A life in Science with the avian embryo

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ABSTRACT My career in research was a second thought. I first (during 8 years) worked as a secondary school teacher and after 4-5 years, during which my two daughters were born, I found a way to escape from what was to be a lifetime job. For two years, my initiation to research was limited to the free time left by my teaching duties. This period of time was a bit "complicated" but not enough to prevent me to realize that research was really what I wanted to do for the rest of my life… And this was when I became acquainted with the chick embryo. This companionship later became extended to another representative of the avian world: the quail (*Coturnix coturnix japonica*). I recall in the following lines a survey of scientific stories that came out from my association with these precious animals, ... not without a feeling of gratitude.

KEY WORDS: *quail-chick chimera, cell marking technique, neural crest, neural stem cell, tolerance to self*

How I became a researcher in Biology

Although my orientation, at high school and during my first year at *la Sorbonne* in Paris, was in literature, I resolutely turned my interest to natural sciences for the rest of my education. This may be due to the influence of a talented science teacher in the last year of my school time. My intention was to pursue the career of secondary school teacher. To reach this goal, one had to go through a four year-*cursus* including physics, chemistry and animal and plant biology, physiology and earth sciences. The next step was to go through a competitive exam (a definite – and small - number of posts were available each year) "*l'agrégation de sciences naturelles*" to get a lifetime position.

After six years teaching in a *lycée* in the city of Caen in *Normandie*, I was able to get a position near Paris, in a school where the teachers were encouraged to perform pedagogical experiments aimed at improving the transmission of knowledge. I liked teaching very much, especially in these conditions where the rules were less rigid than in other schools and where I could dare some pedagogic innovations. However, after some time, I seriously missed the University where one can closely follow science in progress and, even better, where as a researcher, one can dream to participate (even if it is only for a small part) in this progress. I had, in the late 1950s, the great privilege of being introduced to Pr. Etienne Wolff, who accepted to give me access to his laboratory, one of the most dynamic, at that time, in the field of developmental biology, during the free time left by my teaching duties. The name of the laboratory was "Institut d'Embryologie et de Tératologie et Expérimentales" (Fig. 1). It was associated to the *Collège de France*, an institution of excellence where Etienne Wolff had just been appointed as a Professor. It is where I started my training as an embryologist and where I was first acquainted with the chick embryo. This little piece of life was in itself a moving and amazing sight to contemplate as it unfolds and grows on the surface of the egg yolk. Immediately available to experimentation at any time of its development, the avian embryo was clearly an appropriate material for trying to decipher the mechanisms underlying the transformation of the germ into a fully developed organism.

Since I got my first experience in research in Etienne Wolff's laboratory, I shall first evoke some aspects of his science and of the heritage he has left, both being linked to the chick embryo, the subject of this Special Issue of the *Int. J. Dev. Biol.*

Etienne Wolff and the chick embryo: a successful cooperation

From his early time in research, Etienne Wolff benefited from the favourable characters of the chick embryo as an experimental model, since the rational basis of his thesis work of "experimental

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Abbreviations used in this paper: AIP, anterior intestinal portal; ANR, anterior neural ridge; AP, antero-posterior; CA, catecholamine; CNS, central nervous system; CNC, cephalic neural crest; CNCC, cephalic neural crest cell; E, embryonic day; EAE, experimental allergic encephalomyelitis; ENS, enteric nervous system; ET, endothelin; ETR, endothelin receptor; FSNC, facial skeletogenic neural crest; Mab, monoclonal antibody; MS, multiple sclerosis; NC, neural crest; NCC, neural crest cell; NF, neural fold; NT, neural tube; PNS, peripheral nervous system; r, rhombomere; Shh, Sonic hedgehog; SMP, Schwann cell myelin protein.

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Fig. 1. The Institute of Embryology in Nogent-sur-Marne, Paris, France. *(Photo taken in 2005).*

teratology"- totally original at his time- was to try and reproduce the monsters that arise incidentally in humans. The aim being to find how and why they appear, thus getting insights about the mechanisms conducting the normal course of development.

At that time, one thesis was not enough to become a *Doctor-es-Sciences*. A second, subsidiary, work was to be produced to merit this title. The one Etienne presented was more acclaimed than the first one because it happened to be more "modern": it described an entirely new phenomenon, the role on sexual development of the newly discovered œstrogens. He found that, if administered during development, these substances transformed genetically male chick embryos into intersexuals, which developed an ovarian cortex and retained Müllerian ducts.

This first demonstration of a hormonal effect operating in embryonic life has opened a large research avenue in all vertebrate phyla.

My first acquaintance with the chick embryo

When I joined Etienne Wolff in the late 1950ies, the laboratory was at its peak. The main orientations concerned tissue interactions and their role in organ development; the chick embryo was THE experimental model elected by most (if not all) of us. This type of research was common to several laboratories over the world at that time. It revealed the existence of diffusible factors, considered as a sort of language critical for the construction of the embryo, through which the cells communicate with each other. The nature of these signalling molecules was to be discovered later when came the time of molecular biology.

My project concerned the development of the digestive tract, a neglected field at that time.

I first studied the morphogenetic movements of the endoderm and mesoderm leading to the ventral closure of the gut. A prerequisite for the analysis of the development of endoderm-derived organs was to construct a fate map of the endo-mesoderm at the early embryonic stages and to try and evidence its transformation into the digestive tract and glandular appendages.

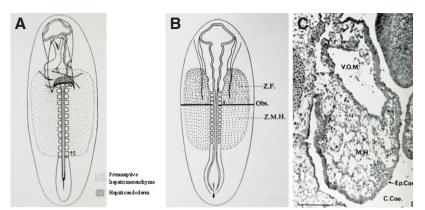Two ways were available to reach this goal; either destroying

definite territories by X-rays and looking at what organs were missing afterward, or labelling the tissues and following *de visu* the movements of the labelled cells. Both techniques could be applied to the chick embryo thanks to its availability during the entire period of development. The X-ray technique was not fully reliable because of the regulation capacities of the embryo at these early stages. In the late 1950ies, a way to label cells was to apply carbon particles that stick firmly to their membrane and are not harmful. The results of such experiments revealed that the construction of the digestive tract involved major and complex morphogenetic movements that were informative for the rest of my enquiry and that I strived to describe (Le Douarin, 1964a,b,c).

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 concentrated my attention on liver and found that the endodermal cells fated to differentiate into hepatocytes (and also those that yielded the glandular component of the pancreas, thyroid and parathyroids) are present in the anterior intestinal portal (AIP) at the 15- to 25-somite-stages. 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. In the 1950-60ies, the relationships that take place between these two original components were under investigation for several tissues and organs. Classically, the mesenchymal and epithelial components of their anlagen were cultured separately in vitro: when isolated, both tissues degenerated rapidly. In contrast when reassembled in the culture dish, they developed into an organ.

The fate mapping experiments that I pursued first, had taught me that, in contrast to the other organs studied, such as the kidney, the salivary glands, and the mammary glands for example, the mesenchymal and endodermal components of the liver were not associated in a definite rudiment containing both tissues: they evolved from different sites in the embryo and met during the ventral closure of the gut and of the general body cavity: while the endodermal cells (in small number), committed to differentiate into hepatocytes, were found to be located in the AIP, the mesodermal liver component, occupied a much larger area. Thus the development of the liver is characterized by the simultaneous development of its epithelial and mesodermal components in different embryonic sites: the former from a small group of cells lying in the AIP, the latter from two large, bilateral, splanchopleural areas that joined in the AIP and that I could delimitate using carbon particles (Fig. 2 A,B). During closure of the foregut, through the regression of the AIP, the endodermal cells fated to become hepatocytes proliferated and invaded the two hepatic lobes that developed simultaneously from the splanchnopleural mesenchyme.

How, in this situation, to find whether the proliferation of the hepatic cords were or not dependent upon the inductive effect of the mesenchyme that participates in the formation of the hepatic lobes? To answer this question, I imagined an *in vivo* microsurgical trick aimed at preventing the colonization of part of the large area of the "liver-splanchnopleural mesenchyme" by the endoderm-derived hepatocytes. This was achieved by interposing an obstacle (a fragment of the shell membrane of the egg) in the somatopleura

Fig. 2. Isolation of the hepatic mesenchyme. (A) *Schematic representation of a chicken embryo at 15-somite-stage. Ventral view showing the respective distribution of the presumptive hepatic mesenchyme (area in stipple) formed by bilateral areas joining in the anterior intestinal portal (AIP) and the hepatic endoderm (hatched area) located in the AIP itself.* (B) *Dorsal view of a 13-somite-stage embryo in which the presumptive areas of the hepatic mesenchyme are represented. An obstacle inserted bilaterally at the level of somite 4 prevents the colonization of the posterior area by the hepatocytes originating from the endoderm of the AIP.* (C) *At E5, the operated embryo shows an area of loose hepatic mesenchyme devoid of hepatocytes, whose progression was prevented by the obstacle.*

within the presumptive territory fated to yield the liver mesenchymal component. The areas located posteriorly to the barrier gave rise to a mesenchymal tissue void of epithelial cells (Fig. 2C) that could be cultured *in vitro,* with or without the endoderm of the AIP of the chick at embryonic days 2 to 3 (E2-E3). If cultured alone as an explant at the air/medium interface, each of these two tissues survived for about 24 hours. The contrast was striking when the small piece of endoderm was placed in contact with the liver mesenchyme. In such an association, the endodermal cells divided actively, colonized the mesenchyme and acquired evident hepatocytic features, of which glycogen storage, enlargement of the nucleolus were the most conspicuous. 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. Further studies demonstrated that the differentiation of endodermal cells into hepatocytes requires the effect of Fgfs (Jung *et al.*, 1999; Zorn and Wells, 2009 for review).

Another question remained in the analysis of liver development: how the specificity of the AIP endoderm was acquired during the early stages of digestive tract development. I found that liver and cardiac development were closely linked and that the commitment of the splanchnic mesenchyme to develop into cardiac tissue preceded that of the endoderm. The "pre-cardiac mesoderm" was then able to induce the endoderm to differentiate into hepatocytes (Le Douarin, 1964c).

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"(designated "*Maître de Conférences*") in a University. This, however, did not seem realistic to me since, at that time, these positions were essentially reserved to men. The function of University Professor was much more prestigious in the early 1960ies than was that of CNRS researcher and, at least in our field, it was the only way to develop an independent research group. It was under the suggestion of Étienne Wolff that I postulated for a position at the University of Clermont-Ferrand where I stayed for one year before going to Nantes where two positions became available
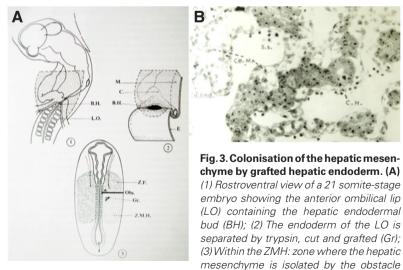
in 1966, which my husband and I occupied.

In 1965, my first year as a *Maître de Conférences* in the Department of Developmental Biology, in addition to teaching, I was given the responsibility of supervising two young students who prepared a Master research project. It is when I was working with them that I had, for the first time, the opportunity of using quail eggs for my experiments.

Devising a cell marking technique in the avian embryo *in ovo*: serendipity allied to curiosity sometimes results in a happy end story

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

In Etienne Wolff's Institute in Nogent, as well as in Clermont-Ferrand, researchers started to use quail instead of chick embryos

Fig. 3. Colonisation of the hepatic mesenchyme by grafted hepatic endoderm. (A) *(1) Rostroventral view of a 21 somite-stage embryo showing the anterior ombilical lip (LO) containing the hepatic endodermal bud (BH); (2) The endoderm of the LO is separated by trypsin, cut and grafted (Gr); (3) Within the ZMH: zone where the hepatic mesenchyme is isolated by the obstacle (Obs). ZF: zone colonized by the hepatocytes produced by the AIP of the host embryo.* (B) *The 5-day host embryo shows cords of hepatocytes, generated by the grafted hepatic endoderm caudally to the obstacle, which colonize the hepatic mesenchyme.*

for some of their experiments. 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 the only one 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 in the ventral body wall or in the intestinal mesenchyme. 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. In these *in vivo* tissue associations, the endodermal cells survived for a while, but failed to proliferate and differentiate. 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 retain my attention at this time.

The following year, I was appointed at the University of Nantes where I was to stay for 8 years and where I established my first independent research group. I developed a project that 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 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 now we know that the molecular pathways involved in developmental processes have been remarkably conserved throughout evolution, but this was not the case, at that time. I then associated in organotypic *in vitro* 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" strokes 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 is the enlargement of their nucleolus. This is related to the increase in protein synthesis, a characteristic of hepatocytes. In the chimeric liver resulting from 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 the observation that I did 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 in embryos and adults as well. I applied Feulgen-Rossenbeck's staining procedure

Fig. 4. Migration of neural crest cells (NCC) in quail-chick chimeras. *Micrograph showing Feulgen-Rossenbeck's staining of DNA, which allows to distinguish quail NCC (arrows) that migrate from the neural primordium of the chick host embryo a few hours after its transplantation at NC pre-migratory stage.*

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 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).

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-E5) (Le Douarin, 1968, Le Douarin and Houssaint, 1968). Epinephrine and norepinephrine are hyperglycemic 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 (NC). This was the beginning of my long-standing acquaintance with this structure for the rest of my research career.

After reading the available literature on the NC, I realized that I had at hand a way to see if the cells, that produced adrenaline in this context, had a NC 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[1]. 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.

These experiments were aside the main stream of the research going on in the lab and I did them on my own just for fun. When I

[1] 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.

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 (Le Douarin, 1973) (Fig. 4). Quail cells were present not only in the grafted spinal cord but also dispersed in many other places of the embryo (at E5-E6): along the nerves, as Schwann cells, in the peripheral ganglia, in the suprarenal gland and within the metanephric 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 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 had, over the previous ones used to label the cells and follow their fate [either tritiated thymidine (^{3}H-TdR), vital stains or carbon particles], the superiority of being stable and unalterable and also, not transmissible to neighbouring 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 appeared immediately considerable for solving a number of problems in embryology.

The neural crest and its derivatives in higher vertebrates

This first experimental acquaintance with the nervous system aimed at seeking an explanation to an intriguing observation concerning liver development seemed to me very attractive. I realized that I could use the quail-chick marker system to document the role of the NC in vertebrate development. Following its discovery by Wilhelm His (1868), this embryonic structure of the vertebrate embryo had essentially been studied in lower forms of vertebrates (see the monograph by Sven Hörstadius, 1950). In 1968, little information was available about its contribution to amniotes embryogenesis and I decided to further document this "chapter of Embryology" by using the quail-chick marker system that seemed perfectly suited to this purpose.

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 NC cells (NCC) was followed during the entire embryonic life and could even be pursued after birth, thanks to the stability provided by this cell labelling technique. Such chimeras were able to hatch and displayed normal growth and behaviour before being subjected to graft rejection (see below the section on immunology).

The experiments that I performed with three young doctoral students, Marie-Aimée Teillet, Christiane Le Lièvre and Josiane Fontaine, were aimed at exploring systematically the fate of the NCC exiting from the various transverse levels of the neural axis. We were able to complete the list of the different cell types arising from this structure, their level of origin along the neural axis and the pathways they follow to reach their destinations. This allowed a fate map of the NC in the avian embryo to be constructed (Fig. 5).

The derivation of the peripheral nervous system (PNS) from the NC was already well known, but determining the level of origin of its different components was of interest: it was striking to see that definite regions of the spinal cord and medulla oblongata were particularly devoted to yield the sympathetic, parasympathetic or enteric ganglia. Certain areas of the NC attracted my attention; one of them extends over somites 1 to 7 and corresponds roughly to the level of emergence of the *vagus* nerve, which innervates the gut and that I designated as "vagal level of the NC". It yields NCC migrating to the gut and invading it down to the cloaca. This showed that the pre- and post-ganglionic parasympathic neurones of the enteric nervous system (ENS) have the same level of origin along the neural axis. This is probably related to the fact that NCC fated to yield the PNS ganglia migrate along the nerves issued from the corresponding preganglionic neurones that remain in the CNS. We found an additional contribution to the ENS from the lumbo-sacral NC located posteriorly to somite 28. These two levels of the CNS thus contain the pre-ganglionic neurons innervating the enteric ganglia that are derived from the NC and which, in the gut environment, differentiate into cholinergic and peptidergic neurons, CA-producing cells being excluded from the ENS.

A third region, located in the spinal cord between somites 18 to 24, was found to provide, apart from the sensory neurons of the dorsal root ganglia, the adrenal cells of the suprarenal gland and the adrenergic neurons of the sympathetic ganglia and plexuses corresponding to this axial level (but does not contribute to the ENS). It was for this reason designated as the "adrenomedullary" level of the NC.

These results were published in the Journal of Embryology and Experimental Morphology in 1973 (Le Douarin and Teillet, 1973).
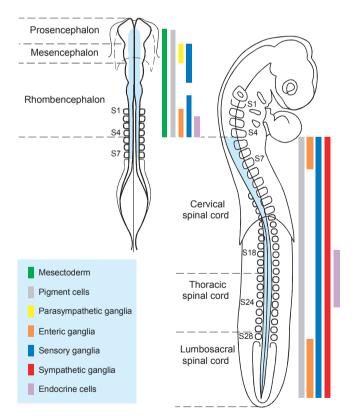

Fig. 5. Fate map of neural crest (NC) derivatives in the avian embryo. *Representation of color-coded NC neural and non-neural derivatives along the neural axis at the cephalic (left, 7-somite stage) and trunk (right, 28-somite stage) AP levels. Mesenchymal derivatives (in green) are restricted to the NC from mid-diencephalon down to r8, corresponding to somite 4,(S4). (Adapted from Le Douarin et al., 2004).*

I happened to know who was 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 gut innervation in the chick several years before (Yntema and Hammond, 1954, 1955), engaged 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 in removing sections of the NC in the chick embryo. The results he had obtained pointed to an origin of most of the neurones from the vagal level of the neural axis, but they were not as precise and clear-cut as those produced by 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 the "Current Contents", where the table of the contents of all the scientific journals were recorded on a weekly basis. Every issue presented and commented one or two articles that had a special interest and were recorded as 'Citation Classics". To our great satisfaction, our paper was one of those. This was very encouraging.

Developmental plasticity of PNS neural precursors

The fate map of the NC (Fig. 5) clearly showed that the NCC exiting from the neural tube (NT) between the vagal and the lumbosacral levels did not contribute to the gut innervation. This striking difference in NCC fate between the "enteric" and the "adrenomedullary" levels, raised the question as to whether the neuronal precursors fated to differentiate into adrenergic or cholinergic neurones were committed prior to their migration. The alternative being that, according to the level of the NT from which they emerge, they could receive specific differentiation signals either during their migration or in 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 NT located at the level of somites 18-24 ("adrenomedullary" level of the NC) up to the 1-7 level ("enteric" level of the NC), showed the capacity of the NCC of this trunk level to colonize the gut and to differentiate into cholinergic (and not to adrenergic) neurones. In the same way, transplantation of the NT from the "enteric" (or "vagal" level of somite 1 to 7) to the "adrenomedullary" level of the neural axis contributed adrenergic neurones and adrenomedullary cells (of vagal origin) to the para-aortic plexus and to the suprarenal gland, respectively (Le Douarin and Teillet, 1974, Le Douarin *et al.,* 1975).

In the early 1970ies, 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 Neurosciences laboratory wherein Edwin Furshpan, David Potter and Paul Patterson were showing on isolated newborn rat sympathetic neurones that their adrenergic phenotype could be switched to cholinergic, through co-culture 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 artefact"* but resulted from a real influence of the environment on the terminal neuronal differentiation concerning the neurotransmitter choice (Patterson and Chun, 1977, Furshpan *et al.,* 1976, Rao

and Landis, 1990).

This series of works were the first demonstration of the developmental plasticity of neuronal progenitors. It was at the origin of a number of projects in various laboratories. Such plasticity was found in other neuronal systems, including the central nervous system (CNS).

The neural crest and the vertebrate head: its role in vertebrate evolution

One of the striking features of the NC is that it is truly multipotent and not only yields neural cell types, melanocytes and endocrine cells, but also mesenchymal cells, which are highly represented in the vertebrate head. The first demonstration of the contribution of the crest to the mesenchyme goes back to the XIXth century. This assumption of a possible ectodermal origin of mesenchymal cells giving rise to bones contended with the generally recognized validity of von Baer's germ layer theory, according to which the skeleton was considered to be exclusively of mesodermal origin. However, further investigations were carried out on lower forms of Vertebrates during the first half of the XXth century, which confirmed and completed Julia Platt's results (for a review, see Le Douarin, 1982).

In Amniotes, the number of embryonic cells becomes rapidly high and the migrating cells 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. In 1966, Malcolm Johnston undertook a series of investigations on craniofacial development in the chick by tracing NCC through the radio-autographic labelling of dividing cells with ^{3}H-TdR (Johnston, 1966). This technique was also used by Drew Noden (1975) for the same purpose. Realizing that neither the specificity nor the stability of the marker were insured by this technique, Johnston, Noden and their colleagues, later on, turned to the quail-chick marker system to investigate the migration and fate of the cephalic NCC (CNCC).

Christiane Le Lièvre and I undertook experiments aimed at deciphering the extent to which the NC contributes to the head skeleton. For this purpose (in contrast to the experiments done before where small fragments of the labelled NT were grafted into unlabelled 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 NC, with a participation of the cephalic mesenchyme (Le Lièvre and Le Douarin, 1974, 1975; Le Lièvre 1974, 1978).

A massive migration of the NCC from their dorsal origin to the surface of the developing brain and the facial and hypobranchial buds was followed. 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 NC origin. Striated myocytes, forming the iridal muscles, are also NC-derived (Creuzet *et al.,* 2005) as well as the ciliary bodies and the corneal endothelium. The meninges of the telencephalon are entirely of NC origin (except for the blood vessel endothelium). These structures are mesoderm-derived in all the other parts of the CNS.

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 NC
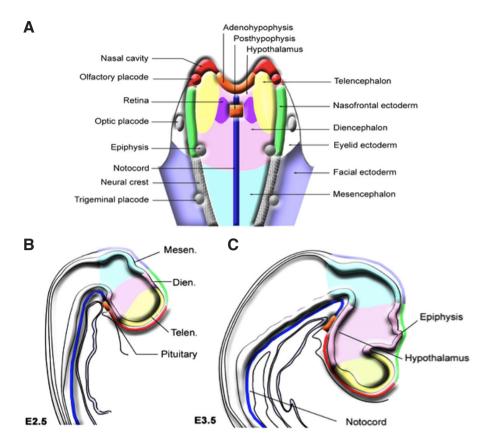
Fig. 6. Fate map of the anterior neural plate. (A) *Transplantation experiments at the 3-somite-stage led to determine the fate map of neural plate territories in the avian embryo. The NC anterior limit is located in the diencephalic NF at the level of the epiphysis anlagen.* **(B,C)** *Diagrams of sagittal views at E2.5 (B) and E3.5 (C) show subsequent developmental growth of neural plate territories. Telencephalic territories (A, yellow) join in the midline and later dramatically expand rostrally to the tip of notochord (B, C, yellow). Dien, diencephalon; Mesen, mesencephalon; Telen, telencephalon. (Adapted from Le Douarin and Kalcheim, 1999).*

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 rostral end of the notocord (Couly and Le Douarin, 1985, 1987).

Olivier Pourquié in his post-doctoral work demonstrated that the differentiation of the paraxial (cephalic and somitic) mesoderm into cartilage and bone depends upon a signal arising from the notocord and later identified as the secreted molecule Sonic hedgehog (Shh) (Pourquié *et al.*, 1993). Thus, the notocord present up to the mesencephalon-diencephalon junction accounts for the formation of the vertebral column and of the occipital region of the skull. The evolution of the vertebrate phylum is characterized by the development of the cerebral hemispheres peaking in 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 (NF) and formation of the encephalic vesicles, these lateral areas are the sites of intensive growth, so that they develop rostrally beyond the tip of the notocord and the hypophysis to form the telencephalon. The hypophysis becomes "burried" 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 NC origin, which formed the forebrain meninges and the skull (optic and nasal skeleton, frontal bone) (Fig. 6) (Couly and Le Douarin, 1985, 1987, Couly *et al.*, 1993). Thus, co-evolution of the anterior brain and of the NC was critical in the development of higher cognitive functions in the most recent forms of Vertebrates.

Further studies of our laboratory revealed that the NC exerts a role of a signalling centre in brain development.

The neural crest: a signalling centre regulating the development of the pre-otic brain

The origin of this work, from which the major influence plaid by the NC on brain development was demonstrated, 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/r2). This means that most cephalic structures develop in a *Hox*-free domain. As far as the NC is concerned, the cells which migrate to the facial primordium and construct the facial skeleton are *Hox*-

was essential for the evolutionary transition from the Protochordates to the Vertebrates. The extant form of Cephalochordates, the *Amphioxus*, generally considered similar to the putative vertebrate ancestor, is devoid of a NC[2], 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 NC and coincides with a change in life style: *Amphioxus* are filter-feeders while Vertebrates became predators. This was made possible thanks to the acquisition of sense organs (vision, smell, audition), which developed from the neural placodes that, as the NC, 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.

By constructing the fate map of the anterior part of the neural plate (Fig. 6) and thus following the development of the early neural primordium, we have shown that, like in its original configuration in jawless Vertebrates (hagfish and lampreys), the anterior most part

[2] This is also the case for the Urochordates (although NC- like cells have been reported in Ascidians by Jeffery *et al.,* 2004).
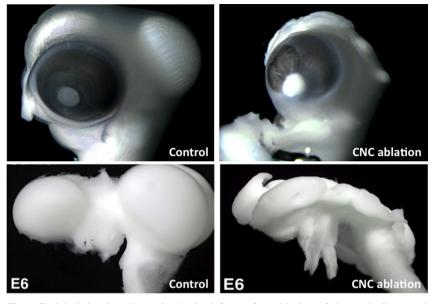
Fig. 7. Facial skeletal and anterior brain defects after ablation of the cephalic neural crest (CNC). *Gross anatomy (upper panels) and whole-mount brain preparations (lower panels) of control (left) and CNC-deprived (right) chicken embryos at E6. The absence of the CNC triggers facial skeletal agenesis and exencephaly with severe alterations of forebrain and midbrain development. (Adapted from Creuzet* et al.*, 2006).*

negative, whereas those forming most of the hyoid cartilage and the so-called "cardiac NC", which contributes to the conotruncus of the heart (Kirby *et al.,* 1985) express *Hox* genes of the first four paralogous groups. Each rhombomere 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 NC derivatives as well as for the development of the vertebral column from the paraxial mesoderm, and of the brain stem from the rhombencephalon (see references in Le Douarin and Kalcheim, 1999).

The *Hox*-negative, anterior domain of the cephalic NC (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 NC) is clearly patterned by a different genetic system[3]. In the cephalic region, the NC is therefore divided into: a rostral, *Hox*-negative, area, and a caudal one expressing *Hox* genes (eg. *Hoxa2*). We showed that NCC originating from the rostral *Hox*-negative region are exclusively able to yield the facial and head skeleton, since they cannot be substituted to do so by the most posterior ones. Accordingly, forced expression of *Hoxa2* in the rostral NC abolished its capacity to participate in head morphogenesis. In the absence of the FSNC (i.e., the NF corresponding to the posterior half of the diencephalon, the mesencephalon and r1/r2) excised prior to the onset of NCC emigration, the facial processes remained empty of NCC and 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 pre-otic brain remained open (Fig. 7).

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[3] This system was shown to involve among others, the vertebrate homologues of the Drosophila Orthodenticle and Emptyspiracle genes: Otx1, Otx2 and Emx1, Emx2, respectively.

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, the NC possesses a high regeneration capacity. For example, a quarter of the endogenous *Hox*-negative NC 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 NC 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 Shh (Brito *et al.,* 2006).

The role of the CNC (or 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 recognized as "*secondary brain organizers*". In the absence of the CNC, 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 Fgf8-soaked beads placed in close contact to the ANR (Creuzet *et al.*, 2006).

It was further shown that Fgf8 production by the ANR is regulated by the CNC, which produces anti-Bmp4 secreted molecules like Noggin and Gremlin from the time they start to migrate onward, Bmp4 being a strong antagonist of Fgf8 production (Ohkubo *et al.,* 2002), the excision of the CNC results in the failure of the development of the telencephalon, the thalamus and the optic tectum, i.e. the brain regions derived from the lateral territories of the rostral neural plate. Fgf8 is critical for this process and the NCC play a regulatory role in the production of this signalling molecule during the early stages of neurogenesis (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 NC also acts as an organising 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 pluripotency and vast distribution of the NCC all over the body are characteristics shared with the blood cells. This led me to undertake an investigation on the developmental capacities of the NCC by using the *in vitro* clonal culture assays that were so successful in the study the hematopoietic cell lineage.

Catherine Ziller, like me a former student of Etienne Wolff, took over the project together with Anne Baroffio and Elisabeth Dupin. They developed a method to culture NCC clonally, while providing them with a medium that, in principle, allowed the full expression of their differentiating capabilities. They showed that, from the earliest migratory stage, the CNCC are a heterogeneous population of multipotent cells endowed with different commitments, together with

fully committed precursors (giving clones with only one cell type).

The CNCC, but not the NC of the trunk level, yield not only derivatives of the "neural" type (neurons, glial cells, melanocytes and endocrine cells) but also cells of the mesenchymal type (cartilage, bone and connective tissues), which in the other parts of the body are of mesodermal origin. The question was therefore raised as to whether these two types of cells belonged to two different lineages or 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 a large proportion of the CNCC. The number of such cells was low in our first experiments (Baroffio *et al.*, 1988, 1991, Dupin *et al.*, 1990) but it increased later on, when we found that the signalling molecule Shh, applied at a defined time course during the differentiation process, enhances significantly the number of cells differentiating into chondrocytes in presence of other cells of the neural type. Moreover, we showed that numerous CNC clonal cultures contained cells expressing *Runx2*, a gene considered as a marker for bone differentiation, together with melanocytes and neurones (Calloni *et al.*, 2007, 2009). Multipotent progenitors with both neurogenic and skeletogenic potentials were later identified in single trunk NCC cultures (Coelho-Aguiar *et al.*, 2013). Therefore, although it does not manifest during *in vivo* development, the ability to produce mesenchymal cell phenotypes does exist in NCC of the trunk region, similarly to their cranial counterparts.

Building on these results, we could propose a cell lineage model that accounts for the diversification of NC derivatives from a multipotent stem cell (Le Douarin *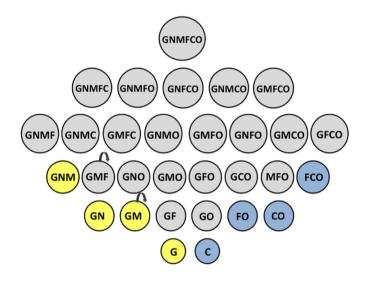et al.,* 2004) (Fig. 8). This was the first proposition of the existence of a "neural stem cell", although, at this time, their self-renewal capacity had not been demonstrated. We could later show that several multipotent progenitors, such as those able to give rise to glial (G), pigment (M, for melanocytes) 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).

Cytokines selectively active on subpopulations of neural crest cells

Another approach of NCC differentiation in our laboratory was the search for growth and survival factors able to promote the proliferation and differentiation of a selected set of precursor cells for each type of NC derivatives. Along this line, in the 1980ies onward, my laboratory engaged in the production of monoclonal antibodies (Mabs) directed against molecular markers for various avian cell types (some being specific of the quail or chick species respectively), including the NCC and their derivatives. Some of these Mabs have been studied in depth. This is the case for example, for the SMP Mab that allowed the cloning of the gene and the purification of the *Schwann cell myelin protein* that it recognizes and which exhibits a strict specificity for oligodendrocytes in the CNS and Schwann cells in the PNS. SMP was discovered and studied by Catherine Dulac, now professor at Harvard University, and by late Patricia Cameron-Curry (Dulac *et al.*, 1988, 1992, Dulac and Le Douarin, 1991).

Our next goal was to identify the factors provided to the NCC by the environment they meet at the term of (or during) their migration and which make them survive, grow and differentiate.

The most interesting of these factors was endothelin 3 (ET3). 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). It was found that ET3 is produced by the superficial ectoderm and by the mesenchymal wall of the gut. It exerts a proliferative effect on the NCC endowed with a specific seven-transmembrane-domain receptor for this peptide. These precursors are essentially of two kinds: those invading the gut and, later on, differentiating into the ENS ganglia, and the future melanocytes that migrate to the skin. We found that these two kinds of NC 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 in both cell types. Laure Lecoin cloned the melanocytic ET3-receptor expressed in chick and in quail, designated ETRB2 (Lecoin *et al.*, 1998). This receptor is encoded by a gene different from the avian ETRB1 expressed in the PNS (Nataf *et al.*, 1996).

In a following work, we demonstrated that 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 glia and melanocytes (GM) or GMF (F for myofibroblasts) types. These cells were shown to be able to 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 re-acquire

Fig. 8. Lineage tree of the cephalic neural crest (CNC) progenitors in the quail embryo. In vitro *clonal analysis of quail CNCC provided evidence for a heterogenous collection of progenitors for glia (G), neurones (N), melanocytes (M), myofibroblasts (F), chondrocytes (C) and osteoblasts (O). A highly multipotent NCC ("GNMFCO") is upstream progenitors hierarchy. The largest population of the clonogenic NCC yields both neural/melanocytic cells (G, N and/or M) and mesenchymal phenotypes (F, C and/or O) (92%; in grey), when compared to progenitors for neural/melanocytic cells only (3.5%; in yellow) or mesenchymal phenotypes only (4.5%; in blue). Self-nenewal (curved arrows) was evidenced in GM and GMF stem cells. (Adapted from Calloni* et al.*, 2009, Dupin* et al., 2010).*

embryonic capacities. The GM and GMF 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 1980ies, 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 labelling the still dividing neuroblasts in the mammalian foetuses with ^{3}H-TdR. In 1988, two groups, using the quail-chick 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.

These explorations were further pursued on the cerebellum by Marc Hallonet. The results reported in his PhD thesis changed significantly the current view on the development of this part of the brain (Hallonet *et al.,* 1990).

The role of cell-cell interactions in *Hox* gene expression in the differentiating neural epithelium

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 rhomb-encephalon, a piece of work undertaken with Anne Grapin in association with Gérard Couly. When we started these investigations, the general assumption was that the expression of *Hox* genes in various levels of the brain was cell-autonomous and nothing was known on the upstream regulation of these genes. By performing a series of heterotopic transplantations of rhombomeres 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 "posteriorising" 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, the 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 posterior to anterior 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 rostrally to 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 r5/r6 were transposed to the level of r7/r8, 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 antero-posterior (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 by 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. The accepted view was that the floor plate is induced by the notocord via the secreted protein Shh. This induction would lead the floor plate cells to produce a transcription factor of the forkhead family, HNF3β, 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) as it regresses along the midline of the embryo. It turned out that the cells of the organizer express HNF3β from the onset of gastrulation onward. Moreover, during its regression, the node yields not only the notocord, as previously recognized, but also the floor plate, designated as the "*node-derived*" floor plate. In addition, the node leaves in its wake a stripe of cells in the dorsal endoderm (Catala *et al.,* 1995, 1996; Le Douarin *et al.,* 1998).

These experiments thus challenged the role of the notocord in floor plate induction during normal development. In fact, a medial floor plate derived from the node itself develops in a cell autonomous manner and the notocord is thus not required for inducing the "primitive" floor plate. Those observations are supported by several genetic data, particularly by zebrafish mutants (like *floating head*) in which a floor plate develops into a normally patterned NT in the total absence of a notocord. Our results therefore led to reconsider the classical notions about the process of gastrulation and neurulation in the vertebrate phylum (Charrier *et al.,* 1999, Le Douarin and Halpern, 2000 and references therein). They have also disclosed a novel role for Shh during neurulation. It turned out that if the development of the notocord and floor plate is prevented by extirpation of Hensen's node, the NT 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 to trigger their cell death program (Teillet *et al.*, 1998a,b, Charrier *et al.,* 2001, 2005, Thibert *et al.*, 2003).

Chimeras in the study of brain functions

For about ten years (from 1985 on), I extended our studies on embryos up to the post-hatching stages after seeing that the neural chimeras were able to hatch and survive. We could then envisage to tackling problems dealing with certain functional aspects of brain development.

In 1983, I had the privilege of being elected as a member of the Neuroscience Research Program (NRP). When I presented our experiments showing that quail-chick neural chimeras could hatch and remain 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 PhD and thinking of a post-doc, was interested by the model. He thought that it could be applied to the problem of "nature versus nurture" in behaviour. What type of behaviour would exhibit a chimera, whose definite 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 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 vocalisation and behaviours 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. Numerous quail-chick 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 behaviours like that of crowing are genetically determined in definite areas of the neural epithelium of the encephalic vesicles. During neurogenesis, the various regions so defined 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 done in another series of experiments that involved a pathological trait determined by a mendelian mutation leading to epilepsy. The work on chicken epilepsy has given rise to several articles (see Teillet *et al.,* 2005 for a review).

From neurobiology to immunology: revisiting the problem of self/non-self discrimination in postnatal chimeras

The experiments that we had performed in the 1970ies to explore the fate of the NCC, 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 post-hatching chimeras.

At the beginning of the venture that started when I had the idea of constructing chimeras to study development, I thought that the technique was particularly suited to follow the fate of migratory cells. In addition to NC, I was also interested in another cell type that occupies multiple locations in the embryo and the adult as well: those of the hemopoietic system. This is why I have studied, with Francine Jotereau and Elisabeth Houssaint, the colonization by hemopoietic stem cells of the thymus and bursa of Fabricius (the organs where T and B lymphocytes are respectively produced in birds), thus closing definitively the controversy that existed at that time about their extrinsic or intrinsic origin. I was therefore familiar

with the field of developmental immunology and particularly interested in one of the most intriguing problem in this domain: self/non-self discrimination.

When a Japanese post-doctoral fellow, Dr. Masae Kinutani, came to the laboratory in 1982, she was enthusiastic about a project that would explore the hatching ability of quail-chick spinal cord chimeras and the fate of the graft after birth, when the host immune system has become fully competent. Marie-Aimée Teillet obtained the first spinal cord chimera that was able to hatch. She gave me a large photograph of this bird that I keep preciously (Fig. 9). Masae Kinutani, under the efficient teaching of Marie-Aimée, became one of the most skillfull "transplanter" I had around and pursued the project.

The rate of hatching was about 1 out of 10 operated embryos. The birds were healthy for a while but, after a few months, they 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 human subjects affected 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, protected by the blood-brain-barrier, have a privileged immunological status, while expressing no (or only little) MHC antigens. It is likely that the first immune attack on graft-derived tissues took place in the peripheral nerves, devoid of the protective blood-brain-barrier present in the CNS. The T cells recognizing neural and glial antigens thus became 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,

Fig. 9. Adult quail-chick chimera after heterospecific transplantation of the neural primordium. *A "neural" chimera, 3 weeks after hatching, shows the brown quail-characteristic pigmentation of feathers in the wings, due to melanocyte migration and differentiation from the quail neural crest cell grafted at the brachial level in a White Leghorn chick host embryo. (From Le Douarin, 2005).*

like myelin basic protein. The chimeric birds thus appeared as an experimental model for MS (Kinutani *et al.,* 1986)[4].

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 non-neural embryonic tissues (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 and all rejected the foreign wing acutely, starting at various times during the two first post-hatching weeks (i.e. when the host's immune function reaches maturity). This reinforced the notion that the widely accepted theory proposed by Burnet and Lederberg in the late 1950ies, according to which the presence of antigens during development is sufficient to induce self-tolerance, was not valid. Moreover, this experiment revealed that, in grafts between animals of the same species, the delay typical for NT grafts in heterospecific combinations was not observed for non-neural tissues. This was confirmed 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. Contrary to the outcome in quail-chick wing chimeras, the grafted wing was generally not fully rejected. However tolerance was not complete. 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 if 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 quail-chick 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 quail-chick 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 were 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 was 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 3rd and 4th pharyngeal pouches substituted to their counterparts 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), out of the 16 birds that hatched[5], 14 showed tolerance of the quail wing. In 3 of them, slight signs of rejection appeared after about 40 days, thus much later than in quail-chick 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. However, substitution of thymic epithelial rudiments was usually incomplete. This technical problem made the experiment even more interesting than expected: tolerance turned out to be induced permanently provided that, at least 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 (Ohki *et al.,* 1987).

This significant finding meant that tolerance to self did not exclusively result from the elimination, in the thymus, of autoreactive T cells, a process known to be incomplete, since T cells with high affinity for self-components are currently found in the periphery and become harmful in autoimmune diseases. The quail-chick double chimeras revealed that a mechanism at work prevented these T cells from being activated in an antigen-specific manner. In contrast to the accepted dogma of the default mechanism, tolerance to self is "*active*" and mediated by a so far unrecognized type of T cells. This work was recorded in a series of papers (for reviews, Le Douarin *et al.,* 1989, Salaün *et al.,* 2005).

At this point, we had animated discussions with immunologists, particularly with Pr. Max Cooper from the University of Birmingham, who was spending a sabbatical year with us, and Pr. Antonio Coutinho, who was leading an Immunobiology 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 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.

The results produced by this collaboration, confirmed and extended those obtained in birds (Salaün *et al.*, 1990). A so far unrecognized mechanism designated "*dominant tolerance*" was shown to play a role in self/non-self recognition (The responsible T cells, whose existence was subsequently confirmed by many other groups (for references, see Le Douarin *et al.*, 1996, Coutinho, 2005), were called "regulatory T cells" (Treg). 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 that expanded vigorously. At the Congress of the International Union

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[4] This paper was published in *Cell* with a chimera on the cover page.

[5] The hatching rate was 1 out of 10 operated embryos.

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 have in transplantation medicine.

Concluding remarks

I have recalled, in these pages, some aspects of the happy years of my scientific life. During all this time, the chick embryo provided me with the possibility to tackle a variety of scientific problems to which I had a chance of bringing a contribution. They go from the development of the liver to the neural crest, hemopoietic organs and the seminal question of how higher organisms are able to protect themselves from harm through the defenses they present to their enemies. These stories, which covered more than five decades, did not move along a pre-established design, neither have they been elected independently from one another. On the contrary, they were most often related, since each developed in order to answer a question raised by the preceding one. It often happened that the results of an experiment had two sides: one was answering the question raised when initiated, the second leading to a novel problem.

In addition to these topics, the possibilities offered by the avian chimeras have led to the study of various other problems in my laboratory, such as the mechanisms underlying certain types of epilepsy, and migration of cells during brain neurogenesis, among others.

I have evoked the main stream of the researches pursued by myself and under my direction, but there are other interesting developments that did not belong to these themes. I would like to mention a project carried out by a post-doctoral fellow, Anne Eichmann, now Professor at Yale University, which led to the discovery of two tyrosine kinase receptors that we called Queck 1 and Queck 2 (Eichmann *et al.,* 1993). These are exclusively expressed by the endothelial cells of the blood and lymphatic vessels, respectively. We proposed that they might correspond to the then unknown receptor for vascular growth factor whose existence was suspected at that time; a suggestion that was fully confirmed later on (Eichmann *et al.,* 1996).

I wish to conclude by mentioning the seminal studies developed in the Nogent Institute by Dr. Françoise Dieterlen and her coworkers, on the early stages of hemopoiesis. A critical experiment aimed at finding whether the hematopoietic stem cells produced in the yolk sac contributed or not to adult hemopoiesis, a subject of hot debate at that time, was based on the construction of embryo/yolk sac chimeras: a quail embryo was grafted on a chick yolk sac *in ovo*. The quail embryo developed normally up to hatching time and delivered an unequivocal answer to the question raised.

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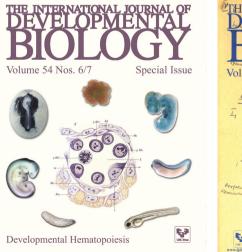http://www.intjdevbiol.com/web/paper/041940cr

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