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Craniofacial development: discoveries made in the chicken embryo

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ABSTRACT The aim of this review is to highlight some of the key contributions to our understanding of craniofacial research from work carried out with the chicken and other avian embryos. From the very first observations of neural crest cell migration to the fusion of the primary palate, the chicken has proven indispensable in facilitating craniofacial research. In this review we will look back to the premolecular studies where "cut and paste" grafting experiments mapped the fate of cranial neural crest cells, the role of different tissue layers in patterning the face, and more recently the contribution of neural crest cells to jaw size and identity. In the late 80's the focus shifted to the molecular underpinnings of facial development and, in addition to grafting experiments, various chemicals and growth factors were being applied to the face. The chicken is above all else an experimental model, inviting hands-on manipulations. We describe the elegant discoveries made by directly controlling signaling either in the brain, in the pharyngeal arches or in the face itself. We cover how sonic hedgehog (Shh) signals to the face and how various growth factors regulate facial prominence identity, growth and fusion. We also review abnormal craniofacial development and how several type of spontaneous chicken mutants shed new light on diseases affecting the primary cilium in humans. Finally, we bring out the very important role that the bird beak has played in understanding amniote evolution. The chicken, duck and quail have been and will continue to be used as experimental models to explore the evolution of jaw diversity and the morphological constraints of the vertebrate face.

KEY WORDS: *craniofacial, facial prominence, morphogenesis, beak development, maxillary, mandibular, frontonasal*

Introduction

In this review craniofacial development in the chicken embryo will be discussed. The focus will be on questions that can be best addressed by using an accessible animal like the avian embryo. Topics we will cover include the origins of facial tissues gleaned from cut-and-paste, interspecific experiments, the morphogenesis of the beak, the participation of specific signaling pathways in different aspects of facial patterning, models of abnormal beak development including spontaneous mutant chicken lines, and evo-devo studies involving the beaks of modern birds and non-avian reptiles. Throughout this review, the common theme is the high degree of conservation between chicken facial ontogeny and other model organisms. Thus the findings made in the chicken are relevant to our understanding of human development and disease.

Techniques to study craniofacial development in the chicken embryo

Before reviewing the craniofacial research that was carried out on the chicken embryo, it is important to provide some technical context. The main way in which the chicken shines as a model organism is that direct interventions are possible at early stages and can be followed until the full structure has been elaborated. However the craniofacial region presents unique challenges compared to other organs such as the neural tube and limb buds. One

Abbreviations used in this paper: BMP, bone morphogenetic protein; FGF, fibroblast growth factor; NCC, neural crest cell; RA, retinoic acid; RCAS virus, replication-competent ASLV long terminal repeat with a splice acceptor; SHH, sonic hedgehog; WNT, wingless-type MMTV integration site.

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of the main issues is that the skull forms relatively late in chicken development (12-14 days). It requires skill to keep the embryo alive for 2 weeks following complex surgical procedures. Also, the heart develops very close to the face, thus lethality is a common complication. Furthermore, the avian embryo turns on its side at the pharyngula stage, making the centre of the face difficult to access. Nevertheless we and others have remained passionate about using the chicken embryo for craniofacial research.

The methods most commonly employed are cutting and pasting of tissues from one embryo to another, either in the same location or an ectopic location. The soaking of microscopic beads in compounds (agonists or antagonists) and then implanting them in the face is also a favorite method to change the levels of signaling with great temporal and spatial precision. Exogenous genes can also be delivered using electroporation or the Replication-Competent ASLV long terminal repeat with a Splice acceptor (RCAS) system (Gordon *et al.*, 2009). Electroporation is very challenging to carry out in the face due to the proximity of the heart. However some labs have had success with this method (Hu *et al.*, 2015). For more global expression changes in a region of the embryo, the RCAS retrovirus is the preferred method. The virus is avian-specific and permits local misexpression in regions of the face while allowing normal development in the rest of the embryo. Viruses may be utilized to introduce an exogenous gene (Abzhanov and Tabin, 2004, Eames *et al.*, 2004, Foppiano *et al.*, 2007, Hu *et al.*, 2008), mutant versions of a gene (Hosseini-Farahabadi *et al.*, 2017) or less commonly, to knock down a gene using an shRNA cassette (Bond *et al.*, 2016). Moreover, since conservation of protein sequence is very high in developmental genes, the exogenous gene used for overexpression may be from the chicken or another species. The advantage of using sequence from another species is that the level of exogenous gene expression can be subsequently measured against the backdrop of endogenous target gene changes from the chicken. We have previously quantified the levels of expression from RCAS viruses (Geetha-Loganathan *et al.*, 2014, Hosseini-Farahabadi *et al.*, 2017, Nimmagadda *et al.*, 2015) and measured gradual increases in expression between 48 and 96h. Interestingly, the overexpression of genes can be used to detect target genes and importantly, to exclude those that are not likely to be involved in a particular pathway. Thus we would argue that the virus overexpression experiments provide physiologically relevant information.

It is more difficult to carry out loss of function experiments in the face of the chicken embryo. Reagents such as antisense Morpholinos (MO) work well in some locations such as the neural tube (Norris and Streit, 2014), but not at all in the face. Other methods to interfere with gene function seem to be more effective. Electroporation of a mutant SHH (Sonic Hedghog) receptor *Patched* has been successfully carried out. This *PTCΔLoop*^{2} construct lacks a domain that prevents signal transduction in the presence of SHH ligand (Hu *et al.*, 2015). Electroporation seems to work in targeting patches of ectoderm such as in the lip fusion zone but is less useful for targeting facial mesenchyme.

As an alternative to retroviruses, we have tested several types of lipid nanoparticles for nucleic acid delivery (Geetha-Loganathan *et al.*, 2009, Kulkarni *et al.*, 2017). These nanoparticles are capable of over expression or knockdown. Plasmids expressing an shRNA construct targeting gallus *WNT11* led to decreased gene expression (Geetha-Loganathan *et al.*, 2014) and rounding of the cells in which it was expressed. The lipid-based transfection method is not global, but is a great way to create a mixed population of labeled and unlabeled cells.

Although the learning curve is steep, the value of locally controlling gene and signaling pathway activity in the face or to use unbiased, grafting methods, offers the chance to address important research questions in highly creative ways that are not available in other amniote models.

Cranial neural crest cells and their contribution to the head skeleton

The chicken and quail were the first animals to be used for long-term lineage mapping of neural crest cells (Le Lievre, 1978, Noden, 1975, Noden, 1983). At the early stages of craniofacial patterning, functionally equivalent cranial neural crest cells derived from the prosencephalon, mesencephalon and rhombomeres 1,2 migrate into the ventral region of the head, filling the pharyngeal arches (Couly *et al.*, 1996, Koentges and Lumsden, 1996) (Fig. 1A). These facial neural crest cells contribute to the skeleton supporting the upper and lower beaks, anterior calvaria (frontal bones) and anterior cranial base (Couly *et al.*, 1993, Le Lievre, 1978, Noden, 1975). Non-skeletal neural crest cell derivatives include melanocytes, connective tissue, smooth muscle, fascia, as well as parts of the peripheral nervous system, just to name a few (Creuzet *et al.*, 2005).

In addition to their interaction with specific germ layers, neural crest cells are patterned by the HOX code found along the anteroposterior axis of animals. The anterior neural tube segments (prosencephalon and mesencephalon) do not express members of the Antennapedia HOX gene clusters, whereas posterior regions of the neural tube do (Fig. 1A)(Couly *et al.*, 1996). Consequently, when HOX-negative neural crest cell progenitors derived from anterior neural tube are transplanted into posterior regions, normal development of ectopic mandibular structures are produced (Noden, 1983). However, in an inverse experiment, when *HOXA2* was artificially introduced into the anterior cranial neural crest via electroporation, jaw development was inhibited (Creuzet *et al.*, 2002). Whether there are factors that actively repress *HOXA2* or other HOX genes in the anterior neural crest is unknown.

Premigratory cranial neural crest cells contain important information controlling the size of skeletal elements. Grafts of quail neural crest cells into the duck embryo changed the size and shape of the beaks to be pointed and smaller; resembling the donor species, the quail (Eames and Schneider, 2008, Fish *et al.*, 2014, Schneider and Helms, 2003, Tucker and Lumsden, 2004). Timing of development is also intrinsically controlled. The quail hatches in 17 days whereas the duck takes 28 days to hatch (Eames and Schneider, 2008). At the beginning of neural crest cell migration, there is a proportionately larger group of cells dorsal to the neural tube in the duck than in the quail (Fish *et al.*, 2014). These early differences lead to more mesenchymal cells in the face and subsequently to larger skeletal elements in the duck. The quail-duck studies also revealed that intrinsic cell cycle times are retained in neural crest-derived cells, even when interspecific grafts are made (Fish *et al.*, 2014). Thus cranial neural crest cells are preprogrammed to contain main elements of the craniofacial pattern with some details to be specified later, such as the arrangements of bones and cartilages (Noden, 1983, Trainor *et al.*, 2002).

The first pharyngeal arch contributions to the face

The next stage of craniofacial development is the entry of neural crest cells into the ventrally positioned pharyngeal arches. The pharyngeal arches are repeated segments and each one contains similar elements (skeletal tissue, musculature, neurovascular bundle) (Fig. 1B) (Graham, 2003, Veitch *et al.*, 1999). It has previously been shown that the segmental characteristics of the pharyngeal pouches develop independently of the presence of the neural crest cells (Veitch *et al.*, 1999). This suggests that at least part of the information for patterning the face could originate from signals within the endoderm (Couly *et al.*, 2002). Indeed a series of studies from the laboratory of Nicole Le Douarin showed that the foregut endoderm of the chicken embryo (which will later line the pharyngeal pouches, Fig. 1B) specifies identity and orientation of the neural crest-derived facial skeleton including mandible (Brito *et al.*, 2006, Couly *et al.*, 2002) and nasal capsule (Benouaiche *et al.*, 2008).

The neural crest-derived mesenchyme within the first pharyngeal arch condenses to form a dorsal condensation (future maxillary prominence) below the eye, and a ventral condensation (future mandibular prominence) below the oral cavity; leaving a maxillo-mandibular cleft representing the "hinge" between the jaws (Depew and Compagnucci, 2008, Tak *et al.*, 2017). The respective prominence condensations ultimately form the skeletal structures of the jaws: the Meckel's cartilage and dentary (embryonic skeleton of the lower jaw) and the palatine, jugal and maxillary bones (embryonic skeleton of the upper jaw) (Fig. 1B-D). However, some aspects of this hypothesis have been recently called into question.

Cerny and colleagues injected DiI into the presumptive "maxillary" and "mandibular" prominences of the first pharyngeal arch of stage 13-14 chicken embryos (Cerny *et al.*, 2004). They found that a single condensation of cells, ventrally located in the first pharyngeal arch, gives rise to both maxillary and mandibular jaw cartilages, and which they refer to as 'maxillomandibular' region. On the other hand, the dorsal condensation of the first pharyngeal arch was found to give rise to the trabeculae cranii, which were thought to be anterior neurocranial structures (Cerny *et al.*, 2004). However, a similar study performed by our laboratory found that the injection of dye in the post-optic region labeled the center of the stomodeal roof where the trabecular cartilages will later form. Thus we have pinpointed the stage where midline mesenchyme

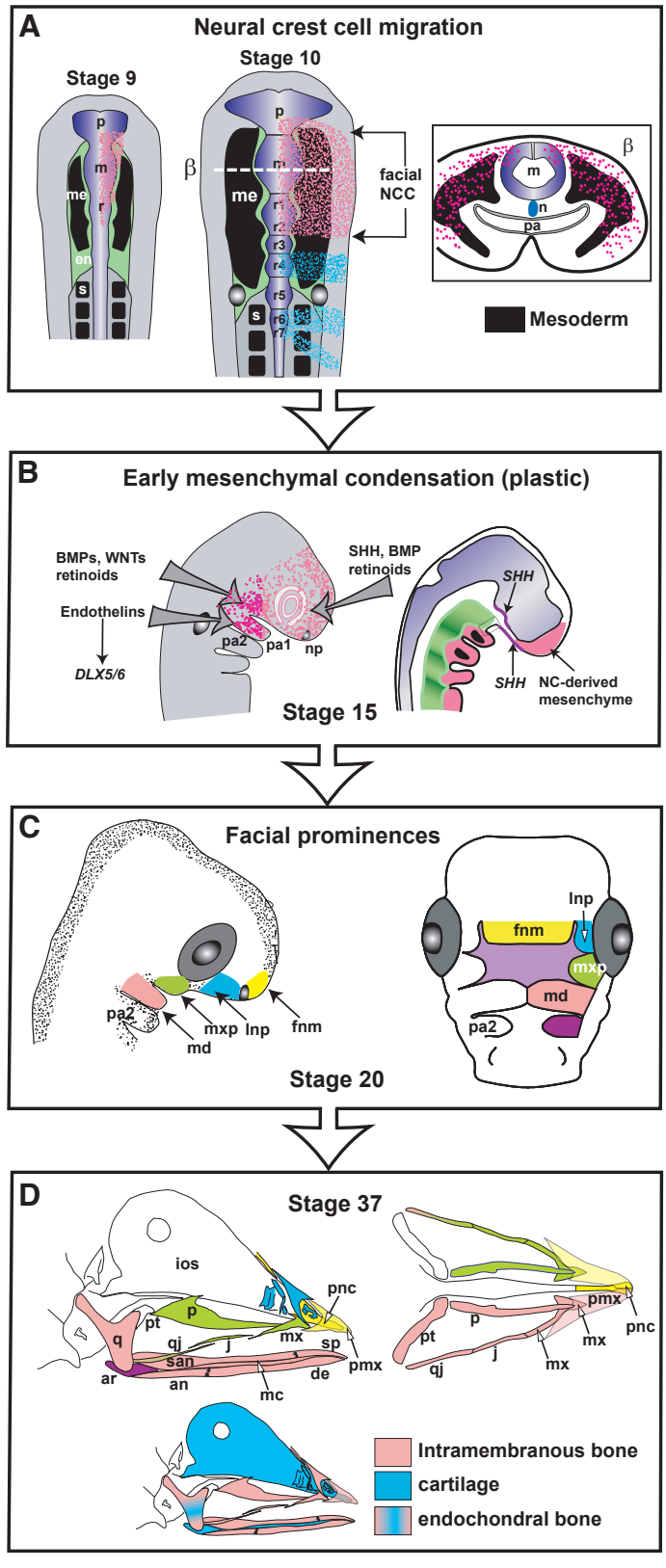

Fig. 1. Overview of craniofacial development in the chicken embryo. **(A)** *Hox-negative neural crest cells (NCC, pink) move out from the primitive brain, through the paraxial mesoderm (black) and into ventral face (inset). Hox-positive neural crest cell streams originate from below r4 (NCC, blue).* **(B)** *On the left is an external view showing areas of face populated by NCC. On the right is a midsagittal slice, showing the position of neural crest-derived mesenchyme (pink) and mesoderm in the pharyngeal arches and ventral to the brain. The pharynx is lined with endoderm (green).* **(C)** *Facial prominences are color- coded to match their skeletal derivatives shown in panel D.* **(D)** *Sagittal and palatal views of the skull. In palatal view, upper half is the fate map, lower half is showing intramembranous bones. Below is an overview of the intramembranous and endochondral bones of the head. KEY: an – angular, ar – retroarticular process, de – dentary, fnm – frontonasal mass, ios – interorbital septum, j – jugal, lnp – lateral nasal prominence, m – mesencephalon, mc – Meckel's cartilage, md – mandibular prominence, mxp – maxillary prominence, mx- maxillary bone, n – notochord, np – nasal pit, p – prosencephalon (in panel A), p – palatine (in panel D), pa – pharyngeal arch, pmx – premaxilla, pnc – prenasal cartilage, pt – pterygoid, q – quadrate, qj – quadratojugal, r – rhombencephalon, r1 (– r7) – rhombomeres 1-7, s – somite, san – surangular, sp – splenial.*

is continuous with and is likely mixing with post-optic mesenchyme. At later stages (stage 24), once the maxillary prominences have formed, there is no more mixing of cells and maxillary cells only contribute to the palate skeleton (Lee *et al.*, 2004). Our work agrees with that of others (Cerny *et al.*, 2004, Shigetani *et al.*, 2000, Tak *et al.*, 2017) that locally, at the maxillo-mandibular cleft, there is movement of cells between the first arch and presumptive maxillary prominence. This sharing of mesenchyme between the future proximal maxillary and mandibular prominence may explain why the global transcriptomes of these two regions are more similar to each other than to that of the frontonasal mass (Buchtova *et al.*, 2010).

Jaw identity is being established during pharyngeal arch development. The post-migratory neural crest-derived mesenchyme in the presumptive face continues to interact with local tissues (epithelium, endoderm) to gradually give rise to characteristic patterns of the upper and lower beak. Experiments that target facial mesenchyme after neural crest cell migration has ceased, but before individual facial prominences have formed, can induce a change in identity from maxillary prominence to frontonasal mass (Cela *et al.*, 2016, Lee *et al.*, 2001, Nimmagadda *et al.*, 2015). We will discuss the signals involved in specification of neural crest-derived skeleton later in this review.

Brain face interactions – the chicken forebrain signals to the face

Due to the close proximity of brain and face, the brain has long been suspected of being an important signaling center that controls facial development (Demyer *et al.*, 1964). Recently, the molecular underpinnings of these interactions have been teased apart using the chicken embryo as a study system. The main molecule involved in this interaction is Sonic Hedgehog (SHH). The earliest *SHH* signals come from the prechordal plate, which then turns on expression of *SHH* in the ventral diencephalon between 6 and 8 somite stages (stage 7) (Brito *et al.*, 2006). If the forebrain is excised after the induction of *SHH* in the diencephalon, only the lower beak forms. However, the addition of a SHH soaked bead to an embryo lacking the forebrain restores the upper beak. This experiment proves that SHH is necessary and sufficient for upper beak formation.

In another series of experiments on slightly older embryos (stage 10), signaling from the forebrain was inhibited by injecting hybridoma cells secreting a SHH-blocking antibody (Marcucio *et al.*, 2005, Young *et al.*, 2010). The cells were injected into the lumen of the neural tube in order to specifically interfere with the brain-derived SHH, rather than that of the foregut or prechordal plate. The treated embryos exhibited a shortened premaxilla, absence of the prenasal cartilage, smaller and medially fused maxillary bones, and smaller and medially shifted palatine bones.

The mechanism by which *SHH* affects the development of the upper face appears to be indirect. The absence of signaling from the brain reduces cell proliferation within the neural crest cell-derived mesenchyme, leading to developmental defects of the upper beak (Marcucio *et al.*, 2005). However, there was also concomitant loss of *SHH* expression in the frontonasal ectoderm, as well as decreases in target genes of *SHH* such as *PTC1* and *GLI1*, in both the ectoderm and adjacent mesenchyme. The aforementioned changes, particularly the loss of *SHH* in the ectoderm could also have contributed to decreased proliferation. As we will discuss later, the ectoderm is required for craniofacial growth.

The nasal placodes are signaling centers for the upper face

An interesting hypothesis, which has recently been gaining experimental support, is that the cranial sensory placodes may serve as organizing centers throughout the craniofacial region (Steventon *et al.*, 2014). The nasal placodes, in addition to forming the olfactory neurons, provide inductive signals for the formation of the face (Szabo-Rogers *et al.*, 2008, Szabo-Rogers *et al.*, 2009). By placing a foil barrier on the lateral side of the nasal placode in the chicken embryo, Szabo-Rogers and colleagues showed that they could prevent signals from diffusing laterally, and thus block specification of the lateral nasal prominence (Szabo-Rogers *et al.*, 2009). Similarly, ablating the olfactory placode at stage 15 by Nile blue sulfate epithelial-stripping prevented formation of the lateral nasal bone and nasal turbinates (Szabo-Rogers *et al.*, 2009).

The nasal placodes have surprising instructive properties as well. When grafted to an ectopic, Hox-negative region of the head, the nasal pits induced supernumerary frontonasal structures and furthermore, did not repress normally occurring skeletal elements (Szabo-Rogers *et al.*, 2009). An earlier study also showed that the olfactory placode is able to form thickened epithelium and neurons in ectopic locations (Bhattacharyya and Bronner-Fraser, 2008). However skeletal changes were not recorded because the endpoint was prior to skeletogenesis. Our study, on the other hand, followed the placodes up to stage 37 and identified characteristic olfactory invaginations in the head as well as accompanying skeletal changes in an ectopic location. Such cut and paste experiments are ideal for testing the instructive properties of a tissue and the supportive nature of the host environment to allow continued development.

Facial prominences formation, fusion and fate

Formation of the face is a complex and precisely timed morphogenetic event that takes place between stages 20-29 in the chicken embryo. After the nasal placodes have formed, they begin to invaginate at stage 20, while the adjacent mesenchyme proliferates to form the craniofacial prominences (Fig. 1C, Fig. 2). The facial prominences bud around the primitive oral cavity (stomodeum), and will eventually grow and fuse with each other to form the intact face. These facial prominences consist of the frontonasal mass (between the nasal placodes), paired lateral nasal prominences (lateral to the nasal placodes), maxillary prominences (lateral to the stomodeum) and the mandibular prominence (inferior to the stomodeum). At stage 27, the prominences have grown out enough to initiate contact. In chicken, the maxillary prominences meet with the distal corners of the frontonasal mass ('globular processes' in chicken or 'medial nasal prominences' in mammals), in order to form a continuous upper lip (Fig. 2B) (Abramyan and Richman, 2015, Abramyan *et al.*, 2015). When prominences make contact, a transient, bilayered epithelial seam (or nasal fin) forms between them, breaking down relatively quickly, and allowing for mesenchymal continuity between distinct prominences (Abramyan and Richman, 2015). Next, the grooves or furrows between the newly fused prominences fill out through a process of merging, to create a smooth surface (Abramyan and Richman, 2015).

The meeting, fusion and merging of craniofacial prominences represents a critical period of development across amniotes. If contact is abnormal, then a cleft will result in the developing upper lip, which

may extend into the palate. Perturbation such as displacement of facial prominences in relation to each other could make it difficult for facial prominences to meet, thus there is strict conservation of morphology across amniotes at "pre-fusion" developmental stages (Young *et al.*, 2014). Nonetheless, we have previously identified variation in the prominences between amniote lineages, adding an extra degree of difficulty in predicting developmental defects resulting from misalignment (Abramyan *et al.*, 2015). Fortunately, humans and chickens utilize the maxillary prominence and the medial nasal/globular processes to make initial contact, making chicken an appropriate model for studies of human clefting (Abramyan *et al.*, 2015).

Each facial prominence has a distinct contribution to the skeleton of the jaw. Pioneering transplantation experiments, where components of the embryonic face were grafted onto host limb buds, allowed a fate map of facial prominences to be determined. The frontonasal mass forms the prenasal cartilage, nasal septum, premaxillary bone and ectodermally-derived egg tooth (Richman and Tickle, 1989, Richman and Tickle, 1992, Wedden, 1987). The maxillary prominences form the bones of the palate (maxillary, palatine) as well as the jugal bone (Lee *et al.*, 2004). The mandibular prominences form the entire mandibular bone, Meckel's cartilage, and the malleus and incus (Richman and Tickle, 1989, Wedden, 1987). The lateral nasal prominences form the nasal conchae (MacDonald *et al.*, 2004) (Fig. 1D).

Through epithelial-mesenchymal exchange experiments between facial prominences, it became clear that jaw identity was determined by the mesenchyme, whereas the epithelium is required for outgrowth (MacDonald *et al.*, 2004, Richman and Tickle, 1989, Wedden, 1987). Another demonstration of the role for epithelium in outgrowth was identified by some elegant grafting experiments performed by Hu and colleagues (Hu *et al.*, 2003). Small strips of frontonasal mass epithelium containing the caudal edge were grafted to the lateral surface of the frontonasal mass or the mandibular prominence. In both cases, branches of the normal skeleton were produced (Hu *et al.*, 2003). This frontonasal ectodermal zone is also an important region for species-specific differences in the form of the midface (Hu and Marcucio, 2009b, Xu *et al.*, 2015).

Proliferation, polarity and other mechanisms driving facial prominence morphogenesis

Facial prominences dramatically change shape during stages when lip fusion is taking place. The frontonasal mass becomes narrower medio-laterally (Fig. 2A-C) and extends in the perpendicular axes (dorso-ventral, cranio-caudal). The maxillary prominences elongate cranio-caudally (Geetha-Loganathan *et al.*, 2014). Proliferation accounts for the enlargement of the prominences but not entirely for the changes in shape. Minkoff and Kuntz carried out some of the original work on cell proliferation in the face, using the chicken embryo as a model (Minkoff and Kuntz, 1977, Minkoff and Kuntz, 1978). They showed that at stage 20, proliferation was similar in all regions of the mesenchyme but then at stage 24-25, the rate of proliferation decreased at the base of the facial prominence, leading to a relatively higher level remaining in the distal parts of the mesenchyme. Such differential proliferation was also described during chicken limb bud outgrowth (Searls and Janners, 1971), as well as the medial side of the maxillary prominences in the chicken embryo (Abramyan *et al.*, 2014), leading to palatal shelf formation and outgrowth. Thus a relative drop in proliferation in adjacent mesenchyme explains most of the outgrowth of facial prominences (rather than a localized addition of cells to the tip). Indeed when the proliferation gradient in the maxillary prominence was disrupted through *WNT11* overexpression in the maxillary mesenchyme, the maxillary buds were smaller in volume, due to a decrease in length in the cranial caudal axis (Geetha-Loganathan *et al.*, 2014). Similarly, introduction of *Noggin* protein into the maxillary prominence also decreased proliferation, causing a gap to form between the frontonasal mass and maxillary prominence (Ashique *et al.*, 2002).

Despite observation of proliferation gradients, early hypotheses assumed that proliferation, which was driving morphogenesis of facial prominences, was undirected and isotropic (Linde-Medina *et al.*, 2016). These traditional models must now accommodate new data identifying cell polarity as a significant part of facial prominence budding and outgrowth (Geetha-Loganathan *et al.*, 2014, Li *et al.*, 2013). In a study of the frontonasal mass, Li *et al.,* quantified polarity in mesenchymal cells by analyzing the position of the nucleus and the Golgi body. Through analysis of static images, they showed that the mesenchymal cells located in the lateral regions of the frontonasal mass were oriented toward the points of outgrowth (i.e. the globular processes) (Geetha-Loganathan *et al.*, 2014, Li *et al.*, 2013). In the maxillary prominence, we utilized measurement of the angle formed by cell's major axis and a reference line to show that cells were preferentially oriented ~30 degrees medial to the cranial-caudal axis (Geetha-Loganathan *et al.*, 2014). In functional experiments performed in vivo, we also showed that the mesenchymal cells will reorient themselves and migrate toward an ectopic source of WNT11. These experiments demonstrated that directional cues, in addition to proliferation, are likely required to drive growth of prominences towards a larger size, the correct

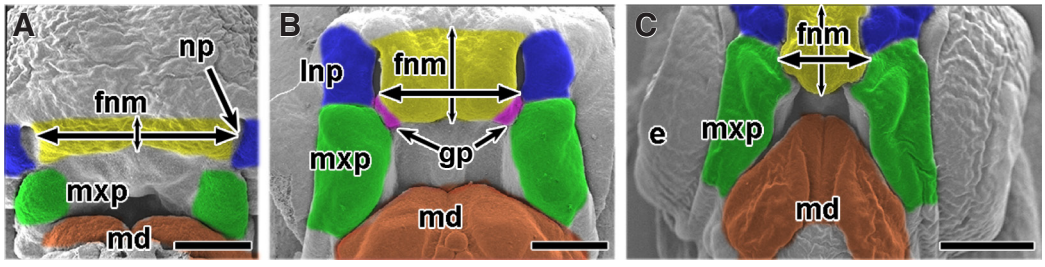

Fig. 2. Craniofacial prominences in the developing chicken. *Scanning electron micrographs of the chicken face. The facial prominences change proportions between stage 20* **(A)**, *3.5 days; stage 24* **(B)**, *4.5 daysand stage 29* **(C)**, *6 days. Note the mediolateral axis of the frontonasal mass becomes narrower relative to the cranial caudal axis. Measurements in our lab show that absolute width decreases but volume increases by stages 24 and 29, particularly due to dorsoventral growth. The maxillary prominences change shape from round buds at stage 20 to elongated, sculpted prominences at stage 29. Lip fusion takes place between the globular processes of the frontonasal mass and the medial maxillary prominences between stages 25 and 27. Key: fnm, frontonasal mass (yellow); gp, globular process (pink); lnp, lateral nasal prominences (blue); md, mandibular prominences (orange); mxp, maxillary prominences (green). Scale bar, 500 μm for stage 20 and 24; 1 mm for stage 29.*

shape, and in the right direction.

In thinking about growth and morphogenesis during development, most developmental biologist focus on the behavior of cells, while the extracellular matrix is generally ignored. In an immunofluorescence study on chicken embryos, Xu *et al.* found that the basement membrane is thinner in regions that undergo extensive shape changes. It makes sense that the basement membrane would have to facilitate budding though mechanical compliance, however this model has not yet been tested functionally in the face (Xu *et al.*, 1990).

Other mechanisms of facial prominence morphogenesis could involve mesenchymal cell rearrangements. This idea has thus far only been studied using DiI injections and static imaging. Since the embryonic face of the chicken is turned on its side (in ovo), data are mainly available for the accessible, lateral nasal prominence, maxillary prominence, lateral frontonasal mass and mandibular prominence (Lee *et al.*, 2004, McGonnell *et al.*, 1998) (Fig. 3). The focally labeled regions expand over the 48h of the experiment due to displacement, migration and cell proliferation. In the lateral maxillary prominence the cells spread out next to the eyes (McGonnell *et al.*, 1998), similar to what we found in transfection experiments (Geetha-Loganathan *et al.*, 2014). The cells labeled at the maxillo-mandibular cleft are distributed on either side of the cleft (McGonnell *et al.*, 1998, Tak *et al.*, 2017). The mandible also has some interesting patterns of cell expansion correlating with beak outgrowth. The midline mesenchyme spreads in a line following the cranial-caudal axis. Mesenchyme near the second pharyngeal arch spread in the medio-lateral plane (Fig. 3). However, more work needs to be done to determine whether these DiI patterns correspond to directed cell movement, oriented cell division or differential proliferation. DiI cannot label individual cells. Therefore, our lab is currently developing live imaging methods to track single mesenchymal cells in the face during organogenesis stages.

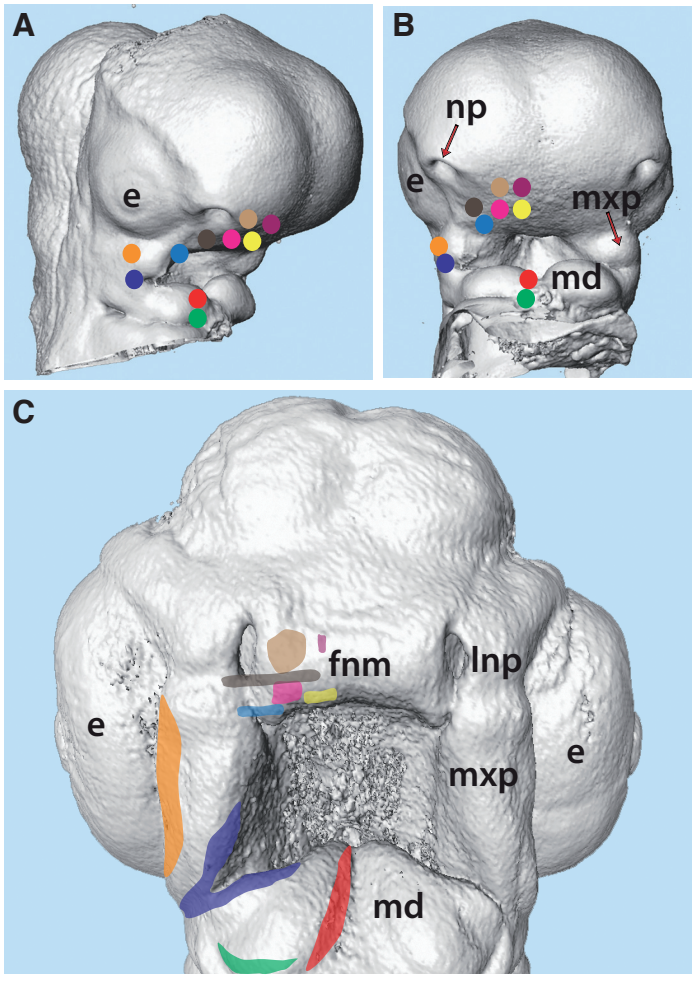

Fig. 3. Summary illustration of experiments from McGonnell *et al.*, 1998. *Optical projection tomography scans of chicken embryo heads. Isosurfaces were generated using Amira software.* **(A,B)** *Side and frontal views of stage 20 embryos with injection sites color coded in each of the facial prominences.* **(C)** *Stage 28 embryo illustrating the various shapes formed by the expanded DiI, 48h after injection at stage 28. Abbreviations: e, eye; fnm, frontonasal mass; lnp, lateral nasal prominence; md, mandible; mxp, maxillary prominence; np, nasal pit.*

Signaling during facial morphogenesis

The implantation of beads that are soaked in agonists and antagonists have been used extensively to examine the roles of signaling molecules such as retinoic acid (RA), SHH, fibroblast growth factors (FGF), wingless related factors (WNTs) and bone morphogenetic proteins (BMPs) during many stages of craniofacial development. Here we will focus on facial prominence growth and lip fusion.

Sonic hedgehog (SHH)

SHH RNA is expressed in epithelium covering the stomodeum and extends to the caudal edge of the frontonasal mass and medial surface of the maxillary prominences (Ashique *et al.*, 2002, Marcucio *et al.*, 2005). Application of beads soaked in a blocking antibody for SHH into the frontonasal mass caused cleft lip in the chicken embryo (Hu and Helms, 1999). Similarly, a more recent approach where electroporation was used to block SHH-signaling specifically in the epithelium also caused clefting (Hu *et al.*, 2015). Therefore, it is likely that the epithelium-derived SHH is required for outgrowth of the frontonasal mass, which is itself a requirement for contact and fusion with the maxillary prominence in order to form an intact upper lip. An increase in SHH levels either in the brain (Hu and Marcucio, 2009b) or directly in the frontonasal mass (Hu and Helms, 1999), was found to have opposite effects. Direct application to the frontonasal mass caused it to become slightly wider, but ultimately major skeletal defects were not observed (Hu and Helms, 1999). When SHH-soaked beads were placed in the brain, the frontonasal mass again grew wider (Young *et al.*, 2010), but upper beak was significantly shorter due in part to a decrease in proliferation (Hu and Marcucio, 2009b). This result is slightly difficult to explain, since normally SHH promotes cell survival as well as expression of *SHH* in the frontonasal mass epithelium. Both of these outcomes should have resulted in a wider prenasal cartilage. Indeed, a more sustained delivery of SHH via a retrovirus within the frontonasal mass caused a bifurcation of the prenasal cartilage and duplication of the egg tooth (Hu and Helms, 1999). Thus it seems that SHH signals coming from the brain are received differently in the frontonasal mass than when supplied directly in the facial mesenchyme. The fact that excess SHH causes a different set of craniofacial deficiencies from the

two types of manipulations may also be due to stage-specific differences in the mesenchymal response.

Bone morphogenetic proteins (BMPs)

Studies of chicken craniofacial development from our lab have implicated BMPs as major players in lip fusion (Ashique *et al.*, 2002). *BMP2* was found to be abundant in the globular process mesenchyme as well as the maxillary prominences (Ashique *et al.*, 2002, Francis-West *et al.*, 1994). *BMP4* and *BMP7* were largely localized in the epithelium. *NOGGIN* (*NOG*), a BMP antagonist, was expressed in epithelial tissue surrounding the globular process at stage 24, but then was downregulated at stage 28. This curious change in expression of *NOG* coincides with the time when the bilayered epithelial seam forms between the frontonasal mass and maxillary prominences. When beads soaked in exogenous NOGGIN protein were implanted into to the globular process to keep NOGGIN levels high, the epithelium became thicker. The increase in cell survival in the epithelium could also have interfered with fusion, had the facial prominences contacted each other, since the degradation of the epithelial seam is a key component of fusion. Concomitant with the epithelial thickening, a reduction in proliferation of the mesenchyme was observed, ultimately leading to a cleft of the upper beak in a similar anatomical position to human cleft lip. These subtle changes are visible in bead-implanted embryos because the diffusion of the protein is only 100-200μm from the bead. Thus the study of a process that is very local, like lip fusion, is ideally suited for the bead implant approach.

Fibroblast growth factors (FGFs)

FGF signaling is critical for a number of proliferative zones in the developing face. Studies from our lab (Szabo-Rogers *et al.*, 2008, Szabo-Rogers *et al.*, 2009) have determined that molecular signals including FGF8 from the nasal pit are necessary for patterning the lateral nasal prominence and frontonasal mass. By implanting beads soaked in a pan-antagonist of FGF receptors (SU5402), we were able to show that proliferative growth had a surprising degree of dependence on FGF signals in some areas of the face and while none at all in others (Szabo-Rogers *et al.*, 2008). A cleft was induced when FGF signaling was disturbed in the cranial part of the prominence. Most unexpectedly, we found that FGF signaling was dispensable in the globular process of the frontonasal mass and medial corner of the maxillary prominence, which are the two regions critical for the process of primary palate fusion (Szabo-Rogers *et al.*, 2008). We concluded that proliferation in the cranial frontonasal mass mesenchyme displaced the globular process caudally, promoting contact with the maxillary prominence. Experiments from others showed that inhibition of FGF signaling in the centre of the frontonasal mass at stage 17 shortened the upper beak and also induced a cleft (Hu and Marcucio, 2009a), confirming the general requirement for FGF in outgrowth of the mesenchyme. Other studies also demonstrated a role for epithelial-derived FGF signaling in regulating frontonasal mass transcriptional responses (Firnberg and Neubuser, 2002).

FGF signaling is also a major element in the formation of the discrete region of facial ectoderm in the chicken embryo such as the frontonasal ectodermal zone (Hu *et al.*, 2003). *FGF8* is required for initial frontal ectodermal zone activity (Hu *et al.*, 2003), but other data indicate that *FGF8* needs to be downregulated for normal development of the frontonasal region of the head (MacDonald *et al.*, 2004). While FGF8 on its own is not sufficient to induce outgrowths of the frontonasal mass (Hu *et al.*, 2003), the combination of *FGF8* and *SHH* ectopic expression in the head epithelium induces ectopic outgrowths supported by cartilage (Abzhanov and Tabin, 2004). The frontonasal epithelial zone secretes multiple genes in addition to *FGF8* and *SHH* such as *BMP4* and *NOG* (Ashique *et al.*, 2002) all of which may contribute to upper beak outgrowth.

Wingless-type MMTV integration site (WNTs)

We performed a comprehensive characterization of WNT pathway genes in the chicken embryo in preparation for future functional studies (Geetha-Loganathan *et al.*, 2009). We analyzed the expression of a number of WNT paralogs, as well as other genes within the WNT pathway such as the *DKK* family of antagonists, the *FZD* family of receptors, as well as the signal transduction molecule *CTNNB1* and the transcriptional target, *LEF1*. We examined the roles of *WNT5A* in the development of the chicken skull due to its high level of expression in the frontonasal mass, maxillary and mandibular prominence mesenchyme (Hosseini-Farahabadi *et al.*, 2013). Previous chicken studies had exclusively focused on the function of *WNT5A* on the appendicular and axial skeletons (Hartman and Tabin, 2000). Through a series of *in vivo* and *in vitro* experiments, we showed that *WNT5A* represses canonical WNT signaling on order to allow for the differentiation of craniofacial cartilage. In a recent study, the overexpression of *WNT5A in vivo* was characterized in more detail and compared to mutant version of the gene that causes Robinow syndrome (Hosseini-Farahabadi *et al.*, 2017). Patients with a single allele containing the *WNT5A* variants have major craniofacial and limb anomalies (Person *et al.*, 2010, Roifman *et al.*, 2014). We were able to use the chicken to determine the pathogenicity of 2 missense variants affecting a cysteine residue in *WNT5A*. The wild-type and mutant human *WNT5A* genes introduced into the chicken mandibular prominence caused shortening of the structure. Specifically, the mutant version of *WNT5A* randomized the orientation of Meckel's cartilage chondrocytes. These cell orientation defects, along with reduced cell migration, suggested there were problems in the JNK-planar cell polarity pathway (one of the non-canonical WNT pathways). This study suggests that normal WNT5A signaling is needed for proper chondrocyte stacking in order that Meckel's cartilage elongates.

WNT11 is another putative non-canonical WNT, which we had previously identified as being highly expressed in the maxillary prominences (Geetha-Loganathan *et al.*, 2009). Through a series of knockdown and overexpression experiments, we found that *WNT11* induces a decrease in cell proliferation, thereby preventing lengthening of the facial prominences and inducing a cleft in the developing beak. In this study, we also uncovered a novel role for the WNT11 molecule, showing that it can behave as a chemoattractant to cells in the developing craniofacial prominences. Through detailed analysis of cell migration, cell orientation, as well as luciferase assay data, we showed that *WNT11*, similar to *WNT5A*, is capable of activating a planar cell polarity (PCP)-type of mechanisms in the facial mesenchyme cells (Geetha-Loganathan *et al.*, 2014).

Retinoic acid (RA)

The first molecule tested in a local release experiment in the chicken embryo was RA (Tickle *et al.*, 1982). At the same time as digit duplications were induced, the upper beak was unexpectedly,

completely truncated (Tamarin *et al.*, 1984). The molecular reasons for the upper beak defect were never discovered, but it was clear from epithelial-mesenchymal exchanges between treated and untreated embryos that the main target of RA was the mesenchyme (Wedden, 1987).

Excess RA provides insights into the teratogenic properties of this molecule. However endogenous RA is also required for development. Earlier studies from our lab utilized Citral-soaked beads (a general antagonist of RA synthesis) (Song *et al.*, 2004). Treatment of stage 20 chicken embryos resulted in the loss of derivatives from the lateral nasal prominences. Specifically, resulting in the absence of the nasal bone and nasal conchae. We were able to show that the Citral-induced phenotype was due to reduction in RA synthesis and *FGF8* expression. Exogenous FGF8 or RA application rescued the Citral induced phenotype, implicating FGF8 as a regulator of cell survival in the developing face.

Cross talk between the retinoic acid and bone morphogenetic protein pahways

RA and NOGGIN protein soaked beads placed into the first pharyngeal arch of stage 15 chicken embryos resulted in an unexpected facial phenotype. The maxilla was transformed into a second frontonasal mass; ultimately leading to a partial duplication of the upper beak (Lee *et al.*, 2001, Nimmagadda *et al.*, 2015). The ectopic skeletal elements were located in the palate and consisted of a duplicated interorbital septum, prenasal cartilage and premaxilla. The identity of the transformation was unambiguous due to formation of an ectopic egg tooth (exclusively found on the frontonasal mass). In subsequent studies, NOGGIN protein with greater bioactivity was obtained (Regeneron) and this baculovirus-derived protein was sufficient on its own to drive the formation of an ectopic interorbital septum, prenasal cartilage, premaxilla and egg tooth (Cela *et al.*, 2016). We carried out a gene profiling experiment and found novel mediators of RA signaling (Nimmagadda *et al.*, 2015). We also discovered cross talk between NOGGIN and RA. NOGGIN itself was able to induce several RA pathway molecules, further increasing the levels of endogenous RA synthesis (Nimmagadda *et al.*, 2015). This ability of NOGGIN to activate RA signaling may explain why NOGGIN on its own could elicit the same phenotype as NOGGIN and RA combined (Cela *et al.*, 2016).

Abnormal craniofacial development in spontaneous chicken mutants

While development of transgenic lines has met with limited success in the chicken (with the exception of GFP lines), several spontaneous mutant lines with craniofacial anomalies exist and have been utilized by developmental biologists for several decades. Our lab has studied the *cpp* mutant (*cleft primary palate*) characterized by Ursula Abbott (MacDonald *et al.*, 2004, Yee and Abbott, 1978). The *cpp* mutant carries an unknown recessive mutation that causes upper beak truncation, not dissimilar to the effects of excess RA. A particular challenge with studying the *cpp* mutant is that the mutant phenotype is first manifested at stage 28, which is relatively late in development. To identify mutants at earlier stages, the affected tissue was divided into pieces and grafts were made to host embryos. The normal frontonasal mass gave rise to long cartilage rods whereas the mutant frontonasal mass only formed short nodules of cartilage. Through blind tissue recombination experiments, we determined that the mutation specifically affects the epithelium (MacDonald *et al.*, 2004). The tissue recombination method provides unique information that cannot necessarily be predicted from the genetic sequence. It is a challenge to identify the spontaneous mutations in chicken embryos due to outbreeding that has to be done to keep genetic lines robust (J. Pisenti, UC Davis, personal communication).

The talpid mutants [*talpid, talpid2* (*ta2*)*, talpid3* (*ta3*)] are categorized together due to their shared phenotype of polydactyly and severe craniofacial malformations (Schock *et al.*, 2016). After many years of work, the Edinburgh group who spearheaded many of the early efforts to create genomic resources for the chicken, finally mapped and identified the gene underlying the *talpid3* line (*KIAA0586*) (Davey *et al.*, 2006, Yin *et al.*, 2009). The *ta2* mutation was later identified in the USA (Chang *et al.*, 2014) as being caused by deletions in the *C2CD3* gene. Interestingly, *ta2* has a relatively mild craniofacial phenotype where the facial prominences fail to fuse properly, resulting in a cleft of the primary palate, while *ta3* mutants exhibit a collapse of the facial midline (through reduction of brain floorplate SHH), resulting in hypotelorism (Schock *et al.*, 2016)). Importantly, both of these mutations affect the primary cilia which may explain the similar phenotypes. The chicken mutations ended up affecting the same genes as 2 human craniofacial ciliopathies: Oral-facial-digital syndrome (*ta2*) (Schock *et al.*, 2015) and Joubert syndrome Joubert Syndrome (JS) (*ta3*) (Stephen *et al.*, 2013). Thus the chicken embryo once again proves to be a useful model in which to study human genetic disease.

The chicken beak in studies of evolutionary biology

Avian beaks exhibit immense diversity in shape and size, ranging from relatively small beaks of tits and chickadees of the Paridae family to the extensive beaks of hornbills and toucans. As we previously mentioned, morphological diversity is highly reduced in the craniofacial region of amniote embryos (Young *et al.*, 2014), yet we were able to identify some minor, lineage-specific changes in craniofacial prominence shape, position, and points of fusion (Abramyan and Richman, 2015, Abramyan *et al.*, 2015). By studying primary palatogenesis in the chicken embryo in 3D morphospace, we found that chickens (along with squamates and turtles) form their primary palate across a patent 'choanal groove' that remains open throughout development (Abramyan *et al.*, 2015). A literature search of studies from the early 20th century found that this was also the case in lungfishes and amphibians, suggesting that mammals have a unique, derived character in the transient closure of their choanae during the fusion of their upper lip (Abramyan *et al.*, 2015) while birds retain the ancestral developmental pattern.

At stages following primary palatogenesis, Abzhanov and Tabin performed studies of the natural variation in beak size and shape amongst Galapagos Island finches (Abzhanov *et al.*, 2004). As a classical example of adaptive evolution, Galapagos finches exhibit a gradation in beak size and shape, ranging from the ground finches which have deep and wide beaks, to the fine-beaked cactus finches. Even before phenotypic variation could be observed, *BMP4* expression appears to differ in the two groups (Abzhanov *et al.*, 2004). By using the chicken embryos as an experimental model, they showed that excess *BMP4* can indeed induce an ectopic "growth zone" through increased cell proliferation, resulting in the conversion of the relatively narrow and short chicken beak

into much broader, deeper beak that resembled the bulky Ground finch beak (Abzhanov *et al*., 2004). Later studies of growth zones in songbird beaks showed that while they provide the basis for variation in shape, they also constrain the shape of the beak to a limited morphological parameter space (Fritz *et al*., 2014).

Chuong and colleagues also studied cellular proliferation and gene expression that underlie differences in beak shape, this time between the chicken, duck and cockatiel (Wu *et al*., 2004). They identified zones with higher mesenchymal proliferation, which they called 'localized growth zones', within the frontonasal mass. At stage 28, the two growth zone in the frontonasal mass converged in the chicken embryo while remaining separate in the duck (Wu *et al*., 2004); causing prolonged lateral growth in the wider duck bill as compared to the chicken. These authors also found that an increase or decrease in BMP signaling (*BMP4* expression in particular) was responsible for the disparity in break size and shape between the duck and chicken (Wu *et al*., 2004). In a second study, Wu *et al.* (2006) found that the curvature of the beak correlates with proliferation patterns. The relatively flat and broad duck bill mainly exhibits distal proliferation, while the cockatiel beak exhibits maximal proliferation proximally; resulting in the strong downward curvature characteristic of parrots (Wu *et al*., 2006). Epithelial gene expression also differed in temporal patterns. *FGF8* is typically downregulated in the chicken frontonasal mass after stage 20 (Higashihori *et al*., 2010) but persists in the duck up to stage 23 (Wu *et al*., 2006). The aforementioned studies on birds suggest that minor alterations in the spatiotemporal regulation of signaling molecules (e.g. FGFs, WNTs, SHH, BMPs) is sufficient to evolve novel beak shapes. The use of chicken as a model in evolutionary developmental biology has even extended to analysis of frontonasal architecture across Archosaurs, the ancestral group that includes dinosaurs. By manipulating the midline *WNT* responsiveness in the chicken frontonasal mass, Bhullar and colleagues were able to change the shape of premaxillae and palatine to more of an ancestral shape, resembling crocodilians and extinct archosaurs (Bhullar *et al*., 2015). Thus experimental studies on birds can also be used to model evolution of the jaws in non-avian reptiles as well.

Concluding remarks

Fortunately, natural selection has obligated early stages of development to remain constrained across amniotes, allowing for the broad use of a large number of non-human models in better understand human disease. Indeed the chicken embryo frontonasal mass resembles the human embryo far more than the mouse, which has a very deep midline furrow. The chicken is a low cost model which permits the accumulation of many replicates, something that is difficult to achieve in the mouse. Even though there is variation due to technical issues (bead placement, slight stage differences etc.), a large number of embryos can be collected, increasing the experimental rigor. The power of the chicken embryo is that all steps can be followed easily, thereby connecting genes or signals to phenotypes. Even though the chicken is not a genetic model, there is still great promise in using this avian model to test the pathogenicity of human DNA variants causing a variety of structural birth defects. Therefore we are confident that the chicken will complement the work being done in the mouse and zebrafish and will retain its place as a model organism for craniofacial development.

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