Nicole M. Le Douarin

Born:

Lorient, France
August 20, 1930

Education:

Université, La Sorbonne, Paris
Licence et agrégation de Sciences-Naturelles (1954)
Doctorat d'Etat es Sciences Naturelles, Paris (1964)

Appointments:

Professeur agrégé de Sciences Naturelles (1954)
CNRS Researcher (1960)
Assistant Professor University of Clermont-Ferrand (1965) and Nantes (1971)
Director of the Institute of Cellular and Molecular Embryology of the CNRS and Collège de France (1975)
Professor at the Collège de France, Paris (1988)
Permanent Secretary of the Académie des Sciences, Paris (2001)

Honors and Awards (Selected):

Prix Cognac-Jay de l'Académie des Sciences (1965)
Prix Van Beneden de l'Académie Royale de Belgique (1973)
Member of the Académie des Sciences, Paris (1982)
Member of the Academy of Arts and Sciences (1984)
President of the International Society of Developmental Biologists (ISDB) (1985)
Kyoto Prize in Advanced Technology de la Fondation Inamori, Japon (1986)
Gold Medal of the CNRS (1986)
Foreign Associate of the National Academy of Sciences, USA (1989)
Foreign Fellow at the Royal Society of the United Kingdom (1989)
Award of the Fondation Louis Jeantet de Médecine, Genève (1990)
Louisa Gross Horwitz Prize, Columbia University (1993)
Ross Harrisson Prize of the International Society of Developmental Biology (1997)
Member of the Pontifical Academy of Sciences, le Vatican (1999)
Grand Officier dans l'ordre de la Légion d'Honneur (2002)
The Pearl Meister Greengard Prize, Rockfeller University (2004)
The Edwin Conklin Award of the American Society of Developmental Biology (2005)
Grand' Croix dans l'ordre National du Mérite (2005)
Ralph W. Gerard Prize, Society for Neuroscience, USA (2007)
Prix d'Honneur de l'INSERM (2009)
Grand' Croix dans l'ordre de la Légion d'honneur (2010)

Nicole M. Le Douarin began her research career while a high school teacher. Her Ph.D. work under the supervision of Etienne Wolff was on the development of the digestive tract and liver in the chick embryo. She later devised a cell-marking technique based on the construction of chimeras by combining cells of quail and chick embryos in ovo. Using this technique, she demonstrated that the neural crest is a major player in vertebrate embryogenesis, namely by its role in the development of the head and brain. She demonstrated with her colleagues the existence of a stem cell in the neural crest, the first example of a neural stem cell. She and her group applied the quail-chick chimera system to other problems such as hematopoiesis and demonstrated a novel mechanism of tolerance to self.

Nicole M. Le Douarin

Early Years

I was born in Brittany, a beautiful French province, in the city of Lorient. I spent my childhood in a small village, 30 km away from Lorient, where my mother was a teacher in the public school. My father was in business.

In France, child education was made both free and compulsory by Jules Ferry in the late 19th century. State schools were constructed even in small remote villages so that all children, whatever the financial means of their parents, could have a basic education up to the age of 15. Brittany was, at that time, under the powerful influence of the Catholic Church and, apart from the school provided by the state, there were also Catholic schools to which, especially in country places, the parents were strongly influenced to send their children. In fact, for many decades, a kind of "school war" (*guerre scolaire*) prevailed in several regions in our country and particularly in Brittany. As a result, the number of pupils in the public school was often small and, in small villages, like Locoal-Mendon where we were, there was a single teacher available in charge of pupils from the age of 6 to 15 (the so-called *classe unique*).

My mother allowed me to sit at the far end of the classroom from the age of 3 or 4, at least for part of day, provided that I behaved. As a result, I learned to read by myself without any effort at a very early age, just by listening and following the lessons meant for others. This was the way my primary school proceeded, smoothly and happily with not much difference between school and home.

At the age of 11 came the time for me to go to the secondary school called *lycée* (now designated as college for the first 4 years and *lycée* only for the next three). From the village where we lived, the closest city with a *lycée* was Lorient. But we were at war. The German troops occupied the country and Lorient had been chosen by them to establish a base for submarines, which was the target of frequent attacks by the Allied air force. The inhabitants of Lorient were evacuated and no *lycée* was available there anymore. I had to go to Nantes, 150 km away from home by train, which took 5 hours when everything went well. My father had a car, but during these difficult times, there was no gas except for people with a special permit, such as physicians who needed a car to visit their patients.

The separation from my family was extremely difficult for me and also, I think, for my parents, as I was their only and cherished child. We accepted this situation and my schooling went on smoothly, except that in 1944

Nantes was also the target of heavy bombing by the Royal Air Force. Fortunately the worst of it took place during the summer vacations. The consequence was that the *lycée* was closed and both teachers and pupils were invited to stay home. Thus, for one academic year we communicated by mail. At the "Libération" I was 15 years old. The country was free again. My mother got a teaching position in Lorient and the *lycée* opened again there. I left the Nantes boarding school and spent the 2 years that remained before the *baccalauréat* in the *lycée* in Lorient. The city had been totally destroyed; one house out of ten had been spared by the massive destruction inflicted to it because of its strategic position. My family had been given an "American house" provided by the Marshall Plan. We stayed there several years before the house, which my parents possessed in the city, was reconstructed. This period, when France revived from its long submission to an omnipresent enemy was a very exciting one. It was such a relief not to have the "occupation troops" around anymore and a joy to welcome the American troops, our liberators. These memories have remained vividly in my mind.

After obtaining my *baccalauréat*, the terminal high school examination, I wanted to go on studying and attend a University, a choice that my parents adhered to enthusiastically. The decision was that I should go to La Sorbonne in Paris, although, being in Brittany, the law (not too strict, fortunately) wanted me to register at the University of Rennes, the closest one to my residence. I succeeded in being accepted as a student in Paris.

My orientation in the secondary school was essentially about literature and humanities; I studied Greek, Latin, French literature, and philosophy. However, in the last year I had a very good teacher in biology and this probably explains my decision at the University, where after one year as a student in literature I resolutely turned my interests to natural sciences. My first project was to be a high school teacher. At the age of 24 I passed the competitive exam, called "agrégation de Sciences Naturelles," which, if you succeed, provides you with a permanent position as a "Professeur de Lycée."

At 23, I was already married and mother of my first daughter, Claire. I taught natural sciences (biology, physiology, zoology, botany, etc.) as well as earth science to high school children for 8 years, first in the city of Caen in Normandy and then in a special school where different teaching experimentations were conducted. This was in Montgeron, a city close to Paris, where the conditions were particularly nice: the *lycée* was in in the middle of a large park and the number of children by class was less than in a regular school. I was told that I was offered this position because I had been identified as someone who liked teaching. These 5 years in Montgeron left me with nice memories. However, I was not completely satisfied with my professional life. Although I liked to teach, I missed the University where one follows the science in progress. I felt that transmission of the knowledge to younger generations, although a very valuable task, was a bit frustrating.

Thus, I decided, at the age of 28 to go back to the University and to try and see whether I could find a means to do research.

I was the mother of two little daughters and had a full-time job at the *lycée*. I needed to go on with a job to survive since, although my husband was also a teacher, our two salaries were necessary. Therefore, satisfaction of this ambition was not a simple endeavor. My parents helped me very generously by paying a nanny who took care of our children. Moreover, my teaching duties left me some time every week so I could spare the equivalent of about 2 days to try and see whether research was something I could do and would like to do.

The next step was to find a laboratory in Paris that would accept me as a freelance researcher *à l'essai*, so to speak. This was a serious problem. Research was not very active after the war. Only a few laboratories were on the forefront of international research. Fortunately, the CNRS (Centre National de la Recherche Scientifique) had been created and efficiently contributed to the revival of French science. However, after some unsuccessful attempts to find a laboratory to work in, I realized that I did not have the right profile: I was too old (28 years old), and I had a family and a job. As I was told by someone: that was good enough and OK for me. Why should I want to do more or something else?

I was nearly completely discouraged when I had the chance to be introduced to Professor Etienne Wolff, who was running one of the most dynamic laboratories of experimental biology at the Collège de France in Paris. The Institut d'Embryologie et de Tératologie Expérimentales was located in a superb environment in Nogent-sur-Marne at the border of the Bois de Vincennes on the east side of Paris.

This laboratory has had a great importance in my professional life since it is where I was initiated to research in my young years and a place where I became Director later on for 25 years. For this reason I would like to tell some of its history.

The Institute of Embryology in Nogent-sur-Marne: Origin and Intellectual Heritage

Before being appointed to the Collège de France, Etienne Wolff held a professorship at the University of Strasbourg, where, back from a 5-year stay in a German prisoner camp, he had developed an already large laboratory supported by the CNRS.[1] The Nogent venture was part of a significant effort in the 1950s to re-create a national corpus of research, on the ruins left by the war.

The Nogent estate became a laboratory dedicated to the study of embryology in 1955 when Etienne Wolff was elected as a Professor at the Collège

[1] Centre National de la Recherche Scientifique.

de France, where the chair of "Embryologie Expérimentale" was created for him. The estate had been donated to the Collège de France in the 1940s by the late Pr. d'Arsonval. This wealthy family house sat in a park planted with magnificent trees and ornamental bushes, bordering the residential suburban city of Nogent-sur-Marne in the "Bois de Vincennes."

Given the choice between the CNRS campus in Gif-sur-Yvette and the Nogent site, Etienne Wolff chose Nogent and had it completely renovated, one floor becoming his own apartment, the rest being transformed into what was at that time a modern laboratory. A large part of the building had previously been the private laboratory of Pr. d'Arsonval.

Etienne Wolff was already famous for his finding that the recently discovered estrogens were able to influence the sexual development of the chicken embryo. He showed that these hormones transformed genetically male birds into intersexuals, which developed an ovarian cortex and retained Müllerian ducts. This first demonstration of a hormonal effect in the developing embryo opened a large research avenue brilliantly developed in France, apart from Etienne Wolff's own laboratory, by Alfred Jost in mammals and Louis Gallien in amphibians. Wolff's experiments were carried out just before the Second World War (Wolff, 1936a, 1936b) and were reported in his secondary thesis.[2] This piece of work was experimental rather than the typical analytical assay—an exceptional fact and a testimony to the intellectual dynamism and enthusiasm for biology displayed by this young man whose initial university education was in philosophy.

Etienne Wolff's main thesis, published in 1936 in the Archives d'Anatomie, d'Histologie et d'Embryologie is a monumental study in the field of teratology. How do "monsters," which appear from time to time in human or animal evolution, emerge? Some mechanism must go astray during development. The rational basis of this work was to try and reproduce the morphological abnormalities in the hope of finding cues as to how and why they appear, and, more interestingly, in the hope of acquiring information about normal embryological development. This was long before the time when any gene responsible for developmental malformations was identified. Wolff's thesis, a masterpiece, still yields accurate information for avian embryologists.

In the laboratory that Etienne Wolff created in the College de France in the late 1950s, the number of investigators was already significant. Each was in charge of his own project, teamwork being rare at this time, so that the problems tackled were very diverse, ranging from regeneration in planarians and neural induction in amphibians to teratological effects of various drugs such as thalidomide.

[2] At that time and up to the 1970s the grade of Docteur ès Sciences d'Etat was delivered upon defense of a main and a secondary thesis.

Apart from the persistent interest of Etienne in the effects of ionizing radiations, the major trends concerned, first, sexual differentiation, which was directly related to Wolff's previous findings (namely the effects of steroid hormones on the developing genital tract), and, secondly, tissue interactions occurring during organ development.

From the 1950s onward, one of the trends in physiology and developmental biology has been to dissect processes, previously described in the animal, in simplified systems, that is, in tissues isolated and grown in vitro in organotypic cultures. There, organ rudiments maintain their tridimensional structure in which the interacting tissue players can be separated by filters with adequate pores, allowing or not allowing cell traffic and passage of molecules of various sizes.

With one of his most skilful collaborators, Katy Haffen, Etienne Wolff developed a culture technique with which he himself and his coworkers (among whom were his spouse and colleague Emilienne Wolff) analyzed the responses to steroids of various organs rudiments. This culture technique was based on the incorporation of nutriments in a semisolid agarose gel, the tissues being placed at the interphase between air and medium. It was commonly used by researchers interested in tissue interactions during organogenesis.

My Early Research Experience

I came to Nogent in the late 1950s when the laboratory was at its peak. Broadly, my project concerned the digestive tract. Not much interest was directed to endoderm and its derivatives at that time and the message I received, along with some practical advice, was that I would find something worth investigating in this area.

In the first 2 years of my laboratory experience, I was far from being a full-time researcher. I remained a high school teacher and joined the Nogent-Institute only when my teaching duties gave me free time.

I rapidly realized that research would be my choice for life. Etienne Wolff was very encouraging and wise. He had created, in Nogent, a very well-organized laboratory with a pleasant atmosphere for work. At the end of these years during which I could test my motivation and he could evaluate my capacities for this activity, I was able to switch from teaching to research administration and from 1962 I was paid to do research and thus could devote myself full time to my project.

My first goal was to study the developmental events leading to the formation of the digestive tract and associated glands. As Etienne said, it was a neglected field. I learned from him how to handle early chick embryos, to make current type microsurgery such as grafting tissues into the coelomic cavity or on the chorio-allantoic membrane of host embryos. Organotypic cultures were among the techniques that Etienne Wolff had developed in

the laboratory. Histology and histochemistry were the other methods regularly used by embryologists at that time.

In 1964, I defended my doctorate thesis entitled *Etude expérimentale de l'organogenèse du tube digestif et du foie chez l'embryon de Poulet*, which was published in the *Bulletin Biologique de la France et de la Belgique* (1964c). In this work, I first studied the morphogenetic movements of the endomesoderm, which lead to the ventral closure of the gut. I was fascinated by the spectacular transformations affecting the avian embryo during the first days of its development in the egg. How could a sheet of cells lying flat on the yolk become, within the next 2 to 3 days, a highly structured organism endowed with a head, a trunk, and a beating heart, surrounded by a frame of blood vessels? This appeared as a marvel and I thought that trying to decipher the cell behavior and the mechanistic forces underlying these processes would be an appealing (although very ambitious) challenge.

Arriving in Etienne Wolff's laboratory, I naturally had the curiosity to read his thesis. Although dating from 22 years back (Wolff, 1936a), I found the opus inspiring. In this published memoire entitled *Les bases de la tératogenèse expérimentale des Vertébrés Amniotes d'après les résultats de méthodes directes*, he wanted to demonstrate that the abnormalities and monstrosities exhibited by some unfortunate children at birth were nothing mysterious (some people believed at that time that they were a manifestation of the ire of God) and could be scientifically explained. Developmental genetics had not yet been invented, and the best way to prove the validity of this view was to experimentally reproduce these naturally occurring abnormalities by interfering directly with the development of the embryo.

The availability of the avian embryo in the egg during the entire period of development was particularly suitable for this endeavor. Etienne Wolff was able to destroy selected embryonic areas in the early embryo by subjecting them to heavy and strictly targeted X-irradiation. The chick embryo as a whole was protected by a lead screen placed on top of it in ovo, except for the targeted area which was the only living tissue exposed to the X-rays. The cells of the irradiated zone died after a while and were progressively replaced by the adjacent living cells. Thus, the continuity of embryonic tissues was never interrupted. This allowed Etienne not only to produce some spectacular monsters such as Cyclops but also to discover the presumptive territories fated to yield various tissues and organs.

The precision of his experiments and the logic underlying both their planning and their interpretation were perfect. Coming back to my project, I thought that a prerequisite for the analysis of the development of endoderm-derived organs was to construct a fate map of the endomesoderm at the early embryonic stages and to try and discover its transformation into the digestive tract and its glandular appendages. In order to do so, I followed the path opened by Etienne many years before.

Two ways were available to reach this goal, either labeling the tissues and following de visu the movements of the labeled cells, or destroying definite territories by X-rays and looking at what was missing afterward. I was conscious, however, that the X-ray technique was not fully reliable since the neighboring embryonic cells might partly replace those that had been killed. The "regulation capacity" through which an embryo tends to produce a normal individual after being subjected to various kinds of injuries is actually very efficient at these early stages of vertebrate development.

In the late 1950s, one of the best ways to label embryonic territories was to apply carbon particles to them which, although they adhere to the cell membrane, are not harmful. The relative displacements of the labeled cells can, by this means, be roughly (if not very precisely) visualized and recorded. The use of these methods, namely X-ray destruction and carbon labeling of embryonic areas, led to the construction of embryonic fate maps at different developmental stages. The results showed that the construction of the digestive tract involved major and complex morphogenetic movements that I strived to describe.

My second objective was to determine when the commitment of the endodermal cells at the origin of the glands associated with the digestive tract takes place early in development. I found that endodermal cells fated to differentiate into hepatocytes, pancreas, thyroid, and parathyroid are present in the anterior intestinal portal (AIP) at the 15 to 25 somite stages (15–25 ss). It is only later, when ventral closure of the foregut has occurred, that these cells start to form the corresponding glandular structures. This, however, cannot happen without the cooperation of the splanchnopleural mesenchyme.

I studied the relationships that take place between endoderm and mesoderm during the development of the liver. I showed that the few endodermal cells in the AIP committed to become hepatocytes originated from the coalescence of paired endodermal areas (the prehepatic endoderm) lying on each side of the primitive streak and head process at the presomitic stages. These prehepatic areas of endoderm were juxtaposed to the future cardiac mesoderm.

Two successive steps of induction of the prehepatic endoderm are required for the differentiation of hepatocytes to occur. The first one takes place early (before 5 ss) and arises from the precardiac mesoderm. It turned out to be required for the efficacy of the second interaction, which occurs between the specified hepatic endoderm and the mesenchymal component of the liver. These two steps lead to the growth of the endodermal cells and their differentiation into hepatocytes (Le Douarin, 1963a, 1963b; Le Douarin, 1964a–1964c; Croisille et Le Douarin, 1964; Le Douarin, 1965).

In the early 1960s, the molecular tools required to identify the signaling molecules involved in these processes were not available. Since then, several

groups have pursued investigations on the tissue interactions responsible for the development of glandular tissues of endodermal origin. The two-step induction of the prehepatic endoderm was found to occur also in mouse development. Fgf1 and Fgf2 were shown to trigger the specification effect of the cardiac mesoderm on the prehepatic endoderm. Fgf8 seemed to be responsible for the second induction, which leads to growth and differentiation of the hepatocytes (Jung et al., 1999).

In the chick, I investigated the nature and origin of the mesenchymal component of the liver. It turned out that the liver mesenchyme develops at the same time as it is invaded by cords of epithelial cells from which the hepatocytes differentiate. This is why it had never been described or isolated. I designed a microsurgical technique, through which the mesenchymal component of the liver developed in the absence of its endodermal component, which was prevented from invading it. In such a situation, the *liver mesenchyme* developed as bilateral empty bags connected with the *septum transversum*. It turned out that, in the embryo, the liver mesenchyme was entirely composed of cells belonging to the vascular endothelial lineage.

The technique I devised to isolate the mesenchymal network into which the epithelial cords of endodermal origin progress consisted in interposing an obstacle (a fragment of the shell membrane of the egg) in the somatopleura within the presumptive territory fated to yield the liver mesenchymal component. The areas located posteriorly to the barrier gave rise to the mesenchyme that could be cultured in vitro. The liver mesenchyme was then placed in organotypic cultures, with the aim of exploring more precisely its role in the differentiation of the hepatic-endoderm (Le Douarin, 1964b, 1964c).

The endoderm of the AIP of the chick embryo of 2 to 3 days of embryonic age (E2–3) could easily be isolated by using trypsin or pancreatin. This avoided contamination by mesenchymal cells. If cultured alone as an explant at the interface air/medium, it survived for about 24 hours, but its component cells did not proliferate. The contrast was striking when this small piece of endoderm was placed in contact with the liver mesenchyme taken from an E5–6 embryo, which I operated so that this mesenchyme developed void of hepatocytes. In such an association, the endodermal cells divided actively, colonized the mesenchyme, and acquired evident hepatocytic features: glycogen storage and enlargement of the nucleolus were the most conspicuous. None of the other endodermal anlagen in the E2–3 embryo, with the exception of the epithelium of the AIP, taken from the donor and subjected to the influence of the hepatic mesenchyme, underwent any of these changes. This was indicative of the commitment of the AIP endoderm to hepatic differentiation.

I found that the effect of hepatic mesenchyme was mediated by a diffusible substance since it could take place when a filter preventing cell-to-cell

contacts was interposed between the two reactive tissues. The substance(s) probably belongs to the Fgfs family, as is the case in the mouse.

From Liver Studies to the Quail/Chick Marker System

Being "Docteur ès sciences" in 1964, the "normal" course of my career should have been to continue as a CNRS researcher. The other possibility was to apply for a position of "Assistant Professor" in a University. This, however, did not seem realistic to me since, at that time, these positions were essentially reserved for men. The position of University Professor was much more prestigious in the early 1960s than that of CNRS researcher.

Etienne Wolff, however, encouraged me to apply for a position at the University of Clermont-Ferrand , where I stayed for 1 year before going to Nantes. Two positions became available there in 1966, which my husband and I occupied. We therefore moved the family to this town, where I had spent my high school years in the Lycée Gabriel Guist'hau and where I established my first independent research group.

The respective status of University Professor and CNRS researcher has radically changed since this time. In the 1950s and early 1960s, the laboratory directors were all professors while the CNRS employees, at least in biology, worked under their authority. Moreover, in contrast to the "Education Nationale," the CNRS did not appoint their researchers and technicians to permanent jobs. It was only after 1981, when the left wing won the elections and François Mitterrand became President, that the CNRS researchers acquired the status of civil servants with the same salaries as the University personnel.

With the after-war baby boom, a rush of students submerged the universities. The teaching load of the assistants and professors increased considerably. Moreover, the social changes that followed the 1968 movement, which minimized the prestige of the University Chairman, contributed to switching the research funding provided by the Ministry of Education and Research from the universities to CNRS and INSERM (Institut National de la Santé et de la Recherche Médicale) laboratories. A more accurate way of evaluating the scientific achievements of the laboratories prevailed and, beginning in the 1970s, research subsidies were preferentially distributed to laboratories in which CNRS researchers, free from teaching duties, could devote all their time to their research projects. As a consequence, research and universities tended to be separated with, at term, negative consequences for both. Fortunately, a certain number of productive university laboratories became associated with the CNRS and INSERM so that research activity remained, at least in these, closely associated with teaching and students.

In 1965, I was thus appointed as a Maître de Conférences (now designated as second-class Professor) in Clermont-Ferrand in the Department of

Developmental Biology whose chairman was Hubert Lutz. Hubert Lutz had been Etienne's first student when he started his career at the University of Strasbourg. I was provided with a substantial amount of teaching, which extended over the whole academic year. I was in charge of teaching Biology for the first and second-year students and also in charge of a course in Developmental Biology, two others in General Physiology, and one in Agricultural Zoology! My family was still staying in Paris and I had to travel to Clermont-Ferrand every week by train. In addition to teaching, Hubert Lutz gave me the responsibility of supervising two young students who prepared a master research project. It was when I was working with them that I had, for the first time, the opportunity to use quail eggs for my experiments.

Several laboratories working with avian embryos in France were provided with eggs of quail (*Coturnix coturnix japonica*) by a geneticist working in the CNRS campus of Gif-sur-Yvette in close vicinity of Paris. Dr. Ernst Bösiger was doing experiments on this species to document the concept of "hybrid vigor," developed after the Second World War, with a view of demonstrating that establishing "pure races" by means of inbreeding was not particularly favorable to the species, in contrast to the selective advantages provided by hybridization.

Each sexually mature female quail lays one egg per day, meaning that the large colony that he needed for his investigations produced far more eggs than he could use. He had the generous idea of giving these fresh quail eggs to labs which could use them for their research. In Etienne Wolff's Institute in Nogent, as well as in Clermont-Ferrand, some researchers started to do some experiments with quail instead of chick embryos. This is what I did with my two first students. We published a paper in 1966 (Le Douarin and Chaumont, 1966) in which we described that the mesenchyme of the liver is not unique in being able to promote the growth and differentiation of hepatocytes from the AIP endoderm. The entire splanchnopleural mesoderm can play the same role. If the endoderm of the AIP is grafted into the splanchnopleura of an E3 embryo, a liver lobe develops either in the ventral body wall or in the intestinal mesenchyme. Interestingly, other glandular structures, parathyroid and thymus, are also present at the graft site, meaning that the splanchnopleura is permissive for the expression of all the potentialities contained in the AIP endoderm. The signaling pathways responsible for this effect are still not known. Another result of these experiments was that the dorsal mesenchyme (somitic and unsegmented paraxial mesoderm) associated with the AIP endoderm inhibited its growth and differentiation, while allowing the survival of only a selected set of the grafted cells. Most of these experiments were carried out on quail embryos. I noticed, after a simple hematoxylin-eosin staining, that quail nuclei had a large, densely stained nucleolus, but this did not particularly capture my attention at this time.

The following year I was appointed at the University of Nantes, where I was to stay for 8 years. There I established my first independent research group. I was in charge of most of the Developmental Biology course and part of the Physiology one, the latter mainly under the responsibility of my husband, Georges le Douarin. These courses attracted several young students and some, interested in studying development, asked me to be their thesis supervisor. Christiane Le Lièvre, Marie-Aimée Teillet, and Josiane Fontaine, who were among them, have been working with me from the beginning, when I decided to apply the quail-chick marker system to the ontogeny of the neural crest and its derivatives, and for many years onward.

Devising a Cell-Marking Technique in the Avian Embryo In Ovo

Serendipity allied to curiosity sometimes results in a story with a happy ending. Before telling this story I will describe the framework of the institution in which I established my first independent research.

Nantes had possessed a University in historical times. Such an institution was created by a papal bull in 1490 but was abolished by the French revolution. The new University dated from the early 1960s. When we arrived, no active research laboratory had been established yet in the Life Sciences. Both teaching and research had to be created there by the newly arriving professors.

Georges Le Douarin was also a former disciple of Etienne Wolff and was interested in the development of the heart. During his postdoctoral stay with the well-known physiologist Edouard Coraboeuf, he became interested in the physiological aspects of cardiomyocytes differentiation. We joined our efforts to create a laboratory in which the two groups, his and mine, worked side by side on different subjects. I developed an embryology project which was the continuation of my thesis work on liver development. I was intrigued by the strong effect of the hepatic mesenchyme on the differentiation and growth of the AIP endoderm, and I decided to concentrate on the mechanisms underlying these tissue interactions. As a side project and since quail eggs were available, I decided to see whether the induction of growth and differentiation of the hepatic endoderm by the homologous mesenchyme could take place if these tissues belonged to two different species. This seems evident today since we know now that the molecular pathways involved in developmental processes have been remarkably conserved throughout evolution, but it was not so sure at that time. I then associated in organotypic culture the hepatic mesenchyme of a quail embryo with the AIP endoderm of a chick (and vice versa) in the same way I previously did with the two components belonging to the chick. I saw similarly that a lobe of hepatic tissue, in which mesenchymal cells and hepatocytes were closely associated, developed in the culture dish.

A "detail" struck my eyes, however, when I observed the microscopic sections of these chimeric liver lobules resulting from the association of chick AIP endoderm with quail liver mesenchyme. One of the conspicuous changes taking place in the endodermal cells when they become hepatocytes was the enlargement of their nucleolus. This is related to the increase in protein synthesis, a characteristic of hepatocytes. In the chimeric liver resulting from the association of quail mesenchyme with chick endoderm, the chick hepatocytes looked the same as in normal chick liver, but the mesenchymal cells also exhibited a large nucleolus, a character not expected in this cell type.

This probably reminded me of the observation that I made in Clermont-Ferrand of the large size of this organelle in quail cells. At that time, it remained in my mind in a subliminal state, but in the context of the hepatic tissue it occurred to me that it was something strange since no particular quantitative increase in protein synthesis was known to justify it in hepatic mesenchymal cells.

I decided to make some enquiry on this observation, just to satisfy my curiosity. First, I had a look again at diverse quail tissues, and I noticed that the large nucleolus of the hepatic mesenchymal cells was, in fact, a general feature of all quail cells. I applied Feulgen-Rossenbeck's staining procedure for DNA to various cell types, including the chimeric liver lobes that had developed in culture. I also tested the RNA component of the nucleoli through the Unna-Pappenheim staining method. To my surprise, I saw that the quail nucleolus was mostly composed, not of RNA as is classically the case, but of DNA (Le Douarin, 1969, 1973a, 1973b).

Notably, in the mesenchymal cells of the liver lobes, the amount of RNA of the nucleolus was as small as in the same cells of the chick. The enlargement of this organelle was due to the presence of heterochromatin, a feature common to all quail cell types in embryos and adults as well. Some variations in the quantitative and spatial relationships of DNA and RNA in the nucleolus existed, however, in the different cell types as reported in an article I published in 1973 (Le Douarin, 1973b).

In the late 1960s, an in-depth structural and dynamic study of the nucleolus was being performed in the laboratory of Wilhelm Bernhard in a CNRS laboratory at Villejuif, a Paris suburb. I decided to visit Dr. Bernhard and to show him the unusual nucleoli of quail cells. He was interested and invited me to report this observation at a meeting that he was organizing and which was going to take place a month later in Hungary, close to Lake Balaton. He found 15 minutes for me in the already finalized program.

This introduced me into the biology of this important cellular organelle whose role in the molecular biology of protein synthesis was being actively studied at that time. I was very interested and thought for a while to embark into this field with the aim of finding why the nucleoli of quail cells accumulated so much heterochromatin while in the chick, mouse, and humans, for

example, heterochromatin was more or less evenly dispersed in the nucleoplasm with merely some aggregates called chromocenters always distinct from the nucleolus. Having been interested in zoology and biodiversity during my training years at the University, I wondered whether this particularity of the quail was a rare occurrence or if it was common in the avian world.

I bought as many birds as available in specialized shops and decided to look at the distribution of their DNA in the nucleus. The problem was that I had to kill these beautiful little birds and found myself unable to do it. A colleague in the lab graciously replaced me in this nasty work.

I found that domestic birds had the same type of nucleolus as the chicken (with a very restricted amount of DNA component). Among wild birds, many had quail-like nucleoli. This particularity turned out to be a generic character. Within a given genus, like *Sturnus* for example, all the species I could look at had the same DNA-rich nucleolus (Le Douarin, 1971). These zoological investigations were only a short parenthesis. I rapidly resumed my research on the mechanisms of development and fully devoted my interests to this fascinating field.

I was still working on the cell-to-cell interactions that lead endodermal cells to differentiate into hepatocytes. At that time nothing was known about the signaling molecules (like the Bmps, Wnts, Fgfs, noggin, chrodin, etc.), which are responsible for determining dorsoventral polarity in the vertebrate embryo. What was suspected, however, was that this polarity corresponded to inverse gradients of still hypothetical dorsalizing and ventralizing substances along the dorsoventral axis of the embryo (see, for example, Toivonen and Saxen, 1955).

I had observed that any ventral type of mesenchyme (belonging to the lateral plate, either somatopleural or splanchnopleural) in the 2- to 4-day-old embryo (E2–4) was permissive for the development of the endodermal glandular tissues. In contrast, the paraxial, dorsal mesoderm was clearly inhibitory. I was thus curious to see what would be the effect of the still undifferentiated intermediate mesoderm in similar circumstances. At that time, several laboratories were investigating the development of the metanephros in the mouse and in the chick. I therefore knew how to isolate the chick metanephric mesenchyme before it had been induced by the ureteric rudiment to differentiate into kidney tubules. I therefore cultured this mesenchyme organotypically in association with the AIP endoderm in the same way I had done before with the liver mesenchyme.

The result was striking. The metanephric mesenchyme promoted the growth of the endodermal epithelium, which produced epithelial cords of hepatocyte-like cells but with a surprising characteristic: they were totally devoid of glycogen (Le Douarin and Houssaint, 1968; Le Douarin, 1968).

The capacity of these cells to produce glycogen, however, was not definitively lost. The lobe of pseudo-liver (made up of kidney mesenchyme associated

with the AIP endodermal cords of cells) was co-cultured with pure liver mesenchyme. Hepatocytes arising from the proliferation of the endodermal cells present in the metanephric environment invaded rapidly the hepatic mesenchyme. In this context these liver cells accumulated glycogen, although they were derived from the endodermal cells which had first developed in the metanephric mesenchyme.

The question was then the following: why was the glycogen absent in the hepatocytes that colonized the metanephros? I finally could prove that, in the chick, catecholamine (CA)-producing cells were present in the metanephric rudiment at the time I was removing it from the embryo (E4–5) (Houssaint and Le Douarin, 1968; Le Douarin, 1968). Epinephrine and norepinephrine are hyperglycaemic hormones. I thus inferred that they were depleting the glycogen from the hepatocytes present in the explant. I wanted next to prove that these CA-producing cells revealed by a simple histochemical technique were bona fide adenomedullary-like cells, hence derived from the neural crest.

After reading the available literature on the neural crest, I realized that I had at hand a way to see whether the cells that I suspected were producing adrenaline had a neural crest origin, as they should if my hypothesis was correct. The tool I had in hand for approaching this problem was the capacity to trace embryonic cells by constructing quail-chick chimeras. I then started to construct such chimeras by replacing the neural tube of the chick by that of a stage-matched quail at the level from which I supposed the medullary cells of the suprarenal glands originate.[3] I extended these grafts caudally since it had been reported in the literature that some "extra suprarenal glands" represented by adrenergic cell aggregates existed along the dorsal aorta in birds. In mammals such accessory adrenergic tissues are also present and even form islets of adrenergic cells on the surface of the urinary bladder.

These experiments were lateral to the main stream of the research going on in the lab, and I did them on my own just for fun. When I first saw a chimeric embryo whose sections had been treated with the Feulgen-Rossenbeck's staining procedure (allowing distinction between quail and chick cells by their nucleolus), the result was striking. Quail cells were present not only in the grafted spinal cord but also were dispersed in many other places of the embryo (at E5–6): along the nerves, as Schwann cells, in the peripheral ganglia, in the suprarenal gland and within the metanephritic mesenchyme. It was a shock, a real joy which has no equivalent, which I wanted to share immediately with the other members of the lab. They were invited to watch by themselves, at the microscope, what could be considered as the visualization

[3] I had an idea about that from the fate maps I had constructed for my thesis work where I destroyed transverse sections of the embryo through X-ray irradiations.

of the migration of cells within the embryonic body, a phenomenon that had so far merely been inferred from fragmentary experimental proofs. It was clear that this technique, compared to the previous ones used to label the cells and follow their fate (either H^3-TdR, vital stains, or carbon particles), was superior due to its being stable and unalterable and also not transmissible to neighboring cells. Its degree of precision thus depended upon the skill of the microsurgeon who was substituting the quail (or chick) tissue to that removed in the host of the other species. The potential use of this cell-marking technique for solving a number of problems in embryology immediately appeared to be considerable.

During my Ph.D. work, I had been working abundantly on the embryo in vivo, and I was well trained for doing microsurgery on many systems. Along the rest of my career in research I taught this technique, with many variants, to a number of younger research students and also to colleagues who came to Nogent for a sabbatical stay with us. Among many others, Marie-Aimée Teillet acquired remarkable skills in in vivo manipulations. We have worked together on a number of developing systems during 30 years. Christiane Le Lièvre was one of my first students in Nantes. For her thesis work, she completely revisited the role of the neural crest in vertebrate head development (Le Lièvre, 1978). This subject was later on pursued by Gérard Couly and myself with more refined methods and is presently taken over by Sophie Creuzet.

Others, such as Josiane Fontaine-Pérus, who developed her own lab at the University of Nantes, derived from the original quail-chick chimeras a new technique in which she constructed mouse-into-chick chimeric embryos and was thus able to study certain developmental problems in an original way. For her thesis work, Christiane Le Lièvre explored the fate of the cephalic neural crest and was the first to demonstrate that the facial skeleton is *entirely* derived from the neural crest. This was made possible because she performed large grafts of segments of the quail encephalic vesicles into chick embryos leading to cartilages and bones entirely made up of quail cells.

My long-term association with Gérard Couly deserves to be mentioned. When he came to the lab to undertake experimental work on the embryology of the face, he was a young surgeon at the Hospital Necker-Enfants-Malades in Paris. He was trained in odontofacial surgery and was essentially using his skills to repair congenital or traumatic malformations or damage in the faces of babies and children. He was intrigued by the origin of the distortions from normal morphogenesis that he had to deal with in the unfortunate children born with facial defects, a situation which occurs with a rather high frequency. He had heavy duties at the hospital and, although I fully acknowledged his curiosity and ambition to undertake experimental work on avian embryos, I was not so confident that he would be in a situation to meet the constraints involved in such an endeavor.

The reason he happened to know about my work is because I was asked to give a series of lectures on the neural crest to medical students. Although not a student anymore, he attended these lectures and developed a desire to be part of this venture.

Christiane Le Lièvre was in charge of teaching him how to handle the avian embryos. He thus started to switch from surgery on babies to microsurgery on small chick and quail embryos whose size at the stages he was concerned with was much smaller. He impressed us by the regularity with which he attended his microsurgery sessions in the lab. He regularly came one full day per week during which he was stuck to the dissecting microscope. When we were convinced that he was really taking it seriously, I managed to have him assisted by a technician to collect the embryos and perform histology on them when he was back at the hospital.

He became passionate for this work, and we engaged in a close collaboration that led to his becoming an embryologist particularly gifted in microsurgery while still being a doctor and eventually a Professor of Medicine at the University of Paris V in the Necker-Enfants-Malades hospital. Gérard Couly's grafting procedures were very refined. They concerned very small fragments of the anterior neural primordium and even of its lateral border, the neural fold at the head level, which were exchanged between quail and chick embryos, with the objective of discovering their actual fate during embryogenesis and their contribution to the adult brain and head.

Anne Grapin, now in EPFL (Ecole Polytechnique Fédérale de Lausanne), followed him in this venture for her thesis work. This approach gave rise to several articles in which many basic features of vertebrate head development were established (Couly and Le Douarin, 1985, 1987, 1988, 1990a, 1990b; Couly et al., 1992, 1993, 1994, 1995, 1996, 1998, 2002; Etchevers et al., 2002; Creuzet et al., 2005). This work is now pursued by Sophie Creuzet and myself with a new goal: discovering the molecular control of facial and brain development (see below) (Creuzet et al., 2006; Creuzet, 2009).

The Neural Crest and Its Derivatives in Higher Vertebrates

As related before, my first work on the neural crest was to seek an explanation for an intriguing observation concerning liver development. This first experimental acquaintance with the nervous system seemed to me very attractive, since I realized that I could achieve something worthwhile by using the quail-chick marker system to document the role of the neural crest in vertebrate development.

This transitory structure had been discovered by the German histologist, Wilhelm His, in 1868, and had been mostly studied in lower forms of vertebrates during the first half of the 20th century. This early work had been summarized in 1950 in a well-acknowledged monograph by Sven Hörstadius entitled *The Neural Crest* (Hörstadius, 1950).

When I became interested in this structure, in 1968, only a little information was available about the contribution of the neural crest to embryogenesis in higher vertebrates (birds and mammals). Moreover, knowledge about the neural crest in lower vertebrates was only partial. It seemed therefore that the identification of the derivatives of the neural crest in amniotes was a chapter of embryology that had to be further documented and the quail-chick marker system was perfectly suited to do so.

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 cells was followed during the entire embryonic life and could even be pursued after birth thanks to the stability provided by this cell labeling technique. Such chimeras were able to hatch and displayed normal growth and behavior before being subjected to graft rejection (see later discussion).

The experiments that I performed with young doctoral students, Marie-Aimée Teillet, Christiane Le Lièvre, and Josiane Fontaine, were aimed at exploring systematically the fate of the neural crest cells exiting from the various transverse levels of the neural axis. We could thus establish the following: *(1)* the cell types arising from the neural crest, *(2)* their level of origin, and *(3)* the pathways they followed to reach their destinations. This allowed a fate map of the neural crest to be constructed.

The derivation of the peripheral nervous system (PNS) from the neural crest was already known. However, it was striking to see that regions of the spinal cord and medulla oblongata were particularly devoted to yield the sympathetic, parasympathetic, or enteric ganglia. Certain areas of the neural crest were particularly interesting to me in this respect. One of them was the so-called vagal level of the neural crest (extending over somites 1 to 7 and corresponding roughly to the level of emergence of the vagus nerve, which innervates the gut), from which the neural crest cells migrated to the gut and invaded it down to the cloaca. This showed that the pre- and postganglionic parasympathic neurons of the enteric nervous system (ENS) have the same level of origin along the neural axis. We found an additional contribution to the ENS from the lumbosacral neural crest located posteriorly to somite 28. These two levels of the central nervous system (CNS) thus contain the preganglionic neurons innervating the enteric ganglia, which are derived from the neural crest and which, in the gut environment, differentiate into cholinergic and peptidergic neurons, catecholamine (CA)-producing cells being excluded in the ENS.[4]

[4] This is true for birds at all developmental stages. In the mouse, the neural crest cells invading the gut give rise to a transient wave of adrenergic cell differentiation in the fetus. No adrenergic neurons, however, persist after birth.

A third region of interest is located in the spinal cord between somite 18 to 24. It provides the suprarenal gland with adrenal cells and yields the adrenergic neurons of the sympathetic ganglia corresponding to this axial level (but does not contribute to the ENS). It was for this reason designated as the "adrenomedullary" level of the neural crest. As mentioned earlier, accessory aggregates of catecholaminergic glandular cells located along the dorsal aorta are also produced by the neural crest of the more caudal levels.

These results were published in the *Journal of Embryology and Experimental Morphology* in 1973 (Le Douarin and Teillet, 1973). I happened to know the referee of this paper, since I met him in Woods Hole where I was invited during the summer as a Lillie Fellow. Pr. Yntema, who had worked on the origin of the gut innervation in the chick several years before (Yntema and Hammond, 1954, 1955), engaged in a discussion with me on the subject and said that he enjoyed reading the article when he was invited to act as a referee for the JEEM. The method he had used several years before to investigate how the ENS developed consisted of removing sections of the neural crest in the chick embryo. The results he obtained pointed to an origin of most of the neurons at the vagal level of the neural axis, but they were not as precise and clear-cut as those that were produced from the use of a cell marker. At that time (before Internet) Eugene Garfield, the founder in Philadelphia of the "Institute of Scientific Information," published every week *Current Contents*, where the table of the contents of all the scientific journals were recorded on a weekly basis. Every issue presented and commented on one or two articles that had a special interest and that were recorded as "Citation Classics." To our great satisfaction, our paper was one of those.

Developmental Plasticity of PNS Neural Precursors

The fate map of the neural crest clearly showed that the neural crest cells exiting from the neural tubte between the vagal and the lumbosacral levels did not contribute to gut innervation. This striking difference in neural crest cells fate between the "vagal-lumbosacral" or "enteric" on the one hand and the "adrenomedullary" levels, on the other hand, raised the question as to whether the neuronal precursors fated to differentiate into adrenergic or cholinergic neurons were precommitted prior to their migration. In such a case, this prespecification would be responsible for their migratory behavior. The alternative was that, according to the level of the neural tube from which they emerge, they could be led through defined migration pathways to a particular destination. Their differentiation into one or the other neuronal types would in this case depend upon signals that they would receive either during their migration or later at their site of arrest or both.

Experiments carried out to test these alternatives demonstrated the initial pluripotency of the autonomic neuroblasts. Thus, transplantation from

quail to chick (or vice versa) of the fragment of the neural tube located at the level of somites 18–24 (adrenomedullary level of the neural crest) up to the 1–7 level (enteric level of the neural crest), showed the capacity of the neural crest cells of this truncal level to colonize the gut and to differentiate into cholinergic (and not to adrenergic) neurons. In the same way, transplantation of the neural tube from the "enteric" or "vagal" level (somite 1 to 7) to the "adrenomedullary" level of the neural axis contributed adrenergic neurons and adrenomedullary cells (of vagal origin) to the para-aortic plexus and to the suprarenal gland, respectively (Le Douarin et Teillet, 1974; Le Douarin et al., 1975).

In the early 1970s, I was invited to lecture in several Canadian universities, and I related these observations before they were published. When I was visiting Pr. Jack Diamond, at McMaster University, Pr. Edwin Furshpan from Harvard was in the audience and saw, in my results, a support for experiments going on at that time at Harvard Medical School in the famous neuroscience laboratory where Edwin Furshpan, David Potter, and Paul Patterson were showing in isolated newborn rat sympathetic neurons that their adrenergic phenotype could be switched to cholinergic through coculture with glial cells. The plasticity of the neuronal progenitors that we demonstrated in vivo reinforced their conviction that the phenomenon they were seeing was not an in vitro artifact but resulted from a real influence of the environment on the terminal neuronal differentiation concerning the neurotransmitter choice (Patterson and Chun, 1977; Patterson et al., 1975; Furshpan et al., 1976; Rao and Landis, 1990). This series of work was the first demonstration of the developmental plasticity of neuronal progenitors. It was at the origin of a number of projects in various laboratories. Such a plasticity was found in other neuronal systems, including the CNS.

Paul Patterson continued to work on this capacity of sympathetic neuroblasts to switch their phenotype from adrenergic to cholinergic and identified a substance able to mediate this effect (Yamamori et al., 1989) that was already known for its action on hematopoietic cells, LIF (for leukemia inhibitory factor).

The Problem of the Origin of the Endocrine Cells Associated with the Digestive Tract: The APUD Cells

In the 1970s a great deal of attention was devoted to aminergic and peptidergic-secreting cells that turned out to be common in the nervous system as well as in the gut epithelium and its derivatives. One way to demonstrate such cells was to show that they were able to take up various precursors of monoamines and to decarboxylate them. Hence, this rather heterogeneous group of cells is designated by the acronym APUD (for amine precursor uptake and decarboxylation), the name given to them by a British histochemist, Anthony

Pearse (1969). Being decarboxylated, these amines were fluorescent if subjected to the appropriate wavelength of ultraviolet light. Pearse put forward the hypothesis that all the endocrine cells of that type (including the adenomedullary catecholaminergic cells [AM cells], the dopaminergic cells of the carotid body, several cells of the intestinal epithelium [e.g., the enterochromaffin cells], the pancreatic islet cells, the calcitonin-producing cells of the ultimobranchial body) were all neural crest derivatives. This was established for the AM cells, but the fact that all these cells had some histochemical common characters could not be considered as a proof of their common origin. With Josiane Fontaine and Christiane Le Lièvre, we traced the origin of the cells of the carotid body and of the calcitonin-producing cells of the ultimobranchial body back to the rhombencephalic neural crest (Le Douarin and Le Lièvre, 1970, 1971; Le Douarin et al., 1972, 1974, 1978; Pearse et al., 1973; Polak et al., 1974; Fontaine and Le Douarin, 1975). In contrast, the endocrine cells of the pancreas and of the gut epithelium were found to be endodermal in origin (Le Douarin, 1988; see also Le Douarin, 1982).

The Neural Crest and the Vertebrate Head

One of the striking features of the neural crest is that it is truly pluripotent and yields not only neural cell types, melanocytes, and endocrine cells but also mesenchymal cells that are highly represented in the vertebrate head. The first demonstration of the contribution of the crest to the mesenchyme goes back to the 19th century when Kastchenko (1888) and Goronowitsch (1892, 1893) observed cells of mesenchymal types arising from the neural crest in Selacians and Teleosts. At the same time, Julia Platt, watching the development of *Necturus* under the microscope, noticed that cells originating from the dorsal midline of the neural anlage travelled ventrally to participate in the formation of the lower jaw (Platt, 1893). This assumption of a possible ectodermal origin of mesenchymal cells giving rise to bones contested the generally recognized validity of von Baer's germ layer theory, according to which each of the three germ layers was devoted to yielding definite types of derivatives. In this context, the skeleton was considered to be exclusively of mesodermal origin. It thus appeared (at that time) "impossible" that the jaw could have an ectodermal origin via the neural crest. Following this observation, numerous investigations were carried out during the first half of the 20th century, essentially on lower vertebrates, which confirmed and completed Julia Platt's results (for reviews, see Hörstadius, 1950; Le Douarin, 1982; Hall, 1999).

Very little work had been devoted to the neural crest in the higher forms of vertebrates when I started to be interested in this structure. This is related to the fact that in amniotes the number of embryonic cells becomes

rapidly very high and the migrating cells thus become undistinguishable from those of the tissues through which they move. A way to label them in a reliable and stable manner was actually required for this purpose.

Until such a method became available, researchers performed ablation experiments in which excision of fragments of the neural primordium in chick embryos were followed by the absence of certain structures (Hammond and Yntema, 1953; see Le Douarin, 1982, for a review). However, this technique is not totally reliable, since at the stage of development elected for these experiments, the capacity of the embryo to regulate the deficiencies due to the ablations is high.

In 1966, Malcolm Johnston undertook a series of investigations on craniofacial development in the chick by tracing neural crest cells through the radio-autographic labeling of dividing cells with tritiated thymidine (^{3}H-TdR) (Johnston, 1966). This technique was also used by Drew Noden in 1975 for the same purpose. Realizing that neither the specificity (due to contamination of neighboring cells by the marker released by the dying labeled cells) nor the stability (because the label becomes diluted as the cells divide) of the marker were insured by this technique, Johnston, Noden, and their colleagues turned to the quail-chick marker system to investigate the migration and fate of the cephalic neural crest cells. These authors clearly confirmed the participation of the cephalic neural crest to the mesenchyme of the head and the jaws (see Le Douarin, 1982, for a review).

Christiane Le Lièvre and I undertook experiments aimed at deciphering the role of the cephalic neural crest cells on morphogenesis of the head from the early 1970s onward. Our aim was to see to what extent the neural crest contributes to the head skeleton and what was the respective part of the ectoderm (via the neural crest) and the mesoderm in both cephalic skeleton and connective tissues. For this purpose (in contrast to the experiments done before, where small fragments of the neural tube labeled with ^{3}H-TdR were grafted into unlabeled chick embryos), entire encephalic vesicles were exchanged between stage-matched quails and chicks. This allowed us to see whether the bones were entirely or only partly derived from the neural crest (Le Lièvre and Le Douarin, 1974, 1975; Le Lièvre 1974, 1978).

A massive migration of the neural crest cells from their dorsal origin to the surface of the developing brain and the facial and hypobranchial buds was observed. Apart from the striated muscles of the branchial arches and the vascular endothelium of all the blood vessels, which were derived from the host mesoderm, the connective and skeletal tissues of the face and of the frontal and parietal part of the skull were of neural crest origin. Striated myocytes, forming the iridal muscles, have recently been shown to also be neural crest derived (Creuzet et al., 2005) as well as the ciliary bodies and the corneal endothelium. The meninges of the telencephalon were entirely of neural crest origin (except for the blood vessel endothelium). These structures are

mesoderm-derived in all the other parts of the CNS (Le Lièvre, 1974; Le Lièvre and Le Douarin, 1975).

The Neural Crest and Vertebrate Evolution

These embryological results together with other considerations led Gans and Northcutt (1983) to put forward their concept of the vertebrate "New head," according to which emergence of the neural crest is essential for the evolutionary transition from the protochordates to the vertebrates. The extant form of cephalochordates, the amphioxus, generally considered to be similar to the putative vertebrate ancestor, is devoid of a neural crest. This is also the case for the urochordates (although neural crest-like cells have been reported in ascidians (Jeffery et al., 2004). Amphioxus has a poorly developed encephalic vesicle and is devoid of the sense organs that characterize the vertebrate head. According to Gans and Northcutt, the development of a head in the chordate phylum is linked to the appearance of the neural crest and coincides with a change in lifestyle. Amphioxus is filter-feeder, while vertebrates became predators. This was made possible thanks to the acquisition of sense organs (vision, smell, audition), which developed from the anterior part of the neural primordium from placodes that, like the neural crest, are vertebrate innovations. The vertebrate brain became more and more complex and efficient through the development of associative neural structures, especially in the forebrain, midbrain, and cerebellum.

The work that we did in the late 1980s and early 1990s with Gérard Couly and Olivier Pourquié, and which consisted in constructing the fate map of the anterior neural plate, brought about interesting data along this line. By constructing the fate map of the anterior part of the neural plate and thus following the development of the early neural primordium with Gérard Couly, we have shown that, as in its original configuration in jawless vertebrates (hagfish and lampreys), the anterior-most part of the early neural primordium corresponds to the diencephalon (thalamus, hypothalamus, and pituitary gland) with only a modest development of the telencephalon dorsally. The diencephalon corresponds to the anterior end of the notocord, a vertebrate-organizing center that plays an important morphogenetic role at different steps of body plan patterning.

Olivier Pourquié in his postdoctoral work demonstrated that the differentiation of the paraxial mesoderm (cephalic and somitic) into cartilage and bone depends upon a signal arising from the notochord (Pourquié et al., 1993), later identified as the secreted molecule sonic hedgehog (Shh). The notocord present up to the mesencephalon-diencephalon junction thus accounts for the formation of the vertebral column and the occipital region of the skull. The evolution of the vertebrate phylum is characterized by the development of the cerebral hemispheres, which peaks in nonhuman primates and humans. We focused our attention on the early developmental

steps of the cerebral hemispheres and showed that they arise from the lateral areas of the anterior neural plate. After fusion of the neural folds and formation of the encephalic vesicles, these lateral areas are the site of intensive growth, so that they develop rostrally beyond the tip of the notocord and the adenohypophysis to form the telecephalon. The latter becomes "buried" inside the stomodeal cavity while maintaining its close relationships with the floor of the diencephalon (which yields the hypothalamus). Due to the absence of notocord and mesoderm at the telencephalic level, no skeleton of mesodermal origin develops to cover the "new brain" that appeared and enlarged during the course of evolution. This new brain was covered by cells of neural crest origin, which formed the forebrain meninges and the skull (optic and nasal skeleton, frontal and parietal bones). Thus, coevolution of the anterior brain and of the neural crest was critical for the development of higher cognitive functions in the most recent forms of vertebrates. Notably, this considerable development of the forebrain has been accompanied by the emergence of sense organs, the eyes that originate from the diencephalon and the smell organs whose precursors cells we have localized in the anterior neural fold. The fate map of the neural plate that we published in 1985 and 1987 (Couly and Le Douarin, 1985, 1987) has served as a reference for many investigations of the forebrain in mammals, birds, as well as *Xenopus* by other groups. Acquisition of higher brain functions in vertebrates was matched by a change in lifestyle as compared to the filter-feeder ancestors, the cephalochordates. Vertebrates became able to seek their food and later even became predators. Their facial skeleton, which is entirely derived from the neural crest (see Le Douarin, 1982), involves the first organ of predation, the jaw, which is already well developed in some teleost fishes. Evolution of the vertebrate head is therefore characterized by an increased participation of ectoderm via the neural primordium. The latter not only generates the brain but also the neural crest, which is critical to construct the face and yields a large part of the heart and of the head vasculature (endothelium excluded). In conclusion, the embryological analysis of neural crest ontogeny has shed new light on the evolution of the vertebrate phylum. This was recently confirmed by a series of studies that we performed on the role played by the neural crest on the development of the vertebrate brain.

The Neural Crest: A Signaling Center Regulating the Development of the Preotic Brain

The experimental demonstration that the neural crest has a major influence on brain development had not been reported before a series of studies carried out in my laboratory in recent years. I wish to describe how and why the experiments which led to this important notion were undertaken.

The origin of this work stems from the observation of several authors (see Le Douarin and Kalcheim, 1999, for a review) that *Hox* genes, which play a critical role in patterning the body in all bilateria, have, in vertebrates, their anterior limit of expression between the two rostral-most rhombomeres (r1 and r2). This means that most cephalic structures develop in a Hox-free domain. As far as the neural crest is concerned, the cells that migrate to the facial primordium and construct the facial skeleton are *Hox*-negative, whereas those forming most of the hyoid cartilage and the so-called cardiac neural crest, which contributes to the conotruncus of the heart (Kirby et al., 1985), express *Hox* genes of the first four paralogous groups. Each rhombomere (or pair of rhombomeres) is characterized by a combinatorial *Hox* gene expression (also designated as *Hox*-code). Mutational analyses carried out in the mouse have shown that *Hox* genes are critical for patterning these neural crest derivatives as well as for the development of the vertebral column from the paraxial mesoderm and for the development of the brain stem from the rhombencephalon (see Le Douarin and Kalcheim, 1999, and references therein).

The Hox-negative, anterior domain of the cephalic neural crest (CNC), which is responsible for the construction of the face and part of the skull (Couly et al., 1996), designated as FSNC (for facial skeletogenic neural crest) is clearly patterned by another genetic system which was shown to involve among others the vertebrate homologs of the *Drosophila Orthodenticle* and *Emptyspiracle* genes: Otx1, Otx2 and Emx1, Emx2, respectively.

The question that we raised was the following: is it possible to get a normal development of head and face skeleton if a fragment of the Hox-positive domain of the premigratory cephalic neural crest (e.g. r4–r8) is substituted for the anterior Hox-negative one? In previous work we had shown that the rostral-most neural fold down to the level where the epiphysis is formed (i.e., at the mid-diencephalic level) does not undergo the epithelio-mesenchymal transition (EMT) and therefore does not produce neural crest cells (Couly and Le Douarin, 1985, 1987). The problem was raised as to whether, after excision of the FSNC down to r3 excluded, the remaining rostral and/or caudal portion of the neural fold (NF) that were left in situ would regenerate neural crest cells able to construct the head skeleton. The result was that, in the absence of the FSNC (the NF corresponding to the posterior half of the diencephalon, the mesencephalon and r1 + r2) excised at 5–6 ss, that is, prior to the onset of neural crest cells emigration, the facial processes remained empty of neural crest cells and did not grow. Hence, a complete absence of facial structures ensued. Moreover, the brain was the site of major defects resulting in anencephaly. The telencephalon was severely reduced as well as the thalamus and optic tectum, which did not develop since the preotic brain remained open.

If a fragment of the Hox-positive NF was grafted rostrally to replace the rostral domain, morphogenesis of the head was equally disrupted. In contrast,

within the *Hox*-negative domain, including the diencephalic, mesencephalic, and anterior rhombencephalic levels (r1–r2), the neural crest possesses a high regeneration capacity. For example, a quarter of the endogenous Hox-negative neural crest (i.e., belonging to the FSNC), whatever its level of origin (e.g., diencephalic, mesencephalic, or rhombencephalic), is able to reconstitute the entire facial and lower jaw skeleton and to restore brain morphogenesis as well (Creuzet et al., 2002). This means that the information encoding any particular element of the facial skeleton does not belong to the neural crest proper but is imposed on it by extrinsic cues. The foregut endoderm was shown to play a role in this morphogenetic process (Couly et al., 2002), and we could demonstrate that one of the signals involved is *Sonic hedgehog* (*Shh*) (Brito et al., 2006).

Moreover, gain of function experiments, in which *Hox* genes were selectively electroporated into the Hox-negative domain of the neural crest, have shown that expression of these genes inhibits the differentiation of the facial cartilages and bones (Creuzet et al., 2002). The molecular mechanism of this inhibition is presently under investigation in our laboratory.

The role of the cephalic neural crest (FSNC) in brain development was further investigated at the molecular level. One of the immediate effects of FSNC excision was to dramatically reduce the production of Fgf8 in the anterior neural ridge (ANR) and to a lesser extent in the isthmus, two regions of the brain anlage that have been recognized as "brain organizers" through the production of this signaling molecule. In the absence of the cephalic neural crest, the branchial arch ectoderm is also deprived of *Fgf8* mRNA. The effect of Fgf8 on both facial and brain development is therefore critical, and the dramatic phenotype resulting from FSNC removal can be rescued by exogenous Fgf8 provided to the operated embryos through Sephadex beads placed in close contact to the ANR (Creuzet et al., 2006).

In still ongoing experiments, it has been shown that Fgf8 production by the ANR is regulated by the cephalic neural crest, which produces anti-Bmp4 secreted molecules like *Noggin* and *Gremlin* from the time they start to migrate and onward. Because Bmp4 is a strong antagonist of Fgf8 production (Ohkubo et al., 2002), the excision of the cephalic neural crest results in the failure of the development of the telencephalon, the thalamus, and the optic tectum, that is, the brain regions derived from the lateral territories of the rostral neural plate. Fgf8 is critical for this process, and the neural crest cells play a regulatory role in the production of this signaling molecule during the early stages of neurogenesis (Le Douarin and Creuzet, 2009).

Therefore, the role of the CNC in head development is not restricted to providing the cells that build the skeleton and connective tissues of the face. The neural crest also acts as an organizing center able to regulate the activity of both the ANR and the isthmus.

Cell Culture of Neural Crest Cells: Toward the First Demonstration of a "Neural Stem Cell"

The experiments aimed at disclosing the fates of the neural crest cells emerging from the various levels of the neural axis illuminated two characteristics of these cells: their capacity to give rise to a large number of different cell types and the fact that they migrate all over the developing embryo to settle in many places where they differentiate into a large variety of derivatives. In fact, there are virtually no organs or tissues in the vertebrate organism devoid of neural crest cells since, as Schwann cells, they line the nerves that travel with the smallest blood vessel ramifications.

This pluripotency and vast distribution all over the body are characteristics also shared with the blood cells. This led me to undertake an investigation on the actual developmental capacities of the neural crest cells by using clonal culture assays as had been so successfully done to study the hematopoietic cell lineage.

When I arrived in Nogent in 1975, I found appropriate conditions to develop in vitro cultures of the neural crest cells. Catherine Ziller, like me a former student of Etienne Wolff, took over the project together with Anne Baroffio, a postdoctoral fellow, and Elisabeth Dupin, a graduate student at that time. They developed a method that allowed clonal cultures of neural crest cells and provided them with a medium that, in principle, allowed the full expression of their differentiating capabilities. This led us to show that, as early as the migratory stage, neural crest cells from the cephalic level of the neural axis (CNCC) form an heterogeneous population of cells with different commitments. Fully committed precursors, giving clones with only one cell type, were found, as well as pluripotent progenitors, in respective proportions of about 20% and 80%.

Our problem raised by the CNC is that it yields not only derivatives of the "neural" type (such as neurons, glial cells, melanocytes, and peptidergic cells) but also cells of the mesenchymal type (cartilage, bone, and connective tissues), which in other parts of the body are of mesodermal origin. The question was therefore whether these two types of cells could be found in the progeny of single CNCC. Analysis of the phenotypes represented in the colonies showed that the capacities to produce cells of the neural and mesenchymal lineages coexist in the CNCC. The number of the neural crest cells recorded as being able to yield elements belonging to these two cell lineages in their progeny was low in our first experiments (Baroffio et al., 1988, 1990, 1991a, b; Dupin et al., 1990) but increased recently for two reasons: first, because we could show that the signaling molecule Shh, applied at a defined time course during the differentiation process, enhances significantly the number of cells differentiating into chondrocytes in the presence of other cells of the neural type. Secondly, we showed that numerous

cultures contained cells expressing *Runx2*, a gene considered as a marker for bone differentiation (Calloni et al., 2007, 2009).

Moreover, as also shown by the Graham group (McGonnell and Graham, 2002), the trunk neural crest which in vivo does not yield cells of the mesenchymal lineage turned out to be able to do so in appropriate culture conditions.

Building on these results, we could propose a cell lineage model that accounts for the diversification of neural crest derivatives from a multipotent stem cell (Le Douarin et al., 2004). This first identification of a "neural stem cell" (although at that time, the self-renewal capacity of this cell had not been demonstrated) in the nervous system was confirmed by other groups in the United States and was followed by the demonstration of neural stem cells in the CNS.

This work is now pursued in our group by Elisabeth Dupin and several coworkers. We showed that several pluripotent progenitors such as those able to give rise to glial (G), pigment (M), and smooth muscle (F) cells (GMF precursors), or only glial cells and melanocytes (GM precursors), are endowed with self-renewal capacities and thus can be considered bona fide as "stem cells" (Trentin et al., 2004; Le Douarin et al., 2004). It was obvious that a parallel could be established between the segregation of cell lineages in the neural crest and in the hematopoietic system.

This led to the search for growth and survival factors able to promote the proliferation and differentiation of a selected set of precursor cells in each type of neural crest derivatives. The identification of molecular markers specific for the different cell types arising from neural crest progenitors has been a prerequisite for the analysis of the phenotypes present in the cultures. In the 1980s onward, my laboratory engaged in the production of monoclonal antibodies directed against molecular markers for the avian neural crest cells and their derivatives and also for other cell types. Some of these monoclonal antibodies have been studied in depth. This is the case for example, for the SMP monoclonal antibody, which allowed the cloning of the gene and the purification of the Schwann cell myelin protein, which exhibits a strict specificity for oligodendrocytes in the CNS and the Schwann cells in the PNS (Dulac et al., 1988, 1992; Dulac and Le Douarin, 1991). SMP was discovered and studied by Catherine Dulac, now professor at Harvard University, and by the late Patricia Cameron-Curry who died of acute lymphocytic leukemia 1 month after being diagnosed at the age of 35. Patricia came to our laboratory as an Italian postdoctoral fellow and decided to stay in France. She had just been appointed to an INSERM position when she suddenly fell sick. This was for us a terrible shock and a great loss.

Other monoclonal antibodies were useful in our studies on chimeras because they recognized species and cell type–specific antigenic determinants and thus allowed us to recognize quail from chick cells as well as their differentiated phenotypes in chimeric birds.

Cytokines Selectively Active on Subpopulations of Neural Crest Cells

Our next goal was to identify the factors provided to the neural crest cells by the environment they meet at the term of (or during) their migration and which make them survive, grow, and differentiate. Among the secreted factors we studied, endothelin 3 (ET3) was the most interesting. Laure Lecoin, Ronit Lahav, Valérie Nataf, and Elisabeth Dupin worked very successfully on this project (Lahav et al., 1996, 1998; Nataf et al., 1996, 1998a, b; Lecoin et al., 1998), which is presently pursued actively by Elisabeth Dupin and myself.

We could show that ET3 exerts a proliferative effect on the neural crest precursors, which are endowed with a specific seven-transmembrane-domain receptor for this peptide. These precursors are essentially of two kinds, those which invade the gut and later on differentiate into the ENS, and the future melanocytes which migrate to the skin. ET3 is produced by the superficial ectoderm and by the mesenchymal wall of the gut. We found that these two kinds of neural crest progenitor cells express two different (although closely related) ET3-receptor genes in avian species, while in the mouse and humans only one ET3-receptor exists on both cell types called ETRB. Laure Lecoin cloned the melanocytic ET3-receptor in chick and quail that she designated ETRB2 (Lecoin et al., 1998), which is encoded by a different gene from the avian ETRB (designated as ETRBI) expressed in the PNS (Nataf et al., 1996).

In the avian embryo, the precursors of melanocytes exit from the neural tube later than those which have taken the dorsoventral migration pathway. They were found to express ETRB2 and not ETRB1. If the early migrating crest cells are cultured in vitro, they express in their majority the ETRB1 gene. In the presence of ET3, a switch of ETRB1 to ETRB2 expression takes place as soon as 24 hours of exposure to the cytokine. One day later it appears that the cells have proliferated much more actively in presence of ET3 than in control cultures and they massively differentiate into melanocytes in the following days. The presence of neural crest cells expressing transiently both receptors suggests that ET3 is able to activate the ETRB2 genes both in culture and in the skin but not in the gut environment where ETRB1 remains the only ET3 receptor gene activated (Lahav et al., 1998).

In following work, we were able to demonstrate that the ET3 receptors (ETRB1 or ETRB2) were either continuously expressed or at least inducible in fully differentiated melanocytes and glial cells (like Schwann cells). If subjected to ET3 in vitro, these cells were induced to proliferate and to dedifferentiate into precursors of the GM or GMF types which could self-renew and later on yield melanocytes (M), glial cells (G), and/or myofibroblasts (F) (Dupin et al., 2000, 2003; Real et al., 2005, 2006).

These experiments were a striking demonstration of the capacity of differentiated cells to dedifferentiate and reacquire embryonic capacities. The GM and GMC precursors are able to self-renew. Therefore, under the proliferative stimulation exerted by ET3, melanocytes and glial cells are able to yield cells that have acquired a stem cell status.

Following Cell Migrations and Morphogenetic Movements during Neurogenesis of the Brain

In the late 1980s, the dominant thinking was that cell migrations in the encephalic vesicles were essentially radial. Tangential migrations had not been described, whereas radial migration of the neuroblasts had been clearly demonstrated by labeling the still dividing neuroblasts in the mammalian fetuses with tritiated thymidine. In 1989, two groups, using the Q/C chimera system (Balaban, Teillet, and Le Douarin) or LacZ carrying retroviruses as cell markers (Connie Cepko's group at Havard), published side by side two articles in *Science* (Balaban et al., 1988; Walsh and Cepko, 1988). Both showed that extensive tangential migrations actually occur during neurogenesis in the mammalian and avian forebrain, including the cerebral hemispheres, a notion now widely accepted.

In my laboratory, these explorations were further pursued on the cerebellum by Marc Hallonet, a graduate student. The results described in Marc's Ph.D. thesis changed significantly the current view about the development of this part of the brain. First, it was established that, in contrast to prior belief, the cerebellar cortex did not originate entirely from the metencephalic vesicle. An important contribution from the mesencephalon was demonstrated, such that the cerebellar primordium encompasses the territory expressing the homeobox gene *En2*, and indeed, as later shown, agenesis follows the targeted mutation of this gene in the mouse. The construction of chimeric brain showed that the cerebellum arises from a territory equally distributed on each side of the constriction known as the midbrain-hindbrain junction. In addition, the assumption according to which cells of the molecular layer originate from the external granular layer was found to be incorrect. We could show that these cells undergo an inward-outward migration from the ventricular epithelium, in the same way Purkinje cells do (Hallonet et al., 1990).

The Role of Cell–Cell Interactions in Gene Regulation within the Differentiating Neural Epithelium

A Japanese group headed by Harukazu Nakamura, a former postdoctoral fellow in my laboratory, and the French team of Dr. Alvarado-Mallard in Paris, using the Q/C transplantation technique, discovered that the midbrain-hindbrain junction is an important organizing center in brain development.

The two groups showed that transplantation of neuroepithelial grafts from this region to the diencephalon or rhombencephalon resulted in the induction of the *En2* gene in the host's neighboring neural epithelium and in the differentiation of tectal or cerebellar structures depending on the site of implantation. This led to the discovery by Dr. Salvador Martinez and colleagues (Crossley et al., 1996) that the critical factor in this induction is FGF8, naturally produced in the midbrain-hindbrain junction and fated to become the *isthmus*.

The fact that transcription factors expressed in the brain during development can be environmentally regulated prompted me to investigate the regulation of *Hox* gene expression in the rhombencephalon, a piece of work undertaken by Anne Grapin in association with Gérard Couly. When we started to investigate the problem of gene regulation in the hindbrain, the general assumption was that the expression of *Hox* genes in various levels of the brain was cell-autonomous and nothing was known about the upstream regulation of these genes. By performing a series of heterotopic rhombomere transplantations at the early neurulation stages between quail and chick embryos, we have shown that in fact *Hox* gene expression at the various rhombencephalic levels obeys extrinsic cues and is regulated by signals transmitted through the neural epithelium itself and the paraxial mesoderm. Retinoic acid acts on this process by "posteriorizing" the anterior levels of the brain through *Hox* gene induction (Grapin-Botton et al., 1995).

Our experiments have also revealed that the potentiality to express *Hox* genes extends up to the prosencephalon. Indeed, when transplanted posteriorly, prosencephalic neuroepithelium was induced to express *Hox* genes corresponding to the position of the transplant along the neural axis. Thus, in normal development either the inductive signal is not present in the prosencephalic and mesencephalic vesicles, or it is inhibited by antagonistic cues. Several experimental results point to the distribution of this signal as a postero-anteriorly decreasing gradient, a notion compatible with the hypothesis that retinoic acid could be involved in mediating *Hox* gene induction.

Transposition of rhombomeres along the neural axis does not modify *Hox* gene expression in transplants grafted more rostrally than their normal position. In contrast, caudal transposition results in "posteriorisation" of the neuroepithelium as far as expression of *Hox* genes is concerned, thus illustrating in vertebrates the "posterior dominance" demonstrated in insects. When rhombomeres 5/6 were transposed to the level or rhombomeres 7/8, this change in the *Hox*-code was followed by a homeotic transformation of the transplant phenotype. The transplant differentiated neural structures corresponding to its new AP position.

These experiments thus indicated that regulation of the *Hox*-code at each AP level of the neural axis depends upon positional cues and showed

the crucial role of *Hox* genes in neural specification at the rhombencephalic level.

Neurulation in Amniotes Revisited

From the work of Hans Spemann, neurulation was considered to result from an induction of the ectoderm by the "organizer" and its derivative, the notocord. The resulting neural plate was thought to be originally formed by a homogeneous sheet of epithelial cells in which the notocord introduces heterogeneity by inducing the neuroepithelial cells to become the floor plate, a medial structure that plays an important role in patterning the neural tube. The accepted view was that the floor plate is induced by the notocord via the secreted protein sonic hedgehog (shh). This induction would lead the floor plate cells to produce a transcription factor of the forkhead family HNF3bβ which in turn would control the production of Shh protein by floor plate cells. By using the quail-chick marker system in the embryo in ovo, it was possible to trace the fate of the avian organizer (Hensen's node cells) throughout the entire neurulation process. It turned out that the cells of the organizer express HNF3bβ from the onset of gastrulation onward. Moreover, they yield not only the notocord, as previously thought, but also the floor plate which accordingly is derived, not from the ectodermal but from the mesodermal layer and a stripe of cells in the dorsal endoderm. During gastrulation and neurulation, Hensen's node moves along the anteroposterior axis according to the process called "regression" and leaves in its wake these three mediodorsal structures (Catala et al., 1995, 1996; Le Douarin et al. 1998). This and other experiments thus challenged the role of the notocord in floor plate induction during normal development. In fact, the floor plate develops in a cell-autonomous manner, and the notocord is not required for inducing the floor plate. Those observations are supported by several genetic data, particularly by the existence of mutants in Zebrafish (like floating head) in which a floor plate develops into a normally patterned neural tube in the total absence of a notocord.

Our results therefore led to a reconsideration of the classical notions about the process of gastrulation and neurulation in the vertebrate phylum (Charrier et al., 1999; Le Douarin et Halpern, 2000 and references therein). They have also disclosed a novel role for the protein Shh during neurulation. We have shown that, if the development of the notocord and floor plate is prevented by extirpation of Hensen's node, the neural tube and the paraxial mesoderm are the site of massive cell death. The Shh morphogen thus acts as a survival factor, and one of the primary roles of these midline structures in the vertebrate embryo is to prevent the neural and mesodermal cells from triggering their cell death program (Charrier et al., 2001). This is further attested by the fact that after the removal of Hensen's node in the

six somite-stage (6 ss) embryo, the development of its posterior part can be rescued if it is provided with an exogenous source of Shh.

In collaboration with Dr. Patrick Mehlen and coworkers at the University of Lyon (France), we could demonstrate, in this system, that the Shh receptor Patched (Ptc) belongs to the family of "dependence receptors" characterized by the fact that in absence of their ligand, they induce cell death by activating cellular caspases through the liberation of a "death domain" from the intracellular part of their molecule (Thibert et al., 2003).

Therefore, one of the major roles of the notocord-floor-plate complex at these early stages of neurulation is to insure the survival and induce the growth of the cells of the neural tube and of the paraxial mesoderm. This analysis of neurulation through the use of quail/chick chimeras has profoundly revised current notions about neurulation in amniote vertebrates (Teillet et al., 1998a, 1998b; Thibert et al., 2003; Charrier et al., 2005).

Chimeras in the Study of Brain Functions

For about 10 years (from 1985 on), I extended our studies on embryos up to the posthatching stages. After seeing that the neural chimeras were able to both hatch and survive, we could tackle problems dealing with some functional aspects of brain development.

I had the privilege of being elected as a member of the Neuroscience Research Program (NRP) in 1983. This Program was founded in 1962 by Francis O. Schmitt (1903–1995), then Professor at MIT, and chaired by him until 1974. The NRP is an informal college of scholars and research scientists whose purpose was and still is to promote study of the brain. When Francis O. Schmitt retired, the NRP was transferred from Boston to the Rockefeller University in New York and its new chairman became Gerald Edelman. Members of the program met twice a year for a few days at the Rockefeller University in New York, where they presented their new results or listened to invited scientists. When I presented our experiments showing that Q/C neural chimeras were able to hatch and remained in a healthy condition for a long period after birth, Peter Marler, a specialist in bird ecology, then a NRP member, was in the audience. He reported these experiments to the fellows of his laboratory, one of whom, Evan Balaban, already a Ph.D. and thinking of a postdoc, was interested by the model. He thought that it could be applied to study the problem of "nature versus nurture" in behavior. What type of behavior would a chimera exhibit when regions of the brain had been replaced by those from another bird?

When he came to Nogent, Evan Balaban chose to study the species-specific crow (or call) induced in young birds by testosterone implants. He found that crows were different in young quails and chickens. Moreover, movements of the head accompanying crowing were also different between the two species. Evan decided to look for the brain site(s) driving vocalization

and behaviors linked to crowing. Marie-Aimée Teillet took part in the project and taught Evan how to exchange specific neuroepithelial areas between E2 quail and chick embryos. At that time encephalic vesicles are not yet vascularized and fusion of graft and donor neuroepithelium takes place rapidly. Numerous Q/C brain chimeras hatched, and it appeared that the crowing-specific area was in the mesencephalon. A chicken grafted with a quail mesencephalon emitted the quail typical crowing. However, simultaneous head movements were of the chicken type. When he returned to the United States and established his own laboratory, Evan was able to assign the origin of the typical head movements, accompanying the crow in the quail, to the brain stem (Balaban et al., 1988; Balaban, 2005).

This work, very demanding as far as the quality and precision of the microsurgery was concerned, showed that complex behaviors like crowing are genetically determined in definite areas of the neural epithelium of the encephalic vesicles a long time before neuronal differentiation takes place. Thus, at the early stages of neurogenesis, the neural plate is far from being a homogeneous structure with interchangeable regions. On the contrary, the neural epithelium is already the site of an underlying structuralization that involves genetic and species-specific inbuilt characteristics. During neurogenesis, the various regions so defined acquire their functionality together with their differentiation in the context of the connections that they establish with neighboring structures.

These experiments showed that the quail regions which define the crow as well as the head movements develop in a species-specific manner, whether they are on their own or in a foreign environment. Moreover, these regions develop autonomously, even though the connections they establish with other foreign brain structures are critical for the full expression of their functional characteristics.

The same observation was made in another series of experiments that involved a pathological trait determined by a Mendelian mutation leading to epilepsy.

The Epileptic Chickens

Seeing the possibility of exchanging parts of the brain between two embryos, Pr. Robert Naquet, a highly recognized specialist in epilepsy, wanted to use the avian chimera model to try and define the origin of an autosomic Mendelian recessive form of epilepsy in a strain of chicken discovered in Canada by Roy Crawford in 1972. Genetic forms of epilepsy are known in humans and are characterized by seizures triggered, among other things, by visual or auditory stimuli (e.g., intermittent light). They are considered to be a subcortical type of epilepsy. However, what generates seizures and what brain area(s) is (are) involved in this process was not well known, though it had been proposed that the focus is located in the brain stem.

As mentioned earlier, the embryonic grafts of neural epithelium are rejected in interspecific chimeras 1–3 months after the young bird has become immunologically competent, an event which takes place about 2 weeks after birth. During our work on the host's immunological response to neural grafts, we made an observation that opened a novel research avenue. Neural grafts between MHC-different chickens were permanently tolerated. This led our group to develop a series of experiments in collaboration with Pr. Robert Naquet and Dr. Cesira Batini, a neurophysiologist from the CNRS in Paris. The disorder that affected the homozygous epileptic chickens *(epi/epi)* consisted of seizures of the "grand mal" type following either light or sound stimulation. By transplanting entirely or partly either the prosencephalic, mesencephalic, or rhombencephalic brain vesicles, the neural pathways involved in the onset of the photic or audiogenic forms of epilepsy could be determined (Teillet et al., 2005). It was shown that the *nucleus mesencephalicus* (pars dorsalis) triggers the seizures after processing either stimulus (sound or light). The "*epi"* chicken mutant can thus be considered as a unique animal model for the so far poorly understood subcortical types of human epilepsy. The work on chicken epilepsy has given rise to several articles (Guy et al., 1992; Fadlallah et al., 1995; Teillet et al., 1995; Batini et al., 1996).

From Neurobiology to Immunology: Revisiting the Problem of Self/Non-Self-Discrimination in Postnatal Chimeras

The experiments that we performed in the 1970s to explore the fate of the neural crest cells exclusively concerned events taking place during embryogenesis. When I gave talks, however, I was often asked about the immunological status of the grafted quail cells and their numerous progeny in posthatching chimeras.

When a Japanese postdoctoral fellow, Dr. Masae Kinutani, came to the laboratory in 1982, she was enthusiastic about a project that would explore the hatching ability of Q/C spinal cord chimeras and the fate of the graft after birth, when the host's immune system has become fully competent. Marie-Aimée Teillet obtained the first hatching of a chimera. She gave me a large photograph of this bird that I keep as a precious remembrance. Masae Kinutani, under the efficient teaching of Marie-Aimée, became one of the most skillful "transplanters" in the laboratory and pursued the project.

The rate of hatching was about 1 out of 10 operated embryos but, once born, the chimeras generally were healthy. Their health did not last for long, however, as birds with a graft at the brachial level developed a paralysis of the wings and in most cases eventually died. A few animals, however, recovered complete motility and survived. The grafted segment of spinal cord appeared infiltrated with macrophages and lymphocytes, and the inflammatory process further invaded the host nervous tissues. These lesions were

similar to those observed in animals with induced experimental allergic encephalomyelitis (EAE) and to those occurring in humans afflicted with multiple sclerosis (MS). In addition, the chimeric birds developed neuritis in the peripheral nerves derived from the grafted spinal cord.

This pathology of the chimeras was clearly the manifestation of graft rejection but, curiously, the first signs appeared long after immunological maturity of the host. We interpreted this delay by the fact that CNS tissues have a privileged immunological status on one hand and express no (or only a little) class I and class II MHC antigens on the other. It is likely that the first immune attack on graft-derived tissues takes place in the peripheral nerves, devoid of the protective blood–brain barrier present in the CNS. The T-cells recognize neural and glial antigens and thus become activated and able to attack the CNS in the same way as in EAE, where T-cells become activated by systemic injection of neural antigens, for instance myelin basic protein (MBP). The chimeric birds thus appeared to be an experimental model for MS (Kinutani et al., 1986).

As far as developmental immunology is concerned, these results were at odds with the common assumption, according to which antigens present during development of the immune system are later recognized as self (for a discussion, see Coutinho, 2005). To understand whether the delay observed before rejection of the graft was due to the privileged status of the nervous tissue or whether it was related to the presence of the graft in the embryo, we undertook, with Claude Martin, Maurice Belo, Catherine Corbel, and a young Japanese researcher, Hiroko Ohki, a series of studies in which quail nonneural tissues, the limb bud and the bursa of Fabricius, were grafted into chick embryos. In both cases, the graft pursued its development in the chick, reaching its species-specific size according to its own timing.

Many chimeras of this type were obtained. All of them rejected the foreign wing acutely, starting at various times during the first two posthatching weeks (i.e., when the host's immune function reaches maturity). The wing was totally destroyed after 2 months. Thus, the delay typical for neural tube grafts was not observed for nonneural tissues. This was confirmed by Maurice Belo for bursal grafts (Belo et al., 1985; Corbel et al., 1987).

At that point, it appeared relevant to investigate the immunological status of wing bud grafted between chickens of different MHC. These strains were available from the INRA laboratory of genetics belonging to the Institut National de Recherche Agronomique (INRA). The grafted wing was not rejected, contrary to the outcome in Q/C wing-chimeras. However, tolerance was not complete since rejection crises occurred, separated by episodes during which inflammation regressed (Corbel et al., 1990).

Inspired by a paper in *Nature* (Fehilly et al., 1984), I was anxious to see whether manipulating the immune system of the chimera could induce permanent tolerance of the quail wing by the chicken host. On the front page of the journal was a strange animal, a sheep-goat chimera in which the tissues

from the two species formed a patchwork while entertaining peaceful relationships. What were the differences between the Q/C wing-chimeras and the sheep-goat chimeras? As mammalian chimeras result from the aggregation of two (or more) morulae, there is a chance that virtually all tissues of the animal contain cells from the two species. There is no telling the host from the recipient in this case while, in Q/C wing, bursa, or neural grafts, recipient tissues were clearly *dominant* over donor tissues.

I reflected that chimerism in the blood or in the thymus could be decisive for the reciprocal tolerance in sheep-goat chimeras, as thymus is the site of T-cell differentiation and as mouse blood chimeras produced at birth are tolerant to skin grafts of the MHC-type of the injected foreign blood cells in allogeneic combinations (Billingham et al., 1953). I knew, however, from a conversation with Milan Hasek that, in birds, this kind of tolerance is not induced by heterospecific blood chimerism. Consequently, we devised an experimental design in which the donor quail embryo provided not only a wing bud but also the four thymic epithelial anlagen of the third and fourth pharyngeal pouches substituted for their counterpart in E4 chick embryos, host and donor being stage matched.

The thymus operation was delicate. Claude Martin's skills in microsurgery were decisive, and she taught Hiroko Ohki and Maurice Belo. As described in our paper in *Science* (Ohki et al., 1987), one out of 10 animals hatched. Out of the 16 birds that hatched, 14 showed tolerance of the quail wing. In 3 of them slight signs of rejection appeared after about 40 days, much later than in Q/C limb-only chimeras. Two did not show tolerance. The complete thymic lobes of all these double limb-thymus chimeras were studied for chimerism analysis: quail cells appeared to form the thymic epithelial stroma in all the chimeras that were tolerant and not in the others that were not tolerant. However, substitution of thymic epithelial rudiments was usually incomplete. The proximity of the branchial arch arteries made the operation difficult because hemorrhages were fatal. This technical problem made the experiment even more interesting than expected. Tolerance turned out to be induced permanently if only one-third of the thymic lobes was chimeric (Belo et al., 1989). This meant that chick T-cells differentiating in contact with the quail thymic epithelium not only failed to aggress the quail graft but were able to "protect" the graft from the chick T-cells that had differentiated in the purely chick thymic lobes. When no tolerance was induced, none or very few thymic tissues were found to be chimeric.

This significant finding meant that tolerance to self did not result exclusively from the elimination of autoreactive T-cells in the thymus. The process was shown to be incomplete: T-cells with high affinity for self-components are currently found in the periphery and become harmful in autoimmune diseases. The Q/C double chimeras showed that a mechanism at work prevented these T-cells from being activated in an antigen-specific manner.

Tolerance mediated by these cells is "active," differently from the widely accepted dogma of the default mechanism, that is, autoreactive cell elimination in the thymus. This work was recorded in a series of papers (see for review Le Douarin et al., 1989, and Salaün et al., 2005).

At this point we had animated discussions with immunologists, particularly with Pr. Max Cooper from the University of Birmingham in Alabama, who spent a sabbatical year with us, and Pr. Antonio Coutinho, who was leading an immunobiology research unit at the Pasteur Institute. Although a number of important concepts in fundamental immunology were inspired by experimental results obtained in the chick, in modern times the classical model in this science is the mouse. Therefore, we have had to convince immunologists that we had made an observation of general interest. Open-minded and creative, Antonio Coutinho was immediately interested by the quail/chick chimera model and by the insights it was yielding about the mechanisms of self/non-self-recognition. We decided that we should try and reproduce these experiments in the mouse model. Josselyne Salaün in our laboratory successfully carried out this project together with Antonio Bandeira from the Pasteur group. Josselyne Salaün had been combining in vivo and in vitro studies on the mouse embryo in order to sort out the successive ontogenic steps of T-cell differentiation (Salaün et al., 1986) and was well prepared to tackle this problem.

Since microsurgery on the early mammalian embryos is not feasible, athymic nude mice were elected as recipients for isogeneic or allogeneic thymic epithelium grafting. Nude mice are devoid of T-cells and therefore immuno-incompetent. In both combinations, T-cell function was rescued by HC from the host, which colonized the grafted thymic epithelium, differentiating into T-lymphocytes that migrated to the periphery. We demonstrated that, in allogeneic combinations, the nude mice engrafted with the thymic epithelium were tolerant to both skin grafts of their own haplotype and of the thymus haplotype while rejecting third-party grafts. Yves Modigliani, a graduate student in Antonio Coutinho's laboratory, showed that this tolerance was mediated by CD4 T-cells. Antonio Coutinho designated it as "dominant" tolerance. These T-cells, whose existence was subsequently confirmed by many other groups (see Le Douarin et al., 1996, for a review, and Coutinho, 2005) and are called "regulatory T-cells" (Treg), had to be characterized. The first important step toward their molecular characterization was due to Sakaguchi and colleagues (1982), who found that such cells express as a surface marker CD25 in addition to CD4. A new research avenue in immunology was thus initiated, which is now expanding vigorously. At the Congress of the International Union of Immunological Societies held in Montreal on July 16 to 22, 2004, where I was invited to give a lecture on the contribution of my laboratory to immunology, several symposia were devoted to regulatory T-cells and the importance they may have in transplantation medicine.

Concluding Remarks

I wish to say that I consider it an honor to be invited to write about my own scientific experience in this prestigious series of autobiographies. I received this invitation in 2004 and accepted it with great pleasure. However, when I started to write about the past, I realized that I was so busy and had so many ongoing projects, that I did not feel able to pursue this task at that time. I put my writing aside and nearly forgot it. Fortunately, Pr. Larry Squire did not forget and encouraged me to go on. I wish to thank him for his persistence.

Now, time has passed and I can ponder my contribution to science with some composure. As a developmental biologist, I had the chance to make an observation that led me to devise a technique which turned out to be useful for deciphering some of the developmental features of many systems in the vertebrate embryo. Among those, the nervous and hematopoietic ones have been my favorites. About the former, I could show that the neural crest, a transitory and very unobtrusive structure of the early embryo (and a more or less abandoned topic at that time), is in fact a major player in vertebrate embryogenesis and even in the evolution of this phylum. It plays a critical role in building the head and, as shown recently, the brain. Several aspects of neural development were highlighted in this work: the plasticity of the neuroblasts and the influence of environmental factors on the final phenotypes they exhibit at the end of the differentiation process; the existence of a stem cell in the neural crest (at a time when stem cells were not the popular subject they are today), indeed the first example of "neural stem cells" ever mentioned; the evidence of the real cell movements involved in neurulation in amniotes. These cell movements have disclosed mechanisms that had not, so far, been correctly interpreted.

The fact that I led parallel research projects on the nervous and the hemopoietic systems led me with my colleagues to bring about the concept of *dominant tolerance*, now well illustrated by the large current of work on regulatory T-cells.

In conclusion, these series of experiments are characterized by the combination of a large diversity of themes, together with an underlying unity. This unity lies not only in the technology, a combination of classical embryological methods and molecular biology, but also in the rationale underlying the problems tackled and the experimental approaches elected to unravel them. This work was accomplished thanks to the collaborations of many gifted scientists, Ph.D. students, postdoctoral fellows, and colleagues, to whom I wish to express my heartfelt gratitude.

Selected Bibliography

Balaban E. Brain switching: studying evolutionary behavorial changes in the context of individual brain development. *Int J Dev Biol* 2005;49:117–124.

Balaban E, Teillet MA, Le Douarin N. Application of the quail-chick chimera system to the study of brain development and behavior. *Science* 1988;241:1339–1342.

Baroffio A, Dupin E, Le Douarin, NM. Clone-forming ability and differentiation potential of migratory neural crest cells. *Proc Natl Acad Sci USA* 1988,85: 5325–5329.

Baroffio A, Dupin E, Le Douarin NM. Cell lineage studies in avian neural crest ontogeny. In Lauder J ed. *Proceedings of the Second Conference of the Institute of Dev. Neuroscience and Aging Society for Developmental Neuroscience on Molecular Aspects of Development.* New York: Plenum Press, 1990; 53–62.

Baroffio A, Dupin E, Le Douarin NM. Common precursors for neural and mesectodermal derivatives in the cephalic neural crest. *Development* 1991a;112: 301–305.

Baroffio A, Dupin E, Le Douarin, NM. Clone-forming ability and differentiation potential of migratory neural crest cells. *Proc Natl Acad Sci USA* 1991b;85: 5325–5329.

Batini C, Teillet MA, Naquet R, Le Douarin NM. Brain chimeras in birds: application to the study of a genetic form of reflex epilepsy. *Trends Neurosci* 1996;19: 246–252.

Belo M, Martin C, Corbel C, Le Douarin NM. A novel method to bursectomize avian embryos and obtain quail/chick bursal chimeras. I. Immunocytochemical analysis of such chimeras by using species-specific monoclonal antibodies. *J Immunol* 1985;135:3785–3794.

Belo M, Corbel C, Martin C, Le Douarin N.M. Thymic epithelium tolerizes chickens to embryonic graft of quail bursa of Fabricius. *Int Immunol* 1989;1:105–112.

Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. *Nature* 1953;172:603–606.

Brito JM, Teillet MA, Le Douarin NM. An early role for sonic hedgehog from foregut endoderm in jaw development: insuring neural crest cell survival. *Proc Natl Acad Sci USA* 2006;103:11607–11612.

Calloni GW, Glavieux-Pardanaud C, Le Douarin NM, Dupin E. Sonic Hedgehog promotes the development of multipotent neural crest progenitors endowed xith both mesenchymal and neural potentiels. *Proc Natl Acad Sci USA* 2007;104:19879–19884.

Calloni GW, Le Douarin NM, Dupin E. High frequency of cephalic neural crest cells shows coexistence of neurogenic, melanogenic, and osteogenic differentiation capacities. *Proc Natl Acad Sci USA* 2009;106:8947–8952.

Catala M, Teillet, MA, Le Douarin NM. Organization and development of the tail bud analyzed with the quail chick chimaera system. *Mech Dev* 1995;51: 51–65.

Catala M, Teillet MA, De Robertis EM, Le Douarin NM. A spinal cord fate map in the avian embryo: while regressing, Hensen's node lays down the notochord and floor plate thus joining the spinal cord lateral walls. *Development* 1996;122: 2599–2610.

Charrier, JB, Teillet MA, Lapointe F, Le Douarin NM. Defining subregions of Hensen's node essential for caudalward movement, midline development and cell survival. *Development* 1999;126:4771–4783.

Charrier JB, Lapointe F, Le Douarin NM, Teillet MA. Anti-apoptotic role of Sonic Hedgehog protein at the early stages of nervous system organogenesis. *Development* 2001;198:4011–4020.

Corbel C, Belo M, Martin C, Le Douarin NM. A novel method to bursectomize avian embryos and obtain quail/chick bursal chimeras. II. Immune response of bursectomized chicks and chimeras and post-natal rejection of the grafted quail bursas. *J Immunol* 1987;138:2813–2821.

Corbel C, Martin C, Ohki H, Coltey M, Hlozanek I, Le Douarin NM. Evidence for peripheral mechanisms inducing tissue tolerance during ontogeny. *Int Immunol* 1990;2:33–40.

Couly GF, Le Douarin NM. Mapping of the early neural primordium in quail-chick chimeras. I. Developmental relationships between placodes, facial ectoderm, and prosencephalon. *Devel Biol* 1985;110:422–439.

Couly G, Le Douarin NM. Mapping of the early neural primordium in quail-chick chimeras: II. The prosencephalic neural plate and neural folds: implications for the genesis of cephalic human congenital abnormalities. *Devel Biol* 1987; 120:198–214.

Couly G, Le Douarin NM. The fate map of the cephalic neural primordium at the presomitic to the 3-somite stage in the avian embryo. *Development* 1988;103: 101–103.

Couly G, Le Douarin NM. Head morphogenesis in embryonic avian chimeras: evidence for a segmental pattern in the ectoderm corresponding to the neuromeres. *Development* 1990a;108:543–558.

Couly G, Le Douarin NM. Mapping of the cephalic ectoderm at the neurula stage in avian chimeras. In Le Douarin N, Dieterlen-Lièvre F, Smith J, eds *The avian model in developmental biology: from organism to genes*. Paris: Editions du CNRS, 1990b; 11–24.

Couly G, Coltey P, Le Douarin NM. The developmental fate of cephalic mesoderm in quail-chick chimeras. *Development* 1992;14:1–15.

Couly G, Coltey P, Le Douarin, NM. The triple origin of skull in higher vertebrates. *Development* 1993;117:409–429.

Couly G, Coltey,P, Chéron G, Abadie V, Martelli H, Le Douarin NM. Rhombomères, code *Hox*, crête neurale et malformations de la face. *Méd Sci* 1994;10:151–162.

Couly G, Coltey P, Eichmann A, Le Douarin NM. The angiogenic potentials of the cephalic mesoderm and the origin of brain and head blood vessels. *Mech Dev* 1995;53:97–112.

Couly G, Grapin-Botton A, Coltey P, Le Douarin NM. The regeneration of the cephalic neural crest, a problem revisited: the regenerating cells originate from the contralateral or from the anterior and posterior neural fold. *Development* 1996;122:3393–3407.

Couly G, Grapin-Botton A, Coltey P, Ruhin B, Le Douarin NM. Determination of the identity of the derivatives of the cephalic neural crest: incompatibility between *Hox* gene expression and lower jaw development. *Development* 1998;125:3445–3459.

Couly G, Creuzet S, Bennaceur S, Vincent C, Le Douarin NM. Interaction between *Hox*-negative cephalic neural crest cells and the foregut endoderm in patterning the facial skeleton in the vertebrate head. *Development* 2002;129:1061–1073.

Coutinho A. The Le Douarin phenomenon; a shift in the paragdim of development self-tolerance. *Int J Dev Biol* 2005;49:131–136.

Creuzet S, Couly G, Vincent C, Le Douarin NM. Negative effect of *Hox* gene expression on the development of the neural crest-derived facial skeleton. *Development* 2002;129:4301–4013.

Creuzet S, Couly G, Le Douarin NM. Patterning the neural crest derivatives during the development of the vertebrate head. *J Anat* 2005;207:447–459.

Creuzet SE, Martinez, S, Le Douarin NM. The cephalic neural crest exerts a critical effect on forebrain and midbrain development. *Proc Natl Acad Sci USA* 2006;103:1433–1438.

Creuzet S. Regulation of pre-otic brain development by the cephalic neural crest. *Proc Natl Acad Sci USA* 2009;20:751–759.

Croisille YL, Douarin N. Development and regeneration of the liver. In Organogenesis, De Haan, and Ursprung eds. Holt-Rinehardt and Winston, New York,1964; 421–446.

Crossley PH, Martinez S, Martin GR. Midbrain development induced by FGF8 in the chick embryo. *Nature* 1996;380:66–68.

Dulac C, Le Douarin NM. Phenotypic plasticity of Schwann cells and enteric glial cells in response to the microenvironment. *Proc Natl Acad Sci USA* 1991;88: 6358–6362.

Dulac C, Cameron-Curry P, Ziller C, Le Douarin NM. A surface protein expressed by avian myelinating and nonmyelinating Schwann cells but not by satellite or enteric glial cells. *Neuron* 1988;1:211–220.

Dulac C, Tropak MB, Cameron-Curry P, Rossier J, Marshak DR, Roder J, Le Douarin NM. Molecular characterization of the Schwann cell myelin protein, SMP: structural similarities within the immunoglobulin superfamily. *Neuron* 1992;8:323–334.

Dupin E, Baroffio A, Dulac C, Cameron-Curry P, Le Douarin N.M. Schwann cell differentiation in clonal culture of the neural crest as evidenced by the anti-Schwann cell myelin protein monoclonal antibody. *Proc Natl Acad Sci USA* 1990; 87:1119–1123.

Dupin E, Glavieux C, Vaigot P, Le Douarin NM. Endothelin 3 induces the reversion of melanocytes to glial through a neural crest-derived glial melanocytic progenitor. *Proc Natl Acad Sci USA* 2000;97:7882–7887.

Dupin E, Real, C, Glavieux-Pardanaud C, Vaigot P, Le Douarin NM. Reversal of developmental restrictions in neural crest lienages: transition from Schwann cells to glial-melanocytic precursors *in vitro*. *Proc Natl Acad Sci USA* 2003; 100:5229–5233.

Etchevers HC, Couly G, Le Douarin NM. Morphogenesis of the branchial vascular sector. *Trends Cardiov Med* 2002;12:299–304.

Fadlallah N, Guy N, Teillet MA, Schuler B, Le Douarin NM, Naquet R, Batini C. Brain chimeras for the study of an avian model of genetic epilepsy: structures involved in sound and light-induced seizures. *Brain Res* 1995;675, 55–66.

Fehilly CB, Willadsen SM, Tucker EM. Interspecific chimaerism between sheep and goat. *Nature* 1984;307:634–636.

Fontaine J, Le Douarin NM. Recherches sur le métabolisme des monoamines fluorigènes au cours de la différenciation des cellules endocrines du pancréas embryonnaire d'oiseau. *CR Acad Sci Paris* 1975;280:1885–1888.

Furshpan EJ, MacLeish PR, O'Lague PH, Potter DD. Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: evidence for cholinergic, adrenergic, and dual-function neurons. *Proc Natl Acad Sci USA* 1976;73:4225–4229.

Gans C. Northcutt RG. Neural crest and the origin of vertebrates. A new head. *Science* 1983;20:268–274.

Grapin-Botton A, Bonnin MA, McNaughton LA, Krumlauf R, Le Douarin NM. Plasticity of transposed rhombomeres: Hox gene induction is correlated with phenotypic modifications. *Development* 1995;121:2707–2721.

Goronowitsch N. Die axiale und die laterale Kopfmentamerie der Vögelembryonen. Die Rolle der sog. « Ganglienleistein » im Aufbaue der Nervenstämme. *Anat Anz* 1892;7:454–464.

Guy N, Teillet, MA, Schuler, B, Le Gal La Salle G, Le Douarin NM, Naquet R, Batini C. Pattern of electroencephalographic activity during light induced seizures in genetic epileptic chicken and brain chimeras. *Neurosci Letters* 1992; 145:55–58.

Hall BK. *The neural crest in development and evolution*. New York: Springer-Verlag, 1999.

Hallonet ME, Teillet MA, Le Douarin NM. A new approach to the development of the cerebellum provided by the quail-chick marker system. *Development* 1990; 108:19–31.

Hammond WS, Yntema CL. Deficiencies in visceral skeleton of the chick after removal of cranial neural crest. *Anat Rec* 1953;115:393–394.

Hörstadius S, *The neural crest: its properties and derivatives in the light of experimental research*, London: Oxford University Press, 1950.

His W. Untersuchung über die ersteanlage des Wirbeltierleibes. Die erste entwickling des Hühnchens. Leipzig: Vogel, 1868.

Jung J, Zheng M, Goldfarb M, Zaret KS. Initiation of mammalian liver development from endoderm by fibroblast growth factors. *Science* 1999;284:1998–2003.

Jeffery WR, Strickler AG, Yamamoto Y. Migratory neural crest-like cells form body pigmentation in a urochordate embryo. *Nature* 2004;431:696–699.

Johnston MC. A radioautographic study of the migration and fate of cranial neural crest cells in the chick embryo. *Anat Rec* 1966;156:143–156.

Katschenko N. Zur Entwicklungsgeschichte der Selachierembryos. *Anat Anz* 1888; 3:448–467.

Kinutani M, Coltey M, Le Douarin NM. Postnatal development of a demyelinating disease in avian spinal cord chimeras. *Cell* 1986;45:307–314.

Kirby ML, Turnage KL, Hayes BM. Characterization of conotruncal malformations following ablation of "cardiac" neural crest. *Anat Rec* 1985;213:87–93.

Lahav R, Ziller C, Dupin E, Le Douarin NM. Endothelin 3 promotes neural crest cell proliferation and mediates a vast increase in melanocyte number in culture. *Proc Natl Acad Sci USA* 1996;93:3892–3897.

Lahav R, Dupin E, Lecoin L, Glavieux C, Champeval D, Ziller C, Le Douarin NM. Endothelin 3 selectively promotes survival and proliferation of neural crest-derived glial and melanocytic precursors *in vitro*. *Proc Natl Acad Sci USA* 1998;95:14214–14219.

Le Douarin N. Rôle du mésenchyme dans l'histogenèse hépatique chez l'embryon de Poulet. *CR Acad Sci Paris* 1963a;257:255–257.

Le Douarin N. Action inductrice précoce du mésoderme de l'aire cardiaque sur l'endoderme hépatique de l'embryon de Poulet. *CR Acad Sci Paris* 1963b; 257:1357–1360.

Le Douarin N. Induction de l'endoderme pré-hépatique par le mésoderme de l'aire cardiaque chez l'embryon de poulet. *J Embryol Exp Morphol* 1964a;12:651–676.

Le Douarin N. Isolement expérimental du mésenchyme propre du foie et rôle morphogène de la composante mésodermique dans l'organogenèse hépatique. *J Embryol Exp Morphol* 1964b;12:141–160.

Le Douarin N. Etude expérimentale de l'organogenèse du tube digestif et du foie chez l'embryon de poulet. *Bull Biol Fr Belg* 1964c;98:544–674.

Le Douarin N. La différenciation de l'endoderme hépatique étudiée en culture *in vitro*. *CR Soc Biol* 1965;159:90–96.

Le Douarin N. Etude ultrastructurale du foie sans glycogène résultant de l'association d'hépatocytes avec le mésenchyme métanéphrique de l'embryon de Poulet. *CR Acad Sci Paris* 1968;267:886–888.

Le Douarin N. Particularités du noyau interphasique chez la Caille japonaise (Coturnix coturnix japonica). Utilisation de ces particularités comme « marquage biologique » dans des recherches sur les interactions tissulaires et les migrations cellulaires au cours de l'ontogenèse. *Bull Biol Fr Belg* 1969;130:435–452.

Le Douarin N. A biological cell labeling technique and its use in experimental embryology. *Dev Biol* 1973a;30:217–222.

Le Douarin NM. A Feulgen-Positive Nucleolus. *Exp Cell Res* 1973b;77:459–468.

Le Douarin NM. The embryological origin of the endocrine cells associated with the digestive tract: experimental analysis based on the use of a stable cell marking technique. In Bloom SR ed. *Gut Hormones*. Edinburgh: Churchill Livingstone, 1978; 49–56.

Le Douarin N. *The neural crest*. Cambridge: Cambridge University Press, 1982.

Le Douarin NM. On the origin of pancreatic endocrine cells. *Minireview Cell* 1988;53:169–171.

Le Douarin N, Chaumont F. La différenciation morphologique et fonctionnelle de l'endoderme hépatique en présence de mésenchymes hétérologues. *CR Soc Biol* 1966;160:1868–1871.

Le Douarin N, Houssaint E. La synthèse du glycogène dans des hépatocytes de Mammifère adulte associés expérimentalement au mésenchyme du métanéphros d'embryons de Poulet et d'embryons de Souris. *CR Soc Biol* 1968;162: 1196–1199.

Le Douarin N, Le Lièvre C. Démonstration de l'origine neurale des cellules à calcitonine du corps ultimobranchial chez l'embryon de Poulet. *CR Acad Sci Paris* 1970;270:2857–2860.

Le Douarin N, Le Lièvre C. Sur l'origine des cellules à calcitonine du corps ultimobranchial de l'embryon d'Oiseau. *CR Ass Anat* 1971;152:558–568.

Le Douarin N, Teillet, MA. The migration of neural crest cells to the wall of the digestive tract in avian embryo. *J Embryol Exp Morphol* 1973;30:31–48.

Le Douarin NM, Teillet MA. Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological cell marking technique. *Dev Biol* 1974;41:162–184

Le Douarin NM, Kalcheim C. *The neural crest.* 2nd ed. Cambridge: Cambridge University Press, 1999.

Le Douarin NM, Halpern M. Origin and specification of the neural tube floor plate: insights from the chick and zebrafish. *Curr Op Neurobiol* 2000;10:23–30.

Le Douarin NM, Creuzet SE. Craniofacial patterning. In *The skeletal system*. Cold Spring Harbor Laboratory Press, 2009;53:117–147.

Le Douarin N, Le Lièvre C, Fontaine J. Recherches expérimentales sur l'origine embryologique du corps carotidien chez les Oiseaux. *CR Acad Sci Paris* 1972;275:583–586.

Le Douarin N, Fontaine J, Le Lièvre C. New studies on the neural crest origin of the avian ultimobranchial glandular cells. Interspecific combinations and cytochemical characterization of C cells based on the uptake of biogenic amine precursors. *Histochemistry* 1974;38:297–305.

Le Douarin NM, Renaud D, Teillet MA, Le Douarin GH. Cholinergic differentiation of presumptive adrenergic neuroblasts in interspecific chimeras after heterotopic transplantations. *Proc Natl Acad Sci USA* 1975;72:728–732.

Le Douarin NM, Teillet MA, Ziller C, Smith J. Adrenergic differentiation of cells of the cholinergic ciliary and Remak ganglia in avian embryo after *in vivo* transplantation. *Proc Natl Acad Sci USA* 1978,75:2030–2034.

Le Douarin NM, Corbel C, Martin C, Coltey M, Salaün J. Induction of tolerance by embryonic thymic epithelial grafts in birds and mammals. *Cold Spring Harbor Symp Quant Biol* 1989;54:777–787.

Le Douarin NM, Teillet MA, Catala M. Neurulation in amniote vertebrates: a novel view deduced from the use of quail-chick chimeras. *Int J Dev Biol* 1998;42: 909–916.

Le Douarin NM, Creuzet S, Couly G, Dupin E. Neural crest cell plasticity and its limit. *Development* 2004;131:4637–4650.

Le Lièvre C. Rôle des cellules mésectodermiques issues des crêtes neurales céphaliques dans la formation des arcs branchiaux et du squelette viscéral. *J Embryol Exp Morph* 1974;31:453–477.

Le Lièvre CS. Participation of neural crest-derived cells in the genesis of the skull in birds. *J Embryol Exp Morphol* 1978;47:17–37.

Le Lièvre C, Le Douarin N. Origine ectodermique du derme de la face et du cou, montrée par des combinaisons interspécifiques chez l'embryon d'Oiseau. *CR Acad Sci Paris* 1974;278:517–520.

Le Lièvre C, Le Douarin N. Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. *J Embryol exp Morph* 1975;34:125–154.

Le Lièvre CS, Schweizer GG, Ziller CM, Le Douarin NM. Restrictions of developmental capabilities in neural crest cell derivatives as tested by *in vivo* transplantation experiments. *Dev Biol* 1980;77:362–378.

Lecoin L, Sakurai T, Ngo MT, Ab, Y, Yanagisawa M, Le Douarin NM. Cloning and characterization of a novel endothelin receptor subtype in the avian class. *Proc Natl Acad Sci USA* 1998;95:3024–3029.

McGonnell, IM, Graham A. Trunk neural crest has skeletogenic potential. *Curr Biol* 2002;12:767–771.

Nataf V, Lecoin L, Eichmann A, Le Douarin NM. Endothelin-B receptor is expressed by neural crest cells in the avian embryo. *Proc Natl Acad Sci USA* 1996;93: 9645–9650.

Nataf V, Amemiya A, Yanagisawa M, Le Douarin, NM. The expression pattern of endothelin 3 in the avian embryo. *Mech Dev* 1998a;73:217–220.

Nataf V, Grapin-Botton A, Champeval D, Amemiya A, Yanagisawa M, Le Douarin NM. The expression patterns of endothelin-A receptor and endothelin 1 in the avian embryo. *Mech Dev* 1998b;75:145–149.

Ohki H, Martin C, Corbel C, Coltey M, Le Douarin NM. Tolerance induced by thymic epithelial grafts in birds. *Science* 1987;237:1032–1035.

Ohkubo Y, Chiang C, Rubinstein JLR. Coordinate regulation and synergistic actions of BMP4, SHH and FGF8 in the rostral prosencephalon regulate morphogenesis of the telencephalic and optic vesicles. *Neuroscience* 2002;111,1–17.

Patterson PH, Reichardt LF, Chun LLY. Biochemical studies on the development of primary sympathetic neurons in cell culture. *Cold Spring Harbor Symp Quant Biol* 1975;40:389–397.

Patterson PH, Chun LLY. The induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neurons. I. Effects of conditioned medium. *Dev Biol* 1977;6:263–280.

Pearse AEG. The cytochemistry and ultrastructure of polypeptide hormone-producing cells of the APUD series and the embryonic, physiologic and pathologic implications of the concept. *J Histochem Cytochem* 1969;17:303–313.

Pearse AG, Polak, JM, Rost FW, Fontaine J, Le Lièvre C, Le Douarin N. Demonstration of the neural crest origin of type I (APUD) cells in the avian carotid body, using a cytochemical marker system. *Histochemie* 1973;34:191–203.

Platt JB. Ectodermic origin of the cartilages of the head. *Anat Anz* 1893;8:506–509.

Polak JM, Pearse AG, Le Lièvre C, Fontaine J, Le Douarin NM. Immunocytochemical confirmation of the neural crest origin of avian calcitonin-producing cells. *Histochemistry* 1974;40:209–214.

Pourquié O, Coltey M, Teillet MA, Ordhal C, Le Douarin NM. Control of dorsoventral patterning of somitic derivatives by notochord and floor plate. *Proc Natl Acad Sci USA* 1993;90:5242–5246.

Rao M, Landis SC. Characterization of a target-derived neuronal cholinergic differentiation factor. *Neuron* 1990;5:899–910.

Real C, Glavieux-Pardanaud C, Vaigot P, Le Douarin NM, Dupin E. The instability of the neural crest phenotypes: Schwann cells can differentiate into myofibroblasts. *Int J Dev Biol* 2005;49:151–159.

Real C. Glavieux-Pardanaud C, Le Douarin NM, Dupin E. Clonally cultured differentiated pigment cells can dedifferentiate and generate multipotent progenitors with self-renewing potential. *Dev Biol* 2006;300:656–669.

Sakaguchi S, Takahashi T, Nishizuka Y. Study on cellular events in post thymectomy autoimmune oophoritis in mice. I. Requirement of Lyt-1 effector cells for oocytes damage after adoptive transfer. *J Exp Med* 1982;156:1565–1576.

Salaün J, Calman F, Coltey M, Le Douarin NM. Construction of chimeric thymuses in the mouse fetus by *in utero* surgery. *Eur J Immunol* 1986;16:523–530.

Salaün J, Corbel C, Le Douarin NM. Regulatory T cells in the establishment and maintenance of self-tolerance. *Int J dev Biol* 2005;49:137–142.

Teillet MA, Guy NTM, Schuler B, Le Gal La Salle G, Batini C, Le Douarin N.M, Naquet R. Transfer of a genetic form of epilepsy in the chicken by embryonic brain grafts. Symposium on Developmental Mechanisms. *CR Acad Sci Paris* 1993;316:1164–1176.

Teillet MA, Lapointe F, Le Douarin NM. The relationships between notochord and floor plate in vertebrate development revisited. *Proc Natl Acad Sci USA* 1998a;95:11733–11738.

Teillet MA, Watanabe Y, Jeffs P, Duprez D, Lapointe F, Le Douarin NM. Sonic hedgehog is required for survival of both myogenic and chondrogenic somitic lineages. *Development* 1998b;125:2019–2030.

Teillet MA, Naquet R, Batini C. Transfer of an avian genetic reflex epilepsy by embryonic brain graft: a tissue autonomous process? *Int J Dev Biol* 2005;49:237–241.

Thibert C, Teillet MA, Lapointe F, Mazelin L, Le Douarin NM, Mehlen P. Inhibition of neuroepithelial patched-induced apoptosis by sonic hedgehog. *Science* 2003;01:843–846.

Toivonen S, Saxen L. Uber die Induktion des Neuralrohrs bei Trituruskeimen als simultane Leistung des Leber-und Knochenmarkgewebes vom Meerschweinchen, *Ann Acad Sci fenn Ser A VI* 1955;30:1–29.

Trentin A, Glavieux-Pardanaud C, Le Douarin NM, Dupin E. Self-renewal capacity is a widespread property of various types of neural crest precursor cells. *Proc Natl Acad Sci USA* 2004;101:4495–4500.

Yamamori T, Fukada K, Aebersold R, Korsching S, Fann MJ, Patterson PH. The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. *Science* 1989;246:1412–1416.

Yntema CL, Hammond WS. The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. *J Comp Neurol* 1954;101:515–541.

Yntema CL, Hammond WS. Experiments on the origin and development of the sacral autonomic nerves in the chick embryo. *J Exp Zool* 1955;129:375–414.

Walsh C, Cepko CL. Clonally related cortical cells show several migration patterns. *Science* 1988;241:1342–1345.

Wolff E. Les bases de la tératogenèse expérimentale des Vertébrés Amniotes d'après les résultats de méthodes directes. *Arch Anat Histol Embryol* 1936a;22:1–382.

Wolff E. L'évolution après l'éclosion des Poulets mâles transformés en intersexués par l'hormone femelle injectée aux jeunes embryons. *Arch Anat Histol Embryol* 1936b;223:1–28.