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Insights into neural crest migration and differentiation from experimental embryology

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In this essay, I discuss two studies published in the *Journal of Experimental Embryology and Morphology* that represent how experimental embryologists began to deal with the issue of the vertebrate body plan. In one such study by Nicole Le Douarin and Marie-Aimée Teillet, the neural crest was unequivocally identified as being the origin of the chick enteric nervous system through careful chimeric experiments and histological analyses. In the second, Michael Rickmann and colleagues showed how to combine immunohistochemical and experimental techniques in a study of the segmental patterning of the spinal nerves of the chick embryo.

Introduction

The strategy used to construct the vertebrate body plan is most vividly exemplified by the differentiation of the neural crest, as well as by the arrangement of repeated embryonic elements that result in iterative anatomical patterns. Amongst these patterns, the peripheral nervous system and tissues derived from the somites (or that are patterned in association with somites) have been studied most extensively. These studies acted as the basis, for example, for the concept that the Hox code – an evolutionarily conserved molecular mechanism of specification – connects comparative and evolutionary morphology (Burke et al., 1995). According to this concept, the vertebrate trunk can be described as an array of somitic segments (vertebrae), each of which differentiates into a specific morphological identity, depending on its positional values as defined by the Hox code.

When developmental biologists began to investigate the concept of the body plan – the developmental blueprint for how the animal body is laid out – in the new context of cell and molecular biology, they had to learn how to examine the anatomical fabric of the embryo not just observationally, but with experimental techniques that provided cellular and mechanistic insights into embryogenesis. The work of the French developmental biologist, Nicole Le Douarin and her colleagues, achieved this transition and is characterized by their analysis of the dynamic mechanism of cell movement and differentiation in the chick embryo. Long before we used the term 'body plan', they performed their research using body plan perspectives, and their contributions provided a crucial link between cell biology and anatomy, and laid the foundation for molecular developmental biology. As I discuss in this essay, one particular study by Le Douarin and Teillet that was published in *the Journal of Experimental Embryology and Morphology* (*JEEM*) in 1973 (Le Douarin and Teillet, 1973) showed that the enteric nervous system (ENS) of the avian embryo arises from a specific axial level of the neural crest, and that the cells that originate from the neural crest appear to migrate a long distance to reach the gut wall. Although a neural crest origin of the ENS had been assumed by comparative embryological observations, this work provided the first direct evidence to support this assumption and also introduced an innovative cell labeling technique that is still widely employed in modern developmental experiments.

The second *JEEM* paper I discuss is by Michael Rickmann, James Fawcett and Roger Keynes (Rickmann et al., 1985). This study also exemplifies the early switch to a molecular-level understanding of the segmental body plan of vertebrates. As a sequel to the experimental approach that elucidated the developmental mechanisms of mesodermal segmentation and its related structures, such as neural crest derivatives, these authors applied immunohistochemical methods to characterize the molecular nature of another segmentation-associated process, in this instance spinal nerve patterning. This study was also based on a precise knowledge of embryonic morphological patterns, and represents a gold standard for the scientific evaluation of immunoreactivity, which nowadays tends to be taken for granted.

The power of avian chimeras

Early experimental embryology developed, more or less, from the invention of cell lineage labeling techniques, as used by Spemann and Mangold in their classic experiment (Spemann and Mangold, 1924), and from the clonal analyses made possible by these approaches. In these early cell lineage studies, chimeric embryos were constructed by transplanting a piece of tissue from one embryo (the donor) to another (the host; Fig. 1). In order to know the developmental fate of the transplanted cells and their interactions with the environment, these cells have to be labeled properly prior to transplantation. Classically, cells were simply deleted from the embryo (ablation) or colored with vital dyes, which tended to become diluted. The discovery of interspecies transplantation, such as Le Douarin and colleagues' use of Japanese quail donor tissue transplanted to donor chick embryos to create chimeric embryos, was a huge technical step forward for the Le Douarin laboratory that initiated a series of very successful studies. Both the chicken, *Gallus gallus*, and Japanese quail, *Coturnix japonica*, belong to the family Phasianinae, but the heterochromatin of quail cells is more compact than that of chicken cells, allowing the two species' cells to be readily distinguished from each other using relatively simple histochemical staining techniques, such as Hematoxylin and the Feulgen DNA staining technique. Indeed, when I tried several other galliform species as possible donors for such cell lineage transplantation studies, including the common pheasant and bamboo partridge, I found that it was only the Japanese quail that showed good staining with the Feulgen method. Thus, it was actually the development of chicken chimeras created by the grafting of Japanese quail tissue, which possessed an enduring, stable and natural genetic marker, that enabled Le Douarin and colleagues to construct chimeric embryos for their neural crest cell-lineage tracing studies.

Using a sharpened 'microscalpel' that was made out of a sewing needle (which Le Douarin's disciples in Japan called 'Issunboushi's sword' after the diminutive hero of Japanese folklore), Le Douarin and Teillet excised pieces of neural tube fragments with the neural

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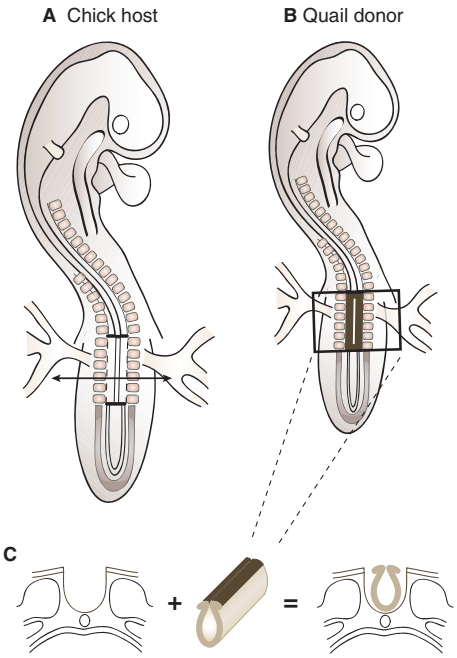

Fig. 1. The construction of avian chimeras. (A,B) Schematics of a chick host (A) and a quail donor (B) embryo at stage 12-13 of development. (**A**) A region of the neural tube, as indicated by the black bars, is removed from the chick host. (**B**) A region of the neural tube that includes the neural crest (dark shading) is removed from the quail donor. It is trypsinized and used to replace the area of excised tissue in the host chick. Owing to the more compact appearance of heterochromatin in quail cells, the donor cells can be distinguished from those of the host in the chimeric embryos. (**C**) The excised neural tube tissue and its transplantation into the host chick embryo (shown in transverse section; arrows in A mark the level of the transverse section). Redrawn, with permission, from Le Douarin (Le Douarin, 1982).

crest still attached to them from various axial levels of quail embryos (Le Douarin and Teillet, 1973). They then transplanted these fragments into chick embryonic hosts of the same stage, into a scar made at an identical axial level to that from which the host neural crest had been removed (so-called isochronic and isotopic transplantations; Fig. 1). Thus, in the host chick embryo, the neuroectodermal component of the neural tube at a certain level was replaced by quail-derived tissue. The transplantation was performed at stages when neural crest cells had not yet migrated, or shortly after they had begun to migrate, so that host neural crest cells were replaced with quail neural crest cells in the chimera. Le Douarin and colleagues grew the chimeric embryos to the stage when quail-specific pigmented feathers could be recognized (showing that the operation had been successful), and then sacrificed them for histological analyses. Le Douarin and Teillet employed this technique to explore the developmental origins of the autonomic nervous system (Le Douarin and Teillet, 1973).

The vertebrate autonomic nervous system is largely classified into sympathetic and parasympathetic systems, of which the former arises at thoracic levels with comparatively short pre-ganglionic axons, and the latter arises from cranial (vagal) or sacral levels with longer pre-ganglionic fibers (Fig. 2). The cranial component of the parasympathetic system, or the vagus nerve, is especially intriguing with its possession of a long intestinal branch (pre-ganglionic fibers) that innervates the gut (see Fig. 2). The post-ganglionic neurons form nerve networks in the gut wall, which are called Meissner's plexus and Auerbach's plexus. Characteristically, the avian gut contains Remak's ganglion made of post-ganglionic neurons (see Fig. 3).

Nine days after the surgery, the digestive tract of transplanted embryos was fixed for histological observation. Alternate paraffin sections were split between two series of slides, so that the Feulgen reaction could be carried out on one series, while the other series could be used for the histological analysis of neurons. Based on a number of chimeric embryos, Le Douarin and Teillet determined the level at which neural crest cells gave rise to the ganglia of the ENS for each part of the digestive tract (Fig. 3).

What was shown by the findings of this paper is that the post-ganglionic neurons of the autonomic nervous system have their origins in specific anteroposterior (AP) levels of the neural crest along the axis. They found that two separate regions of the neural crest, one ranging from somite 1 (or mid-otic) to somite 7, and the other in the unsegmented paraxial mesoderm at the sacral level of the axis, gave rise to ENS neurons in the gut; by contrast, the neural crest outside of these regions did not. Moreover, Le Douarin and Teillet reported that the neural crest cells from the sacral region appeared to migrate later than did the neural crest cells from the more caudal region, so revealing that neural crest development proceeds in a rostral-to-caudal direction. Interestingly, these two axial levels correspond to the cranio-sacral origins of the parasympathetic nerves. The ENS-forming neural crest overlaps with the axial levels of the neural tube from which the pre-ganglionic nerves arise (the same is true for the sympathetic nervous system; see Fig. 3), showing that the autonomic nervous system as a whole might be specified developmentally along the anteroposterior axis as a functional unit, involving the neural tube and the neural crest simultaneously.

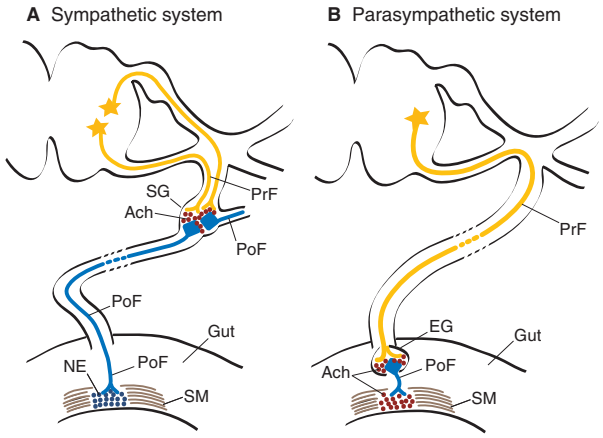

Fig. 2. Anatomy of the gut autonomic nervous system. (A,B) Cross sections of an amniote neural tube (NT, top) to show (A) the sympathetic and (B) the parasympathetic (PS) systems of the vertebrate autonomic nervous system. (**A**) The sympathetic system consists of comparatively short pre-ganglionic (yellow) axons that arise at thoracic levels. (**B**) The PS system has longer pre-ganglionic (yellow) axons that arise at the cranial (vagal) and sacral levels of the axis. The post-ganglionic neurons form nerve networks in the gut wall called Meissner's plexus and Auerbach's plexus. Note the presence of the enteric ganglia in the wall of the gut. Ach, acetylcholine, EG, enteric ganglia; NE, noradrenalin; PoF, post-ganglionic fibers; PrF, pre-ganglionic fibers; SG, sympathetic ganglia; SM, smooth muscles. Redrawn, with permission, from Le Douarin (Le Douarin, 1982).

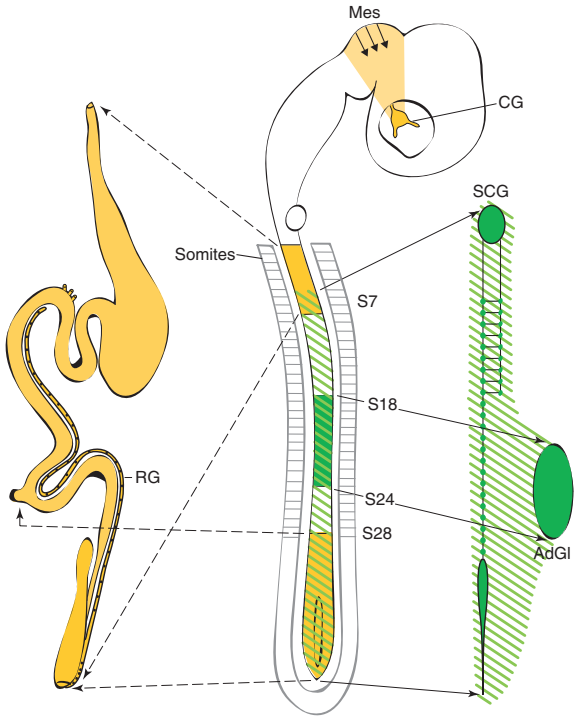

Fig. 3. Neural crest origin of the ENS. Fate mapping of autonomic ganglia along the neural crest, showing the origins of parasympathetic (left) and sympathetic (right) ganglia. The results of Le Douarin and Teillet (Le Douarin and Teillet, 1973) are shown on the left of this figure. (Centre) A schematic of a chick neural axis, anterior to the top, showing the positions of somites (S) along the anteroposterior (AP) axis. The neural crest origins of the sympathetic postganglionic neurons are shaded yellow, and those of the parasympathetic postganglionic neurons are shaded green. (Left) A schematic of the chick stomach and gut. (Right) Peripheral sympathetic nervous system. AdGl, adrenal gland; CG, ciliary ganglion; Mes, mesencephalic crest; RG, Remak's ganglion; SCG, superior cervical ganglion. Redrawn, with permission, from Le Douarin (Le Douarin, 1982).

Thus, the findings of Le Douarin and Teillet did not simply show the fate map of the neural crest, they also showed that the avian body is constructed with a very straightforward and clear 'schematic' in terms of cell lineages, their migration pathways, and their specifications at early embryonic stages. Undoubtedly, their interpretation of avian development has stimulated our realization of the concept of the body plan, which, in the context of genome-wide research today, we think of in terms of gene regulatory networks and cis regulatory elements.

Somitomerism and experimental embryology

The paper by Michael Rickmann, James Fawcett and Roger Keynes examined the segmental development of the spinal nerves against the background of the body plan (Rickmann et al., 1985).

Segmentation is one of the most fundamental features of the vertebrate body plan, which exhibits several different types of segmented structures. For example, the paraxial mesoderm of the embryonic trunk is segmented into somites. The neuroepithelium of the anterior hindbrain and forebrain is also segmented into structures, known as the rhombomeres and prosomeres, respectively. In the digestive tract, the foregut (pharynx) endoderm develops into an iterative series of pharyngeal pouches that divide the pharyngeal wall into segmented pharyngeal arches. Among these structures, it is the anatomical segmental patterns of the somites, their derivatives and other, similarly iterative, associated structures that have been most extensively studied throughout the history of morphology and embryology.

Somites begin to appear quite early in development in the trunk of an embryo, even during gastrulation. Their derivatives, such as the vertebrae, dermis and myotomes, are also segmented. Interestingly, segmentation can also be seen in the pattern of the spinal nerves that innervate the tissues that derive from the somites. Spinal nerves consist of a dorsal (or sensory) root, called the sensory ganglia (spinal dorsal root ganglia), which is derived from the neural crest, and a ventral root that consists of motor nerves, the cell bodies of which are located in the neural tube. Importantly, the segmentation of the spinal nerves correlates with that of the somitic derivatives, i.e. a set of spinal nerve roots is associated with one somitic segment. Curiously, in early development there does not seem to be any overt segmental patterning of the trunk neural crest cells that migrate from the neural tube; the trunk crest cells arise ubiquitously along the entire neural axis.

In amniote embryos, each somite undergoes anteroposterior separation after being segmented from the paraxial mesoderm. By the stage at which trunk neural crest cells begin to migrate ventrally, the somite has differentiated into the lateral dermomyotome (the primordium for the dermis and the trunk skeletal muscles) and the medially located sclerotome (the precursor of the vertebrae). When the segmental distribution pattern of the neural crest cells becomes apparent, prefiguring the segmental distribution of the dorsal root ganglia, the cells are located in positions that correspond to the rostral half of the sclerotomes. Similar to the neural crest cells, motor axons (the precursors of the spinal ventral roots) also grow through the rostral sclerotome.

In the latter half of the nineteenth century and in the beginning of the twentieth century, comparative anatomists and embryologists formulated the idea of the vertebrate body plan by categorizing these iterative structures into an array of somitic segmental schemes. They did this to explain the various shapes of vertebrate species in an evolutionary context, and set about achieving this goal by making morphological observations of the anatomy of embryos and adults, without referring to the morphogenetic mechanisms that lead to the segmental pattern per se.

At the onset of the twentieth century, experimental embryology tried to explain these phenomena by revealing the mechanism of segmentation. There were comparative embryologists, such as Herbert V. Neal (Neal, 1918), who realized the importance of tissue interaction for the generation of segmental patterns. He thought, for example, that the patterning of myelomeres (neuromeres in the spinal cord) is imposed upon them by adjacent somites. This, of course, had to be shown experimentally, and one of the first experiments to reveal the segmentation of peripheral nerves was performed by Samuel Randall Detwiler (Detwiler, 1934), who added and removed somites from salamander embryos and saw parallel changes in the morphology of spinal nerves. Thus, the segmental pattern of spinal nerves appeared to lie downstream of mesodermal segmentation. To further investigate the mechanism of this segmentation, the developmental mechanisms of axonogenesis and neural crest cell migration needed to be distinguished from each other. On a technical level, transplanted cells and host tissues also needed to be distinguished from each other. In addition, methods of observation had to be improved, as most morphological analyses depended on the tedious reconstruction of serially sectioned histological specimens. As such, to make advances in this field possible, several embryological techniques had to be invented, including advances in

cell lineage markers, the construction of chimeric embryos, and immunohistochemical methods to enable cell type identification, which still remain vital methodological tools today.

The mechanism of spinal nerve segmentation was finally clarified by Keynes and Stern in 1984 (Keynes and Stern, 1984). They showed immunohistochemically that a somite-derived factor affected the segmental formation of the spinal nerve motor roots in the chick embryo. When the embryonic neural tube was rotated along the anteroposterior (AP) axis, the patterning of the spinal nerves changed, while that of the peripheral spinal nerves remained unaffected. Thus it was shown that the segmental pattern of somites determined the segmental morphology of the spinal motor roots; the motor roots are segmented, as the axons can only sprout through the rostral halves of somites that are specified even before the segmentation of the paraxial mesoderm.

In their *JEEM* study, Rickmann et al. further questioned the mechanistic process that underpins this phenomenon, using mainly immunohistochemical and microsurgical techniques (Rickmann et al., 1985). By using the monoclonal antibodies NC-1 (a neural crest cell marker) and HNK-1, Rickmann et al. showed that the HNK-1 antibody stains migrating neural crest cells in chick embryos at Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951) stages 13 to 17, in addition to the extracellular matrices surrounding the notochord, including the nerve fibers. During development, these trunk neural crest cells arise in an anterior-to-posterior direction along the whole of the neural axis, but were observed to pass only into the rostral halves of the somites, thus forming a segmental pattern similar to that of the somites themselves.

The authors then ablated the neural crest in the trunk region on one side of the developing chick spinal cord at HH stage 11 to see how motor axonal growth would be affected by the absence of neural crest cells; there was almost no change observed in the pattern of spinal nerve root formation, except that it was slightly delayed, and roots developed normally in the rostral halves of somites (see Fig. 4). Interestingly, these neurites were ensheathed proximally by NC-1^{+}HNK-1^{+} cells, even when there were no neural crest cells around; Schwann cells in the peripheral nerves are generally thought to be derived from the neural crest. The distal parts of the axons were completely unmyelinated, but their distribution was basically normal. Thus, it became clear that it is not the neural crest cells that distribute segmentally in the somites, but rather it is the somitic environment, per se, that restricts motor axonogenesis to a segmental pattern. The next obvious question was, what factor could be functioning in this segmental patterning of peripheral nerves? To address this question, Rickmann et al. resorted to an immunohistochemical strategy to observe the spatial distribution of extracellular matrix components, which were regarded as being important substrates for the distribution and migration of neural crest cells, and for axonogenesis. They used an antibody against fibronectin to observe its distribution pattern within a somite. As had already been reported by other researchers, this molecule was found to be present ubiquitously in the sclerotome and did not appear to be associated with the AP polarity of the somite. Similarly, laminin distribution did not show any differential distribution within the somitic environment.

Historically, this study by Rickmann et al. can be placed at the onset of a phase of studies that used modern experimental embryology, in which embryonic surgical techniques were coupled with immunohistochemistry. It is worth reminding the reader that these studies took place several years before in situ hybridization became available to this field. When re-reading this paper to write this essay, I was struck by the care that the authors placed on the immunohistochemical procedures they used; not only was the technique carefully performed, but so were their observations and discussion. I feel that this meticulous detail is highly relevant to the way we do experiments today and is an approach that we should continue to consider, particularly before jumping in with more sophisticated cutting-edge technologies.

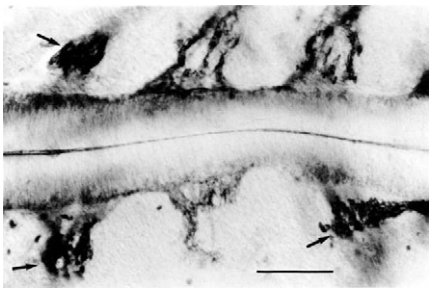

Fig. 4. Neural crest ablation experiment. A figure from the study of Rickmann et al. showing a horizontal section from a chick embryo that had neural crest ablated from it at the 15-somite stage, was killed at stage 19, and then stained with anti-NC-1 antibody. The image shows that the pattern of outgrowth of the ventral root nerve fibres is essentially normal in the neural crest-free somites and is restricted to the rostral half of the somite. Only the middle somite on either side is entirely free of neural crest cells; the surrounding somites have small numbers of stained cells within them (arrows). Scale bar: 1 μm. Reproduced, with permission, from Rickmann et al. (Rickmann et al., 1985).

However, one caveat that hangs over this paper concerns the specificity of the HNK-1 antibody. This monoclonal antibody was originally raised against the human natural killer cell, as implied by its name, and the epitope that it recognizes is found on the carbohydrate moiety of some glycoproteins (such as myelin-associated glycoprotein or N-CAM) that are often found on the cell membrane of neurons and neural crest cells. Rickmann et al. published this paper just before I started to use this antibody to stain neural crest cells in chicken embryos. I have stained sections of, and whole-mount, chick embryos (fixed with Bouin's solution) with this antibody, and have observed that many cell types, including mesenchymal and epithelial cells, are recognized by this antibody. Towards the end of the 1980s, I remember reading many instances where HNK-1 immunoreactivity was overestimated as representing neural crest derivatives.

It is clear that Rickmann et al. (Rickmann et al., 1985) were well aware of these caveats when they used HNK-1 as a neural crest cell and neurite marker. By comparing their findings with data reported in earlier papers on the patterns of neural crest cell distribution, as detected by various labeling studies [see references in Rickmann et al. (Rickmann et al., 1985)], they determined the stage at which HNK-1 immunoreactivity could be safely translated as marking neural crest cells. Immunohistochemistry may not be so difficult, but the critical evaluation of its results is.

The paper by Rickmann et al. was followed by many more experimental studies, and led to later discoveries of the developmental importance of somites for the segmental patterning of other tissues and structures (Stern et al., 1989; Bronner-Fraser and Stern, 1991; Lim et al., 1991). We now know that cell-cell contact-dependent signalling, mediated by Eph and ephrins, functions in neural crest cell migration and their distribution in somites (Krull et al., 1997; Santiago and Erickson, 2002), and that Notch signalling is involved in the oscillating expression of *hairy* and *mesp*, which generate the segmental pattern of somites (reviewed by Maroto and

Pourquie, 2001). Rickmann et al. (Rickmann et al., 1985) can therefore be seen as both the end of the careful histological analysis of embryogenesis and the prelude to a molecular understanding of the segmental body plan of vertebrates.

Conclusion

These studies have impacted the way in which I do research immensely. Of course, Le Douarin strongly influenced me during my formative, early, scientific life, especially in the way I thought of and carried out embryological experiments. As a result, I was (and still am) fascinated by the embryology and evolution of the vertebrate skull, concepts introduced by *The Neural Crest* (Le Douarin, 1982), a book that has taught me a number of modern ideas that I could have never encountered in classical textbooks. This being the case, Nicole Le Douarin's textbooks and papers still sit right beside me, within easy reach. The same is true of the series of studies on somite-spinal nerve segmentation led by Rickmann et al. This theme is one of the best examples of how a specific developmental mechanism results in a particular anatomical patterning of animal bodies. Naturally, it would be very hard for any anatomist to remain unaffected by such works and, in light of what we know now, these studies are of even greater significance.

The papers by Le Douarin and Teillet (Le Douarin and Teillet, 1973) and Rickmann et al. (Rickmann et al., 1985) represent monumental works in the history of experimental embryology, and are still influential as examples that remind us of how modern labeling techniques, which are widely used today, should be applied to embryos and how much care should be taken to interpret the resulting data. Nowadays, we tend use new techniques that produce pretty pictures without taking the time to examine routine data such as immunohistochemistry. We may be busy playing catch-up in this rapidly changing world of science, but I believe it is also a good idea for us to revisit classic papers like these to learn lessons from the past that might strengthen our findings in the future.

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