Sex, differentiation and cancer

An interview with Professor Etienne Wolff, pioneer researcher and promoter of modern developmental biology in France

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Professor Etienne Wolff was the initiator and animator of what was to become a flourishing school of embryology in France. Born on February 12, 1904 in Auxerre (France), he entered research in the early thirties. From the outset, he selected the avian embryo as his experimental material, a choice which, in retrospect, was exceptionally judicious. He first sought to understand the genesis of freaks and, by experimentally manipulating embryos, succeeded in reproducing spontaneous abnormalities at will, thus establishing a number of rules about how errors of development occur.

Turning next to the study of sex differentiation, he was able to show that adult hormones were capable of bringing about partial or total sex reversal in the embryo, influencing the differentiation of the very glands that are responsible for the synthesis of these hormones. He also demonstrated that sex reversal in this class of vertebrates is effected by female, rather than by male, hormones – a discovery that was to receive its full significance later, when it was found that the female is the heterogametic sex in birds.

In order to withdraw the gonads and reproductive tract from environmental influences, E. Wolff devised an *in vitro* organ culture system. This efficient and practical technique later yielded a large corpus of data as he and his collaborators analyzed the tissue interactions at work in most processes of organogenesis. Later he became interested in cancer and, by means of his organotypic culture technique, showed that cancer cells of human origin can be cultured virtually indefinitely when grown in close association with living embryonic organs that provide them with a support and a source of nutrients. The stage was not set yet for identifying the growth factors involved, but Wolff foresaw the importance of this quest and put forward the notion that cancer and embryonic differentiation are closely-related processes.

An interruption in Etienne Wolff's activities was caused by World War II, most of which he spent as a prisoner of war in a German *Oflag*. There he gave conferences on biology to his fellow prisoners and, relying entirely on his mental notes, prepared books that were published shortly after the war.

All Etienne Wolff's research was accompanied by institutional teaching, first at the University of Strasbourg, where he gained a professorial chair in 1945, and 10 years later at the Collège de France, a prestigious institution dating back to Guillaume Budé and King François I, which was created to promote and diffuse recent advances in most fields of human activity. The Collège de France granted him the use of an estate east of Paris, where he created a large laboratory, the Institut d'Embryologie et Tératologie expérimentales, supported to a great extent by the CNRS. Emilienne Wolff, his wife, was a constant help in running the Institute and played a very active part in developing the research themes that were closest to his heart. This laboratory is now headed by Prof. Nicole Le Douarin, a former graduate student of Etienne Wolff's, who has oriented the research themes towards the study of the ontogeny of the immune and nervous systems, but has remained faithful to the avian model.

Prof. Wolff's achievements, as well as his successful efforts to make science accessible to the public, were officially recognized by his successive elections to the Académie des Sciences (1963), the

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Académie Nationale de Médecine (1966) and finally to the Académie Française (1971), where he still participates actively.

In the following interview, held on November 29, 1989, Prof. Wolff discusses some of the factors that helped shape his professional orientation, and traces the high points of a career that has long been recognized internationally as having contributed enormously to the progress of developmental biology.

My first question concerns the manner in which you first became interested in biology. I know that you began by studying philosophy, so how is it that you were diverted from your early aspirations and devoted your entire career instead to the study of the embryo?

Obviously this came on progressively. However, the real turning point that triggered my reorientation was that, in order to continue studying philosophy, it was necessary to study Greek. I knew no Greek at all, but it was possible to substitute knowledge of this language with a science degree, so I began studying science. This was at first very boring to me. At the University of Strasbourg at that time, many of the professors taught mainly systematics.

However, I was soon enraptured by general biology and by meeting Edouard Chatton, whom I was eventually to work under. Edouard Chatton was a great scientist; he may be somewhat forgotten nowadays, but let it merely be said that he introduced André Lwoff to biology and that this influence was certainly instrumental in setting Lwoff off on his dazzling career. E. Chatton and A. Lwoff collaborated a long time together, particularly studying the life cycles of lower crustaceans, protozoa and many other animals. Coming back to your question, it was through necessity that I first studied biology, and the enthusiasm that Edouard Chatton communicated to me then led me on to continue in natural sciences.

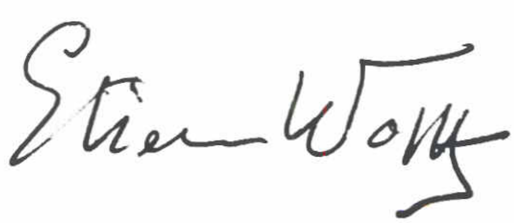
Could you tell us, then, how you became interested in embryology? Your other professors at that time, in particular Ancel and Bouin, surely had an important role in your orientation. What was the state of the discipline in France at that time?

Well, chance always makes careers and evolution. Prof. Ancel, who was the Dean of the Medical Faculty and Professor of Embryology, managed to have an assistant appointed to his unit. He had a *"chef de travaux"*, called Vintemberger, with whom he always collaborated, but he asked Prof. Chatton whether there was a student who might want to pursue his studies in that direction rather than in clinical medicine. The truth actually is that they knew that I was looking for an opportunity of going into embryology. I accepted the job and went to work with Prof. Ancel. At that time in the thirties, the major specialists in experimental embryology were Germans, following in the steps of Hans Spemann, the great scientist whose works are still famous. Two other notable figures were then prominent in Germany. One was Mangold and the other was Vogt, who invented supra vital color markers for the amphibian embryo – a technique used thereafter for all types of embryos. Holtfreter should also be mentioned. He explanted rudiments in an isotonic medium, where they continued their differentiation, using up the vitelline nutriments contained in the cells. I was very excited about the research going on in Germany and wanted to introduce these techniques into France. Ancel and Vintemberger's team was conducting experiments on the determination of the plane of bilateral symmetry, which still serve as a reference today. One day, Ancel, on his daily visit to me, said: "you are certainly tempted to work on the problem of the organizer, but you will only be following in the footsteps of the Germans, and it will be difficult to come up with original findings". One big problem dominated the entire field of embryology: this was teratology, experimental teratology. There had been a few precursors; for instance, Dareste around 1850-1870. Some had incidentally obtained a few freaks but experimental teratology had not started off yet. Ancel suggested that I irradiate local sites of the embryo. And this is how it began. There was in the laboratory an antique apparatus, made of a large bulb with an anticathode, which produced X rays. It was coupled with a current rectifier that produced enormous and maybe dangerous sparks. This primitive apparatus (this was in 1928 or 29) was very useful to several of us. I managed to perform local irradiations by making the rays go through a narrow tube. This is how I began experimental teratology.

Wasn't this line of research interrupted by the war and the time you spent in Germany as a prisoner of war? After this interruption you undertook some novel type of work, I believe.

War did interrupt this work, but a few months before the war I had already reproduced most of the freaks that develop spontaneously in birds and mammals. These experiments were all carried out on chicken embryos, which are easily manipulated and highly accessible. I had thus produced all categories of simple and double freaks that were known in a spontaneous and accidental state but had not previously been obtained experimentally, such as Cyclopeans, Otocephala and Symelians. But in addition, new types of freaks were created in these experiments, such as anterior Symelians, in which the wings are fused in the middle of the back. Intersexes, which I am going to talk about now, were also part of these new freaks.

I had undertaken before the war another line of research, concerning the effect of sex hormones on very young embryos, incubated for 4 to 5 days. At that time the first sex hormones had been isolated by Butenandt and others like Girard in France. I was collaborating with a gynecologist from the medical school, and we had tried out the effect of placental extract on the embryo without success, but this oriented my research towards sex hormones. Delépine, from the Collège de France, provided the first decigram of estrone and then its derivative estradiol, which is even more potent. With my collaborator Ginglinger, we tried it out on the embryo, despite the current views that it had to be impossible for adult hormones to work on embryonic rudiments. The hormone was deposited on the appendages of 5-day embryos. During the course of these experiments, I took a few days off and when I came back and asked my co-worker about the results, he answered: "I don't understand, many embryos are dead and I cannot diagnose their sex". This was a surprise since it is so easy to do so after 8 days of incubation because of the typical asymmetry of the genital tract in female embryos. We autopsied the last 20 embryos and discovered that most were females but also that there was a whole series of intersexed individuals. All intermediates were there. It was impossible to doubt the powerful effect of female sex hormones on



the avian embryo. Among the overnumerous females, some were true females, but the others were completely inverted males.

This discovery immediately led to many similar experiments in France and in other countries, on all classes of vertebrate embryos. All these experiments, notably those of Gallien on amphibians, Raynaud and Jost on mammals, yielded positive results, but not always as clear cut. Salamanders and frogs were the most pliable to sex inversion. Fish were also amenable to sex transformation. On the whole, lower vertebrates were easiest. In birds our results were remarkable but they were only transient and the transformed animals eventually went back to their original sex. In mammals it is even more difficult. Genital ducts are inverted but the gonads are not. Only in marsupial mammals is this possible. Burns in the United States obtained the transformation of females into males. In mammals the sex inversion obtained through hormonal treatment is opposite to that in birds. Females are the ones transformed by male hormones.

This leads into another question. Several times in the course of this interview, you have mentioned luck, like for instance when you were telling us about the incentive that led you to biology. Was it also luck that made you try out the effect of female hormones on bird embryos, since it is now well established that sex determination is driven by female hormones in birds, in contrast to the situation in mammalian embryos, where male hormones are entirely responsible and the female phenotype differentiates in the absence of hormones?

The answer is simple. It is true that luck is often important in the management of research, but one must seize it. In the case of sex differentiation, we tried female hormones because they had been isolated first and were available. Shortly afterwards, male hormones were isolated and they induced the transformation of genital ducts but not of the gonads. Indeed in birds, as my wife Emilienne Wolff and I have shown, the neutral sex, which differentiates when hormones are absent, is the male sex. This is what happens in castration experiments.

Can you tell me how these castration experiments were performed?

The embryos were irradiated very precisely on the region of the genital anlage, at an early age, around day 5 or 6. Later on, inversion is no longer possible, and indeed we were able to demonstrate that female hormones are secreted as early as day 6 to 8. This was the first demonstration of a fact that has been well established since. Sex hormones are secreted at two different points in time. The first occurs very early in development, while the second consists of a tremendous increase beginning with puberty. The hormones are the same during these two periods.

Didn't this question of sex differentiation drive you to devise an *in vitro* culture technique, in which the three-dimensional structure of the embryonic rudiments is maintained? This technique was later applied to the study of the development of other organs.

This is true. I thought it was crucial to be able to follow the development of the gonads *in vitro*, as well as that of the genital ducts. Organ cultures had been initiated in one or two laboratories, in particular that of Miss Fell in Great Britain. Miss Fell obtained very interesting results, but her technique was not a general one, because rudiments were drowned in the nutrient medium containing adult plasma. Plasma was unstable because of the effect of other components in the medium. With Katty Haffen, I devised a solid medium made of agar enriched with nutrients, and endowed with a proper saline balance, depending on the origin of the tissues. This technique was then applied to a variety of organs. For instance, genital ducts from 11-day embryos were cultivated. Female ducts developed, whereas male ducts disappeared. Thus, the influence of male hormone, exerted by the gonad prior to explantation, was responsible for the activation of an enzyme that later digested the ducts. Bone rudiments were cultivated – tibiae from 5 to 6-day embryos, small undifferentiated sticks – which became modeled *in vitro*, displaying after 5 to 6 days typical articular protuberances, epiphysis, etc., so that a typical miniature tibia developed. Articular surfaces differentiated exactly as if the complementary bone was present, thus the capacity for forming these typical structures was seen to be innate. The only organs that could not be cultivated in that fashion were nerve and ganglia, which do not thrive in these conditions. One remarkable organ was studied by my wife. This is the organ responsible for bird song, the syrinx, which develops at the bifurcation of the bronchia and trachea. The male syrinx grows enormously and becomes asymmetric, whereas the female one does not present a similar swelling. Cartilaginous rings support this swelling and they fuse into a tympanic drum. Explanted *in vitro*, syrinx from both sexes develop the characteristic male swelling. We had thus demonstrated that the male phenotype is neutral while the female is the sexualized one.

Thus the organotypic culture technique that you devised with K. Haffen in 1952 demonstrated the capacity of certain organs to differentiate according to plan and also the hormonal requirements that some of them need to achieve their typical differentiation. But another very productive approach was to study the relationships between tissues of different germ layer origin, that is, the induction processes between epithelium and mesenchyme.

Yes, indeed there has been a lot of work performed in my laboratory, first in Strasbourg, and then in Paris when I came to the Collège de France. For example, Philippe Sengel worked out the messages that are exchanged between ectoderm and mesoderm during the differentiation of feathers. The development of various organs was also studied, such as that of the glandular and muscular stomachs of birds, that of the pancreas or the lung, and all were shown to involve induction processes between an epithelium and mesenchyme.

The last phase of your activity was dedicated to the study of cancer growth, using this same organotypic cuture technique.

After cultivating many normal organs, it was natural to wonder whether this technique could not be applied to cancerous tissues. Actually there is a difference, because only embryonic organs can be cultured in this way, not adult ones. But tumors, even if they are constituted by cells from the adult, have very particular growth properties that make them similar to embryonic cells. With Emilienne Wolff, we first chose animal tumors from mice or rabbits or birds, and then human tumors. It should be emphasized that not all tumors demonstrate the same growth potential *in vitro*. Some of them grow very easily. We first used the same medium as for embryonic organs, but this did not work. We finally found out that fragments from embryonic organs promoted the survival and growth of human tumors, in particular the embryonic kidney, or mesonephros. These cultures can grow indefinitely, and we have been able to carry them out for as long as 15 years, so that their outgrowth reached a considerable mass.

Thus, the associated embryonic tissues provided factors that are necessary for the growth of the cancerous cells. We were thereafter able to obtain the same result when the tumor was separated from the embryonic tissues by a dialysis membrane, showing that the nutrient substances are diffusible. We were able to determine which amino acids are necessary, or in some cases peptides. However, a small quantity of embryonic serum was always necessary, meaning that there still was (at least) one unknown factor.

Of all your research, what do you now consider as the most significant?

This is a difficult question, because, during the actual work, the study being carried out always seems the most interesting. As in the case of Jean Rostand, who was always being asked whether he was working on frogs, for a long time people inquired whether I was still making monsters. I enjoyed enormously the experiments on sex hormones. The results were very paradoxical because the very hormones made by the genital glands were operating at a time when these organs are not yet differentiated. However, this is not so surprising, since it suffices that chemodifferentiation of the tissue has occurred for synthesis and secretion to be possible. Organ cultures were also extremely rewarding, if only because it is so spectacular to see a tibia modeling from an undifferentiated rudiment, to give one example. Anyone looking at these minute but perfectly recognizable tibias immediately thought of a macaber dance from the middle ages, like for instance the one in the Saint Maclou cloister in Rouen. To conclude about these organ cultures, I would like to emphasize that Miss Fell was a forerunner, followed by Prof. Gaillard in the Netherlands. However, her technique had its limitations, while the one devised by us has universal use. It can be used for any vertebrate embryonic organs, but also for invertebrates. For instance, we used it to culture organs from *Limulus*, the horseshoe crab, when we were in Woods Hole. Concerning cancer cells, nowadays more sophisticated techniques are used, and in all cases molecular techniques have to complement cytological studies.

Your earlier work, that is, the study of sexual dimorphism, really encapsulates the central problem of embryology – how a small difference between two genomes can program two different morphologies. You pioneered this line of thinking, which is still bearing fruit in the present molecular era.

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