Katie Au
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Developmental Biology
Professor Cruz

Avian Chimeras as Tools for Understanding Craniofacial Development

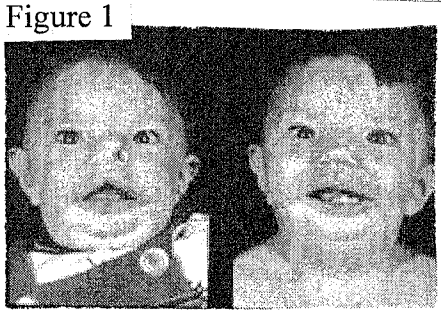
I affirm that I have adhered to the Honor Code.

Craniofacial development requires intricate cell and molecular interactions, many of which involve the neural crest, an ectodermally-derived embryonic cell population. Clinically, craniofacial birth defects are not uncommon, and an understanding of the mechanisms involved in the patterning and development of the head gives insight to the causes, prevention, and treatment of such malformations. The mechanisms by which neural crest cells differentiate and respond to and induce neighboring cell populations are not fully understood. However, through the creation of avian chimeras as a model for understanding craniofacial development, knowledge of the cellular potency, actions, and evolutionary significance of the neural crest has surfaced. This paper examines the use and potential of the avian chimera as a tool for understanding craniofacial development and it highlights some of the key findings attributable to the study of avian chimeras.

Introduction:

Craniofacial development describes the complex tissue partitioning and processes required for the formation of the skeletal, muscular, connective, and nervous tissues that comprise the head and neck. This intricate arrangement involves precise interactions of specific cell populations throughout development and disruptions of these interactions can cause fetal fatalities or congenital craniofacial anomalies (Frances-West *et al.* 2003). Such anomalies are not rare; one out of every five hundred births has a craniofacial deformity (World Craniofacial Foundation). Typical craniofacial deformities may include cleft palate and lip, craniosynostosis, or hemifacial microsomia (Figure 1). These abnormalities have diverse etiologies involving malformations of particular tissues and can be extremely debilitating. Thus, an understanding of the mechanisms and origins underlying craniofacial anomalies not only elucidates an

Figure 1



This child has a cleft lip, one of the most common craniofacial anomalies (World Craniofacial Foundation).

understanding of the developmental processes involved, but has clinical applications for the prevention and treatment of birth defects.

One crucial and multi-faceted cellular population involved in craniofacial development is the neural crest. Neural crest cells are known for diverse migratory and differentiation capabilities and contribute largely to cells of the nervous system, connective tissue, cartilage, and skeleton of the head, thus playing a major role in development (Gilbert 2003). Historically, biologists have long been fascinated by the neural crest's cellular potency, inducing properties, and mechanisms of migration and differentiation (Le Douarin 1982). Through various neural crest experiments, and specifically through the use of avian chimeras, researchers have been afforded the ability to highlight and reveal many properties of the neural crest. It is also important to note, however, that although the neural crest is fundamental in craniofacial development, the mesoderm and ectoderm also have important roles in this process. Thus, it is possible to study craniofacial development through chimeras with non-neural crest transplants, however, much of craniofacial research has centered on the neural crest and its interactions with these tissues. Consequently, this paper will focus on avian chimeras based on neural crest transplantation.

Neural Crest Cells in Craniofacial Development:

The creation of the avian chimera has emerged as a popular technique for studying the neural crest because of its ability to yield information regarding neural crest migration and differentiation in addition to the cell and molecular capabilities that govern

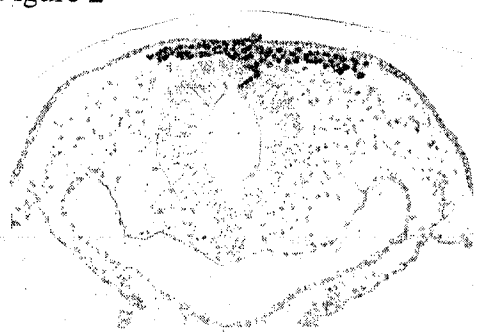
craniofacial development (Le Douarin 2004). The neural crest is an ectodermally-derived embryonic cell population, and ultimately contributes to adult structures which are diverse in cell type, function, and physical location (Gilbert 2003). In order to migrate extensively and differentiate into various cell types, neural crest cells must partake in elaborate signaling and environmental interactions (Carlson 1996). This raises fundamental questions concerning potency and timing of neural crest differentiation; perhaps neural crest cells are pluripotent as they migrate and differentiate by way of their changing environment, or perhaps a neural crest cell's fate is determined earlier and its future consequently dictates its path of migration (Le Douarin 2004). Much research has been directed toward addressing these possibilities, but many of the genes and mechanisms involved in neural crest development have yet to be elucidated. Thus, the actions of the neural crest encompass essential questions of developmental biology and the creation of the avian chimera as a model system to study the neural crest has facilitated the understanding of cell potency and communication.

Qucks, Duails, and Quicks: The Avian Chimera

An avian chimera consists of tissues with different genetic composition and is generated experimentally through transplantation. Traditionally, avian chimeras are made with quails and chicks or quails and ducks through the excision and exchange of neural crest or precursor cells from one bird to another. In 1969, Nicole Le Douarin proved the avian chimera system useful when she discovered a molecular marker of Japanese quail cells (Le Douarin 1982). Quail nuclei have a centronuclear condensation of heterochromatin which makes possible histological differentiation of quail cells from

duck or chick cells (Le Douarin 1982, Figure 2). This noteworthy and fundamental discovery exploits an endogenous quail cell marker and permits fate mapping of donor or host quail cells in the chimera.

Figure 2



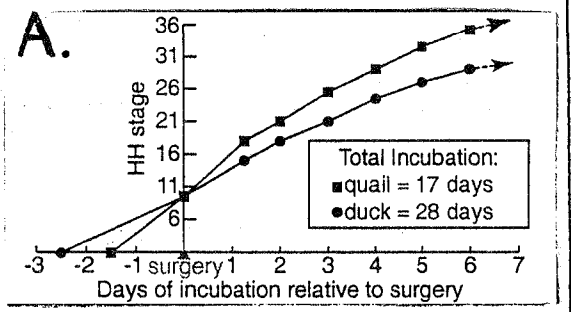
This Feulgen stained cross section of the chimera neural tube highlights the grafted quail cells which are darkly stained (Le Douarin 2004).

In addition to the benefits of the quail nuclear stain, avian chimeras have proved useful and advantageous for several other reasons. Foremost, the use of an avian model is highly appropriate because of the wealth of knowledge already available on avian embryonic development and because of the accessibility of avian embryos for experimental use. Incubators are used to grow embryos in the laboratory and precise developmental stages of the embryos can be predicted and calculated by length and temperature of incubation. Additionally, eggs can easily be windowed for experimental manipulation and after surgery it is frequently possible for the chimeric embryo to develop normally upon incubation. For experimental analysis, avian embryos are collected without difficulty and a number of whole mount, in-situ, and histological markers are available to visualize neural crest fate and gene expression.

Furthermore, phenotypic variation within avian species allows the chimera system to naturally exploit these differences (Schneider and Helms 2003). In particular, quails and ducks are remarkably different in their size, maturation rates, and onset of gene expression. Ducks take longer to reach the same stage of development as the quail and

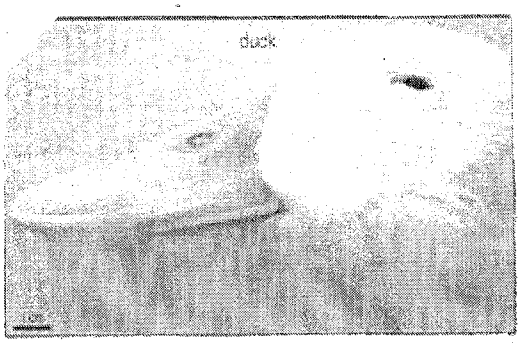
ducks hatch in 28 days as opposed to the 17 days for quail (Hamburger and Hamilton 1951, Schneider and Helms 2003, Figure 3a). Ducks also mature to be much larger animals and have morphologically distinct bills as compared to the beaks of quails (Figure 3b). Thus, exchange of neural crest cells between quails and ducks allows for observation of the contributions and inducing properties of the host and donor birds. Use of such divergent birds is particularly advantageous for experiments focused on morphology and temporal aspects of development and regulation (Schneider and Helms 2003). For this reason, several recent studies involving avian chimeras have used quails and ducks, although quail-chick chimeras are still commonly produced and were the original chimeras introduced by Le Douarin.

Figure 3



A.
Quails and ducks mature at differing rates Quails hatch in 17 days while ducks hatch in 28 days. In this study, quails and ducks were stage matched at HH9.5 for surgery (Schneider and Helms 2003).

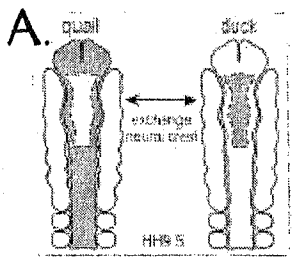
B.
Mature quails and ducks show distinct morphology in beak structure. The size of the adult is also different. Scale bars indicate 1cm (Eames and Schneider 2005).



Avian chimeras may be generated in numerous ways. For example, transplants can be unilateral or bilateral, the donor and host may or may not be developmentally stage-matched, and specific sections of the neural crest can be excised and transplanted to the same or different location on the donor embryo. There are countless possibilities for

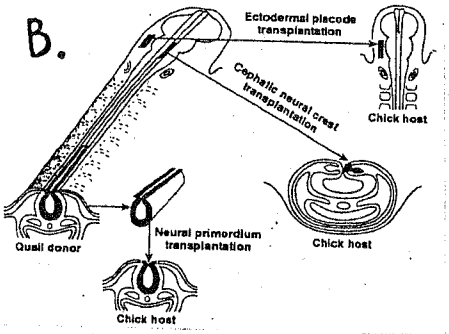
chimeric transplants, but a fundamental creation process can be described. Once a donor bird and host bird are identified, the donor and host are incubated and stage-matched to the developmental stage desirable for surgery. When the embryos are ready for surgery, the transplant tissue is surgically excised from the donor and the graft placed in the desired location of the host embryo (Figure 4). Post-surgery, the eggs may be collected and fixed for analysis at later developmental stages.

Figure 4



A) The exchange of neural crest cells between quail and duck at HH stage 9.5 (Eames and Schneider 2005).

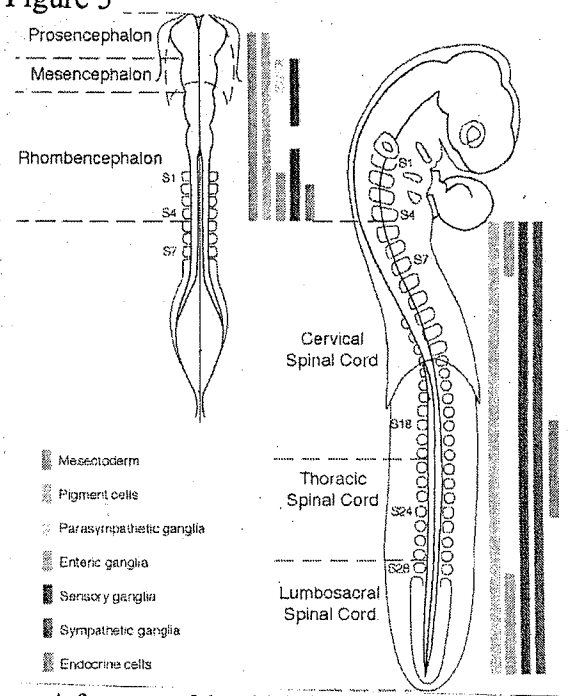
B) A schematic representation of possible experimental approaches to the creation of a chimera (Le Douarin 2004).



Lessons Learned from Avian Chimeras

Several important and innovative studies using avian chimeras have generated pivotal data toward our understanding of neural crest and craniofacial development. Nicole Le Douarin's discovery of the quail nuclear marker has enabled her to study the neural crest for the past three decades, creating a

Figure 5



A fate map of the chick (Le Douarin 2004).

library of knowledge on the neural crest. Le Douarin's work, with help from Catherine Le Lièvre, has generated what some regard as the most comprehensive fate map of cranial neural crest cells in any species (Hall 1999, Figure 5). Drew Noden of Cornell University has built on Le Douarin's studies and has done significant research on the muscular components of the head and general interactions between mesoderm and the neural crest. More recent work conducted by Richard A. Schneider at the University of California San Francisco has explored the advantages of the quail-duck "quck" system and the effects of neural crest transplants on morphologically distinct structures, such as the quail beak and duck bill and the patterning of feather buds. The work of these key researchers illustrates not only the progression of information gleaned about craniofacial development, but also the diversity of results and applications available through the avian chimera system.

Le Douarin, the pioneer of the avian chimera and quail nuclear stain, has published numerous works focused on fate mapping of both cranial and trunk neural crest and in 1982 published a comprehensive book of her work, The Neural Crest. In addition to tracing cell lineage, Le Douarin elucidated the importance of neural crest cells to the development of the nervous system. Interpretations of these results have implicated the evolutionary importance of the neural crest in the development of complex neural and head structures (Hall 1999).

In a paper entitled "The fate map of the cephalic neural primordium at the presomitic to the 3-somite stage in the avian embryo," Le Douarin and Gerard Couly exhibit a classic example of her work (Couly and Douarin 1988). Her experiments

include six quail-chick transplants, each with a grafting site specific to a region of the anterior neural primordium of the prosencephalon (Couly and Douarin 1988, Figure 6a). Histological analysis of the resulting chimeras reveal the future of the cell populations at each grafting site, thus allowing for a fate map of the neural primordium at the 3-4 somite stage (Couly and Douarin 1988, Figure 6b). Such a map makes possible a projection of these fates to a later developmental stage of the avian and may apply to analogous structures in humans (Couly and Douarin 1988, Figure 6c). In this study, Le Douarin and Couly linked the lateral neural folds to the presumptive skin area of the forehead, and regions of the medial neural plate to the telencephalon and diencephalon (1988).

Figure 6

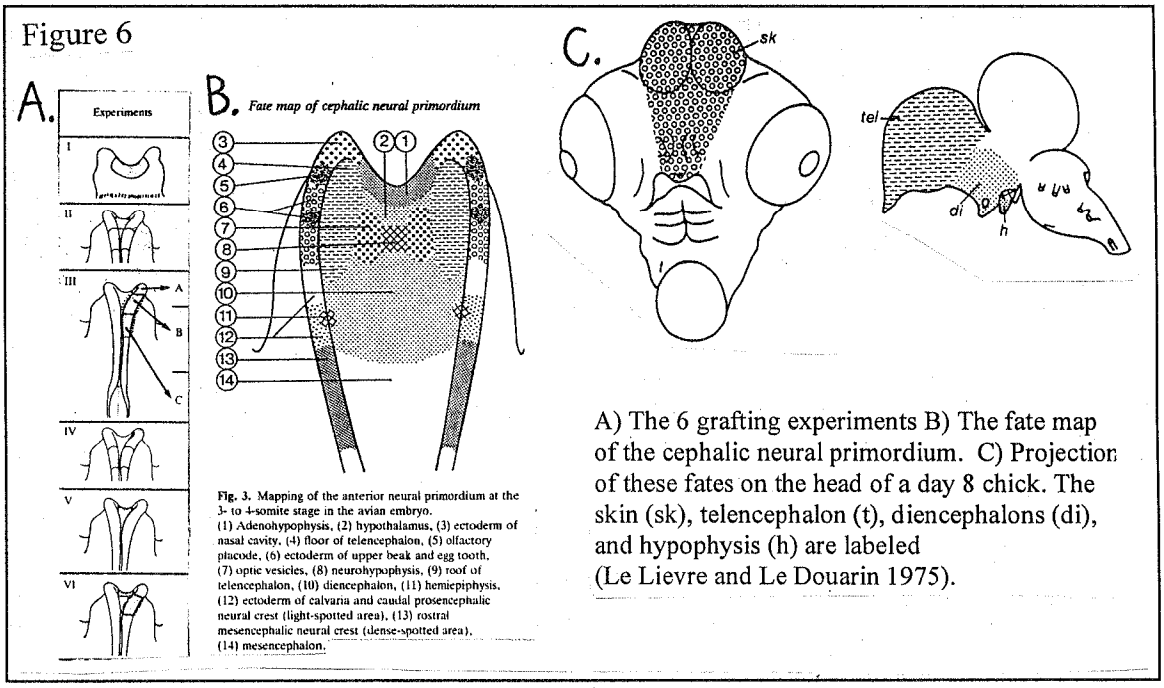


Fig. 3. Mapping of the anterior neural primordium at the 3- to 4-somite stage in the avian embryo. (1) Adenohypophysis, (2) hypothalamus, (3) ectoderm of nasal cavity, (4) floor of telencephalon, (5) olfactory placode, (6) ectoderm of upper beak and egg tooth, (7) optic vesicles, (8) neurohypophysis, (9) roof of telencephalon, (10) diencephalon, (11) hemiepiphysis, (12) ectoderm of calvaria and caudal prosencephalic neural crest (light-spotted area), (13) rostral mesencephalic neural crest (dense-spotted area), (14) mesencephalon.

A) The 6 grafting experiments B) The fate map of the cephalic neural primordium. C) Projection of these fates on the head of a day 8 chick. The skin (sk), telencephalon (t), diencephalons (di), and hypophysis (h) are labeled (Le Lievre and Le Douarin 1975).

These results, and Le Douarin's additional mapping of head structures, are of particular interest to the understanding of craniofacial anomalies. Persons with DeMyer's syndrome, for example, have telencephalic and diencephalic defects associated with nasofronto-premaxillary hypoplasia (Couly and Douarin 1988). Thus, certain craniofacial anomalies may be explained by positional interaction of presumptive

structures in the embryo (Couly and Douarin 1988). In other significant fate-mapping studies, Le Douarin and Le Lièvre demonstrated that the neural crest contributes to the meninges of the forebrain and when absent, the forebrain undergoes apoptosis as the head mesenchyme cannot compensate for the loss of neural crest cells (1975). For this reason, some have projected evolutionary significance on the neural crest for its role in the advancement of the primitive brain and growth of the forebrain (Frances-West, et al. 2003).

Drew Noden has furthered the fate mapping work of Douarin and adapted the avian chimera system to study the necessary interactions between the neural crest and the mesoderm involved in head formation. In his paper, "The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissue," Noden makes some momentous observations regarding the potency and migration of neural crest cells in the branchial arches (1983). Noden notes that each of the brachial arches give rise to morphologically distinct skeletal, connective and muscular tissues which are derived from the neural crest (1983). Thus, Noden used the quail-chick chimera system to transplant presumptive first arch neural crest to areas of presumptive second and third arch neural crest cells. Noden found that the host birds developed arch irregularities and that the beak-like structures normally found in the first arch developed instead in the second or third arch neck region (1983). Thus, it appears that although the first arch cells were transplanted to a different region, they executed a native developmental program in a novel environment, thereby creating duplicate structures in the host bird. However, further analysis revealed that even though the transplanted cells carried out an intrinsic program, grafted cells migrated in pathways which corresponded to the arch in which

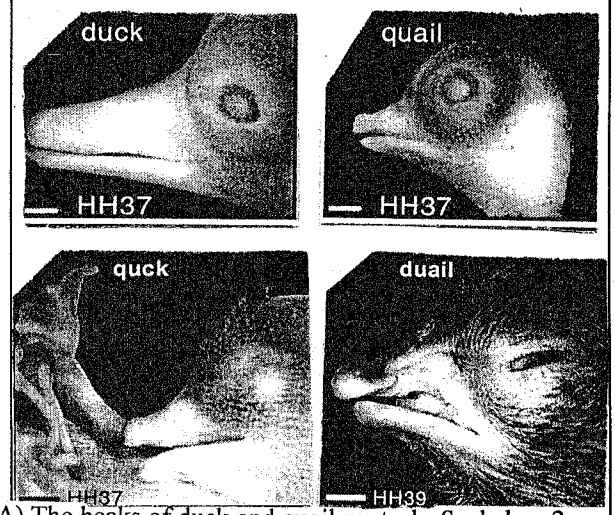
they were transplanted (Noden 1983). These results proved extremely interesting and addressed questions regarding neural crest potency and migration. Noden concluded that the patterning of branchial skeletal and connective tissues was ultimately determined by a neural crest cell population before migration and not by the pharyngeal pouches (1983). Thus, in addition to contributing to Le Douarin's work on neural crest fate, Noden has demonstrated a role for the neural crest as an inducer of neighboring tissues after migration. Noden's work and his conclusions have been furthered by more recent studies and the advances which have allowed for the study of gene expression in the avian chimera.

In the past few years, Richard A. Schneider has expanded the work of both Le Douarin and Noden by implementing genetic analysis and the use of quails and ducks in the avian chimera system. Recent technologies in cell and molecular biology have made histochemical investigation of gene expression in chimeras possible and the use of distantly related birds has facilitated the examination of morphological and temporal aspects of development. Schneider's unique decision to use the quail-duck chimera, or "qucks" and "duails," has generated fascinating information on the origins of beak morphology, a topic which also relates to evolution and interspecific variation (Schneider and Helms 2003).

In his paper with Jill Helms, "The Cellular and Molecular Origins of Beak Morphology," Schneider exchanged neural crest cells of the presumptive beak region from both quails and ducks to study the effects of transplant on beak morphology (2003). Ducks normally have long, broad bills and quails have short, convex beaks (Schneider and Helms 2003). However, when quail neural crest was transplanted to a duck host, the

duck developed a quail-like beak and duck neural crest grafted to a quail host developed a duck-like bill (Schneider and Helms 2003, Figure 7a, Figure 7b).

Figure 7



A) The beaks of duck and quail controls. Scale bar, 2mm
B) The transformed bills and beaks of qucks and duails. Also note the feet of the quck remains the webbed like a duck. (Schneider and Helms 2003).

These findings concur with Noden's observations of transplanted neural crest executing autonomous programming (Noden 1988). However, Schneider also observed that non-neural crest derived structures of the beak and fronto-nasal process were also transformed to resemble that of the donor bird (2003). Thus, not only do neural crest cells self-determine their morphology, but they influence neighboring tissues as well. Molecular analysis through in-situ hybridization also showed that quck mesenchyme expresses genes prematurely; this demonstrates that the host duck mesenchyme gene expression was accelerated by the quail neural crest (Schneider and Helms 2003). Thus, the differing maturation rates of the duck and quail reveal the extent to which neural crest regulates non-neuronal adjacent tissues and causes a temporal shift in gene expression. In this study, the modification of the avian chimera system to use only quails and ducks offered an innovative way to study the timing of gene expression and also shed light on interspecific beak morphology.

Schneider's paper acknowledges our understanding of beak evolution as a product of natural selection, but notes that a developmental basis for variation within species has not been described (Schneider and Helms 2003). He proposes, as evidenced by the

neural crest regulation of beak morphology, that perhaps it is the neural crest which plays a primary role in the emergence of interspecific variation (Schneider and Helms 2003). Changes in neural crest and the regulation of adjacent cell populations are responsible for the accomplishment of interspecies differences such as the duck bill and quail beak. Schneider's work, with credit to the extensive work of his predecessors, imparts a new height to the possibilities of craniofacial studies using avian chimeras and genetic analysis.

Conclusion

With careful use of the avian chimera system, researchers have been able to assign fates to neural crest cell populations, understand the derivatives of complex adult craniofacial structures, answer questions regarding cellular potency and induction, analyze the regulation of genes required for head development, and hypothesize evolutionary roles for the neural crest. Though the avian chimera is created with a simple microsurgical procedure, it has proved an invaluable asset to development biology, molecular genetics, the craniofacial sciences, and clinical medicine. Some suggest the head is the most anatomically sophisticated structure of the vertebrate and unfortunately, complex developmental processes present opportunity for errors which can lead to various malformations and birth defects (Gilbert 2003). Thus, as the avian chimera facilitates our understanding of craniofacial development, it is the hope that our knowledge will someday include improved understandings of birth defects and affect our abilities to prevent and treat these anomalies.

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World Craniofacial Organization. "About Us." < http://www.worldcf.org/about_us.cfm>