

# Developmental hematopoiesis: historical background and perspectives - an interview with Nicole Le Douarin

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**ABSTRACT** Nicole le Douarin has shown a long lasting interest for developmental hematopoiesis. Starting from her early research experience, we travel along the main discoveries and concepts that have shaped the modern view of developmental hematopoiesis. All through, we survey the seminal contribution of the "Ecole de Nogent" about lymphocyte homing and the discovery of endothelial-specific tyrosine kinases. This interview is a promenade through the past and present of developmental hematopoiesis narrated by an exceptional personality and an outstanding scientist.

**KEY WORDS:** *quail, chick, liver, thymus, bursa of Fabricius, bone marrow*

## Introduction

Prof. Nicole le Douarin started her research career by exploring the development of the liver in the avian embryo. She demonstrated that hepatic differentiation requires two induction events coming from the mesoderm. By associating quail mesenchyme to chick hepatic endoderm, Nicole Le Douarin made an exceptional observation: the quail nucleus, in contrast to the chick one, contained a huge amount of heterochromatin, centronucleolar and associated with ribosomal RNA. Thus, she discovered a novel experimental approach, designated as the quail/chick system that was going to revolutionize Developmental Biology by allowing to follow the fate of cells or tissues during organogenesis and cell migrations. From 1969, working at the Faculty of Science in Nantes till 2005 when she was at the head of the Nogent Institute and Professor of Developmental Biology at the College de France, Nicole Le Douarin and her colleagues produced seminal contributions that have increasingly improved our knowledge on neural crest cell differentiation, neural development and immune system. Nicole Le Douarin's scientific achievement covers more than 450 articles and several books which made her world famous and unanimously recognized as a major driving force in modern developmental biology. She is the recipient of distinguished international awards such as: Kyoto Prize of Advanced Technology, Jeantet Prize for Medicine, Gold Medal of CNRS.

She is a member of many academies including: the French Académie des Sciences, Royal Society (UK), US National Academy and Doctor Honoris Causa of several highly regarded Universities. She is honorary Permanent Secretary of the Académie de Sciences, Paris.

## When did your interest in developmental hematopoiesis start?

My interest in this field started when I decided to prepare my secondary thesis (a state of the art analysis) on embryonic hematopoiesis (Le Douarin, 1966). For this piece of work, my supervisor was Prof. Louis Gallien, a famous French embryologist, member of the French Academy of Science, who worked on the amphibian embryo. I focused this thesis on the work of pioneer embryologists (Dantshakoff, Brachet...) who dedicated their work to the origin of blood cells in the amphibian embryo. They showed that hematopoietic cells originated from the ventral blood islands, functionally the equivalent of the extraembryonic mesoderm in amniotes. This work stimulated my interest in comparative embryology and the role of the extraembryonic mesoderm in the development of the blood system.

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*Abbreviations used in this paper:* AIP, anterior intestinal portal; BM, bone marrow; HSC, hematopoietic stem cell; VEGF, vascular endothelial growth factor.

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The concept and techniques for my future work on hematopoiesis were acquired during my first research experience on the digestive tract. I initially studied the development of the liver, a tissue of endodermal origin and discovered that, early in ontogeny, a small number of endodermal cells originating from the Anterior Intestinal Portal (AIP) initiate their hepatocyte differentiation. I wondered about the control of this hepatocyte induction and unraveled the critical role of the mesodermal component (Le Douarin, 1964 a,b). If the endoderm was cultured alone, it remained poorly developed, whereas if it was associated with the hepatic mesoderm, it gave small hepatic lobes. The same differentiation could be obtained *in vivo* if the presumptive hepatic endoderm was associated with lateral mesoderm (either somatopleural or splanchnopleural mesoderm). If the association was made with the paraxial (somatic) mesoderm, hepatic differentiation did not occur. I also tried to associate endoderm with intermediate mesoderm. In this particular experimental scheme, formation of hepatic-like cords occurred, but glycogen (a hallmark of hepatic differentiation) was not synthesised. Instead, the cells contained electron-dense granules typical of adrenal gland differentiation. I thought at that time that these cells could originate from the neural crest. At the same time, I discovered the quail/chick marker and was thus able to demonstrate the neural crest origin of the adrenomedullary cells. I was fascinated by the possibilities offered by the quail/chick marker to study the broad dispersion and sites of migration of the neural crest and I naturally oriented my work towards this major biological question.

I would like to open a parenthesis here. I believed (and I still believe) that the best model to explore these complex biological questions is the avian embryo. Indeed, the use of the quail/chick chimera system (Le Douarin, 1969) made morphogenetic movements obvious and allowed investigators to follow the migration and fate of grafted cells *in vivo*. The avian embryo has been a unique tool to solve many embryological problems, in particular the question of the origin of the hematopoietic system. Its use as an experimental model relies on several cogent reasons: first it is easily accessible throughout the developmental period, which is not the case for viviparous species. Birds are homeothermic vertebrates like mammals; moreover they are amniotes. Here I want to highlight one important discovery, which relied on a specific anatomical trait of birds; the existence of B and T cells, the two types of immune cells. In 1960, Jacques Miller at the Walter and Eliza Hall Institute in Melbourne, Australia, demonstrated that ablating the thymus in new-born mice in the three days following birth caused a profound immunodeficiency characterized by the absence of graft rejection and immune response against viral infection. Antibody production persisted, but only against specific antigens (Osoba and Miller, 1964). Thus there were clearly two classes of lymphocytes, one responsible for production of circulating antibodies, another for cellular immunity. The latter class appeared clearly associated with the thymus. The discovery of antibody-producing lymphocytes was achieved in birds, because they have a specific anatomical site, absent in other vertebrate classes, where antibody-producing lymphocytes differentiate. The bursa of Fabricius is appended at the caudal end of the digestive tract. In 1956, an American endocrinologist, Bruce Glick, was working on the effects of testosterone in avian embryos. When Glick injected testosterone during the first period of avian development, the most remarkable effect was the complete

loss of the bursa of Fabricius. The hatched individuals were particularly vulnerable to pathogen infections because plasmacytes were totally absent (Glick, 1966). Plasmacytes are cells derived from small lymphocytes indistinguishable from those of the thymus, which evolve to produce antibodies. These lymphoid cells are called B lymphocytes to recall the bursal origin. In Mammals, B cells are produced in the bone marrow.

My interest in developmental hematopoiesis came from a paper I wrote in tribute to one of the most prominent figures of histology, Mr. Manfred Gabe. Mme. Arvy, an MD-PhD who asked me to write this tribute, had warned me about the question of the origin of T lymphocytes, a problem totally unsolved at that time. I realized that the quail/chick system could help to work out this fundamental question. Since the beginning of my research experience, I was attracted by the process of cell migration during embryogenesis. Because I had studied formation of the pharynx in detail (the fetal liver is a pharynx derivative), I had a fair knowledge of this region and I knew where thymic rudiments and those of the other glands (e.g. thyroid, parathyroid) were located. I first decided to graft into a chicken host a quail thymic rudiment composed of endoderm and associated mesoderm. At that time (late sixties), the mesodermal origin of blood cells was widely accepted, but it was not clear if T (and B) cells followed the same rule and, in particular, whether they were produced by the thymic anlagen themselves or by another tissue, yet to be defined, before they migrated to the thymus. In this experimental scheme, lymphocytes appeared to be of chicken origin demonstrating that neither the thymic endoderm nor the associated mesoderm were able to generate T cells, the latter likely coming from the blood. This question had, in fact, been investigated several years before by Moore and Owen using parabiotic embryos. These investigators had proposed that B and T lymphocytes originated from the blood. To investigate how the hematopoietic system formed, these authors applied an array of experimental approaches: irradiation/restoration, parabiosis (Moore and Owen, 1965, 1967) and grafting of rudiments (thymus, spleen, bursa of Fabricius) onto the chorioallantoic membrane. To follow the origins of the cells, they used the pair of sexual chromosomes as a marker. These experiments brought about very important insights about the genesis of the hematopoietic system, the most important being that extrinsic Hematopoietic Stem Cells (HSC) colonized all the hematopoietic rudiments, with the exception of the yolk sac. But the experimental design also had several drawbacks: the chromosomes could be observed in only 5% of the cells blocked in metaphase after colcemid treatment, which caused high embryonic mortality. Thus with Francine Jotereau, then a young student, we decided to reinvestigate the question in the light of the quail/chick model. We grafted quail thymic rudiments in chicken embryos and analysed the origin of the T cells. Our striking conclusion was that all T cells (and B cells as well) had an extrinsic origin (Le Douarin *et al.*, 1975a; Jotereau *et al.*, 1980). In complementary experiments with Bruno Péault, we were able to study some cellular aspects of thymus colonisation. With the use of an MB1/QH1 monoclonal antibody (Pardanaud *et al.*, 1987; Péault *et al.*, 1988), we visualized endothelial and hematopoietic cells and studied their specific interactions during thymic colonisation. Pursuing the study, we described precisely the existence of distinct cyclic waves of thymus colonization, whereas a single colonization wave characterized bursal development (Jotereau,

1973; Le Douarin *et al.*, 1975b, 1976, 1977; Houssaint *et al.*, 1976; Le Douarin and Jotereau, 1975; Jotereau and Le Douarin, 1982). In order to study cyclic colonisation, we designed a specific experimental scheme in which the quail thymic anlage before colonisation was grafted onto a chicken host for different periods of time, then withdrawn from the embryonic environment and placed in culture and eventually grafted back into a quail embryo. The rudiment was colonized by HSC that further differentiated into T cells. If the quail rudiment was taken out one day later, when colonisation of the thymic rudiment had initiated, chicken HSC were prevented from migrating into the quail thymic rudiment. We then had the impression that the thymus behaved like a door. In some instances the door was closed, whereas in some others, it was open, allowing lymphocytes to enter. In fact, we discovered that the thymic rudiment had to be completely filled with T cells before draining and initiating a new phase of colonisation. Thus, it was the number of colonizing cells rather than the timing, which was responsible for the signal. It also appeared that the thymus (and the bursa) displayed attractive periods followed by refractory ones. Using the grafting/ablation system, we were able to precisely establish the colonisation schedule for the thymus and the bursa of Fabricius.

**You have performed another experiment which, we believe, has been instrumental in defining the role of the microenvironment. We refer to the association of quail yolk sac mesoderm with a naïve chick thymus resulting in T cell colonisation and differentiation.**

Yes! It means that yolk sac HSC are similar to those found in the embryo proper, i.e. they are able to respond to environmental cues emitted by the thymus, but the yolk sac microenvironment limits their potential. At the same time, yolk sac HSC are used to make something else, in particular red blood cells and macrophages. Another very important demonstration had been to show that, even at a very early stage at the time of gastrulation, HSC are already determined and expressed receptor II for VEGF (VEGFR-2). By cultivating these cells in different VEGFR-2 conditions, they can be oriented either towards the endothelial or the hematopoietic fate (Eichmann *et al.* 1997) and you, Thierry, (Jaffredo *et al.*, 1998) have also demonstrated that endothelial cells are able to produce blood cells. Thus, there is clearly a community between the two cell types, a mother cell that is the basis of the edification of the system. This story is also instrumental in showing the close collaboration between two groups in the Nogent Institute (see Int. J. Dev. Biol. Special Issue dedicated to the Nogent Institute, Le Douarin, 2005) and the permeability between the discovery of VEGFR-2 (Eichmann *et al.*, 1997) and the application of this discovery to the demonstration that endothelial cells in the aorta were able to convert into hematopoietic cells.

**From a historical point of view, what were the main steps supporting the concept of hematopoietic stem cells?**

In my opinion, the origin of the HSC concept is linked to the ability to reconstitute the whole hematopoietic system after transplantation.

Following the use of the atomic bombs in Hiroshima and Nagasaki, people died from the blast and from burns. Irradiated people died within 15 days of marrow aplasia. In the meantime, the bone marrow (BM) was known to contain immature blood cells

and the self-renewal ability of the blood system had been known since a long time. Thus, the main question was: is BM a site of blood cell production? Scientists addressed this question by irradiating mice at lethal doses. They first observed that, as in humans, irradiated mice developed profound marrow aplasia. Transplantation of BM cells from donors to irradiated mice allowed some of these transplanted mice to recover, because of the presence of mature cells in the grafted cell population and also because donor cells were able to fully reconstitute the blood system of recipient mice. This assay, used in many laboratories in the world nowadays, is the gold standard protocol for the identification of HSC. It should be emphasized here that the first transplantations in human were achieved in France in the fifties when BM cells were injected into Yugoslavian researchers who had been irradiated.

The ultimate question then was: is there a stem cell for each blood cell lineage or is there a multipotent HSC able to give rise to all blood cell types? Till and McCulloch (1952) transplanted cells from the bone marrow into lethally irradiated mice. 10-14 days after transplantation, they observed nodules in the spleen of the recipient mice. Analysis of these colonies revealed the pres-



**Fig. 1. Nicole Le Douarin at the microscope in 1980.**

ence of hematopoietic cells from different lineages (e.g. macrophages, erythrocytes, megakaryocytes), with each multilineage colony likely derived from one single cell that had settled in the spleen. Their observations first demonstrated that the bone marrow contains very rare HSC having the ability to self-renew and to produce all hematopoietic lineages.

### If you would have to start a new PhD, what would be your favorite subject of investigation?

I think I would focus on the bone marrow. As an example to illustrate my point of view, I would like to tell you that I was always interested in the formation of blood vessels within developing tissues. For instance, I had observed that during early limb bud development, there were no vessels. The vascular network organized in the limb buds latter on during precise developmental periods. But there is a tissue completely lacking vascular networks: that is the cartilage. So the questions were: why is cartilage, in contrast to bone (which is closely related to the cartilage), a repulsive tissue for blood vessels? and why does a tissue that does not contain any blood vessels suddenly attract them at precise developmental points? During a meeting, I had the pleasure of meeting Dr. J. Folkman. He was presenting data showing that cancer cells are very attractive for blood vessels. At the end of his talk, we started discussing about the capacity or incapacity of some tissues to attract blood vessels. A few years later, Dr. Folkman and his colleagues identified a repulsive agent for blood vessels from cartilage. Then, my group identified two tyrosine kinase receptors, VEGF-R2 and VEGF-R3 that mediated an attractive signal for blood vessel assembly (Eichmann *et al.*, 1993), a discovery confirmed slightly later on by other groups.

Bone marrow is a very complex organ containing HSC and their descendants, as well as stromal cells and neural cells. BM is a reservoir for several types of adult stem cells: blood stem cells, mesenchymal stem cells and neural crest stem cells (giving rise to Schwann cells). Mesenchymal stem cells are known to produce osteoblasts, adipocytes, vascular smooth muscle cells and fibroblasts. Furthermore, BM stromal cells constitute a supportive microenvironment for HSC maintenance and differentiation in the adult. As an embryologist, I am interested by the architecture and polarity of this extremely complex and dynamic structure. How do blood, mesenchymal and neural cell types interact and how do they organize within the bone marrow? How is the polarity (the bone-blood vessel axis) of bone marrow established during development? What are the molecules involved in the cross-talks between HSC and stromal cells, HSC and neural cells or HSC and Schwann cells? I think that answering these fundamental questions should considerably improve our understanding of HSC biology and, maybe, their further manipulation for therapeutic purposes.

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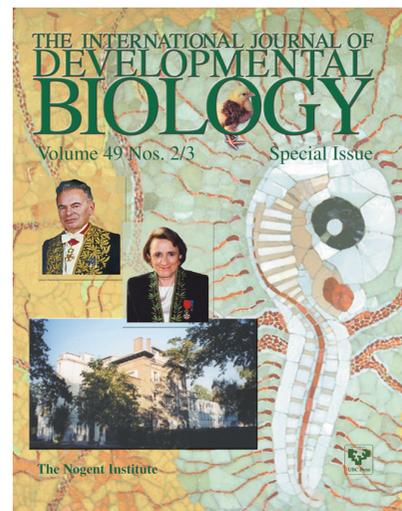
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