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Review

Nicole Le Douarin and the use of quail-chick chimeras to study the developmental fate of neural crest and hematopoietic cells

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ABSTRACT

The quail-chick chimera marking system, devised in 1969, gave a new impetus to the analysis of cell migrations and interactions in the developing nervous, immune and hematopoietic systems. The method is based on the observation that the constitutive heterochromatin in all embryonic and adult cells of the quail is condensed in one large mass in the centre of the nucleus and is associated with the nucleolus, making this organelle strongly stained with the Feulgen–Rossenbeck reaction. The association of cells or rudiments from two avian species, advocated as a means to identify cells that migrate during embryogenesis, was rapidly recognized in this context as a useful tool for the study of many developmental biology problems. This article summarizes the fundamental contribution of Nicole Le Douarin to the discovery and the application of this technique over the last 40 years.

1. Background

Nicole Marthe Le Douarin (Fig. 1) was born in Lorient, France, on August 20, 1930. In 1954, she graduated from the Sorbonne with a degree in Natural Sciences. In the late 1950s, N. Le Douarin was introduced in the laboratory of Etienne Wolff (1904–1996), professor at the Collège de France, and founder of the Institut d'Embryologie Expérimentale et de Tératologie du Centre National de la Recherche Scientifique (CNRS) et du Collège de France, in Nogent sur Marne on the east side of Paris. In 1964, N. Le Douarin discussed the doctoral thesis entitled "Etude expérimentale de l'organogenèse du tube digestif et du foie chez l'embryon de Poulet", which was published with the same title in the "Bulletin Biologique de la France et de la Belgique" (Le Douarin, 1964c). In these years, N. Le Douarin studied the morphogenetic movements of the endoderm and mesoderm leading to the ventral closure of the gut (Le Douarin, 1964a, b, c). In 1965, N. Le Douarin was appointed as "Maître de Conférences" at Clermont-Ferrand in the Department of Developmental Biology directed by Hubert Lutz. In 1966, N. Le Douarin was appointed at the University of Nantes where she established her first independent research group.

2. A Feulgen-Positive nucleolus

N. Le Douarin was interested in the study of the effect of hepatic mesenchyme on the differentiation and growth of anterior intestinal portal (AIP) endoderm. She associated in organotypic co-culture the hepatic mesenchyme of a quail embryo with the AIP endoderm of a chick (and vice versa). When she observed the microscopic sections of these chimeric liver lobules resulting from the association of chick AIP endoderm and quail (*Coturnix japonica*) liver mesenchyme, the chick hepatocytes looked the same as in normal liver, whereas the mesenchyme exhibited a large nucleolus. As N. Le Douarin has remembered: "I noticed the presence of a large nucleolus in all embryonic and adult cell types of this species of bird and further found that the unusual size of this organelle resulted from association of a mass of heterochromatin with the nucleolus proper (essentially made up of RNA). This particularity is of rare instance in the animal kingdom and does not exist in the chick. "(Le Douarin and Dupin, 2018).

N. Le Douarin applied Feulgen-Rossenbeck's, thereafter referred as Feulgen, histological nuclear staining procedure for DNA and the Unna-Pappenheim staining method for RNA component of the nucleoli and demonstrated that the quail nucleolus was mostly composed, not of RNA, but of DNA (Le Douarin, 1969, 1973a, b). She discovered that Feulgen allows to distinguish chick cells from quail cells. Feulgen stained quail cells have interphase nuclei with condensed nucleolar-associated heterochromatin that's not found in chick interphase nuclei (Fig. 2). The intimate association of the heterochromatin and the nucleoli in quail cells can be strikingly seen at ultrastructural level. This makes the cells of the two species easy to distinguish on sections stained for DNA with the Feulgen-reaction and suggested to N. Le Douarin the

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Fig. 1. A port trait of Nicole Le Douarin.

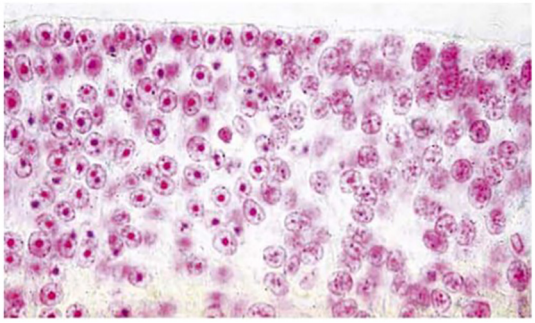

Fig. 2. Feulgen staining of DNA shows a large mass of heterochromatin in the centre of the nucleus, which is associated with the nucleolus in quail cells (left). In chick cells, the heterochromatin is evenly distributed (right).
(Modified from Le Douarin, 2004)

idea of constructing chimeras between these two species of birds, by associating quail and chick cells in the embryo *in ovo*. As she said: "Quail and chick cells, side by side in chimeric tissues can therefore be easily identified, since the natural nuclear labeling of quail is conspicuous enough to enable identification of a single quail cell located in chick tissues, provided that the section includes the nucleolus." (Le Douarin and Dupin, 2018).

3. The construct of chimeras applied to the study of neural crest derivatives

Chimeras resulting from the replacement of definite regions of the embryo of one species by their counterparts from stage-matched embryos of the other, develop normally, suggesting that this system can provide reliable information on the behavior and fate of the grafted cells. N. Le Douarin started to construct chimeras by replacing a fragment of the neural tube of the chick embryo prior the onset of neural crest cells emigration at this level of the neural axis by that of a stage-matched quail embryo at the same developmental stage. The same type of grafts was performed in both directions, i.e. from quail to chick and from chick to quail (Fig. 3).

When she saws a chimeric embryo whose sections had been treated with the Feulgen-Rossenbeck's staining procedure, she demonstrated that quail cells were present not only in the grafted spinal cord but also were dispersed in other places, along the nerves, as Schwann cells, in the peripheral ganglia, in the suprarenal glands and within the metanephritic mesenchyme. This technique, compared to the previous ones used to label the cells (either H^3-TdR, vital stains, or carbon nanoparticles), was superior due to its being stable and unalterable and also non transmissible to neighboring cells. By constructing quail-chick chimeras, in which part of the neural primordium of the host embryo was substituted by its counterpart taken from a stage-matched donor of the other species, the migration and fate of the neural crest was

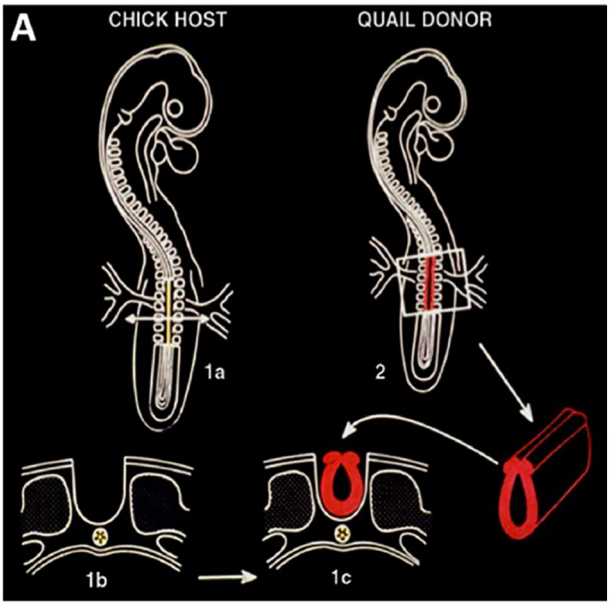

Fig. 3. Quail-chick chimeras for investigating the fate of avian neural crest cells. (A) Schematic of the construction of quail-chick chimeras of the neural tube at "adrenomedullary" trunk level (somites 18–24). The neural tube was removed from chick host (1a, 1b) and replaced by its equivalent (1c), previously taken at the same level from a quail donor of the same developmental stage (2).
(Modified from Le Douarin and Dupin, 2018)

followed during the entire embryonic life and could even be pursued after birth thanks to the stability provided by this cell labeling technique. Through this experimental approach, N. Le Douarin established the cell types arising from the neural crest; their level of origin and the pathways they followed to reach their destinations (Fig. 4) (Le Douarin, 1980, 1982).

4. The construct of chimeras applied to the study of the immune system

The quail/chicken labeling system was also applied to the study of the development of the thymus and the bursa of Fabricius and allows through different grafting schemes to identify the origin of every single cell in early rudiments (Le Douarin et al., 1984). As N. Le Douarin said: "With Francine Jotereau, then a young student, we undertook experiments which showed that lymphocytes are derived from hemopoietic stem cells (HSC) which invade the thymic epithelial rudiment during cyclic waves. These HSC also give rise to the dendritic cells in the medulla of the thymus. In contrast, the development of the bursa of Fabricius is characterized by a single invasion of HSC which lasts 5 to 6 days and generates B cell precursors for the entire life of the bird (Le Douarin and Joterau, 1973; Le Douarin et al., 1975, 1976a, b, 1977; Jouterau and Le Douarin, 1982; Houssaint et al., 1976)" (Le Douarin, 2005). After grafting the early thymic rudiment from a 3 days quail embryo into the chick somatopleure, N. Le Douarin demonstrated that the thymus developed on this ectopic site and that all the lymphocytes it contained, were derived not from the graft, but from the chick host. In contrast, if the quail thymus was removed from the donor later in development, then the lymphocytes that differentiated in the graft were of the quail type. This demonstrated that the lymphocytes that developed in the thymus were of extrinsic origin. Furthermore, N. Le Douarin demonstrated that hemopoietic cells entry to the thymus was preceded by successive waves separated by non-receptive periods (Fig. 5) (Jouterau and Le Douarin, 1982).

Furthermore, N. Le Douarin demonstrated that the thymus was colonized by HSCs, non-endothelial cells of blood vessel walls,

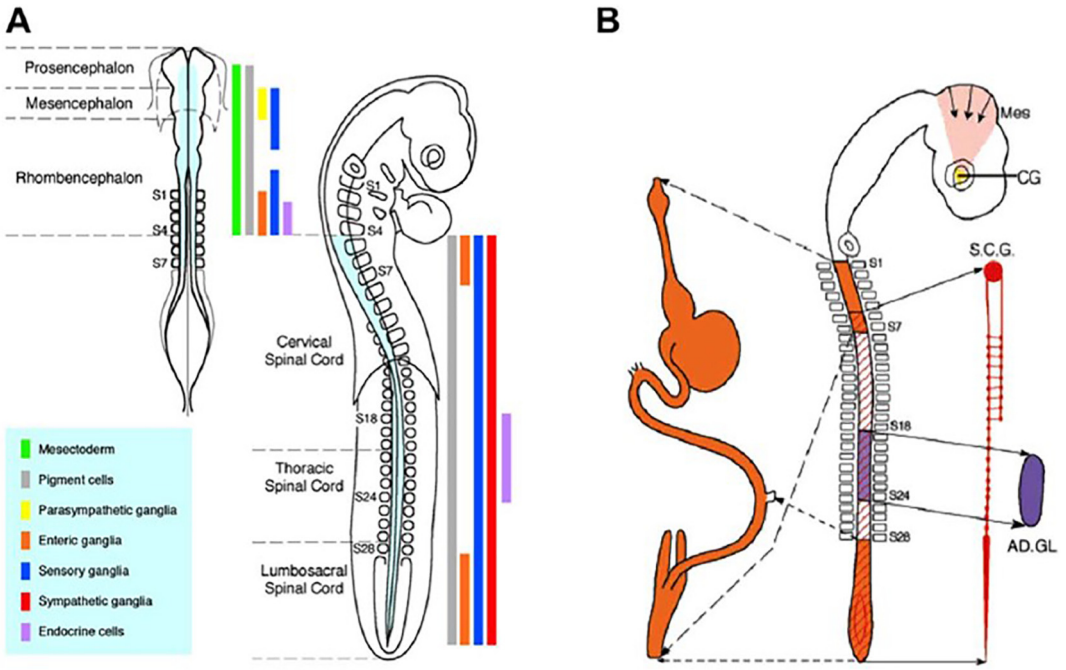

Fig. 4. Fate map of neural crest derivatives in the avian embryo as determined by quail-chick chimeras. (A) Fate map of neural and non-neural derivatives of the neural crest along the neural axis, represented in the cephalic neural crest (left, 7-somite stage embryo) and trunk neural crest (right, 28-somite stage embryo). The rostro-caudal levels of origin of the various neural crest phenotypes are shown with color-coded vertical bars. While the whole (cephalic and trunk) neural crest gives rise to pigment cells (grey bars), the origin of mesenchymal derivatives (including skeletal and connective tissues) (green bar) is confined to the cephalic neural crest from mid-diencephalon down to r8, corresponding to the level of somite 4 (S4). Definite regions of the neural crest yield peripheric nervous system derivatives, including sensory (blue bars), parasympathetic (ciliary ganglion) (yellow bar), sympathetic (red bar) and enteric (orange bars) ganglia. Endocrine (adrenomedullary) cells originate from the trunk neural crest between somites 18 and 24 (purple bar). (B) Schematic representation of a chick embryo of 28 somite-stage, showing various regions of the neural crest along the neural axis and their respective derivatives (same color-coding as in A) in enteric nervous system plexuses (orange), sympathetic ganglia including the superior cervical ganglion (SCG) (red), parasympathetic ciliary ganglion (CG), and in the medulla of the adrenal gland (AD.GL.). (Reproduced from Le Douarin and Dupin, 2018)

including pericytes and smooth muscle cells, derived from the neural crest (Etchevers et al., 2001). Moreover, it was established that endothelial cells in the head and neck derived from cephalic paraxial mesoderm (Couly et al., 1995), and that the time when colonization by HSCs begins in the mouse thymus, by associating the cultured early rudiment with fetal liver, as HSCs donor (Le Douarin et al., 1984).

The quail/chick chimeras could hatch and live a few weeks or months indicating that the graft was tolerated. However, between two weeks and three months after birth, xenogeneic tissue grafts were immune rejected. In the case of the bursa of Fabricius, signs of rejection can be the immune attack of the stroma occurs within the first weeks after birth (Corbel et al., 1987). By using Major Histocompatibility Complex (MHC) matched animals, it was possible to overcome the problem. Within the chicken species, transplantation of limb buds between MHC-mismatched embryos led to virtually complete tissue tolerance not only of the grafted limb, but also of adult skin from the same MHC type as the limb (Corbel et al., 1990). Embryonic chick wings are well tolerated (with only slight signs of pathology without ultimate rejection) by the quail and as in chick-to-chick MHC mismatched combinations. It appears from these experiments that the quail immune system consistently has a capacity to tolerate tissues of relatively closely related species, a capacity that does not exist in the chick.

A further step in the work of N. Le Douarin was the characterization of the monoclonal antibody MB1/QH1, which recognize an antigenic determinant common to the endothelial and white blood cells of the quail at the exclusion of any cell type of the chick (Péault et al., 1983; Pardanaud et al., 1989) (Fig. 6). As N. Le Douarin said: “Our early work on hemopoietic stem cells, carried out by Bruno Péault with the skillful

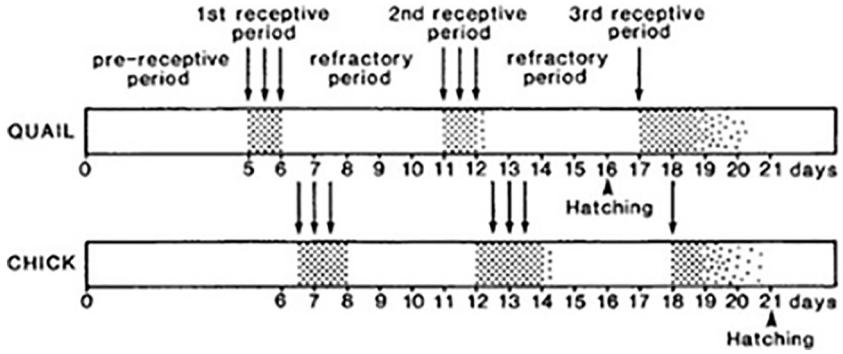

Fig. 5. Successive waves of hematopoietic cell immigration into the thymus in quail and chick embryos and newly hatched birds. (Reproduced from Le Douarin, 1988)

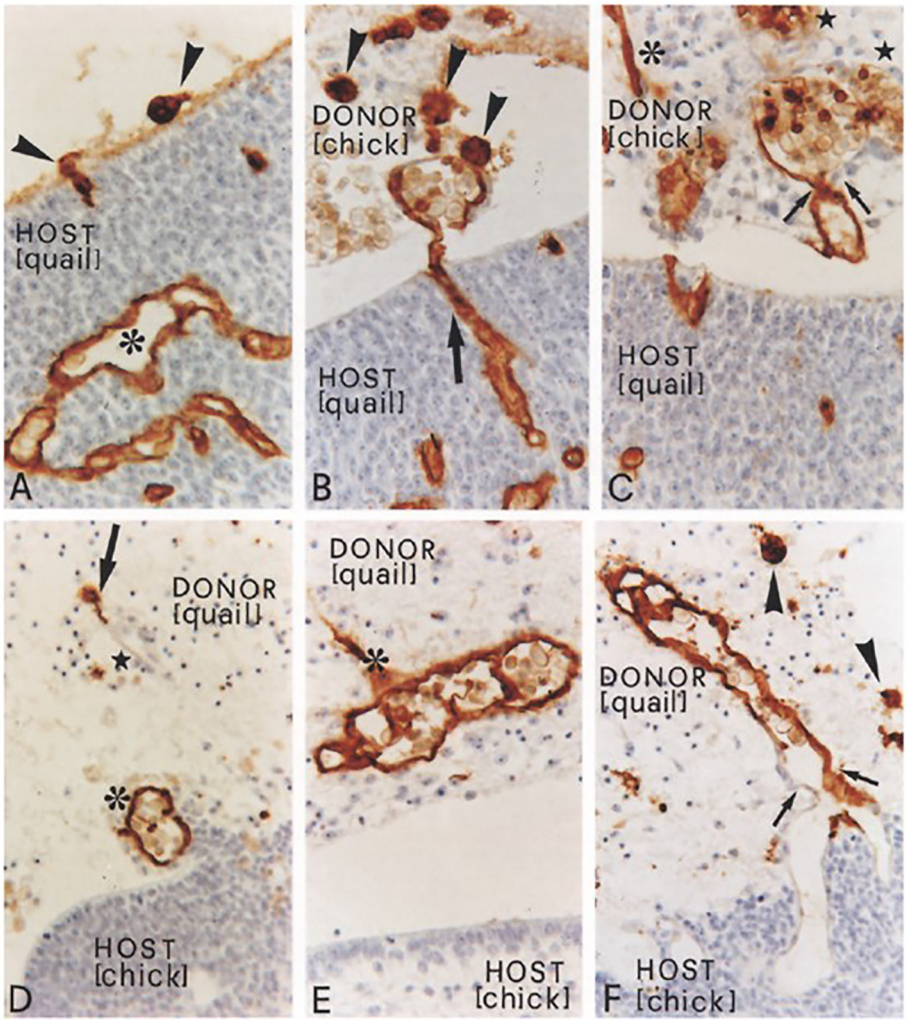

Fig. 6. Chick (Donor) to quail (Host) neural graft. MB 1 immunostaining; 24 h after grafting. The immunostained vascular network of the host tectum (A asterisk) gives rise to a vertical twig (B arrow) penetrating the ventricle and reaching the transplant. In the latter (C), donor-native (stars), host-derived (asterisk) and chimera (arrows) microvessels can be seen. Immunostained host-derived macrophage-like cells (arrowheads) are recognizable within and on the host neuroepithelium (A) and in the graft (B), also in a perivascular position. D–F Quail (Donor) to chick (Host) neural graft. MB 1 immunostaining; 24 h after grafting. D A unlabeled host microvessel (star), with a labelled perivascular cell (arrow) is recognizable within the donor tissue; an immunostained donor microvessel is close to the host tectum (asterisk). E A immunostained microvessel of the graft has a perivascular cell which invades the surrounding tissue (asterisk). F A immunostained donor-native microvessel grown towards the host tectum anastomoses with the chick unlabelled microvasculature, forming a vascular chimera (arrows). Immunostained donor-derived macrophage-like cells are indicated by arrowheads.
(Reproduced from Roncali et al., 1996)

technical help of Monique Coltey, has attracted our attention to the close relationships between endothelial and blood cells in the yolk sac blood islands. This was made possible by using the MB1-Mab, produced by Bruno, which was the first of the numerous species- and cell type-specific antibodies that we further prepared to analyze the chimeras." (Le Douarin, 2005).

Staining of early quail embryos with the MBl/QHl-Mab revealed that endothelial cells are present in the avian cephalic mesoderm from the second day of the incubation period, prior to the onset of heart beating and the appearance of major blood vessels (Pardanaud et al., 1987; Poole and Coffin, 1988), thus supporting the assumption that the cephalic mesoderm is endowed with angiogenic capacities.

Using the quail-chick chimera system, Noden (1991) had claimed that some of the angiogenic potencies in the mesoderm located from the mesencephalon down to the level of the fifth somite are destined to provide the endothelial wall of the cardiac outflow tract and to form the endocardial cushions. Furthermore, he noted a highly invasive behavior of the grafted endothelial cells (Noden, 1989).

Pardanaud et al. (1989) demonstrated that primordial differentiation of the endothelial network required two sets of endothelial cells, one associated with the somatopleural (dorsal) mesoderm giving rise only to endothelial cells and one associated with the splanchnopleural (ventral) mesoderm capable of giving rise to endothelial cells and blood. Furthermore, Pardanaud and Dieterlen-Lièvre (1989) grafted quail somites into the chick hosts and followed the progeny of quail endothelial cells. They demonstrated that the body wall was vascularized by somite-derived endothelial cells, whereas internal organs developed vascular networks with angioblasts emerging from the splanchnopleura.

5. Concluding remarks

An embryonic chimera is an animal that has two or more populations of genetically distinct cells that originated in different embryos of the same or different species. Chimeras have been obtained in the avian embryo following the observation of the interphase nucleolus in the Japanese quail. Making quail cells readily distinguishable from those of the chick where the constitutive heterochromatin is evenly dispersed in the nucleus.

These structural differences have been used to device a cell-marking technique through which cell migrations and cell interactions during embryogenesis can be followed in the embryo *in ovo* by grafting quail cells into chick embryos or vice versa. This method was successfully applied by N. Le Douarin and her co-workers in the study of the ontogeny of the derivatives of the neural crest and of the immune system.

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