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Piecing together the vertebrate skull

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Summary

In a 1993 *Development* paper, the quail-chick chimera system was applied to decipher the embryonic origin of the bones of the head skeleton of the avian embryo. The data reported in this article, together with those from previous works, allowed us to assign a precise embryonic origin to all the bones forming the avian skull. It turned out that their major source is the neural crest, with additional contributions from the head paraxial mesoderm and the first five somites, laying to rest a long-standing debate about the origin of the skull.

The germ-layer theory of skeletal development

Whether vertebrate skeletal tissues can originate from both mesodermal and ectodermal layers has been a controversial issue since the early times of embryology. The germ-layer theory formulated by Karl von Baer in 1828 (von Baer, 1828) firmly pointed out that 'homologous structures in different animals are formed of material from corresponding layers'. Hence, it was considered that, like most of the vertebrate skeleton (i.e. that of the trunk and limbs), the skull vault and facial bones had to obey the same rule and be derived from the mesodermal germ layer.

von Baer's concept had the merit of systematizing various observations and, for this reason, was rapidly considered as a 'law'. It was, however, based on insufficient morphological data and had not been tested experimentally. As a consequence of this belief in von Baer's systematization, the claim put forward by several authors at the end of the 19th century (Kastchenko, 1888; Goronowitsch, 1892; Goronowitsch, 1893) that some mesenchymal cells in the head were derived from the ectoderm via the neural crest (NC), was far from unanimously accepted and even led to a violent controversy. The disagreement was amplified by Julia Platt's report that not only ganglia and nerves but also the cartilage of the visceral arches and teeth dentine were derived from ectoderm in the salamander *Necturus* (Platt, 1893; Platt, 1897). Platt persisted and coined the term 'mesectoderm' to distinguish the mesenchyme of ectodermal origin from the mesoderm-derived mesenchyme. In the following years, several authors denied the capacity of ectoderm to generate mesenchymal cells and, a fortiori, cartilage, bone and teeth (see Landacre, 1921).

It was only from the 1920s that the mesectoderm (also designated 'ectomesenchyme') problem was properly analyzed. Numerous investigations carried out primarily in amphibians and fish in the first half of the 20th century confirmed the existence of ectomesenchyme in all vertebrate species studied and demonstrated its role in the construction of the visceral arch skeleton and also of the rostral part of the trabeculae cranii.

The neural crest

Although the NC had been identified in the chick embryo by Wilhelm His in 1868 (His, 1868) as a band of cells lying between the neural tube and the future epidermal ectoderm, most early investigations of the NC were carried out on amphibians and fish and not on amniotes. Thorough experimental analyses of the development of the cranial NC in the amphibian embryo were performed by Hörstadius and Sellman (Hörstadius and Sellman, 1941; Hörstadius and Sellman, 1946) and de Beer (de Beer, 1947). The monograph written by Sven Hörstadius in 1950 (Hörstadius, 1950) remains a milestone on the road to understanding the role of this transitory and rather elusive structure of the vertebrate embryo. In amniotes, the large number of embryonic cells made following the migration of these cells difficult, particularly in the absence of a reliable and stable cell marker. This only became possible in the late 1960s and, among amniotes, the chick embryo appeared to be the most suitable for investigations on NC cell fate and migration. Vital dye tissue staining or tritiated thymidine ([^{3}H]TdR) radio-isotopic labeling of dividing cells could be used as cell markers. These procedures suffered several drawbacks, one of which being that the labeling they provided was unstable and not strictly specific, as it could spread to neighboring cells that should be unlabeled. Moreover, they were not devoid of a certain level of toxicity. The advent of the quail-chick chimera technique (Le Douarin, 1969; Le Douarin, 1973) opened a new avenue for the study of cell migration during the development of the avian embryo. This technique is based on the difference in the structure of the cell nucleus in two species of birds: the quail and the chick. In all the cell types of the quail, a large mass of heterochromatin is associated with the nucleolus and can be visualized by the Feulgen reaction. It is also possible to recognize quail from chick cells using monoclonal antibodies that react to species-specific antigens. This technique was readily used, not only in my own laboratory but also in others', namely those of Malcolm Johnston and Drew Noden in the USA and by Andrew Lumdsen and collaborators in England (e.g. Johnston et al., 1979; Noden, 1978; Köntges and Lumsden, 1996).

Our first attempt, with Christiane Le Lièvre, to decipher the role of the NC in the development of the head skeleton goes back to the early 1970s (Le Lièvre and Le Douarin, 1975). It involved substituting the chick cephalic vesicles (including both the neural tube and the neural folds) with their quail counterpart (and vice versa) in embryos just prior to the onset of NC cell (NCC)

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A *Development* classic

The year 2012 marks 25 years since the journal *Development* was relaunched from its predecessor, the *Journal of Embryology and Experimental Morphology* (*JEEM*). In 2008, we fully digitised our *Development* and *JEEM* archives, and made them freely available online. At the same time, we took the opportunity to revisit some of the classic papers published in *JEEM*, in a series of commentaries (see Alfred and Smith, 2008). Now, to mark a quarter century of *Development*, we have been looking through our archives at some of the most influential papers published in *Development*'s pages. In this series of Spotlight articles, we have asked the authors of those articles to tell us the back-story behind their work and how the paper has influenced the development of their field. Look out for more of these Spotlight papers in the next few issues.

migration. Owing to the stability of the nuclear marker provided by quail cells, the migration and ultimate localization of the crest cells could be established. The results obtained in my laboratory and in that of Johnston and Noden (Johnston et al., 1979; Noden, 1978) clearly showed that, when the implant contained the prosencephalic and mesencephalic vesicles plus rostral rhombencephalon, the facial part of the skull was of graft origin. Transplantation of the rest of the rhombencephalon resulted in the labeling of the hyoid cartilage by donor cells (see Le Douarin, 1982; Le Douarin and Kalcheim, 1999).

In these experiments, Christiane and I concentrated our attention essentially on the facial skeleton. The embryos were sacrificed at stages that turned out to be too early for the skull vault skeleton to be fully completed. Still, our experiments suggested that, apart from the anteriormost part of the frontal bone, which was fully NC derived, the skull vault appeared to be of mixed mesodermal and ectomesenchymal origin. Our work showed that, in addition to cartilage and bone, the ectomesenchyme produces a large variety of tissues in the head and neck.

These experiments, therefore, demonstrated a complex intermingling of cells from various origins in head morphogenesis. The major message was that the ectoderm was the main contributor to the embryogenesis of the vertebrate head. The mesenchyme forming the dermis of the facial and ventral neck area was shown to be of NC origin, as was the mesenchymal component of the pituitary gland and of the glands arising from the pharynx and buccal epithelium (salivary glands, thyroid, parathyroid and thymus).

The ontogeny of the thymus in the quail-chick chimeras that had received rhombencephalon grafts particularly attracted our attention. The host endodermal buds arising from the 3rd and 4th branchial pouches were surrounded by quail cells that invaded, together with blood vessels, the mass of chick epithelial cells forming the thymic rudiment. The blood vessel endothelium was always of host origin and derived from the mesoderm of the head, whereas pericytes were NC derived. Such was the case in all blood vessels of the head. After the thymus became lymphoid, all the lymphocytes and dendritic cells it contained were of host type; the latter were shown to invade the thymic rudiment at a precise time of development (for details, see Le Douarin et al., 1996). These data were the foundation of further studies on the mechanisms of tolerance to self (Coutinho et al., 1993). Another important result from these experiments concerned the ontogeny of the striated muscles of the head and neck. The central core of the branchial arches, which will form striated muscle, is colonized by NC cells, which differentiate into the muscle connective tissue. In quail-chick chimeras, these connective tissue cells were derived from donor cells, whereas the vascular endothelium of the intra- and peri-muscular blood vessels was of host type.

This brief overview shows that our first attempts to analyze the fate of the cephalic NC (Le Lièvre, 1974; Le Lièvre, 1978; Le Lièvre and Le Douarin, 1975) were highly productive as, even in the early studies in lower vertebrates summarized by Hörstadius in 1950 in his famous monograph entitled *The Neural Crest* (Hörstadius, 1950), the contribution of the ectomesenchyme to the variety of tissues and structures in the head had not been described.

This series of analyses, however, left a lot of space for further investigations. I started my career as an independent researcher in the University of Nantes and, in 1976, I moved to Paris where I succeeded my former supervisor Etienne Wolff as director of the Institute of Experimental Embryology and Teratology at Nogent-sur-Marne. Some time later, Gérard Couly, a Doctor of Medicine and surgeon who specialized in the facial repair of children at Necker-Enfants Malades Hospital, joined my laboratory part time (one day per week). He was eager to investigate the early steps of facial and head development, even though our model was not human – not even a mammal, but a bird! He considered, as I did, that it would be interesting to investigate with more precision the origin of each of the bones of the head skeleton: not only its facial components, but also the skull vault and the occipital and otic regions that our previous work with Christiane had not explored in depth. In the earlier work, grafts had been made from the 5- to 6-somite stage onwards and constituted not only the neural fold, which gives rise to the crest cells, but the whole encephalic vesicles comprising neural tube and neural fold. This procedure often generated developmental defects in the skull vault, and we had examined the embryos too early to analyze the skull in detail. Gérard thus decided to revisit this problem. He started by constructing a series of fate maps of the different tissues that participate in head morphogenesis. He performed grafts of very minute embryonic territories belonging to the neural fold, the neural plate (Couly and Le Douarin, 1985; Couly and Le Douarin, 1987), the cephalic ectoderm (Couly and Le Douarin, 1988; Couly and Le Douarin, 1990) and the cephalic mesoderm (Couly et al., 1992).

[Couly] was eager to investigate the early steps of facial and head development, even though our model was not human – not even a mammal, but a bird!

According to our previous results, we expected the posterior part of the frontal and parietal bones to be of donor type when definite regions of the head paraxial mesoderm were substituted with their counterpart in chick recipients. This was not the case (Couly et al., 1992). Another series of experiments involving the transfer of the quail/chick cephalic neural fold in 3-somite-stage embryos showed that, in fact, the skull vault was also of NC origin. Finally, the precise contribution of the NC, the cephalic paraxial mesoderm and the first somites to the head skeleton was clearly established for the first time in the 1993 paper (Couly et al., 1993), revealing a triple origin of the head skeleton in the avian embryo: all the facial skeleton, the frontal, parietal and squamosal bones, and part of the otic capsule are derived from the NC (Fig. 1, red); the bones labeled by quail cells in the experiments involving the graft of the cephalic paraxial mesoderm were the corpus sphenoidalis and the otic capsules (partly) (Fig. 1, blue); and the first five somites contribute to the occipital and pars ampullaris of the otic capsule (Fig. 1, green).

Later studies in the mouse using the Cre-Lox system and promoters of genes expressed in the early NC (e.g. *Wnt1* and *Sox10*) to selectively label certain cell types confirmed the results obtained in the avian embryo (Chai et al., 2000; Jiang et al., 2000; Jiang et al., 2002; Matsuoka et al., 2005). However, a discrepancy on the origin of the parietal bone in birds and mammals arose in some of these experiments (Jiang et al., 2002).

What followed on from these findings?

The recognition of the segmental structure of the rhombencephalon, which is divided from the early stages of neurogenesis into eight rhombomeres (r), was a landmark event in research on NC ontogeny (see Lumsden and Keynes, 1989; Lumsden, 1990). Subsequently, the fact that rhombomeres are characterized by the expression of definitive sets of regulatory

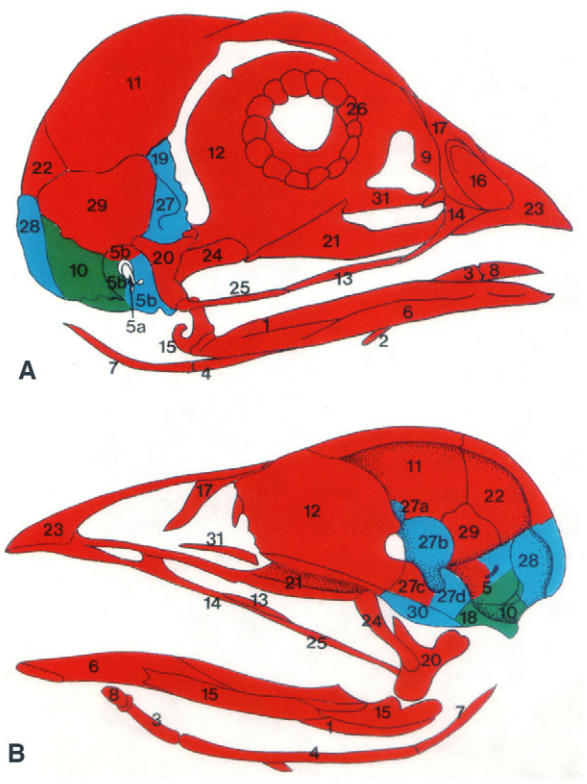

Fig. 1. Schematic drawing of cephalic skeleton of bird taken from the 1993 paper (Couly et al., 1993). (**A**) Right external view. (**B**) Right internal view. Red, skeleton of NC origin; blue, skeleton of cephalic mesoderm origin; green, skeleton of somitic origin.

genes, such as Hox genes (for a review, see Krumlauf, 1994), led to renewed interest in the contribution of NC cells to the complex processes of head morphogenesis. The fate of the midbrain and hindbrain NC was re-examined. To this end, definite fragments of the chick neuroepithelium, corresponding either to the mesencephalon (anterior or posterior half) or to neuroepithelial segments coinciding with individual presumptive rhombomeres, were replaced in chick embryos with their quail counterparts (Köntges and Lumsden, 1996; Couly et al., 1996; Couly et al., 1998). These experiments demonstrated that the cephalic NC is divided into two domains.

The rostral portion, extending from the mid-diencephalon down to the boundary between r2 and r3, produces the cells forming the cranial vault and the entire facial skeleton down to the anterior half of the basihyal bones. This corresponds to a region of the vertebrate body in which the genes of the Hox complex are not expressed; this region was designated the 'facial skeletogenic NC' (FSNC). Forced expression in the FSNC region of Hox genes normally expressed in the rhombencephalic NC (e.g. *Hoxa2*, *Hoxa3*, *Hoxb4*) abolished the capacity of the FSNC to differentiate into skeletal tissues (Creuzet et al., 2002). Posteriorly to r3, the cartilages of the hyoid apparatus develop from Hox-expressing cells. This is also the case for the vertebrae and for the limb skeleton. This striking result, showing that Hox gene expression has opposite effects on skeletal tissue differentiation according to the level of the body, remains an unexplained phenomenon. This is presently the subject of research in our laboratory and others.

Another observation was very intriguing: surgical ablation of the FSNC, the part of the neural fold generating the NC cells that form the facial and skull vault skeleton, results not only in absence of these structures but also in severe malformations of the brain. Moreover, similar abnormalities were observed following the forced expression of *Hoxa2* in the same FSNC territory (Creuzet et al., 2002; Creuzet et al., 2004; Creuzet et al., 2006). It was found that this part of the NC exerts a strong regulatory control on the development of the pre-otic brain by producing bone morphogenetic protein (BMP) antagonists that control the amount of Fgf8 secreted by the anterior neural ridge (ANR), one of the two 'secondary brain organizers'. This newly discovered function of the cephalic NC (Creuzet, 2009) constitutes a strong argument in favor of the 'neural crest and the new head' concept formulated in 1983 by Gans and Northcutt (Gans and Northcutt, 1983; Le Douarin et al., 2012; Le Douarin and Dupin, 2012).

The 'new head' concept and the role of the NC in the evolution of vertebrates

In 1983, Gans and Northcutt proposed, on the basis of embryological and comparative anatomical data, that the NC played a major role in vertebrate evolution by permitting the construction of a 'new head', which was absent in their protocordate ancestors (Gans and Northcutt, 1983). The only extant cephalochordate, Amphoxius, is generally considered to be very similar to the putative ancestor of vertebrates. It is devoid of NC, has a small vesicle at the rostral end of its hollow neural tube, and has no sense organs and no skeletal tissues. Considering the structures that are novel in vertebrates, Gans and Northcutt noted that these are essentially the brain and associated sense organs derived from the ectodermal placodes, which, like the NC, are vertebrate innovations. These structures appeared to result from an addition to the chordate primitive trunk rather than a transformation of its anterior part. In extant Amphoxius, the notochord reaches the extreme anterior end of the body, forming the rostrum with which the animal burrows into the sand. In vertebrates, the forebrain with its associated sense organs lies rostral to the tip of the notochord. Thus, the transition between cephalochordates and vertebrates involved the apparition of a 'new head' mostly derived from the ectoderm via the neural epithelium, the NC and the ectodermal placodes.

The neural crest played a major role in vertebrate evolution by permitting the construction of a 'new head'

These transformations allowed a change in lifestyle, going from filter feeding to predatory behavior. The vertebrate brain developed considerably and the embryological data discussed here showed that the NC played multiple roles in this process. Through its ability to yield skeletal tissue (see Le Douarin and Dupin, 2012), the NC provided protection for the developing fore- and midbrain. It also provided the forebrain with meninges. Last, but not least, the cephalic NC exerts a regulatory role on the growth and patterning of the preotic brain.

Concluding remarks

Our 1993 article, now selected as a *Development* classic, was one important step in a journey that was initiated when the quail-chick cell-marking technique was devised in 1969. This technique allowed cell migrations and fates to be reliably followed during the entire course of embryogenesis in the avian embryo. This retrospective discussion of that work gives me the opportunity to

stress that the knowledge of a complex developmental process, resulting from precise descriptive studies, may provide unexpected and far-reaching perspectives. This story of the NC and the evolution of the vertebrate phylum is one such example.

It also gives me the occasion to recall with emotion the teamwork from which these notions emerged, and the importance of my colleagues in this patient and long enquiry of how the vertebrate head develops. I wish to acknowledge the pioneering efforts of Christiane Le Lièvre; the exceptional competence of Prof. Gérard Couly, his skill for microsurgery and his passionate interest for deciphering the arcanes of the building of the head skeleton, which, as a surgeon, he had to repair in children born with craniofacial anomalies; the skills and intelligence of Dr Anne Grapin; and, more recently, the accomplishments of Dr Sophie Creuzet.

Finally, to a question asked by the editor in commissioning this piece: why did we choose *Development* to publish this work? The answer is straightforward: a large part of the work of my laboratory was published first in the *Journal of Embryology and Experimental Morphology*, which became *Development* 25 years ago. This non-commercially driven journal has always been selective for the quality of research. It is a science-dedicated publication, truly devoted to the advancement of knowledge.

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