
OMIM Online *Mendelian Inheritance in Man*
p Probability (p value)
PBS Phosphate buffered saline
PCP Planar cell polarity
PD Proximal-distal
PDZ Postsynaptic density 95, discs large, zonula occludens-1
PFA Paraformaldehyde
PTCH1 Patched homolog 1
Rac Ras-related C3 botulinum toxin substrate 1
RCAS Replication-competent ASLV LTR with a splice acceptor
RCASBP RCAS bryan polymerase
RCASBPY RCASBP gateway
RFP Red florescent protein
Rho Rhodopsin
ROCK Rho-associated kinase
ROCKi Rho-associated kinase inhibitor
RS Robinow syndrome
SD Standard deviation
SHH Sonic hedgehog
SOX Sex determining region Y-box
TBX T-box transcription factor
TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling
WNT Wingless
wt Wild-type
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Thank you!
Chapter 1: Introduction

1.1 Embryonic facial development

1.1.1 Brain –face interactions - Shh signaling and the first signal to establish the facial midline

In this thesis, we will be focusing on morphogenesis of the midface. The main molecular pathway that was discovered to be required for midface formation is SHH (sonic hedgehog). Loss of SHH signaling is connected to several human midfacial and brain deficiencies that are together grouped under the term holoprosencephaly (HPE) (Belloni et al., 1996; Roessler et al., 1996). The spectrum of phenotypes in HPE ranges from cyclopia (one eye, proboscis instead of nose), to hypotelorism (narrow set eyes) with a small nose, sometimes with a single nostril (Richieri-Costa and Ribeiro, 2010), to midline clefts with agenesis of the premaxilla, to the mildest form, solitary median central incisor (Nanni et al., 2001). Cyclopia and hypotelorism are due to a deficiency in SHH from the prechordal plate (anterior to the notochord). The prechordal plate lies beneath the eye fields and without sufficient levels of SHH, the eye fields will fail to completely separate (Roessler and Muenke, 2010). In most cases of HPE (except for solitary median central incisor), there are structural brain abnormalities (Winter et al., 2015). The telencephalon fails to completely separate into the right and left cerebral hemispheres (Muenke and Beachy, 2000; Roessler and Muenke, 2010). The lack of SHH signaling from the prechordal plate also results in incomplete development of the ventral floorplate of the forebrain. It is thought that signals from the floorplate induce facial expression of $SHH$, thus promoting facial midline growth (Marcucio et al., 2005; Young et al., 2010). The early disruption of SHH signaling within the brain prevents mid-facial development, mimicking HPE (Cordero et al., 2004; Marcucio et al., 2005; Young et al., 2010). The key point about HPE is that all the phenotypes can be classified as deficiencies of midline structures rather than an excess of tissue.
1.1.2 Cranial neural crest cells and their fate

At the same time as the eye fields are being patterned and the telencephalon is forming two cerebral hemispheres, the neural crest cells (NCC) are beginning to develop. In mammals, including humans, NCC are specified at the edges of the neural plate due to a series of interactions with the surface ectoderm (Bronner, 2012). Shortly after specification, the neural crest cells break away from the neural epithelium and transform into migratory mesenchymal cells through epithelial-mesenchymal transformation (Strobl-Mazzulla and Bronner, 2012). Cranial neural crest cells (CNCC) are considered the group of cells that originate from the anterior forebrain down to rhombomere 2 (anterior hindbrain). These cells are all interchangeable within this region as shown by quail-chicken chimeras where neural folds were exchanged between the two animals (Creuzet et al., 2005). However cranial and more caudal neural crest cells are not interchangeable (Noden, 1978; Trainor et al., 2002; Tucker and Lumsden, 2004). One of the reasons for this distinction between CNCC and other populations is that there are no HOX genes expressed above R2. Ectopic expression of HOX genes inhibits facial development (Creuzet et al., 2005). A key feature of neural crest cells is their ability to migrate through the paraxial mesoderm into the presumptive face (Le Douarin et al., 2007; O’Rahilly and Muller, 2007; Santagati and Rijli, 2003) (Achilleos and Trainor, 2012). The migratory neural crest cells encounter signals from the brain (Creuzet et al., 2006), the foregut endoderm (Benouaiche et al., 2008; Brito et al., 2006; Couly et al., 2002) and the ectoderm (Brito et al., 2008; Graham, 2003). Due to these interactions with surrounding tissues, neural crest cells give rise to structures that are characteristic for that region (Schneider, 2018). The size and shape of the jaws is programmed into the neural crest cells (Fish and Schneider, 2014; Fish et al., 2014).

The NCC are considered the 4th germ layer (Hall, 2009) since they arise after the 3 canonical germ layers are established, ectoderm, mesoderm and endoderm and they give rise to diverse derivatives (Dupin and Le Douarin, 2014; Dupin and Sommer, 2012). The cell types formed by a single CNCC include osteoblasts, chondrocytes, neurons, glia, smooth muscle cells and melanocytes (Dupin and Le Douarin, 2014). CNCC are able make skeletogenic cells unlike trunk NCC (Abzhanov et al., 2003). Although chicken-quail studies were informative about the fate of neural crest cells, birds do not have teeth and have different jaw anatomy compared to mammals (for example no temporomandibular joint and an unfused palate). Fate mapping studies
were carried out using the Wnt1-Cre transgenic line which drives expression of LacZ in neural crest cells. This allowed tracing of cells until all the tissues had differentiated. The craniofacial region is filled with neural crest-derived mesenchyme that gives rise to the frontal bone, the bones of the face including maxilla and mandible, cartilages of the nose, Meckel’s cartilage, the connective tissue of the face, dental pulp, periodontal ligament and dentin (Chai et al., 2000; Jiang et al., 2002; McBratney-Owen et al., 2008; Morriss-Kay, 2001). Thus, facial development is entirely dependent on successful initiation, migration and differentiation of neural crest cells. A decrease in neural crest cells due to environmental (Sulik, 2005) or genetic factors (Trainor et al., 2009) leads to major craniofacial abnormalities.

### 1.1.3 Facial prominences, lip fusion and merging

Once the neural crest cells have surrounded the primitive oral cavity, they begin to fill the pharyngeal arches and the facial buds or prominences (Fig. 1.1). The facial prominences are buds of mesenchyme covered by ectoderm that will give rise to the upper and lower jaws (Abramyan and Richman, 2015). The first pharyngeal arch forms the mandibular prominence and part of the maxillary prominence (Lee et al., 2004). The rest of the facial prominences are not derived from pharyngeal arches but instead arise from the frontonasal process (Sperber and Sperber, 2018). From grafting studies in the chicken embryo, we know that the facial prominence mesenchyme contains all the information about face shape and size of jaws whereas the ectoderm is required for outgrowth (Abramyan and Richman, 2018). The frontonasal process is divided by symmetrical nasal placodes. Thereafter the tissue medial to the nasal placodes form the frontonasal mass (in birds) or medial nasal prominences (in mammals). The frontonasal mass or medial nasal prominences form all the midline structures of the face including the midline of the nose, the nasal septum, the columella, philtrum, premaxilla and 4 incisors (Sperber and Sperber, 2018). Lateral to the nasal placodes, the lateral nasal prominences will develop, due in part to supportive signals from the placodes (Szabo-Rogers et al., 2008). The maxillary prominences form the upper jaw (except for the premaxilla) and palate. The lateral nasal prominences form the nasal turbinates and alae of the nose (MacDonald et al., 2004). The mandibular prominences form the entire lower jaw (Abramyan and Richman, 2015; Richman and Tickle, 1989). The facial prominences and their
derivatives are conserved through evolution thus, what we learn from experimental models such as chicken is relevant to human development.

Morphogenesis of the facial prominences is critical for lip fusion. The frontonasal mass, maxillary and lateral nasal prominences contact and fuse to form the upper lip (Error! Reference source not found.). The process of lip fusion is largely conserved in amniotes but there are species-specific differences in the relative sizes and shapes of the prominences (Abramyan et al., 2015). The first point of contact during the lip fusion process for chicken and human is between the globular process which is part of the lateral corner of the medial nasal prominence and maxillary prominence. In the mouse, the lateral nasal prominence also participates in fusion. There are 5 steps involved in lip fusion: 1) outgrowth of the prominences 2) adhesion of the epithelial surfaces, 3) formation of a bilayer epithelial seam, 4) formation of a mesenchymal bridge and 5) filling out of remaining furrows via merging (Richman and Vora, 2017). The adhesion step is thought to be most susceptible to gene variations, leading to non-syndromic cleft lip(Hammond et al., 2017; Khandelwal et al., 2016; Leslie et al., 2016; Parada-Sanchez et al., 2017). Premature adhesion between facial prominences is prevented due to a flat, outermost layer of epithelium, called periderm (Richardson et al., 2014). Therefore, cleft lip may be due to persistence of periderm beyond the time when it should be degraded. The secondary palate also forms due to adhesion of adjacent epithelial surfaces (Hammond et al., 2017) followed by the subsequent steps of fusion. In contrast to the lip and palate, all other grooves between the facial prominences are smoothed out via merging, not fusion. Merging involves differential proliferation and potentially migration of mesenchyme to smoothen the deep furrow between neighbouring facial prominences (Richman and Vora, 2017). Several places in the face undergo merging, the nasolacrimal groove between the lateral nasal and maxillary prominences, the midline groove between the medial nasal prominences and the midline of the mandibular prominence. These areas are rarely affected by orofacial clefting.

Typical cleft lip is a separation between the premaxilla and maxilla with the cleft extending up into the nares (Figure 1.1 C). These forms of cleft lip are distinct from midline clefts of HPE where the medial nasal prominences have not formed (Richieri-Costa and Ribeiro, 2010). In addition, orofacial clefts are not associated with structural brain abnormalities. The most common form of orofacial clefting is non-syndromic or isolated cleft lip with or without cleft palate (NSCLP) which occurs in 1 in 700 births (Lewis et al., 2017). The causes of NSCLP are a
combination of multiple genes (identified in GWAS or by targeted sequencing) and influences from the environment (Richman and Vora, 2017).

1.2 Syndromic conditions affecting midfacial morphogenesis are distinct from holoprosencephaly

During development of the face there are major changes in shapes of the facial prominences at the same time as the lip is fusing. Initially the nasal pits are at the extreme lateral edges of the frontonasal prominence (37-42 days) (Jiang et al., 2006; Richman and Vora, 2017). Then at 44-48 days gestation, the nasal slits relocate towards the center of the face. By 48-51 days, the facial midline consists of a narrow segment between the nasal slits. All these steps occur after the ventral floorplate of the brain has induced SHH in the face. If the narrowing process does not occur, then the eyes will be wider apart than normal (hypertelorism), the nose may have a midline groove, the tip of the nose may be cleft, and the nasal bridge may be wider. The narrowing of the midline may be connected to lip fusion as some studies have found an association between hypertelorism and orofacial clefting (Weinberg et al., 2016).

Examples of craniofacial syndromes with hypertelorism include Frontonasal dysplasia, (#613451), Craniofrontonasal dysplasia (#304110), Opitz GBBB (#145410) and Robinow syndrome (#180700; 616331, 616894, 268310). We hypothesize that the underlying basis of these facial differences is incomplete merging of the medial nasal prominences, leading to greater separation between the nasal slits.
(A,B,C) Frontal view of the embryonic human face in the 5th (A) and 7th (B,C) weeks of development. (A) The medial nasal and the maxillary prominences make epithelial contact before fusion. (B) Normal development; the maxillary and medial nasal prominences are fused. (C) Unilateral left-sided cleft; the medial nasal and maxillary prominences are fused on the right and not fused on the left side. Key: e – eye, ln - lateral nasal process, md – mandibular prominence, mn – medial nasal prominence, mx – maxillary prominence

Figure 1.1. Early development of the human facial prominences
1.3 General morphogenetic mechanisms

1.3.1 Proliferation and apoptosis role in shaping the facial prominences

Proliferation has long been thought of as the main mechanism underlying growth of buds such as the limb buds and facial prominences. There are two main ways in which proliferation contributes to outgrowth. The first is that during budding, the basal mesenchyme decreases relative to the mesenchyme inside the bud. Detailed DNA labeling studies in chicken embryos during the development of lateral nasal and frontonasal prominences demonstrated that there was a decline in proliferation of the mesenchyme next to the brain, however the facial mesenchyme retained the original, higher rate of proliferation. These differential proliferation rates were evident during the phase of lip fusion, helping to bring the frontonasal mass in contact with the maxillary prominence (stages 26-27) (Minkoff and Kuntz, 1977). A more recent study conducted in our lab found the same trend of dropping proliferation in the base of the nasal pit with maintenance of higher proliferation in the frontonasal mass and lateral nasal prominence (Abramyan and Richman, 2015). These differences are increasing the depth of the nasal slit while the face grows out. In another study from our group on the maxillary prominence, the basal maxillary mesenchyme gradually decreased the proliferation rate while the mesenchyme in the palatal shelves remained relatively high in the chicken embryo (Abramyan et al., 2014). However, in the turtle, the mesenchymal rates of proliferation on the medial sides of the maxillary prominences were equal to those of the lateral maxillary prominence mesenchyme. These data correlate with the lack of palatal shelves in the turtle. Once the facial buds have formed there is evidence of higher proliferation directly under the epithelium (Minkoff and Kuntz, 1977, 1978). Thus, the consistent finding is that facial prominence outgrowth in specific regions is due to relative drops in proliferation in the deeper mesenchyme relative to the peripheral mesenchyme.

Proliferation patterns in the frontonasal mass have been mapped by our group (Abramyan et al., 2015; Higashihori et al., 2010; MacDonald et al., 2004; Szabo-Rogers et al., 2008) and others (Abzhanov et al., 2004; McGonnell et al., 1998; Wu et al., 2006; Wu et al., 2004) using BrdU incorporation. Here the patterns appear to be regional. At stages prior to lip fusion, there is high proliferation close to the nasal slits, relatively low proliferation in the center and then at the corners of the frontonasal mass or globular processes there is an area with low proliferation. It appears that
the mesenchyme in the globular process is being pushed by proliferation of more basal layers of mesenchyme (Szabo-Rogers et al., 2008). Later when the beak begins to form the prenasal cartilage, there is very little proliferation in the center, instead the tip of the beak is proliferating at a relatively high level (Wu et al., 2006). The importance of the proliferative zone was recognized when comparing bird beaks with different shapes. The area with the highest relative proliferation was positioned more dorsal or ventral in birds with broader or pointed beaks respectively (Fritz et al., 2014; Wu et al., 2006).

In addition to the levels of proliferation within the mesenchyme, the direction of cell division contributes to morphogenesis. If daughter cells are preferentially produced in a particular axis then it is possible to elongate the entire prominence (Linde-Medina et al., 2016). The most detailed study on role of oriented cell division is that of Kaucka et al., (2016). These investigators performed a clonal analysis which is the most rigorous way to determine the angle of daughter cells relative to a particular axis. Most facial mesenchyme cells appeared to be dividing in the dorso-ventral (proximo-distal) axis or the direction of snout outgrowth. Their study was not able to look at specific facial prominences, due to the random nature of the cell labeling technique. Thus, the question of whether oriented cell division is important for facial morphogenesis is unresolved.

Apoptosis functions through cell elimination in specific regions to sculpt the tissue. The best study on apoptosis in the face was carried out by McGonnel and Tickle (1998). There were very few regions with apoptosis. These included the cartilage forming regions in the mandible, the nasolacrimal groove between the maxillary and lateral nasal prominences and a small area in the center of the frontonasal mass. Our group have also examined apoptosis using TUNEL and agree with the majority of these findings (Abramyan and Richman, 2015; Hosseini-Farahabadi et al., 2017). The role of apoptosis is minor relative to that of proliferation in shaping the face.

1.3.2 Rearrangement of cells, directed cell migration and cell orientation

The facial prominences are packed with neural crest-derived mesenchyme so one might predict that the cells could have retained some ability to migrate. In addition, there are major organs growing next to the face such as the nasal cavity, the brain and the eyes. There may be forces on
the facial prominences that lead to passive displacement of cells. There are several potential intrinsic mechanisms during facial morphogenesis responsible for a relatively quick re-shaping of facial prominences (Error! Reference source not found., A-D). We have observed the midface appears to contract in the mediolateral axis so displacement may be a mechanism driving this aspect of morphogenesis. In order to attempt to measure mesenchymal cell rearrangements, several authors have placed labels into the mesenchyme and followed the embryos over several days. The first study was carried out by Patterson et al. (1984). Cells in the lateral nasal, maxillary and frontonasal mass were carefully labeled with a sable hair dipped in tritiated thymidine. The labeled cells did not cross from the lateral nasal to the maxillary prominence which suggests a boundary may be present at the position of the nasolacrimal groove. Some cells labeled in the maxillary prominence appeared to relocate into the midline of the frontonasal mass, most likely due to fusion of the lip. The cells in the frontonasal mass appeared to relocate to a more lateral position. Note that with this method, the tritium is taken up by dividing cells so some of the relocation of cells is due to proliferation. Characteristic expansion of group of labeled cells was also reported by McGonnell and Tickle (1998) but this time using DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate). These authors microinjected the lipophilic dye into discrete locations of the face at stage 20 and fixed embryos after 48h of growth. The dye spread much more in some areas of the face such as the midline of the mandible, the medial edge of the maxillary prominence and the lateral frontonasal mass mesenchyme closest to the nasal slit. As with Patterson et al. (1984), the authors could not distinguish between expansion of marked cells due to cell proliferation, passive displacement or active migration or a combination of all three.

Additional studies were carried on using cell labeling techniques where GFP plasmids were transfected into local regions of the maxillary prominence. Here is was possible to resolve the orientation of the cell by visualizing the longest axis. Groups of maxillary cells were oriented along the axis of growth of the prominence (Geetha-Loganathan et al., 2014). Cells could be reoriented towards a source of WNT expressing cells suggesting that growth factors could be important for organizing the polarity of facial mesenchyme. This, by Geetha-Loganathan et al. (2014) was the first study to identify a predominant orientation or polarity to facial mesenchyme cells. Others have since measured cell orientation using the Golgi as a marker organelle. Again, frontonasal mass cells appeared to be oriented preferentially along the major axis of growth (Li et al., 2013). Neither study was able to distinguish the leading from trailing edge of the cell, nor was
cell movement confirmed. It would be necessary to watch the cells moving in real-time to know for certain which direction they were heading or to know whether movement had taken place.

1.3.3 Molecular control of cytoskeleton - small GTPases

At a fundamental level, most data on intrinsic mechanisms of cell behavior are derived from in vitro cell culture experiments. Here, cells are plated on plastic dishes, a scratch is created and the migration into the gap is observed. Cell biology studies have shown that leading-edge protrusions result from individual combinations of signaling and actin cytoskeletal proteins (Ridley, 2011). Actin polymerization generates lamellipodia or thin extensions of the cytoplasm that form the leading edge of migrating cells (Petrie and Yamada, 2012). The RhoA family of small GTPases (Rac1, Cdc42 and RhoA) are all active at the leading edges of cells (Machacek et al., 2009). Rho kinases are the effectors of the small GTPases. ROCK (Rho-associated coiled-coil containing kinase) plays a major role in mediating rearrangements of the actin-myosin cytoskeleton that control cell shape and polarity (Amano et al., 2010). Uncoupling of the GTPases from their effectors prevents cells from forming focal adhesions and migrating (Julian and Olson, 2014). More relevant to this study are the in vivo data studying the function of RhoA GTPases or ROCK in mouse models. The conditional expression of a dominant negative version of ROCK in neural crest cells severely inhibits midfacial morphogenesis (Phillips et al., 2012). This study suggests a more detailed examination of how RhoA-GTPases affect cell migration within the face is necessary.

1.3.4 Convergent extension- intercalation, planar cell polarity

Midfacial narrowing presents unique challenges in terms of morphogenesis. This narrowing occurs at a time when proliferation is high at the lateral edges of the frontonasal mass. Therefore, it would appear based on the proliferation, that the frontonasal mass should retain its original mediolateral size. However, if we begin to consider that mesenchymal cells may be able to move in the perpendicular axes, then it is possible for cells from the lateral edges to be relocated
to a more dorso-ventral or cranio-caudal location, perhaps intercalating with cells in the midline as they move (Error! Reference source not found. D). Such movements are defined as convergent extension, where one axis narrows while the perpendicular axes extend. The best examples of convergent-extension movements are during embryonic axis elongation (Tada and Heisenberg, 2012). In the chicken embryo, several studies were carried out using dye labeling. The work of Schoenwolf was critical in defining how cells from the epiblast move towards the midline and ingress through the primitive streak (Lawson and Schoenwolf, 2001a, b). Cells labeled with two colors of dye formed a column in the midline, presumably via intercalation, but the resolution was not sufficient to confirm these results (Lawson and Schoenwolf, 2001a). More recent studies using multi-photon live imaging was carried out with GFP-expressing epiblast cells confirmed the intercalation of labeled cells into primitive streak (Voiculescu et al., 2014). Convergent-extension movements carry on in later stages of tail elongation with very complex sliding of the presomitic mesoderm between the ectoderm and mesoderm (Benazeraf et al., 2017). It is unknown whether mesenchymal cells in the frontonasal mass converge towards the midline and whether then diverge in a perpendicular axis. More reliable cell-specific labelling techniques combined with live imaging are necessary to test this hypothesis. Without live imaging, it is impossible to be certain of the direction of movement.
Figure 1.2. **Intrinsic mechanisms in morphogenesis**
Examples of mesenchymal cell behavior during morphogenesis. Epithelial signals can orient the cells along the leading edge (A), stimulate an oriented cell division (B), orient the cells on a particular axis or induce the convergence of cells along a specific direction (D). Cell cytoskeleton include actin filaments that give the cell its shape and guide the organization of cell's components while they provide a basis for movement and cell division where actin is regulated by small GTPases of the Rho family (A).
1.4 Extracellular matrix

Mesenchymal cells have the potential to generate pulling forces through their connection with the extracellular matrix scaffold (Ingber, 2006). The extracellular matrix (ECM), the main constituent of the extracellular microenvironment, forms a three-dimensional network that supports cells and tissues. ECM is comprised of collagen and non-collagenous proteins such as laminins or fibronectin, glycosaminoglycans, proteoglycans, and associated molecules (Rozario and DeSimone, 2010). The composition of ECM varies widely throughout development and is reflected in the assembly of specialized ECMs (Rozario and DeSimone, 2010). One of the main functions of ECM is in facilitating cell migration. ECM–integrin binding interactions regulate cell adhesion to substrate (Dzamba and DeSimone, 2018). Cycles of cell adhesion interconnect with the cytoskeleton contractions to produce traction forces on an ECM, resulting in cell movement (Rozario and DeSimone, 2010). For instance, neural crest cell movements require cooperation between fibronectin and distinct integrins that regulate different events of cell adhesion and migration (Testaz et al., 1999). Specific components of the ECM such as versican and aggrecan have antagonistic effects on the neural crest cell migration, with aggrecan being characteristic for impenetrable embryonic zones, in contrast with versican that favors the directed movement of the cells (Perissinotto et al., 2000).

1.5 Methods of observing morphogenesis

1.5.1 Live imaging of morphogenesis at the cellular level

Morphogenesis of complex tissues with single cell resolution has only been possible due to two advances, the first being the ability to label cells with GFP either via transfection (Krull, 2004; Teddy et al., 2005; Voiculescu et al., 2008) or by genetic means (Huss et al., 2015; Sato et al., 2010). These advances in long-term labeling of cells days, combined with the ability to image embryos at high resolution using confocal or other forms of microscopy have led to major discoveries about dynamic cell movements. The developing chicken embryo was one of the first organisms to be studied because the embryo is very flat and relatively transparent at early stages of development. Electroporation of plasmids is best done using ex-ovo culture which supports
growth up to 10 days (Luo and Redies, 2005). Most live imaging studies using electroporation are generally restricted to very early gastrulating embryos (Voiculescu et al., 2007), the tail bud (Roszko et al., 2007), neural crest cells (Sauka-Spengler and Barembaum, 2008; Teddy et al., 2005) or the brain (Luo and Redies, 2005). The best results with delivering transgenes to mesenchyme is in the limb bud and here the authors placed the plasmid DNA into the lateral plate mesoderm (Gros et al., 2010). In contrast, to these other regions, electroporation of the face is very challenging. The main reason is that the heart is very close to the electrodes. Thus, for now there is no suitable ways to label or image the face even in the ex-ovo culture system (Trinh le and Fraser, 2015).

The importance of intrinsic cell labeling techniques is that it is possible to track cells over time. Thus direction, velocity can be calculated. With successful labeling of large numbers of cells, it should in theory be possible to measure global changes in cell position across an organ. The face consists of a number of facial prominences that are relatively thick, and each has their own shape characteristics. The face is not really just one organ but the confluence of 5 facial prominences. The review by Trinh and Fraser (2015) highlights the large deficit in knowledge we have about facial prominence development or later stages. Currently the published data end when neural crest cells migrate into the pharyngeal arches. Some of the barriers that need to be overcome are methods to culture facial organs while keeping them still enough for time lapse imaging. A method of labeling as many cells as possible in a stable manner is needed if we hope to observe such behaviors as convergent extension. A high-resolution microscope is needed to separate individual cells in such a dense tissue as facial mesenchyme. Once these basic conditions are established, we will be poised to introduce other methods such as multicolored labeling that will allow lineage tracing at the same time as cell tracking.
1.6 Hypotheses

1.6.1 Coordinated cell rearrangements are required for facial morphogenesis
1.6.2 Cytoskeletal remodeling mediated by Rho GTPases is necessary for facial morphogenesis.

1.7 Aims/Objectives

1.7.1 To develop innovative techniques for improved static and live-cell imaging on the developing embryonic face during early stages of morphogenesis.

1.7.2 To characterize the normal cell movement and cell shape during critical stages of facial morphogenesis.

1.7.3 To characterize the effect on facial convergence/divergence when the face is treated with a Rock antagonist.
Chapter 2: Methods

2.1 Animals and dissection of tissues

Fertilized white leghorn chicken eggs were purchased from the University of Alberta, Edmonton. Eggs were incubated for 3-5 days covering stages 20, 24, 28 and 29 (Hamburger and Hamilton, 1951). Embryos were used for immunostaining or organ culture.

For the organ cultures, chicken eggs were incubated for four days to stage 24. Frontonasal masses including/excluding the eyes and the brain were carefully dissected in 1x Hanks’s solution on ice. Dissections either included all the facial prominences (Fig. 2.1, dashed line a) or just the frontonasal mass and nasal slits (Fig. 2.1 dashed line b). In all cases, the epithelium was left intact to retain signaling.

For Optical Projection Tomography (OPT), embryos were fixed in 10% formaldehyde and processed into Benzyl alcohol: Benzyl benzoate clearing solution as published (Abramyan et al., 2015). Embryos were scanned with a wavelength that excites autofluorescence in formalin fixed tissues. A total of 8 embryos per stage (N=32) were scanned with OPT and reconstructed using NRECON software. Image stacks were exported to Amira software. Each frontonasal mass was segmented manually so that volume could be calculated. The distance between the nasal slits was manually measured from slice views.

2.2 Immunofluorescence staining

Embryos between stages 24 and 28 were sectioned in the frontal or coronal planes. Cells undergoing mitosis were stained with a phospho-histone 3 (pH3) antibody (Rabbit polyclonal, Cell signaling #9701, 1:400). Presumptive chondrocytes were stained with anti-SOX9 (Sigma-Aldrich, 1:200, #HPA001758). Goat anti-rabbit IgG labelled with Alexa-Fluor 488 was used as the secondary antibody (Life Technologies 1:200). Nuclei were counter stained with 10 μg/ml Hoechst (#33568, Sigma). Prolong Gold antifade was applied to all slides (Life Technologies #P36930).

The total number of mitotic cells labeled with pH3 antibody were counted in the lateral edges of the frontonasal mass using the cell counter plugin in ImageJ. Three adjacent sections were
used to obtain the number of dividing cells per embryo and there were 3 embryos studied per stage. The proportion of cells in metaphase, anaphase, telophase or cytokinesis out of the total number of mitotic figures was determined. The angle at which cells were dividing was determined relative to the nasal slit in the frontal sections. A second set of embryos were sectioned coronally and the proportion of mitotic cells in prophase, anaphase, metaphase and cytokinesis was calculated.

2.3 Organ culture system

Base media consisted of DMEM: F12, 1:1, 1X L-glutamine, 10% Fetal bovine serum (FBS), 1:100 antibiotic-antimycotic (Penicillin/streptomycin/amphotericin B). For static imaging of organ cultures over time, embryonic faces were placed on top of Nucleopore membrane (Whatman® Nucleopore, Sigma-Aldrich), epithelium side up and the membrane was supported by a wire mesh. Cultures were grown at the air-liquid interface as described in organ culture dishes (Falcon™ Organ Culture Dish, Fisher Scientific ) (Hu and Helms, 2001). Photographs of the cultures were taken once a day for up to 96h.

For live imaging, frontonasal mass and lateral nasal prominence explants were transferred to 8 chamber Ibid micro slides (#80821). The organs were flipped so that epithelial surface was contacting the glass (Figure 2.1). Matrigel (100%, 80 µl, ThermoFisher) was added on top of the cultures and forceps was used to keep the explant in contact while the gel set (30 minutes in incubator). Base media containing Hoechst 33342 (1µM) plus or minus 10 µM ROCKi was added to each well (150 µl). Organs were cultured in an incubator inside the multiwell slide for 1-2h prior to imaging. HEPES media (15 mM) was included so that when the cultures were imaged inside the confocal microscope environmental chamber, the pH of the media would remain stable in room air.

In order to determine the lowest dose that still gives us a phenotype we performed a dose-response experiment using 1, 2.5, and 5 µM ROCK inhibitor (Y-27632) which is a cell-permeable, highly potent and selective inhibitor of Rho-associated, coiled-coil containing protein kinase (ROCK) (Figure 2.1). Others have used concentrations up to 500 µM for cell culture and organ culture experiments (Duess et al., 2016; Li et al., 2009; Nishimura et al., 2012; Takehara et al., 2008). The dose was adjusted once live imaging of nuclei was carried out. Here, it was possible to see dying cells with the 100 µM concentration. We found both 2.5 µM and 10 µM concentrations
avoided cell toxicity. The lower concentration of 2.5 µM was insufficient to cause cell behavior changes that could be measured. The 10 µM concentration gave measurable differences in cell movements so we added 10 µM for all live imaging and repeats of organ culture experiments.

Figure 2.1. Schematic of the organ culture and areas dissected from the face
A) A blue line outlines the full face plus eyes that was used in some cultures. The b red line outlines the smallest region that could be cultured and still observe facial narrowing. B) The typical organ culture used for stereomicroscope observations. The face is upright in the dish and rests at the air-liquid interface (+/- Y27632); Key: fnm- frontonasal mass.

2.4 Lipid nanoparticle delivery

Marking of the cells was done by using lipid nanoparticle transfection of GFP or RFP plasmids. LNP-pDNA formulations were prepared as previously described (Leung et al., 2015). Amino-lipids, helper lipids, cholesterol and PEG-DMG were dissolved in ethanol at a molar ratio of 50/10/39/1. PCAX-eGFP (expressing eGFP) was dissolved in 25 mM sodium acetate pH 4 to 0.116 mg/ml. The solutions were mixed using a micromixer. Transfection was carried out at time zero and observed either with live imaging starting at 16h for a further 12h of culture or with a stereoscope. Positional injections were carried out using a pulled, glass needle. For lateral injections, small groups of cells were labelled at the midpoint of the nasal slit. For midline injections, cells were labeled at the superior or inferior edge of the frontonasal mass. Confocal
microscopy with 20x magnification and 5 μm z-stacks of 20 slices allowed an imaging penetrance of approximately 100 μm. Stacks of images were collected every 15 or 30 minutes.

2.5 Live cell imaging

Images were taken on a Leica SP5 inverted microscope with an environmental chamber (37°C). Confocal time-lapse microscopy with 10X, 20X or 63X oil objectives was carried out (Fig. 3.3). Stacks of images were collected every 10 minutes for 4-6 hours. Image sequences were saved as LIF files in Leica software. Images were captured at 1024 X 1024 or 2048 X 2048 resolution, 400 Hz, UV 405 laser (10% power). We imaged a minimum of 3 independent cultures at each magnification and for each experimental condition.

2.6 Drift correction and data processing

LIF files produced from Leica software were imported into Fiji (Fiji Is a variation of ImageJ) using the Bioformats plug-in (https://www.openmicroscopy.org/site/support/bioformats5.4/users/imagej/). The separate organ cultures (OC) were separated into individual stacks and saved as TIF files. Images were edited by using Maximum Z Projection on 4-5 slices, each one micron thick, in the stack to produce clear images. Images were drift corrected using the Linear Stack Alignment with SIFT plug-in (https://imagej.net/Linear_Stack_Alignment_with_SIFT). Linear Stack Alignment with SIFT (Scale-Invariant Feature Transform) uses a modified version of JavaSIFT (http://fly.mpi-cbg.de/~saalfeld/Projects/javasift.html) to align stacks and can be used to correct for any cellular drift due to extrinsic factors. The program selects multiple points and follows them through the image to correct for drift due to extrinsic factors.

2.7 Cell tracking and data processing

Using the Manual Tracking plug-in on Fiji, nuclei were tracked in different regions of the frontonasal mass using a grid. At 10X approximately 800 cells were tracked, at 30X between 150-180 cells were tracked and at 63 between 120 and 130 cells were tracked (See Table 5.1). Tracked image data (XY coordinates at each 10-minute interval) was exported with the options “Dots &
Lines” and “Overlay Dots” into TIF files. Raw data was run through excel using macros developed by Gorelik and Gautreau (2014). These macros were used to measure mean square displacement, speed, and directional autocorrelation of the cells as well as used to create plot-at-origin graphs for cell tracks. Mathematical analysis of cell tracking data using MatLab is described in appendix 1.

2.8 Statistical analysis

Statistical analyses for organ culture morphometrics, were done using one-way analysis of variance (ANOVA), followed by Tukey’s or Fisher’s least significant difference (LSD) post-hoc test for multiple comparisons. Statistica software version 6.0 was used.
Chapter 3: Results – development of time-lapse, 3D, live imaging methods

3.1 Chicken model to study face development

During early development, the avian face is more similar to the human face than the mouse. Additionally, in the human and chicken, the upper jaw appears to be continuous while in the mouse it has a large midline depression. In the avian, similar to the human face, the morphology of the frontonasal mass is relatively flat with prominent extending globular processes. The mouse lacks globular processes and has a deep furrow between the medial nasal prominences. The chicken embryo offers numerous advantages for studying the normal development of the face and the pathogenesis of various birth defects. One of these advantages is that we can access chicken embryos in vivo at various stages of development until they reach full skeletal differentiation. We can target early face development and the embryos when face morphogenesis takes place. Furthermore, induced facial defects have a better reproducibility in contrast to mouse models. Different signaling interferences in specific facial prominences can be induced by implanting beads or cells releasing agonists or antagonists. Organ culture obtained through embryo dissection may be optimized for time-lapse light microscopy to visualize cell behaviors. Furthermore, recent progress made in cell labeling combined with imaging analyses expanded our tools of investigation. Fluorescence dyes and reporters can be delivered into single or multiple cells allowing us to trace cell behaviors within the cultures. Additionally, avian-specific, replication competent retroviruses (RCASBP) can be cloned to express coding sequences (Gordon et al., 2009) or interference RNA (Harpavat and Cepko, 2006). Moreover, novel non-viral methods based on liposomes to deliver nucleic acids to the facial prominences are now available (Geetha-Loganathan et al., 2011; Kulkarni et al., 2017).

3.2 Developing a culture system compatible with 3D morphogenesis

Observing embryonic facial growth in real time in amniotes during organogenesis requires new methods of culturing and imaging. We have developed a novel organ culture method to allow the observation of midface development in early morphogenesis. While the chicken embryo is
readily accessible in the egg, facial development cannot be visualized in ovo. We have addressed this knowledge gap by developing a three-dimensional (3D) organotypic culture of the face. This culture system allowed us direct access and observation of midface development during these critical stages. Importantly, we tested the long-term viability of sub-regions of the face in 3D culture system where it maintained its ability to form an egg tooth after cultured for 72 hours (Figure 3.1, Figure 3.2). The next step in the process was to trim the tissue to isolate the face from the eyes and brain. We wanted to address whether the eyes and brain were required for facial narrowing. We did not test whether the epithelium was needed for normal morphogenesis. Numerous epithelial-mesenchymal recombination experiments by the supervisor had shown that minimal growth occurs in the absence of epithelium (MacDonald et al., 2004; Matovinovic and Richman, 1997; Richman et al., 1997; Richman and Tickle, 1989, 1992). For this reason, we left the epithelium in place for all organ cultures. Following the confirmation that morphogenesis occurred independently of the eyes or brain, we focused on the cells within the frontonasal mass.

**Figure 3.1. Face organ culture system**

(A). Top view of “face” organ culture placed into the center well of the organ tissue culture dish, on top of a membrane sitting on stainless-steel mesh (A’-B’’). Face cultures were grown at 37°C, in humidified chamber, 5%CO2, F12/DMEM culture media. Cultures formed an egg tooth after developing for more than 72h in culture (A’ insert); Images in B-B’’ were taken by removing the culture from the mesh to make the face clearer. Key: fnm (frontonasal mass) at time zero (B) was serially photographed at 24h (B’) and 48h (B’’) of culture.
Figure 3.2. In vitro organ culture of the facial prominences, only fnm and ln

In vitro organ culture of the facial prominences. A-G’) Serial photographs of organ cultures taken at stage 24, and 24h later. Dashed lines highlight the frontonasal mass. The facial prominences reached approximately stage 27-28 after 24h of culture. A’-G’) Narrowing occurs in vitro to a similar extent as seen in vivo. This is quantified in H. * p<0.05, determined with ANOVA and Tukey’s post hoc test. Fnm, frontonasal mass; ln, lateral nasal; ns, nasal slits
3.3 Use of dyes to label areas of mesenchyme

A difficulty we next faced was how to visualize individual mesenchymal cells in such a dense tissue. Our first attempts used DiI (DiIC18(3)), a lipophilic dye that binds to cell membranes. This method had been used before by others in the face (Cerny et al., 2004; He et al., 2008; McGonnell et al., 1998) and our own lab (Lee et al., 2004). DiI was focally injected using a pulled needle in all these studies so small groups of cells were labeled (Figure 3.3).

![Fronto-nasal DII cell marking](image)

**Figure 3.3. Marking group of cells in fnm with DiI and observing after 24h**
Stereo microscope photographs of whole face organ cultures. The red staining is visible immediately after injection. Minimal spread of the dye is seen between time zero and 24h (A,A’, B,B’).

The main advantage in using the dye method is that the marked cells can be observed immediately, and the dye is resilient to photo bleaching. In order to test the movement of the cells in our organ culture system DiI injections were photographed starting at time 0, 24 and 48h, overlapping the stages when the midface undergoes narrowing using a stereomicroscope. We labeled specific areas of the frontonasal mass, lateral, close to the nasal slits and central, closed to
the midline (Figure 3.3). The purpose was to observe if marked cells are moving in a predominant direction during the narrowing of the midface. Unfortunately, the dyes showed minimal spread during the time period examined. More importantly, using macroscopic imaging it was not clear if more cells absorbed the dye or the spread was due to cell proliferation or displacement. For these reasons, this method of cell marking was not considered suitable to answer our questions.

3.4 High throughput imaging

Instead of using the stereomicroscope, we turned to confocal microscopy so we could resolve single cells over time. An inverted Leica SP5 confocal microscope was used with an environmental chamber to maintain temperature and humidity. To overcome the movement of cultures during imaging we placed facial prominences in Matrigel. The epithelial side was facing the imaging interface (Figure 3.4). This method of culture system provided the stability needed for long term imaging. A variety of objectives were available (air 10X, 20X and oil immersion 63X). The SP5 microscope also had digital zoom and 405, 543 and 633 laser lines. In most experiments, Z-stacks of 10-15 slices of 1 μm thickness were collected. We tested several different time intervals and found that 10 minutes was just long enough to detect changes in nuclear position while not losing sight of the target from one time point to the next. Live imaging for extended periods of time was possible due the use of 15 mM HEPES buffer which can maintain pH in room air, however beyond 4 hours there was a considerable degree of growth in the Z axis which made it very difficult to maintain the focal plane. Our microscope did not have automatic refocusing.

Figure 3.4. Setup for live imaging
Tissues were place into a multiwell chamber slide with the epithelium side facing down. Key: fnm – frontonasal mass
The number of biological replicates in live imaging experiments is often very low due to the large amount of time needed to analyze just a single specimen. We wanted to increase the number of biological replicates in order to be certain that the observations were generally applicable. There could be many variables influencing our experiment, from the stage of embryo dissected, to the length of time elapsed between dissection and imaging, to variation in the contact of the culture with the glass and the volume of tissue included in each culture.

3.5 Tests of transfection in the frontonasal mass

The DiI method of labeling was not pursued. Instead we tested a method of local transfection with GFP and RFP-expressing plasmids to visualize individual cells in the developing cultures. We had published studies previously where lipid nanoparticles containing GFP or RFP plasmids (Geetha-Loganathan et al., 2014; Geetha-Loganathan et al., 2011; Kulkarni et al., 2017) were used to transfect cells in the face or limb. Transfection was carried out at time zero and observed with stereoscope or with live imaging starting at 16h for a further 9-12h of culture (Figure 3.5).

**Figure 3.5.** Stereoscope imaging of double-labeled GFP and RFP-LNP cells in fnm at 16 and 24h post-transfection
Stereo microscope images of the frontonasal mass following transfection. Lipid nanoparticles containing RFP or GFP were placed at lateral edges or closer to the midline respectively. After 24h, the distance between the labels has decreased in proportion to the narrowing of the frontonasal mass.
The biggest strength of the transfection method was that the full mesenchymal cell could be seen, complete with most of the filopodia. We did not use membrane bound GFP but the cytoplasmic signal was strong enough to see the cell processes. Unfortunately, the disadvantages of the method outweighed the advantages. The delay in expression (16h before signal could be seen) made it difficult to predict where the cells would be once imaging started. Many cultures had to be started to find a few that by chance labeled the area of interest. Furthermore, the additional time spent in culture waiting for GFP expression meant the frontonasal mass had changed shape by the time we started imaging. Each culture grew to a slightly different extent which increased variability in the experiment (Figure 3.6). Another disadvantage of this method was that only superficial cells could be imaged. The number of transfected cells was highly variable and of these, only a selected number of them could be imaged (Figure 3.6, Table 3.1). The limited number of cells that were labeled made it difficult to measure movements of cells relative to their neighbors. For example, intercalation could not be observed since we might have missed quite a few cells in the labeling process. Our attention was redirected to finding a method in which all cells could be seen and reliably tracked over time.

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<th>Total number of cultures transfected</th>
<th>Number of optimal transfections</th>
<th>Number of transfected cells visible for tracking</th>
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<tr>
<td>Control n=12</td>
<td>n=3</td>
<td>24,27,13</td>
</tr>
<tr>
<td>ROCKi n=8</td>
<td>n=4</td>
<td>21,22,25,30</td>
</tr>
</tbody>
</table>

Table 3.1. Transfection efficiency with LNP in fnm organ culture
Staining of nuclei as a way to identify individual cell

We turned to vital nuclear stains which were non-toxic and could penetrate the tissues evenly. We selected the DNA stain, Hoechst 33342. This dye stains AT-rich sequences over the CG-rich sequences of DNA strands (Chazotte, 2011). Light emits only after it attaches to DNA. Outlines of the whole nucleus can be observed in vitro in the organ cultures. Several concentrations of Hoechst were tried based on reports of cytotoxicity at 0.5, 1 and 2 μM (Purschke et al., 2010). Fortunately, the nuclear stain was very helpful in separating cells as compared to the GFP labeling of the cytoplasm where cell processes overlapped considerably. Now that the nuclei of all cells were stained, the next challenge was how to visualize individual nuclei in a dense, non-transparent tissue. To improve transparency, I micro-dissected the frontonasal mass and thinned out the tissue with scissors (Figure 3.2). The face continued to develop in these more highly trimmed cultures (Figure 3.2). I also attempted to use a tissue chopper for dorso-ventral slices, which allowed me to obtain thin slices (100-30 microns) of living tissue. The tissue chopper was not adequate for the

Figure 3.6. Time lapse of GFP transfected individual cells in fnm during face narrowing
A, B) GFP-LNP transfected cells medial to the nasal slit (arrowheads). Images were taken 16h post transfection or time zero for the start of imaging. Cells receiving the ROCK antagonist (A-A’’’) decreased their migration significantly (indicated by X). Control cells (B’-B’’’) migrate towards the midline for 12 hours tracking. Panels are taken at 0, 6, and 9 hours from the start of imaging. Key: fnm – frontonasal mass

3.6 Staining of nuclei as a way to identify individual cell

We turned to vital nuclear stains which were non-toxic and could penetrate the tissues evenly. We selected the DNA stain, Hoechst 33342. This dye stains AT-rich sequences over the CG-rich sequences of DNA strands (Chazotte, 2011). Light emits only after it attaches to DNA. Outlines of the whole nucleus can be observed in vitro in the organ cultures. Several concentrations of Hoechst were tried based on reports of cytotoxicity at 0.5, 1 and 2 μM (Purschke et al., 2010). Fortunately, the nuclear stain was very helpful in separating cells as compared to the GFP labeling of the cytoplasm where cell processes overlapped considerably. Now that the nuclei of all cells were stained, the next challenge was how to visualize individual nuclei in a dense, non-transparent tissue. To improve transparency, I micro-dissected the frontonasal mass and thinned out the tissue with scissors (Figure 3.2). The face continued to develop in these more highly trimmed cultures (Figure 3.2). I also attempted to use a tissue chopper for dorso-ventral slices, which allowed me to obtain thin slices (100-30 microns) of living tissue. The tissue chopper was not adequate for the
full frontonasal mass however since it was difficult to control the angle of the blade relative to the face. Thus, we settled on Hoechst staining for 1 hour prior to imaging. This was far more rapid than waiting for the plasmids to be expressed and meant imaging started within a few hours of being removed from the embryo.

3.7 Confocal magnification – step by step improvements

Initially we began live imaging using the 20X objective which was able to capture nuclear signals. The advantage of the air 20X objective was that we could move around the chamber slide to capture different biological replicates in each imaging experiment. Therefore, much of our initial data was collected with this magnification plus a 1.5X zoom making the total magnification 30X. While theoretically, we were able to collect stacks of 20 slices of 5 microns giving a depth of approximately 100 μm, practically this depth was too great for the time interval we selected. Stacks of images were collected every 10-30 minutes but finally we settled on 10 minutes in order to not lose sight of tracked nuclei between frames. We also attempted to use a 63X oil objective. While increased resolution was possible, the oil could not be moved between wells so only one culture at a time could be imaged. The loss of high-throughput imaging dramatically slowed down progress. The last modification we used was 10X in which the entire frontonasal mass could be seen in one field of view. Initially the resolution was too low but then by capturing the maximum resolution possible (2048X2048 pixels) and a thinner stack depth, we were able to see individual nuclei. The advantages of seeing the entire frontonasal mass were many. We could then analyze more global cell movement patterns as well as symmetry. The location of the cells relative to the entire structure was highly relevant to our understanding of local signaling centers in the face. We were also able to use right-left symmetry as a kind of technical replicate in the experiments using the 10X objective.

3.8 Manual versus automatic cell tracking

We considered using automatic cell segmentation followed by cell tracking since nuclei are a consistent size and shape. However initial tests relied on the computer algorithm recognizing whole nuclei over 220 frames of imaging (Jaqaman et al., 2008). This proved to be unreliable
compared to the human eye. Thus, for all of the work reported here we used manual tracking where only nuclei that could be identified with certainty as being from the same cell were followed over the length of the movie. As many nuclei as could be followed over time were included in the sample with an attempt to fully cover the field of view. In this way, patterns of movement across the tissue could be observed with an unbiased approach. Raw data was run through excel using macros such as those developed by Gorelik and Gautreau (2014). These macros were used to measure directional autocorrelation of the cells as well as used to create plot-at-origin graphs for cell tracks.

3.9 Optimal configuration for 2D cell tracking

Using a standard confocal microscope, high-quality X-Y data was obtained. The method can be adapted for imaging other organs as long as they are relatively flat. Imaging of organs should be carried out prior to cell differentiation. Other areas of the face that can be studied include the lip fusion zone where the steps have never been directly observed in real time. Multiple cultures can be studied in the X-Y plane but not in the Z axis. The next technical improvements are to use a 2-photon confocal where Z axis information can be collected. Advanced software packages that allow realistic segmentation of nuclei prior to tracking would be a huge advantage (Imaris). However, it is still necessary however for a human eye to watch the movies and confirm that the same cells are indeed being tracked.
Chapter 4: Results – in vitro static cultures show narrowing is intrinsic

4.1 Introduction

The formation of the face involves not only budding of the prominences but also more complex changes such as lip fusion and midfacial narrowing. All these events are coordinated to give rise to species specific form. The nasal placodes form on either side of the central facial prominence, the frontonasal process and gradually the placodes are relocated to the center of the face, on either side of the midline. At the same time, the eyes relocate from the side of the head to the front. The consequences of a delay or incomplete narrowing is an increase in separation of the eyes (hypertelorism), broad nasal bridge or a groove in the nose. In humans, insufficient midfacial narrowing is a part of several different syndromes such as Opitz GBBB (#145410) and Robinow syndrome (#180700; 616331, 616894, 268310).

There are epithelial signals known to regulate outgrowth of the facial prominences such as SHH and FGF8 (Hu et al., 2003; Richman et al., 1997). Changing the level of growth factors such as BMPs, FGFs and WNT11 inside the mesenchyme alters the shape of the prominences (Ashique et al., 2002; Geetha-Loganathan et al., 2014; Szabo-Rogers et al., 2008). Interestingly, experiments using a BMP or FGF antagonist soaked-bead placed into frontonasal mass mesenchyme (Ashique et al., 2002; Szabo-Rogers et al., 2008) did not impact facial narrowing. With a lack of supportive evidence, we need a more fundamental approach to study the process of frontonasal morphogenesis, one that does not rely on candidate signaling pathways.

It is important to distinguish early patterning of the midline from that taking place during facial prominence formation. In humans, the brain hemispheres and eyes are established between 22-23 days post-conception. At the same time, cranial neural crest cells begin to migrate (O'Rahilly and Muller, 2007). Neural crest cells contain the information related to jaw size as shown in duck and quail interspecific chimeras (Eames and Schneider, 2008; Schneider, 2018). The earliest signal required for midline development is SHH which is secreted by the prechordal plate (Brito et al., 2006). Loss of this signal either genetically (Chiang et al., 1996) or with antagonists such as cyclopamine (Cordero et al., 2004) leads to a failure of the eye fields to separate fully which is accompanied by structural brain abnormalities. In the most extreme condition, the medial nasal prominences fail to form. Humans lacking SHH signaling also develop holoprosencephaly.
(Muenke and Beachy, 2000). Thus, the early disruption of midline development always results in hypotelorism whereas later abnormalities in midfacial morphogenesis lead to hypertelorism. Virtually nothing is known about the mechanisms of the later phase of midfacial morphogenesis. Indeed, it is difficult to explain the normal reduction in midline width using the usual mechanisms, such as differences in cell proliferation. Other mechanisms are likely involved such as directed migration, cell rearrangements such as intercalation and oriented cell division (Linde-Medina et al., 2016). Extrinsic or biophysical forces caused by growth of the eyes and brain could also displace mesenchyme towards the midline.

The major objective was to investigate the extrinsic and intrinsic factors that influence midfacial morphogenesis in the chicken embryo model. The chicken frontonasal mass has globular processes and slight swellings at the lateral edges adjacent to the nasal slits similar to human embryos. In contrast, the mouse has a prominent midline furrow which ultimately is represented by a notch in the center of the upper lip (Abramyan et al., 2015; Hu and Marcucio, 2009). The chicken is accessible for imaging and is suitable for experimental manipulations. Here we studied the midface in organ culture in order to isolate the face from surrounding influences. We also timed the experiments to begin prior to the onset of midline cartilage condensations which will be driving later outgrowth. The frontonasal mass epithelium is being preserved on the dissected organ cultures to maintain the signaling needed to support mesenchymal outgrowth.
4.2 Results

4.2.1 In vivo 3D morphometrics - normal narrowing

Our goal was to investigate the key window of development when narrowing is taking place in the frontonasal mass and to determine in an unbiased manner the changes in shape that take place over time. First, we collected intact embryos at defined stages and measured the size of the facial prominences using standard morphometrics and 3D, landmark-based geometric morphometrics. We used slice views to measure the distance between the nasal slits at two different levels (the superior and inferior of the nasal slit; Figure 4.1, A’-C’). In absolute terms the inferior-most distance between the nasal slits decreases significantly in a transaxial plane between stage 24 compared to stages 28 and 29 (Figure 4.1, A’-C’, D, E). During the same period, volumetric changes were measured using segmented volumes and was found to increase significantly at all stages (Figure 4.1, F). In order to understand the shape changes that occur independent of size changes, landmarks were placed on the segmented surfaces of the frontonasal mass (Figure 4.1, G,H,I). Following Procrustes superimposition, we determined that the frontonasal mass was diverging in the cranial-caudal axis (Figure 4.1, G) and the dorsoventral axis (Figure 4.1, H) while there was convergence in the mediolateral axis (Figure 4.1, I).
Figure 4.1. In vivo development of the face in the chicken embryo

A-C) Isosurfaces of the individual chicken embryos at stages 24, 28, and 29. The isosurfaces reconstruction used 1 in every 5 slices. This is visible as ridges on the surface of the embryo. A’-C’) Slice views through the frontonasal mass. The dashed lines show where the frontonasal mass width was measured at the maximum and minimum width. Inserts in A’-C’ show the plane of section for the virtual slices. D) Maximum and E) minimum distance between the nasal slits was measured. F) Volumetric increases in the frontonasal mass. The greatest change in volume is seen between stages 28 and 29. G-I) A total of 8 homologous 3D landmarks were placed. Light blue wire frame represents the mean shape of 8, stage 20 embryos and dark blue is the average of 8, stage 29 embryos. The largest differences in the wireframes were in the cranio-caudal axes (I). Key: e – eye, fnm – frontonasal mass, ln – lateral nasal prominence, md – mandibular prominence, mxp – maxillary prominence, np
4.2.2 In vivo data on oriented cell division and differentiation

One of the major proposed mechanisms for morphogenesis is oriented cell division. We hypothesized that if the frontonasal mass is primarily narrowing in the mediolateral axis, while extending in the perpendicular axes that the daughter cells would primarily be produced in either the dorsoventral or anterior posterior axis. We therefore examined embryos at stage 24, 26 and 28 (a 24h incubation period) and stained mitotic figures with pH3 antibodies. We correlated proliferation patterns with the onset of chondrogenesis using SOX9 antibody staining. The patterns of proliferation across the frontonasal mass were distinct with very few mitotic figures in the center of the frontonasal mass at all stages (Figure 4.2, A-C). These data agree with previously published data for similar stages (Szabo-Rogers et al., 2008). The region of low proliferation aligns with the area that will ultimately express SOX9 by stage 26 (Figure 4.2, D-F). This is the future site of the prenasal cartilage. The lateral edges of the frontonasal mass were scored for the angle of the metaphase plate relative to the nasal slit. Measurements were made for all cells in metaphase, anaphase, telophase and cytokinesis. Cells in prophase could not be scored. Only 19% of the mitotic cells could be scored in frontal plane with the remainder of mitotic figures being either in prophase or dividing in the perpendicular plane. To gather enough cells, it was necessary to add up the values across 4-5 sections per animal. The left side angles were inverted so data could be compiled. All mitotic figures were oriented at 90° to the nasal slit (Figure 4.2, A’ B’C’; Figure 4.3 ,A,B). There were no differences in angulation of the cells between stage 24 to 28 (data not shown). In other words, the progeny would be added in the medio-lateral axis as opposed to the cranial-caudal axis. Our study suggests that most of the cell division is oriented in the mediolateral plane but that once chondrogenesis begins, new cells are added preferentially to the lateral edges of the frontonasal mass, likely under the stimulation of signals from the nasal slit. To determine whether the mediolateral axis was indeed the preferred division plane, we scored the proportion of mitotic cells that were in metaphase, telophase or cytokinesis in the coronal plane. Independent of stage examined, there were significantly fewer cells that were dividing in perpendicular, dorso-ventral plane of the section 7.3% ±1.84). Thus, we conclude that data obtained from frontal sections captured most of the oriented cell division in the frontonasal mass.
**Figure 4.2. Mapping of mitotic cells across the frontonasal mass**

A-C) Phospho-Histone 3 antibodies were used to stain sections of the frontonasal mass in the frontal plane. The midline of the frontonasal mass is sparsely populated by pH3 stained cells (arrowheads in A,B). At higher power cells in mitosis or just after cytokinesis are visible (double ended arrows). Cells in prophase cannot be scored. D-F) adjacent sections stained with SOX9 antibodies. The complimentary expression of SOX9 (arrowheads) in non-proliferating regions can be seen in B,E and C,F. The globular processes also have a site of SOX9 positive cells (F). Scale bar = 200 µm in A-C, D-F. Bar for A’-C’ = 20µm. Key: b – brain, fnm – frontonasal mass, gp – globular process, pnc – prenasal cartilage.
We next asked whether facial morphology changes in chicken could be due to forces from brain or eyes. The full face was cut out including the eyes and the forebrain and placed into organ culture. During 48h there was significant decrease in width between the nasal slits (N = 12; a decrease of 30% by 48h compared to 0h; Figure 4.5, A-A’, D). Surprisingly, when the eyes and

**Figure 4.3. Angular measurements for metaphase and telophase cells in the stage 24 frontonasal mass**

A) In metaphase 75 cells were measured across 3 replicates (approximately 20 per replicate). B) In telophase 185 cells were measured across 3 replicates (approximately 60 per replicate). The angle of the metaphase plate is shown to be parallel to the nasal slit or the cranio-caudal axis. However, the angle of the daughter cells would be 90 degrees to this axis (inset schematic). The means for each replicate are shown in red.

4.2.3 **In vitro data – organ cultures with and without eyes and brain**
the brain were removed, the narrowing rate was not changed significantly (N = 12; Figure 4.5, B-B’, D). We also removed the maxillary and mandibular prominences, leaving just the nasal slits, frontonasal mass and lateral nasal prominences. Again, narrowing still took place (Figure 3.2, N = 7). Thus, intrinsic factors within the frontonasal mass are likely mediating the narrowing of this prominence which gives rise to the upper facial midline.

Figure 4.4. Dose response quantification of midfacial narrowing blocked by ROCKi treatment of face cultures

In vitro organ culture of the facial prominences. A-C’’) Serial photographs of organ cultures taken every 24h starting at stage 24 (time zero). The facial prominences reached approximately stage 29 after 48h of culture. Cultures treated with 1 (A,A’), 2.5 (B,B’) and 5µM (C, C’) Y2763, a ROCK antagonist, failed to narrow. D) At 1.0 µM, the frontonasal mass width remained the same. At 2.5 µM, the ROCKi frontonasal mass width remained unchanged from the start of the experiment to time 24h, and increased at time 48h. In the highest concentration (5 µM), the frontonasal mass increased in width between 0 and 24h and between 24 and 28 h. * p<0.05, **p< 0.01, ***p<0.001. ANOVA and Tukey’s post hoc test were used to measure significance. Key: fnm – frontonasal mass, ln – lateral nasal prominence, md – mandibular prominence, mx – maxillary prominence

4.2.4 Treatment with ROCKi blocks facial narrowing

We next tested whether there was a requirement for actin myofilament assembly to be taking place during narrowing. Cultures were treated with an antagonist of ROCK which interferes
with both the Rho and Rac small GTPases. Rho family small GTPases, such as Rho, Rac, and Cdc42, regulate cytoskeletal reorganization in diverse ways (Arnold et al., 2017; Heasman and Ridley, 2008; Zegers and Friedl, 2014). Rho is a regulator of stress fiber formation and cell contraction (Heasman and Ridley, 2008). Furthermore, it modulates microtubule dynamics and cell polarity (Wojnacki et al., 2014). The other GTPases Rac and Cdc42 play a role in the formation

Figure 4.5. In vitro organ culture of the facial prominences
A-C’) Serial photographs of organ cultures taken every 24h starting at stage 24. Dashed lines highlight the frontonasal mass. The facial prominences reached approximately stage 29 after 48h of culture. A-A’) Narrowing occurs in vitro to a similar extent as seen in vivo. This is quantified in D. B-B’) Removing the eyes and brain from the cultures did not interfere with narrowing. C-C’) Cultures treated with 5 μM Y27632 a ROCK antagonist, failed to narrow and even increased in width, which is quantified in D. Schematic shows the location the frontonasal mass and approximate areas dissected for the organ culture. * p<0.05, determined with ANOVA and Tukey’s post hoc test. Key: fmn – frontonasal mass. Scale bar = 1 mm
of lamellipodia and filopodia (Heasman and Ridley, 2008). One of the ways to block ROCK activity is by using an antagonist such as Y-27632. In this way the role of non-muscle myosin and other cytoskeletal components can be examined. The possible effects of inhibiting small GTPases are that the cytoskeleton of the mesenchymal cells would be frozen, impeding movement, elongation and orientation to signals within the frontonasal mass. Cells would also be unable to respond to the extracellular matrix cues. At stage 24, there is primarily type I collagen and fibronectin in the extracellular matrix. No differentiation of cartilage has taken place as shown in Figure 4.3.

We carried out a dose response with 3 concentrations of the ROCK inhibitor (Figure 4.4). Cultures treated with 2.5 µM and 5 µM increased in width throughout the 48h (Figure 4.5, C-C”,D). This suggested that there is a role for actin cytoskeletal remodeling in facial narrowing, but a different, higher resolution approach was required to determine the mechanism.
4.3 Discussion

4.3.1 Midfacial narrowing does not depend on signals from the brain

The organ culture system permitted growth of the face for several days including generating an egg tooth. This was reported by others previously (Hu and Helms, 2001) but was not used subsequently in experimental work. Ours was the first study to tease apart the role of the brain in this process. The literature had previously emphasized a key relationship between the brain and face in establishing the midline (Marcucio et al., 2005). Thus many had assumed that during later organogenesis, the brain continues to play a major role in facial morphogenesis. However we have shown conclusively that after neural crest cells have migrated into the presumptive face, the morphogenesis can continue in the absence of the brain. The signaling centres consisting of the nasal epithelium (Szabo-Rogers et al., 2008), the corners and caudal edge of the frontonasal epithelium (Ashique et al., 2002; Hu et al., 2003; Hu et al., 2015) were preserved in these organ cultures. This may have been crucial to allowing midfacial narrowing to occur. Our previous studies have removed epithelium from the frontonasal mass and growth is extremely reduced unless exogenous FGF is supplied (Richman et al., 1997). Our study did not seek to test individual growth factors and their role in facial narrowing. Instead we took an unbiased approach, by targeting the cytoskeleton.

4.3.2 Oriented cell division does not correlate with the axis of extension

We were surprised that oriented cell division was not a contributing factor to the direction of growth of the frontonasal mass. Others had studied the mouse embryo at a stage after the facial prominences had fused (E12.5) and found there was a correlation with the primary axis of cell division and rostro-caudal (dorso-ventral) growth of the snout (Kaucka et al., 2016). Stage 28 chicken embryos would have been the closest to the E12.5 mouse embryo. In mouse study the authors prepared sagittal sections and did not attempt to study sections in the perpendicular plane as we did here. They concluded that the outgrowth of the upper snout was due to clones of cells aligning their plane of cell division. Our coronal plane would have captured the same information as the study on the mouse embryo and yet we saw an even smaller number of cells undergoing
oriented cell division. The frontal plane of section used in our study did not show cells dividing in the cranial caudal axis which our 3D analysis showed was the major axis of extension. It is possible that the chicken is different than the mouse. The chicken beak extends rostrally a similar way the mouse snout but this extension happens later, at stage 30. Thus it is possible a later phase of oriented cell division may occur.

4.3.3 The role of the cytoskeleton in narrowing

The experiments with the ROCK inhibitor were very informative. Tissue morphogenesis, leading to the three-dimensional architecture of tissues and organs, is determined by biological forces that are characteristically generated by molecular motors, such as non-muscle myosin II (Myo-II) pulling the actin filaments (F-actin) (Heisenberg and Bellaiche, 2013). Cell contractility, microtubule and actin polymerization are important mechanisms for cellular movements (Ridley, 2011). Rho and Rac act via ROCK to phosphorylate myosin light chain for the formation of actin cytoskeletal structures including the formation of stress fibers and lamellipodia (Amano et al., 2010). It has also been established that Rho and ROCK play a role in the planar cell polarity (PCP) pathway, and coordinates collective cell migration in several developmental contexts (Julian and Olson, 2014; Marinissen et al., 2004; Vivancos et al., 2009). By blocking the narrowing of the frontonasal mass, we showed here that cytoskeleton role is essential during this morphogenetic process.
Chapter 5: Live imaging of the frontonasal mass

5.1 Introduction

Species specific shapes in the bird beak are thought to be due mainly to oriented cell division (Li et al., 2013; Linde-Medina et al., 2016), changes in the basement membrane that allow outgrowth in specific regions (Linde-Medina et al., 2016), expansions of cell populations (Kaucka et al., 2017; McGonnell et al., 1998) and differential cell proliferation (Fritz et al., 2014). Some of these same mechanisms are likely operating in mammals as shown by confetti mouse models (Kaucka et al., 2016). In these animals the induction of a neural crest marker is carried out with Tamoxifen leading to a variety of colored alleles of GFP. At low concentrations, a limited number of cells are induced, and their clonal progeny can be followed based on the color. In this mouse model, clones of cells were traced from E8.5 to E12.5, thus during the stages of facial prominence development, fusion and growth of the snout. The authors attempted to model how the clones ended up looking like discrete patches with relatively little mixing. They used mathematical analyses and only when cell movement was minimized did then end up with predictions that resembled the actual data. However, the authors did not study the cells using live imaging. It was therefore still possible that coordinated cell movement could have led to these same patterns. Our own data using the ROCKi suggested that cell movements could be important to midfacial narrowing. The authors of the clonal mouse study (Kaucka et al., 2016) chose to carry out live imaging in the zebrafish embryo instead of the mouse. The likely reason was that the zebrafish is transparent and small. In these rainbow fish, there was evidence of coordinated cell movement.

In order to determine the contribution of cell movement to the process of narrowing it was necessary to observe cells within the frontonasal mass over time. We focused on the start of facial narrowing, prior to the onset of chondrogenesis, stage 24-25. We developed a live imaging system in which to study the movements of mesenchymal cells in real time. This is a challenge because the mesenchyme of older chicken embryos is densely packed. We wanted to visualize all the cells together in order to map movements relative to other adjacent cells. If we had used electroporation or other means of transfection, we would see only a small subset of cells and might have missed collective cell behaviors as has been documented during neural crest cell migration (Szabo and Mayor, 2016). To be able to track cells over many hours in such a dense tissue that has growth in
3D was also a major challenge. We elected to stain the nuclei rather than the cytoplasm so that the interference from overlapping cell membranes were reduced. We saw in organ cultures, there was considerable outgrowth in the Z axis by 48h, so we kept our imaging period to 6 hours. The outgrowth is stimulated by the presence of the epithelium as shown in previous recombination studies (Richman and Tickle, 1989) and by grafting experiments involving just the epithelial edge (Hu et al., 2003). In addition, the nasal slit epithelium provides signals promoting proliferation of the mesenchyme in the lateral frontonasal mass (Szabo-Rogers et al., 2008). To maintain normal signaling we left the epithelium intact over the mesenchyme and imaged the mesenchyme from below with an inverted microscope. The epithelium was lower-most, close to the bottom of the well.

5.2 Results

5.2.1 Patterns observed from manual cell tracking at 10, 30 and 63X magnification

We began the live imaging experiments at a lower power magnification in order to fit the entire frontonasal mass in the field of view. The aim was to assess symmetry of the right and left sides which is a form of technical replicate. We also wanted to observe the midline since this is where cartilage will shortly condense. Large numbers of cells were tracked (Table 5.1).
<table>
<thead>
<tr>
<th>Organ culture</th>
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<th>Number of timepoints recorded</th>
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<tr>
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</tr>
<tr>
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Table 5.1. Number of cells tracked in each organ culture and number of timepoints observed.

Before carrying out the detailed live imaging we confirmed that cells were proliferating in the organ cultures. PCNA staining was carried out on ROCKi and control specimens after 6 hours of culture. Strong signal was present across the cultures indicating there was good cell survival and proliferation during the imaging period (Data not shown). At 10X magnification, distinctive patterns of nuclear movement were observed. The lateral nuclei medial to the nasal slits moved medially towards the centre on both the right and left sides (Figure 5.1, A, A’). The cranial and caudal cells moved in opposition to each other, away from the centre. The central mesenchyme appeared to be stationary as shown by the shorter tracks (see supplementary movies). We
wondered whether the failure of narrowing caused by the ROCKi was correlated with less cell movement. In ROCKi-treated cultures, tracks were shorter and randomly oriented (Figure 5.1, B, B’). It was clear that lateral, cranial, caudal and central frontonasal mass regions had distinct properties and it was necessary to focus on subregions at higher magnification.

The lateral edge of the frontonasal mass was imaged in an independent set of organ cultures, using the top of nasal slit as a landmark. The 30X magnification showed similar trends to those seen at 10X but in a subset of lateral cells. The approximate position of the imaged region could be estimated by the pattern of the tracks. Cranial-caudal divergence was noted (Figure 5.1, C,C’). In the treated animals, some tracks appeared to be shorter than others and direction may have been randomized (Figure 5.1D,D’).

A third set of cultures was imaged at 63X, again keeping the nasal slit at the edge of the field of view. At the higher resolution intercalation of cells was observed (Figure 5.1, E,E’). The ROCKi treated cultures had the appearance of more randomized movement (Figure 5.1, F,F’). It was clear that quantitative analysis would be required to measure differences between culture conditions.
5.2.2 Analysis of mean displacement, POR, autocorrelation and speed reveals differences caused by the ROCKi compared to controls

The regional differences observed at 10X suggested that we should subdivide the frontonasal mass into a grid in order to compare cell behaviour in equivalent anatomical regions. The 10X data proved to be more challenging to analyze so other methods were employed (See section 5.2.3). We started with analyzing the 30X data in which higher resolution of the nuclei was possible. First
tracks were localized according to their initial XY coordinate in the field of view. All tracks were then moved onto a single point of origin to measure the direction and extent of cell displacement over time. The cranial and caudal parts of the grid (5,7 and 7,9) cell tracks were oriented primarily in the cranial and caudal directions (Figure 5.2, A,C). In contrast the centre of the grid (regions 6 and 8) were more radially distributed. The ROCKi treated data were in some aspects similar to the controls (Figure 5.2, B,D). However the tracks appeared shorter and generally more radially distributed rather than polarized in the cranial-caudal axis.

We then measured mean cell displacement from start to finish of the tracks at each time point with data cut off at 175 minutes, focusing only on regions 4,5 and 6 (Figure 5.2, E). The displacement for regions 4 and 6 in controls was significantly greater than for region 5 (grey markers). The ROCKi treated data for regions 4 and 6 (red markers) not significantly different than region 5 of the controls. The ROCKi treated cells from region 5 were almost stationary during the entire culture period.

The mechanism by which cells control persistence of direction during movement is an important question. However, the ratio of displacement to trajectory which measure the directional persistence may be biased due to cell speed influence. An unbiased way to measure it calculates direction autocorrelation as a function of time, depending only on the angles of the vectors that are tangent to the trajectory (Gorelik and Gautreau, 2014). Here, we found no significant difference between control and treated tracks for the entire 320 minutes of the culture (Figure 5.2, F). There were however significant differences between each region of the face with region 6 having the most direct cell movements and region 5 having the least amount of persistence. This central region is where chondrocytes will differentiate in approximately 10-12h. One of the first signs of cell specification to a chondrocytic lineage may be to move less.
Point of origin plot, 30X

A) Control

B) ROCKi

C) 300 mm

D) 300 mm

E) Mean Square Displacement (mean of 3 replicates)

F) Autocorrelation (mean of 3 replicates)
Figure 5.2. Quantitative analysis of 30X tracks
A-D) Point of Origin graphs where the start of each track is moved to the same point of origin A). These graphs show tracks in a grid placed on the frontonasal mass. Regions 4 and 6 migrate in a cranial and caudal direction whereas 5 tracks move out radially. B) The ROCKi reduced track length in regions 4,6 as compared to region 5. Similar polarized directions of tracks in regions 4 and 6 was observed. C) Regions 7-9 are closer to the cartilage condensation, so all the tracks are shorter. The same trends of polarized cell movement was observed in controls. D) The ROCKi data were similar to controls for the equivalent regions. E) MSD measures displacement of cells in the X-Y axis. Region 5 is closest to where the cartilage condensation will form, and this may be why there is less change over time. The ROCKi treatment significantly reduces cell displacement. F) Autocorrelation measures persistence of movement or how direct the cell is in reaching its endpoint. There is no difference in directness of the tracks between control and ROCKi treated embryos. In addition, regions 4 and 6 are similar. Region 5 has less direct movement because cells tend to move in circles.

Figure 5.3. Quantification of order and disorder
A) The cells that are in close proximity to another are more likely to be have similarly because there are fewer cells within a small radius (r). The variation of the cell specific vector (red, v) compared to the norm for the entire group (green, n) is measured as the alpha angle. B) The larger radius increases the likelihood that cells will be moving in random directions. C) If all cells are moving in exactly the same direction, then S will be close to 1. D) If cells move in various random directions, S will be close to zero.

5.2.3 Measuring order and disorder in the frontonasal mass mesenchyme
The high number (many hundreds) of nuclei tracked in the 10X data presented the opportunity to explore patterns of cell behavior in different regions. Furthermore, the whole frontonasal mass was imaged allowing right-left symmetry to be studied. The first step in this analysis was to use the X-Y positions of the nuclei and measure the similarity in direction between neighboring cells. A further enhancement was to measure order and disorder over different sized domains (Figure 5.3, A, B). If nuclei were being displaced in a similar direction in a larger radius this would indicate that there is less randomness or greater order (Figure 5.3, B). A smaller radius would only include a few cells so, even if they have a high degree of alignment close to 1 (Figure 5.3, C), this would be less biologically significant. Many cells with random displacement would have an alignment value closer to 0 (Figure 5.3, D).

We were surprised to find periods of order, followed by disorder and then another peak of order in the cultures, as time progressed (Figure 5.4). At the beginning of the observations the cultures were settling into the dish and, order is significantly increased in the first 20 minutes of imaging (Figure 5.4, A-F). In control cultures, the first peak of order was observed after the first one hundred minutes. Then, the trough where major disorder was observed occurred about 40-50 minutes later. Recovery in order was observed in the last hour of the culture period (Figure 5.4, A-C). This is a very interesting observation, indicating that cell alignment is not linear over time and that successive feed-back is needed for the cells to recover from a period of disorder. Cells appear to communicate (align) over a much larger distance than would be expected by chance.

In the ROCKi-treated cultures there were more peaks and troughs at varying times in the culture and overall level of alignment or order was lower than for controls (Figure 5.4, D-F). The level of order at each peak and trough shows that even when control cultures are disordered, they are still more ordered than ROCKi treated cultures (Figure 5.4, A; -F’). In addition, the radius over which order was detected for ROCKi treated cultures was very small (less than 50 microns (Figure
5.4, D’-F’). In treatment samples, long range communication is reduced which is consistent with a molecular perturbation of signaling within the frontonasal mass.

**Figure 5.4. Alignment across time and space in the frontonasal mass**

Cell alignment for three control cultures (A-C and A’-C’) and a ROCKi treated cultures (D-F and D’-F’) based on the direction of movement vectors. The amount of order (y axis) changes over time (x axis), represented by peaks and troughs (A-F). The alignment drops over distances (x axis) longer than 200 microns radius (blue), particularly in ROCKi treatments (A’-F’). Vertical lines (A-F) represent chosen time points followed over radius (A’-F’). A coefficient of 0.8 (y axis) was considered a threshold for alignment (A-F’). Red circle is 200 microns diameter and blue circle is 400 microns diameter.
5.2.4 Unbiased, k means clustering analysis reveals regional organization

To understand how cell organization contribute to the outgrowth of the midface and to specific shape, we compared how the direction of vectors of cell displacement across the whole frontonasal mass are similar or different. For better visualization we color-coded groups of cells based on the angle of vectors (Figs. 5.4). Cells with a similar angle vector are clustered. Initially we tested out several diameters for clustering based on the order-disorder analysis over time (Figure 5.4). At a radius of 50 microns, there were clusters of cells with some intermixing of cells in non-contiguous areas (Figure 5.5, A). There was less overlap of the clusters at a radius of 200 microns (Figure 5.5, A’). In both the 50 and 200 micron radius there is already a tendency for the cranial and caudal edges to move in opposite directions (as shown by opposite sides of the color wheel (Figure 5.5, A,A’). Treatment with ROCKi resulted in a larger number of clusters sprinkled across the frontonasal mass at 50 microns (Figure 5.5, B). The clustering was improved at 200 microns but was not as tight as for control cultures (Figure 5.5, B’). The size of the frontonasal mass is about 800-1000 µm across. It was not likely from a biological perspective that cells would be communicating across large diameter regions (400 microns) comprising up to half of the frontonasal mass. For this reason, we selected the radius of 100 µm or 200 microns diameter, which was the size of many bead implants placed into the face in previous publications. Signals from a 200 micron bead affected tissues in a similarly sized region (Ashique et al., 2002; Higashihori et al., 2010; Lee et al., 2001; Szabo-Rogers et al., 2008).
We then carried out more detailed K-means clustering for cells in a radius of 100 microns (200 microns diameter; Figure 5.6). Interestingly, there is a correlation between the organization of these regions and the level of order evaluated over time. In the control cultures, there were never more than 6 clusters assigned to a specific vector direction (Figure 5.6, A-C’). In contrast the ROCKi treated cultures had up to 9 clusters and as shown in our test analysis, the cells exhibiting the same vector were not always contiguous (Figure 5.6, D-F’). An interesting observation was that trends in clustering changed over time but then reached a period of stability towards the end of the culture, when order was reached (see movies). Cell vectors appeared to change relatively quickly, perhaps indicating a rapid change in molecular signaling. We noticed slight variations in cellular organization between control samples. Sample C3 was more ordered at all time points and also had different clusters than C1 and C2 (Figure 5.6, C’’ compared to A’’ or B’’). This may be

**Figure 5.5. K-means clustering- unweighted, 50 and 200 micron radius**
K-means clustering of vector direction and velocity at one time point for a control (A, A’) and a ROCKi treated culture (B,B’). Clustering by distance indicate similar clusters at different radii, in contrast to treatments (B,B’). During the ordered time, the clusters of cells on the right and left sides and cranial-caudal axes are moving in opposite directions (see colour wheel). The maximum number of clusters is 7. In contrast the ROCKi clusters are heterogeneous, lack neighbour similarity and are not organized into distinct regions (B,B’). Clusters of cells with the same vector are not located in contiguous regions. Grey arrows are the average vector for that cluster. The black arrows are the raw data for that cell. The more overlap there is, the more that cell is close to the mean for the cluster.
due to the slight variation in stages of development at the time we initiated the imaging or the differences in area dissected.

The ROCKi treatment disrupted the clustering in all cultures. There were patches of non-contiguous cells sharing the same vector (Figure 5.6, D-F’’). The coordination of cells that existed in the controls seemed to be non-existent in the ROCKi treated cultures. Lack of coordination may be preventing shape changes as we saw in the flattened, organ cultures (Chapter 4).
K means clustering by similarity of direction within a radius of 100 microns

$ r = 100$ microns

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Next, we introduced the variable of proximity into the clustering analysis. Arbitrary weighing factors were applied. With no weighting on proximity, the clustering of cells was not as distinct for the controls (Figure 5.7, A) or for the experimentals (Figure 5.7, B). When the maximum weight was placed on proximity and direction of the vector was not considered, artificially smooth clusters were formed (Figure 5.7, A’B’). There were more clusters in the control with full weighting on proximity (Figure 5.7, A’) than when a radius of 100 microns was applied in the clustering algorithm (Figure 5.7, A-C’`). We settled on a weighting of 2 which would reflect a fair level of proximity weighting.

In our control samples during the first period of order, there clusters of cells that mostly were distinct. In the trough where disorder was high, there was intermingling of cells throughout the frontonasal mass. It was impossible to resolve discrete clusters of cells during this period. In the second period of order, clustering with proximity was successful. (Figure 5.8, A-C``). Changes of cell direction and cluster interactions are rapid and coordinated (see movies). The fact that neighbouring cells are moving similarly, reinforces the idea that cells may be communicating, non-cell autonomously (Figure 5.8, A-C``). Neighbour similarity in ROCKi treated cultures appeared totally compromised, a clear indication that cells do not respond to signaling and their direction is random, non-coordinated (Figure 5.8, D-F``).
Figure 5.7. K-means clustering- completely weighted only on the direction of the vector or completely weighted by proximity to other cells
A) The lack of consideration for position, resulted in clusters that intermingled. A’) adding the weighting for proximity made very clear clusters that were not very consistent in the direction of the vector. B) The ROCKi treatment randomized the directions of cells so that no contiguous clusters were formed. B’) The complete weighting on proximity resulted in an artificial grouping of cells that was unlike the previous K-means clustering on similarity of cells within a radius.
K means clustering of vectors by direction, proximity and velocity

Clustered weighting factor of 2

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5.2.5 Interpolation of the data in order to investigate symmetry

The imaging of the entire frontonasal mass presented an opportunity to measure symmetry. We had seen some similar patterns of clustering across the 3 control cultures which suggested that these patterns were due to a conserved molecular mechanism. If these coodintated movements were genetically controlled then there should be symmetry across the midline. In order to make it possible to compare equivalent positions, the raw data was interpolated. The average vector for positions spread 50 microns apart was calculated (See appendix). This calculation took the raw data (Figure 5.9, A,B) and smoothed the differences across the frontonasal mass (Figure 5.9, A’,B’). Interpolation or smoothing was also applied to the ROCKi treated cultures (Figure 5.9, C-D’).
Figure 5.9. Interpolation or smoothing of data
A grid spaced 50 microns apart was placed onto the frontonasal mass. The interpolated vectors (A’-D’) were calculated for that position based on the raw data (A-D). One control specimen (A-B’) and one ROCKi treated specimen (C-D’) are shown here, for ordered (A,A’,C,C’) and disordered states (B,B’,D,D’). After smoothing, the vector length remains proportional to the speed and distance travelled during the preceding 10 minute interval. The centre of the frontonasal mass tends to have shorter tails on the arrows indicating less change in position.
5.2.6 Right-left symmetry is present in the frontonasal mass

We were able to successfully mirror the left side over the right side by matching the contralateral grid positions (Figure 5.10). The blue color indicates that the real angle of the vector is very similar with the virtual (mirrored) angle reflected from the contralateral side. A yellow color indicates a difference in angle between the real and the mirrored angle. Right and left symmetry was more accurate especially during “ordered” timepoints (Figure 5.10, A,A’’, B,B’’,C,C’’), suggesting that cells are periodically recalibrating their coordination to maintain both sides in synchrony. This recalibration happens in a short timeframe of 60 to 100 minutes. However, in the troughs where disorder is found, there is concomitant asymmetry (Figure 5.10, A’, B’). In culture C3 where order was higher throughout the culture period, symmetry is very high throughout (Figure 5.10, C-C’’). The ROCKi treated samples displayed almost no symmetry (Figure 5.10, D-F’’) at any of the timepoints. The lack of symmetry further supports the idea that cell-cell communication is disrupted by the ROCKi.
Figure 5.10. Right-left symmetry across the midline is disrupted by the ROCKi.
Symmetry is shown here by vector angulation comparison between control (A-C') and ROCKi (D-F') treated samples. Different time points are shown for each specimen. Blue dots represent minimal difference in vector angulation between actual and mirror angles at each position reflected on the contralateral side. Yellow dot indicates a significant difference in angulation between real and mirrored angle.
5.2.7 Divergence-convergence patterns at all magnifications is conserved

Our original hypothesis was that convergent extension (CE) takes place in the frontonasal mass. Convergence would occur primarily in the medio-lateral axis and would result in narrowing. Divergence would occur cranio-caudally and dorso-ventrally (Chapter 4). However, our analysis thus far had not allowed us to visualize convergent extension using nuclear tracking. The normalization of data across a grid opened new possibilities to measure sources and sinks within the frontonasal mass. Positive divergence indicates a source located a specific distance away from that group of cells. Negative divergence or convergence would occur where a chemoattractant or sink brings cells towards a signal. Initially we examined divergence and convergence at the same three timepoints that reflected peak order or disorder (Figure 5.11). Surprisingly, all controls started out with a band of convergence crossing from medial to lateral (Figure 5.11, A-C’) in both the first peak and trough. However, by the end of the culture period the trend had reversed, and divergence was present across the frontonasal mass (Figure 5.11, A”, B”, C”). There were also oblique branches of convergence at the lateral edges of the frontonasal mass that were replaced by divergence towards the end of the imaging period. In the ROCKi treated cultures there are no clear signs of divergences or convergence (Figure 5.11, D-F”) fitting with the clustering data.

We also imaged two other sets of cultures at 30 or 63X magnification. When we applied a grid normalizing the data then surprisingly, the patterns of divergence and convergence can be observed (Figure 5.12, Figure 5.13). This analysis confirms once more that cells are receiving external cues allowing them to rearrange in a coordinated manner, to induce frontonasal mass reshaping (Figure 5.12, A-C”). The ROCKi treated cultures had no CE (Figure 5.12, D-F”). However, higher magnifications show that short-range coordination of cells it is still possible in ROCKi samples (Figure 5.13, D-F”). Thus, when examining smaller regions, it was possible to see a degree of coordination, although this may not have a major contribution to morphogenesis. One of the main benefits of imaging at 63X was that we could obtain an accurate measurement of instantaneous speed. The ROCKi reduced cell speed by half. The loss in speed could explain why vectors were more randomly oriented at lower magnifications (Figure 5.10, D-F”). It would have been harder to measure velocity on an essentially stationary cell.
5.2.8 Consistent patterns of divergence-convergence in the frontonasal mass

The original analysis of divergence-convergence at the three representative timepoints had been focused on peak order and disorder. However we wanted to know how long the periods of convergence versus divergence lasted and whether these intervals coordinated with order or disorder. We therefore plotted in a qualitative manner the transitions from one extreme of convergence to the greatest level of divergence. These peaks were determined from looking through all of the timepoints in a qualitative manner. Unlike our previous analysis (Figure 5.11), all the control cultures began the culture period with divergence (Figure 5.14, A-C). The divergence lasted for at least the first hour of the culture period. The middle period of the culture was characterized by convergence across the frontonasal mass (approximately another hour). The last third of the culture period was characterized by a return to divergence. This dynamic switching occurred rapidly, within 10-20 minutes. This analysis strongly suggested a rapid signal that was oscillating between 2 states (such as on and off).
Figure 5.12. Divergence and convergence at 30X
A smaller area of the lateral frontonasal mass was imaged. Data were interpolated over a grid of 20 µm. A-E’) At 30X magnification, there were mainly mediolateral bands of divergence in all 5 control cultures, replicating the 10X magnification data. F-H’) The ROCKi caused a rotation of the line of divergence in two of the cultures (R5, R6). The remaining culture, R4) had a similar mediolateral line to that of controls. Thus, the ROCKi caused a global change in cell rearrangement but patterns were still visible unlike the 10X magnification. See supplementary movies.
Convergence-divergence analysis over time 63x

**Order**
- C9: 100 min
- C10: 150 min
- C11: 70 min
- R7: 100 min
- R8: 60 min
- R9: 70 min

**Disorder**
- A: 140 min
- B: 170 min
- C: 120 min
- D: 160 min
- E: 90 min
- F: 90 min

**Legend**
- Divergence
- Convergence

**Cell speed 63X**
(mean of 3 replicates)

- Control
- ROCKI
Figure 5.13. Divergence and convergence in the frontonasal mass at 63X
A smaller region of the frontonasal mass was imaged, and the cranio-caudal location varied in each culture. Data were interpolated to the average value across a 20 µm distance, around a cell. A,A’)
In culture C9 a vertical band of convergence, in the dimension that is typically narrowing in the organ cultures was seen. This was the only culture in which a clear line of convergence was seen. B-C’)
In the remaining control cultures, a primarily horizontal band of divergence was seen, perhaps capturing a similar region to those imaged at 10X and 30X. D-E’) The ROCKi disrupted most of the patterns of convergence and divergence. F,F’). There was a horizontal line of divergence similar to controls. G) Instantaneous cell speed was measured and there was a significant reduction caused by ROCKi. See supplementary movies.
Figure 5.14. Oscillations in divergence and convergence movements in frontonasal mass
Qualitative plots of the fluctuations in divergence and convergence over time. The line was
drawn to reflect the maximum levels of convergence or divergence. Representative vector plots
are shown at several time intervals. There is a reproducible pattern of oscillation (black line)
in time in all three specimens. There is a correlation between a high degree of order and
divergence. All cultures start out with divergence for the first 60-80 minutes. Then a period
of convergence lasts about 100 minutes before moving to divergence for the end of the culture.
5.3 Discussion

5.3.1 Symmetrical coordination of cell rearrangements suggests conserved signaling between cells may be taking place

The imaging at 10X magnification was highly informative about overall morphogenesis in the frontonasal mass. There were two striking results revealed in the 10X cultures. The first was the tendency for the cells to be ordered (moving in the same direction), then disordered and then returning to order. The fact that these trends are eliminated in ROCKi-treated cultures suggest coordination is an intrinsic feature of the frontonasal mass and not an artifact of culturing. Further analysis using unbiased clustering on a radius of 100 microns revealed 6-7 clusters in controls and these were consistent between cultures and over several time intervals. Had there been no organization across regions of the frontonasal mass, the number of clusters would have been smaller and intermingled. During the period of disorder, there were more clusters and cells with similar vectors could be spread over the frontonasal mass. However there was still more organization in the controls even in the time of maximum disorder compared to cultures treated with ROCKi. Weighting on proximity and velocity gave similar results but here we could actually map the location of cells according to the anatomical region of the frontonasal mass showed that cells of a similar speed and direction were generally close to each other in controls. We started to recognize trends that in controls peripheral cells were moving towards the centre in the first period of order, but then cells were moving outwards from the centre at later timepoints. Generally it seemed that opposite edges of the frontonasal mass were going in opposite directions. The proof of these trends required smoothing of the data over grids.

The interpolation followed by mirroring of the data validated our observations on the non-interpolated raw vectors. The tremendous degree of symmetry within each control specimen strongly supports a the genetic control of facial morphogenesis. The right and left sides of the frontonasal mass derive from separate streams of neural crest cells. Thus the patterning information was established during migration and after interacting with the local environment. Treatment with ROCKi disrupted the patterning across the midline suggesting that there is an interference not only with cell structure but also with molecular signaling. During human facial
development, such perturbation of signals may translate into asymmetric cell behavior in one side of the frontonasal mass leading to congenital anomalies. For example, a lack of coordinated extension of the frontonasal mass in the cranio-caudal axis may mean the contact cannot be made with the maxillary prominence on one side. This would lead to a unilateral cleft lip which is one of the most common craniofacial congenital anomalies (Lewis et al., 2017). By blocking symmetry with the ROCKi, we possibly interfered with molecular signaling that regulates this coordination between cells.

5.3.2 Sources and sinks change dynamically across the frontonasal mass

The normalized data was used to map the locations of putative sources and sinks in the frontonasal mass. We had expected, based on the organ culture data, that we would have seen convergence towards the centre of the frontonasal mass. However when we looked across time, all of the control cultures moved from divergence to convergence and then back to divergence. We had captured a dynamic switch from source to sink and back to source. The organ cultures were grown over a much longer period of time than those used for live imaging. Therefore we had captured about the first 4 hours of frontonasal mass development during the period of narrowing. It is possible that longer imaging would have detected another period of convergence. We were limited in the period that could be imaged due to several factors: 1) the small amount of media in the well, 2) the lack of CO2 buffering, 3) the tendency for growth in the Z axis and 4) only having a conventional confocal microscope as opposed to a 2-photon system.

The suprising result of the convergence, divergence analysis was that the switches in direction were so rapid. This suggests that a quick molecular signal was changing, one that did not require protein synthesis but phosphorylation. Indeed the ROCKi would have blocked conversion of GTP to GDP and convergence/divergence was completely disrupted. Further studies are needed with real-time reporters of GTPse activity are needed to test this hypothesis.

The general pattern of convergence/divergence suggested that sources and sinks were located in the medio-lateral axis. However we also saw many oblique branches off the main line of convergence/extension at the lateral edges of the frontonasal mass. These observations may be
related to the nasal slits that undergo considerable morphogenesis between stages 24 and 28. The nasal slits become angled with the superior end being more lateral than the inferior end.

What is the purpose of alternating convergence and divergence in the frontonasal mass? These contractions and expansions may be necessary to reshape the frontonasal mass over a rapid period. Contractile forces act in bringing non-neighboring cells together, and at the same time cells need to withstand compression, or express stiffness, as they push the tissue along the axis of extension (Lecuit and Lenne, 2007). Stretching the tissue along the cranio-caudal axis may lead to cell intercalation which we did see at 63X.

Rho small GTPases such as RhoA, Rac1 and Cdc42, are the main regulators of cellular structure by controlling actin polymerization and myosin II activity (Heasman and Ridley, 2008). Cells continuously adjust to mechanical stress and strain while preserving the normal structure of organs. Forces are sensed and transduced to neighboring cells by intercellular junctions and ECM adhesions, which are coupled to the actomyosin cytoskeleton (Martin, 2010). Thus, Rho GTPases may affect intrinsic cell structure but also the relationships between cells and the extracellular matrix. Our work in wholemount organ cultures suggests that there is a loss of structure in the tissue leading to flattening. Therefore, one reason for the disrupted patterns of cell movement in the live imaging is that cells have lost their ability to form adhesive junctions with the supporting extracellular matrix.

The live imaging of the frontonasal mass has led to a different model for morphogenesis than originally hypothesized. Instead of narrowing where the main axis of convergence would be cranio-caudal, the main axis of change is in the medio-lateral. Thus, although we can measure a decrease in mediolateral width in the frontonasal mass over a 48h period, this phase is preceded by a dynamic rearrangement of cells. During this early phase cells have a tendency to first move towards the center of the frontonasal mass but then the direction changes and cells move outwards. This second wave of cell movement correlates with the subsequent craniocaudal growth spurt. It is interesting that we seem to have found a time where cell number decreases in the center of the frontonasal mass. However in vivo it is likely cells from deeper layers flow up towards the epithelium to fill the gap. We did not image the Z axis but future studies with better microscopy are the next step.
Chapter 6: General discussion

6.1 Important technical advances

Our new technique for observing developing midface during a critical morphogenetic stage from the frontal view comes with an important advantage over other ways of observing only the lateral side of the face in a living embryo or examine early developmental stages when the size and the transparency are not critical downsides. Amniotes share a relatively similar mechanism of midface narrowing during these stages of development (Young et al., 2014). It is very important to directly observe these morphogenetic cell movements to understand how reshaping takes place in such a relatively short time interval. Additionally, mesenchymal cell behavior is generally less studied due to several limitations, such as too many layers of cells making the tissue less transparent, the epithelial layer covering the mesenchyme or the difficulty of marking these cells (Nguyen and Currie, 2018). Our initial challenge was to be able to maintain the morphogenesis in vitro, on a culture system, and then to find a suitable marker to track the cells during the developing process. To our knowledge, this is the first study to image mesenchyme from an older embryo and still see all the cells. One of the important advantages of our investigation was having a ubiquitous nuclear label, thus we were able to visualize all the cells in the fnm, so relative movements could be tracked. This is different than marking only a few cells and extrapolate for the entire facial prominence. Furthermore, observing cells at a ten-minute interval showed us how dynamic this morphogenetic process is, in contrast to static observations where the progression of the events is missed. We successfully interfered with the midface narrowing in our system while observing cell behavior changes. Thus, new opportunities of studying how known genetic defects affecting the midface are translated at the cell level to impact the normal morphogenesis may emerge.

6.2 High throughput system increases experimental rigor

An important technical advance made in this work was the use of a high throughput system. Live-imaging embryos in a repeatable, high-throughput method is key for understanding the cellular dynamics (Donoughe et al., 2018). Donoughe et al. (2018) engineered small agarose molds
in which to hold dozens of insect larvae. However, these molds are so far unsuitable for chicken embryo organ cultures. The multi-well chambers we have been using achieve the same goal. The only downside of the multiwell chamber slide is that the amount of media is very small. In long term cultures, the media constituents would be exhausted. Here we used serum-containing media with Hepes buffer. We estimate that this media supports growth for up to 6 hours, based on cell behavior in the live imaging studies. Chambers in which media can be exchanged would be an advantage. Alternatively, larger chamber slides could have been used to increase the volume of media. A newer alternative would be to use flow cells such as those used to generate tissue-on-a-chip. The flow of media could introduce movement into the system that would be difficult to correct. As cell-tracking was our aim in this study, it was imperative to keep the cultures as still as possible.

In the end we were satisfied with the quality of the data obtained from the 10X magnification of several cultures at once. We discovered that there were subtle differences due to the stages of the embryos, the length of time to get the cultures set up and the dissection technique. High-throughput imaging is essential to achieve a high degree of experimental rigor.

6.3 **ROCK-sensitive pathways are active during facial development in vivo**

We have taken an unbiased approach in this project, choosing to work with a signal transduction step that is fundamental to multiple signaling pathways, the Rho-family small GTPases (Denk-Lobnig and Martin, 2019). There are indeed two related experiments that support the possibility that RhoGTPases are involved in midfacial narrowing in vivo. The first type of experiment is a conditional knockout of Cdc42. This small GTPase was knocked out in neural crest cells with P0-Cre (Oshima-Nakayama et al., 2016) or Wnt1Cre (Fuchs et al., 2009; Liu et al., 2013). There was also a cross to the Prrx1-Cre by another group which drives expression in facial mesenchyme (Aizawa et al., 2012). Embryos did not survive and had a severe facial phenotype, particularly for the neural crest-deleted animals. The studies had in common that the embryos had a midline cleft that involved the nasal septum which is derived from the medial nasal prominences (or frontonasal mass in chicken)(Fuchs et al., 2009; Liu et al., 2013; Oshima-Nakayama et al., 2016). The premaxillary and maxillary bones were wider in the mutant in the Oshima-Nakayama
et al study (2016) consistent with a lack of midfacial narrowing. The conditional knockout of Rac1 using Wnt1-Cre (Thomas et al., 2010) also resulted in medial nasal prominences that failed to merge in the midline and ultimately a cleft developed.

The second type of genetic study on mice was the expression of a dominant negative version of Rho-kinase which would interfere with both isoforms (Rock1 and Rock2)(Phillips et al., 2012). These authors used the Wnt1-Cre driver to target Rho GTPases function in neural crest cells and their derivatives. Single knockouts of Rock1 or Rock2 survived but double knockouts presumably die since they have not been described. The effect of the dnRock was to specifically block Rho binding activity. The phenotypes included a midfacial cleft and a marked hypoplasia of the premaxilla similar to the P0-Cre knockout of Cdc42. The main mechanism for the midline cleft was thought to be excessive cell death. Thus, from these multiple conditional knockouts or transgenic expression of dnRock, there is a strong link between midfacial narrowing in vivo and the requirement for RhoA-GTPase activity.

6.4 Candidate growth factors that may regulate convergence extension of the frontonasal mass

Small GTPases are mediating non-canonical WNT, Planar-Cell polarity signaling (Kim and Han, 2005) as well as several other growth factor signaling pathways such as the non-canonical TGFβ pathway (Ungefroren et al., 2018) and the PI3Kinase pathway to name a few. Of all the growth factors expressed in the face, the most likely candidate that could be mediating the cranio-caudal divergence is WNT5A. In previous studies from the Richman lab, the location of WNT5A and most of the other WNT ligands was documented with in situ hybridization (Geetha-Loganathan et al., 2009). WNT5A is strongly expressed in a horizontal band across the frontonasal mass at stage 24, correlating with medio-lateral axis of convergence-divergence seen in live imaging (Figure 6.1, A, Figure 6.2, A-C). Studies in the chicken (Geetha-Loganathan et al., 2014) and mouse have shown that Wnt5a is a chemoattractant (He et al., 2008) and thus the band of WNT5A could act as a sink in the frontonasal mass (Figure 6.2, A).

At stage 28, expression resolves into the lateral thirds of the frontonasal mass, with an area of low expression where the prenasal cartilage is differentiating (Figure 6.1, A’). These later stages
of WNT5A expression correlate with the times when the frontonasal mass is growing extensively in the cranio-caudal and dorso-ventral axes.

Figure 6.1. WNT expression in the face at stages 24-25 and 27-28 (HH)
Expression of WNT ligands during chicken craniofacial development. Whole mount in situ hybridization with digoxygenin-labeled probes; frontal views of the facial prominences. A) WNT5A expression as a band midway across the medio-lateral axis of the frontonasal mass. A’) Relatively lower expression of WNT5A is seen in the midline of the frontonasal mass, overlapping the future chondrogenic region. B) WNT5B is not expressed at stage 24 but does overlap that of WNT5A at stage 28 (B’). C,C’) Expression of the canonical WNT, WNT9B in in the ectoderm of all the facial prominences. Images taken from Geetha-Loganathan et al. (2009). Key: f, frontonasal mass; gp, globular process; l, lateral nasal prominence; md, mandibular prominence; mx, maxillary prominence; Scale bar = 500 μm.

The second line of evidence supporting a role for WNT5A in midfacial narrowing comes from the Wnt5a mouse knockout. Loss of Wnt5a leads to much wider and shorter snouts (Kaucka et al., 2017) along the lines of the wider frontonasal mass observed in ROCKi-treated organ
cultures (Chapter 4). Finally, there is evidence linking human WNT5A to midfacial narrowing. A series of missense mutations in WNT5A cause autosomal dominant Robinow Syndrome (Person et al., 2010; Roifman et al., 2014; White et al., 2018). The hallmark features of this syndrome are hypertelorism, broad nasal bridge and recessive jaws. The hypertelorism and broad nasal bridge are suggestive of a failure of narrowing of the midface during embryogenesis.

The blocking of small GTPases with the ROCK antagonist in the present experiments would prevent transduction of endogenous WNT signals such as those coming from WNT5A. Signaling in the non-canonical WNT pathway regulates cell polarity and directed cell migration. The downstream block in WNT signaling may be the reason that cells in the culture lose their polarized movements in the cranial-caudal axis when treated with the ROCKi. It would be interesting to see the cell membranes in our experiment in order to measure these other potential effects on oriented cell behavior.

Aside from the WNT pathway, there are also some important signals for facial development that should be considered relative to the patterns of cell movement and growth that we have observed. SHH is expressed along the caudal edge of the frontonasal mass in the epithelium (Fig. 6.2A) (Ashique et al., 2002; Hu and Marcucio, 2009). SHH is a morphogen that controls polarity of the limb digits (te Welscher et al., 2002; Tickle and Barker, 2013). In the face, the addition of a SHH-soaked bead (Hu et al., 2003) or implanting SHH-expressing cells (Brito et al., 2008) is sufficient to induce a branch off the main beak (mainly mediated by increased cell proliferation). Thus, SHH signals could be a sink along the caudal edge (Figure 6.2). There is so far little evidence that SHH acts as a chemoattractant in mesenchyme although it is an important axonal guidance cue (Charron et al., 2003). We could test the idea of SHH inducing chemotaxis of mesenchyme cells by implanting beads soaked in SHH at a distance away from the caudal edge.

FGF8 is strongly expressed in the nasal slit epithelium (Szabo-Rogers et al., 2008) (Figure 6.2, A-C). In the limb, FGFs have been shown to increase the speed of mesenchymal cell movement although the persistence of direction was not improved (Gros et al., 2010). In live imaging experiments carried out on gastrulating chicken embryos it is very intriguing that FGF4 had opposite effects to FGF8 (Yang et al., 2002). In these studies, FGF8 was a repulsive cue for cells moving through the primitive streak while FGF4 was an attractive cue.
Figure 6.2. Overlay of gene expression and proliferation
Overview of gene expression and proliferation in fnm at HH stage 24 during convergence (A) and divergence (B,C). BMP4, SS and FGF8 are expressed in the epithelium (A-C), while WNT5A is expressed in the mesenchyme of fnm mid-fnm in a medio-lateral direction (A). Areas of high (lateral zones of fnm) and low proliferation (globular process) are marked (A-C). During divergence, a repellant cue is expressed across mid fnm, in a medio-lateral direction or two bands of attractant cue are bordering the mid fnm (B), in a medio-lateral direction (C). Non-canonical pathyay signals activated through WNT5A plays an important role in oriented cellular events such as migration, cell division and cell intercalation; actin cytoskeleton is reorganized by PCP effectors. Blocking the effectors of non-canonical Wnt signaling may interfere with convergent extension in the face during midline narrowing (D)

There is no expression of FGF4 in the frontonasal mass, so this leaves FGF8 as a possible repulsive cue. However, context is important and in the formation of the Wolfian duct, FGF8 is an attractive cue (Atsuta and Takahashi, 2015). In our experiments, cells near the lateral edges of the frontonasal mass are moving obliquely towards the nasal slit in certain periods of the culture,
suggesting that FGF8 is a chemoattractant in the face. It would be straightforward to test whether FGF8 can redirect cells away from the nasal slit in bead implant experiments.

We reviewed the literature on classic repulsive and attractive cues used in the central nervous system such as Robo, Slit and Semaphorins but have not found evidence of their expression in the face. Our data suggests that more attention should be paid to mesenchymal cell chemotaxis or repulsion, since coordinated cell movements could be controlled by as yet, unknown molecules (Figure 6.2, C).

6.5 Dynamic changes in cell behavior may be linked to pulsing of signals

One of the more striking findings was the frontonasal mass appeared to pulse between states of order and disorder. These pulses were abolished by the ROCKi suggesting an involvement in RhoA-ROCK GTPases. There is a dynamic cycling of Rho GTPases between an active, GTP-bound state and an inactive GDP-bound state. It is possible that such cycling occurs in the frontonasal mass like the gastrulating Drosophila embryo (Qin et al., 2018). Certainly the time interval of about 10 minutes between convergence and divergence fits with some of these more rapid changes in myosin II. To be sure that these oscillating changes in cell direction were real, further experiments are needed, preferably using live readouts of active versus inactive ROCK signaling.

6.6 The role of oriented cell behaviors in shaping the frontonasal mass

Aside from proliferation, four other mechanisms could help to shape the frontonasal mass: cell polarity (Griffin et al., 2013; Li et al., 2013), cell shape, oriented cell division (Kaucka et al. 2016) and directed cell migration as we has suggested in this study. In the frontonasal mass, Li et al. (2013) showed that the majority of mesenchymal cells have their leading edge (as determined by the position of the Golgi, relative to the nucleus) pointing towards the globular process. The long axis of mesenchymal cells has not been measured in the frontonasal mass but from our brief experiments with transfection of GFP plasmids, there does not appear to be a predominant axis.
We discounted the role of oriented cell division in the frontonasal mass but perhaps at younger stages this may play a role. Certain the mouse data from Kaucka et al. (2016) suggests that clones are born sometime after E8.5, probably around the time that neural crest cells enter the face. At these early stages, there may be a role for oriented cell division contributing to the separation between the nasal pits. Only approximately 5-10% of cells were going through mitosis in our static imaging and M-phase is about 1-2 hours in the stage 24 chicken embryo (Minkoff, 1984). Therefore, we were not able to find many cells in mitosis during a 4-hour movie. The role of oriented cell division seems to be far less of a contributing factor than oriented cell movements.

6.7 Proper boundaries inside tissue are important for normal craniofacial development

During our observations we noticed that in the ROCKi treated samples the delimitation between different clusters was deficient. These clear boundaries formation between neighboring cell population may be required for proper development. EPH ligand (EFN) and EPHRIN receptor (EPH) signaling plays an important role in cell segregation and border formation, as a result of regulation of cell adhesion, repulsion or tension (Taylor et al., 2017). It is interesting that mutations in the gene coding for EFNBI (EphrinB1) were linked to craniofrontonasal syndrome, an X-linked developmental disorder where females have frontonasal dysplasia, craniofacial asymmetry, craniosynostosis, bifid nasal tip, while males typically show only hypertelorism (Twigg et al., 2004; Wallis et al., 2008; Wieland et al., 2004).

Craniofacial morphogenesis requires changes in cell behavior to achieve normal craniofacial structures. Boundaries usually forms inside a tissue through cellular organization due to patterning, cell segregation, cell adhesion and migratory guidance (Kindberg and Bush, 2019). Computer simulations showed that cells expressing EPHB2, or kinase-inactive EPHB2 (kiEPHB2), separate out due to heterotypic repulsion and form a clear border with EPHB1-expressing cells, and this might be disrupted by knockdown of N-cadherin that regulates the difference between heterotypic and homotypic repulsion, and enables homotypic cohesion (Taylor et al., 2017). By comparing computer simulations with experimental models EPH/EPHRIN cell patterning model of balancing adhesion and de-adhesion between interacting cells recapitulates the process of cell-cell segregation and cell cluster formation (Aharon et al., 2014). Conditional
deletion of ephrin B1 in neural crest cells showed that ephrin B1 controls their migration and acts both as a ligand and as a receptor in a tissue-specific manner during embryogenesis (Davy et al., 2004).

It is not known where EPH receptors and EFN ligands are expressed in the avian face, therefore, more work is needed to associate EFN/EPH signaling with facial mesenchyme cell behaviors observed in our study.

6.8 Chondrogenesis – a major driver of later outgrowth of the upper beak

By the time we are starting the imaging experiments, neural crest cells have given rise to several lineages including the chondrogenic cells. It is possible that these cells in the midline are starting to behave differently from those at the periphery. We noted that cells in the center are not directed either caudally or cranially but are more stationary than other cells. The first signs of forming a condensation is to compact the mesenchyme and this would remove extracellular space for cell movement. It is proposed that increased adhesion via N-cadherin is involved in early condensations (Cela et al., 2016; Hall and Miyake, 2000) and it makes sense that increased adhesion would also stop cells from moving. Had we carried on imaging for another few hours, we would have likely encountered the condensation in the midline. Therefore, we have captured an important time during development, where cells are still in a somewhat fluid environment and can move.

In addition to type II collagen, there are other forms of ECM present in the organ cultures at the start of our experiment. These ECM components are possibly directly interacting with the mesenchymal cells, limiting the cell movement. Immunofluorescence staining during early development of chicken embryos revealed that type I collagen has a wide distribution throughout the embryo, observed in epithelial basement membranes and in the extracellular spaces of the mesenchyme (Duband and Thiery, 1987). Furthermore lectins (sugar moieties on the cell surface that bind to proteoglycans) are expressed in the face (Croucher and Tickle, 1989). There is strong lectin staining in frontonasal mass mesenchyme cells suggesting that cells are interacting with proteoglycans (Croucher and Tickle, 1989). ROCKi might disrupt these interactions between the cells and matrix.
6.9 Caveats and concluding remarks

Understanding cell behavior during morphogenesis requires observing cell shape changes in correlation with cell displacement. Thus far we have only visualized the nuclei, however the movements of the cytoskeleton are key to understanding morphogenesis. In static images we found that facial mesenchyme cells could extend processes for up to 20 µm (Geetha-Loganathan et al., 2014). Active movement could have been detected by observing the leading edge of the cell and correlating the location of the extending cell membrane with the direction of movement. Existing approaches to visualize actin are limited. We unsuccessfully tried to mark the actin cytoskeleton in frontonasal mass cultures by using incorporating Lifeact (Invitrogen) sequences in frame with GFP into RCAS, but this construct did not label the target. Nevertheless, the density of the tissue during the observed period may have prevented us from clear observations of actin dynamics due to overlapping structures. We recognize that using the nuclei in our tracking experiments it is a challenge to differentiate active movement from passive displacement. Nevertheless, if the entire tissue was contracting then we would predict cells would all be moving towards the center. If the matrix was expanding, then it is difficult to explain the period of about 2 hours where the culture is diverging. Passive displacement does not fit the ROCKi results. If cells were being displaced, then narrowing would have occurred in the presence of the ROCKi.

6.10 Future opportunities

Our method opened new possibilities of investigating morphogenesis in the face. Having access to observation of the entire face through live imaging can help us understand how various molecular signals translate into modifying cell behavior and indirectly the reshaping of the facial prominences. Until now, there were mostly static studies or live imaging using a limited number of marked cells to simulate the morphogenesis. One of the important future directions is to improve the imaging capability by using a 2-photon microscope to include the 3D movement of cells, as there is considerable dorso-ventral growth. Furthermore, antagonizing specific pathways or delivering attractive or repulsive cues to the face using localized bead implants, followed by live imaging may assist us to understand how signals influence cell behavior. Areas of importance such as lip fusion zone can be observed now with our new method and the underlying mechanism of
normal or abnormal morphogenesis may be analyzed. Finally, incorporation of live readouts for signaling changes in GTPases would be a powerful way to test the role of intracellular signaling cascades in directing morphogenesis.
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