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CROSSOVERS BETWEEN EPIGENESIS AND EPIGENETICS. A MULTICENTER APPROACH TO THE HISTORY OF EPIGENETICS (1901-1975)

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SUMMARY

The origin of epigenetics has been traditionally traced back to Conrad Hal Waddington's foundational work in 1940s. The aim of the present paper is to reveal a hidden history of epigenetics, by means of a multicenter approach. Our analysis shows that genetics and embryology in early XX century – far from being non-communicating vessels – shared similar questions, as epitomized by Thomas Hunt Morgan's works. Such questions were rooted in the theory of epigenesis and set the scene for the development of epigenetics. Since the 1950s, the contribution of key scientists (Mary Lyon and Eduardo Scarano), as well as the discussions at the international conference of Gif-sur-Yvette (1957) paved the way for three fundamental shifts of focus: 1. From the whole embryo to the gene; 2. From the gene to the complex extranuclear processes of development; 3. From cytoplasmic inheritance to the epigenetics mechanisms.

Introduction

Mainstream literature considers the British scientist Conrad Hal Waddington's pioneering insights, at the crossroad of embryological and genetic studies, the first attempt to create a coherent frame of epigenetics in the mid of XX century[1]. Recently, a second parallel origin, referred to the American ciliatologist David Nanney, mainly

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focusing on cell differentiation, has been put in evidence[2]. The rise of epigenetics, as known today, has been somewhat impaired because of the gap between embryology and genetics, which led to two disciplinary oriented connotations related to development. The first, grounded on genetics, refers to the chemical and molecular mechanisms influencing gene expression and not involving changes of DNA sequence. The second relates to epigenesis, i.e. the whole complex of the developmental mechanisms in the embryo[3].

In this article, we will show that those two approaches in analyzing development were theoretically close in origin, but later drifted apart in reason of their different methods and objects of analysis. Since molecular genetics boomed in the mid-1950s, this approach prevailed on the embryological one. This put under brackets the more strikingly epigenic explanation as well as the studies related to the development of form, as originally emphasized by Aristotle and the *Entwicklungsmechanik* and recently rediscovered in cell biology studies[4].

We propose here an alternative to the traditional literature on epigenetics[5], relying on a multicenter view of its development, which can be charted from the early years of XX century until the 1970s, through some key figures (Thomas Hunt Morgan, Boris Ephrussi, David L. Nanney, Mary F. Lyon, Eduardo Scarano), discoveries (X linked genetic traits, extra-nuclear inheritance, X-inactivation and methylation), novel perspectives and shifts of focus. Such analysis provides a new insight about the link between epigenesis and epigenetics through their scientific, historical and philosophical evolution.

The meaning of epigenetics has a complex background: its roots can be traced back to Aristotle's idea of *epigenesis*, an "internal movement" inside the embryo which leads to a gradual process of development from an undifferentiated matter as opposed to the so-called *preformationism*, that is the idea that the embryonic development is nothing more than the unfolding of the organism, already existing

and structured in all its details within the sperm or the egg[6]. These notions have been discussed through the modern age till our days within the various theories of generation and the study of the process of development of the embryo: how do living beings develop? In which conditions and in which form? What are the differentiation mechanisms underlying embryo development? At the beginning of XX century, these questions were the common background of embryology and the novel science of genetics. In this paper, we argue that the disciplinary gap between embryology and genetics can be "bridged", at least theoretically, and that a multiple origin of epigenetics can be shown through the description of three main shifts of focus revealing a hidden history of epigenetics.

The starting point of our analysis is Morgan's emblematic figure, an embryologist who became the founder of genetics, epitomizing the continuity between these domains albeit shifting the focus from the organism to the gene: a "simpler" level of investigation. A second landmark of our research is the crucial debate originated after the international conference on extrachromosomal inheritance, held at Gif-sur-Yvette (France) in 1957 and organized by the French scientist Boris Ephrussi. It paved the way to further studies which clarified the basis of epigenetics both from a developmental and a genetic point of view. This was a second shift of focus: from the gene to the complex extranuclear processes of development. The question of cell differentiation represented a fundamental issue intertwining both fields of study and its understanding has been crucial for the development of some key epigenetic processes. In the last part of the article, the pioneering discoveries of Lyon (X chromosome inactivation) and Scarano (DNA methylation) will be described. Such pivotal findings determined the third shift of focus, from the genetic observation of the nuclear and cytoplasmic mechanisms to the epigenetic ones. These were originally considered exceptional and were left unacknowledged in their significance - until their meaningas regulatory mechanisms of

genes was understood - and eventually labeled as "epigenetic" during the '90s. Since then, epigenetics became an appealing field of study showing elements that are relevant for an in-depth understanding of pathological conditions as well as potential clinical applications[7].

The epigenesis of epigenetics: Thomas Hunt Morgan

> *Yet it is really preformationism that has triumphed for there is no essential difference, but only one of mechanical detail, between the view that the organism is already formed in the fertilized egg and the view that the complete blueprint of the organism and all the information necessary to specify it is contained there, a view that dominate modern studies of development* [8].

The above quote by the American evolutionary biologist and geneticist Richard Lewontin testifies that the long-lasting debate between epigenesis and preformationism is not over[9]. Surprisingly these "labels" are still active and refers to a too frequently partisan or ideological opposition which should be made explicit. Only observing the debate in its historical stages it is possible to understand its complex and changing meanings throughout times. It has been remarked that the antithesis of epigenesis vs preformationism is almost synonymous with the history of embryology[10]. To put it very briefly, since XVIII century, two main trends opposed one another throughout the history of this debate from a theoretical point of view: a vitalistic-holistic approach and a mechanistic one[11]. Between XIX and XX century, an *analytical and experimental dialectic reaction to the former speculative and synthetic approach*[12] arose, giving birth to experimental embryology, especially developed in Germany: the *Entwicklungsmechanik,* turned to the study of the "mechanical laws of development" or the laws of form[13]. However, the German embryologist Hans Driesch, supporting the epigenic approach, underlined that all the epigeneticists were vitalists, revealing that at the beginning of XX century the epigenesis could still be dubbed as a "metaphysical" position[14].

On the other hand, the rediscovery of Mendel's laws and the first researches on chromosomes as the fundamental factors of hereditary transmission shed a new – quantitative – light also on the study of reproduction, development and inheritance[15]. Old comfortable barriers were undermined, many previous ideological positions were overthrown and new epistemological insights emerged. This meant accepting quantitative and abstract tools of research also in biology albeit pushing geneticists to focus on more specific unities of analysis: from the natural embryo to the artificial factors and genes, concealing the broader perspective on complex phenomena[16].

Briefly, two different approaches to development were proposed, focusing on two different cell compartments: the nucleus and the cytoplasm. According to the former point of view (supported for example by Theodor Boveri), chromosomes were the determinants of heredity and the nucleus was the center of hereditary activity[17]. In the other framework, hereditary factors resided in the cytoplasm and the interaction between molecules determined the hereditary patterns of development, rather than the interaction in some preformed structures such as chromosomes. Among others, the American embryologist and geneticist Thomas Hunt Morgan in the earliest phase of his research supported this opinion[18]. The gap widened in 1905 with Nettie Stevens' and Edmund Wilson' studies about sex determination linked to X chromosome, respectively on *Tenebrio* and *Hemiptera*. In 1910, Morgan demonstrated that the traits responsible for *Drosophila* eye color were associated to the X chromosome and, with an interesting theoretical twist, he abandoned his former beliefs becoming one of the strongest advocates of the chromosome theory, to the point that this approach is known as Morgan's chromosome theory of heredity[19].

The refinement of focus in the genetic approach paved the way for a disciplinary gap[20]. According to many historians the publication of Morgan's book "The Theory of the Gene" in 1926 decreed this

divide: genetics was the analysis of the transmission of genes and embryology was the study of their expression[21]. This divergence had an impact on the languages, the techniques and the models used within the two domains[22]. Indeed, when Morgan himself attempted a synthesis of embryology and genetics in 1934[23], he explicitly admitted that the perspective traditionally adopted by embryology had not integrated a stronger focus on the underlying chemical mechanisms, which in his opinion was a fundamental start.

Genetics and embryology were experimentally apart, , but they shared several issues and questions, which needed a more specific approach at the genetic level before they could be adequately framed together[24]. The theoretical continuity between Morgan's embryological studies and his genetic works[25] can be highlighted linking together epigenesis and epigenetics. Morgan chose the term "epigenetic" to define the process of development of some tissues and organs from an undifferentiated matter[26]. He also used the term to draw a parallelism to epigenesis and, conversely, to make clear his critical stance against preformationism, the latter being considered as an ideological and metaphysical position[27].

In 1934, however, Morgan titled his book "Embryology and genetics", underlining the "intimate" background of these disciplines, though the available tools and techniques made the task of their unification quite difficult. Firstly, Morgan supported the idea, attributed to his friend Herbert Spencer Jennings, that physiologists left the problem of development to scholars with a distinct *romantical and mystical* approach and *under the lethal influence of the doctrine that* '*ontogeny repeats phylogeny*'[28]. On the other hand, in the book "Experimental embryology", he pointed out that *the structure of the egg cannot be disregarded if its development, rather than its chemical composition, is the goal to be sought*[29]. Therefore heredity and development should be conceived as a unique theme (top down)[30], dealing with the structure as well as with the organization of the cell[31].

Morgan proposed a "micro-environmental" hypothesis that could provide an experimental explanation of the reaction theory, i.e. a gradual differentiation of the various regions of the embryo. In other words, he desired to ground the traditional embryologic clues on genetic evidence (*i.e.* genetic factors). The starting point was the observation of the first stages of the differentiation of the protoplasm and the progress of the segmentation. The differences increased as a result of the various shifts of the chemical substances contained in the egg. The protoplasm, indeed, contained the materials for the growth of the chromatin and those apt to the elaboration of the substances produced by the genes.

The main theoretical insight underlying his hypothesis was a top-down together with a bottom-up interaction: cytoplasmic regions and genes activities are *mutually influenced*. But the problem, a leitmotiv of genetic research of the time, was that many phenomena could not be analyzed in their bottom up functioning or, on the contrary, could be only observed at the macro-level, as result of the embryological development. A peculiar antinomy ensued: the activity of genes was hidden, "invisible", since it had not yet been provided on the basis of direct observation, while the analysis of embryonic development was "too complex", preventing the application of chemical-physical laws. Morgan "simplified" the question, making it more specific: how and with which new methodology it possible to explain those hidden factors that allow for the reciprocal influence of genes and cytoplasm?

The invisibility of the gene did not prevent Morgan's group from studying the phenomenon as if the gene were a material entity[32]. Morgan's main aim became to establish the mechanisms of the genes that are crucial in determining the chemical-physical activities in the cytoplasm, shaping the development of the embryo[33]. His analysis proposed two issues strictly linked one another: i. the effect of the gene is due to a kind of dynamic action of the gene over the cytoplasm; ii. there is a property of resonance by which the mutation

of a gene produces changes in the whole organism. At that time it was believed that the characters were single and unitary traits which were the determinants factors and for induction, or analogy, it was thought that also every gene would produce a specific effect only on one specific character, i.e. a one to one relation: one gene-one character. When the multiple effects of the mutation of the genes started to become evident, the geneticists necessarily turned to select more identifiable characters, less variable and less environment-directed[34]. According to Morgan the mutations in single genes gave rise to a *popular illusion* that each mutant character is the effect of only one gene, and *more insidious still, that each unit character has a single representant in the germ material. On the contrary, the study of embryology shows that every organ of the body is the end-result, the culmination of a long series of processes*[35].

Along these lines, the responsiveness to environmental factors and its correlation to gene action in the development of the embryo were recognized as heuristic hypotheses. Morgan underlined that for instance in mammals, since the fetus gets the nurture from the mother, reciprocal hybrids could present variations at birth due to the different environments in which they had been bred[36]. Therefore, in the quest of a theory of the genetic basis of epigenesis and of the development of the embryo clearly emerged that there were several different factors, strictly correlated, among which the genes were *only one of the main components*. Indeed, Morgan explained that every adult character is the product of many genes and even of *all* genes, considering the entire history of the individual starting from the egg[37]. This idea linking the interaction between the gene and its expression throughout the individual life is the basis of the genetic explanation of the epigenetic development: the continuity of the individual is explained by means of epigenetic and phenotypic variation. We can briefly remark how variously this notion has been observed throughout the history of genetics. For instance, the biologist Julian

Huxley, expressing his support to Waddington's ideas, stressed that he used the term "epigenetics" to define *the science of the processes of development in general*[38]. Later he described epigenetics *the analytical study of the individual development (ontogenesis) that is a central issue of cell differentiation*[39]. The latter recalls a very recent definition too: the continuity of the individual, in the changing of his whole phenotype, is effect of the continuity of the epigenetic processes that modulate the different and interrelated phenotypic units that constitute the phenotype[40].

Albeit the shift in tools, units of analysis, evidences and theoretical frameworks, we think it is worth to underline this continuity instead of a sterile gap. The experimental disciplinary divide between embryology and genetics cannot go unnoticed: however, we should observe that recently, thanks to the new advances in genetics, old embryological and regeneration studies have been re-assessed in a new light[41], also for their potential clinical application, in particular with the works on stem cells[42]. Moreover, a new trend of studies in genetics is exploring again the domain of the biology of form[43].

The multicenter approach to epigenetics: from hidden to explicit epigenetics

In the last two decades or so, the debate about the origin and the development of epigenetics was lively and remarkably grew as a consequence of both experimental evidences and theoretical studies that uncovered the genetic mechanisms and contributed to create a common field of analysis. The emphasis on Waddington as the "founder" of epigenetics is the *trait d'union* of a multifaceted literature – scientific, historical and epistemological[44]. A (partial) exception is the "dual origin" of epigenetics proposed by the American biologist David Haig[45]: Waddington, and his focus on differentiation during the ontogenetic process represented the first matrix, later supported also by Julian Huxley[46]. David Nanney's

"epigenetic control systems", mostly targeting cell differentiation, are the second one[47].

Haig's suggestive insight may be broadened here. Between the 1940s and 1970s, several key issues at the crossroad of embryology and genetics emerged. In particular the international conference on extra-chromosomal heredity at Gif-sur-Yvette in March 1957, which gathered many of the most prominent scientists of the time such as Boris Ephrussi, David Nanney and Joshua Lederberg, revived the debate on nuclear and extra-chromosomal heredity and their interactions. Other scientists were independently developing similar researches such as Lyon in the U.K. and Scarano in Italy. Therefore, a different history of epigenetics could be mapped by adding a novel approach to the traditional, Waddington-centered, reconstructions. This outlines a sort of hidden epigenetic circle or a "multicenter focus" on epigenetics.

However, Waddington's pioneering insights should not be dismissed: he inspired several students later recognized as pillars of the novel discipline designated as "epigenetics". Briefly, in "The epigenotype", published in 1942, Waddington proposed the first systematic and explicit conceptualization of the notion of epigenetics linking genetics and experimental embryology. According to Waddington, the epigenetic analysis concerns the mechanics of development and represents the English equivalent of *Entwicklungsmechanik*, as it has been underlined by Muller and Ollson[48].

Waddington, moreover, describes the divide between genetics and experimental embryology as the different answers that these two disciplines gave to the same questions. In his perspectives, the two lines of research can be reconnected thanks to the growing knowledge about the effects of the genes and, more specifically, to the development of the

> *epigenetical analysis by which many of the general principles of experimental embryology reveal themselves again […]. As a result, the epigenetical*

> *analysis discloses the processes involved in the mechanism by which the genes of the genotype bring about phenotypic effects […], the causal mechanisms at work, and [the possibility] to relate them as far as possible to what experimental embryology has already revealed of the mechanics of development*[49].

As mentioned, the interactions between nuclear and cytoplasmic factors and their role in heredity, two main issues involved in the development of epigenetics, were at the center of a debate between embryologists and geneticists. A turning point on the issue was the international meeting of Gif-sur-Yvette promoted by the Russian scientist Boris Ephrussi and to which, Francois Jacob, David Nanney, Tracy Morton Sonneborn and Guido Pontecorvo, among the others, took part. Two main contributions – Ephrussi's and Nanney's – emerged. According to Ephrussi, the fundamental observations about genes, such as self-duplication and variation, persuaded some geneticists to consider the nucleus as the ruling material of the cells and lacking of equivalent evidences about cytoplasmic heredity led to interpret "the rest of cell as a by-product of gene activity"[50]. Conversely, others, such as Nanney and Ephrussi, focused on the role of cytoplasmic or extranuclear factors of inheritance. Here are their descriptions:

> *This was a small conference to which Boris Ephrussi, the most prominent European spokesman for 'cytoplasmic inheritance', had invited a few sympathetic leaders of European and American biology. It was basically a strategy conference concerning a disciplinary issue of deep concern to the participants. Much was at stake at the conference, because 'extrachromosomal heredity' appeared to be at a critical juncture [Sapp, 1987]. The double helix had been grasped but not assimilated. Long-standing questions seemed to be on the verge of answers, and the rhetoric of scientific discussion had shifted perceptibly. Previously stable disciplinary positions were being threatened*[51].

> *[…] changes during development do lie within the province of genetics, and I do take the title of this symposium as a sign that is now generally felt that variations in development do pose a problem to the geneticist* [52].

Ephrussi was sincerely determined in reconciling embryology and genetics and, thus, to specify the role of genes during development. Indeed, some questions highlighted by Ephrussi at the conference, in particular those related to inheritance and raised by cell differentiation, were already at the center of his research.
Five years before, in a series of lectures held in 1952 (published a year later as "Nucleo-cytoplasmic relations in micro-organisms") Ephrussi concentrated on the genetics of the micro-organisms and its several links to cell heredity and differentiation. Though assessing the importance of nuclear heredity, he believed that focusing exclusively on it produced a narrow view, restricted to nuclear genes and leading to an impasse in our understanding of development. Mendelian analysis applied in classical genetic studies

> *[…] has confined our attention to the nuclear genes and thus driven us into an impasse with respect to the understanding of development. […] I have tried to show that the cytoplasm is endowed with genetic properties of its own, and that this provides us with a basis for the interpretation of the phenomena of differentiation and development*[53].

According to Ephrussi, the whole organism undoubtedly derives from divisions of one initial cell where the nucleus plays a key role. Nevertheless, further variations and the inheritance of differences between somatic cells could not be explained only by the nuclear body since *the different cell types […] must therefore all possess the same genotype* and there were no tools to induce such specific gene mutations. That's why Ephrussi intended to supply evidences in support of the role of the cytoplasm in cell heredity and differentiation studying various micro-organisms and their functioning (chloroplasts in some flagellates or the reproduction in yeast, *Saccharomyces cerevisiae*, the ciliate *Paramecium aurelia* and the fungus *Podospora anserine*)[54]. Ephrussi, as discussed at Gif-sur-Yvette, underlined that some cell variations observed in microorganisms, were inherited and

produced stable differences between cell lines, although they did not follow Mendelian rules[55].
On the other hand, Nanney was one of the first to emphasize the term "epigenetic" in the title of an article and to pioneer a theoretical synthesis of the major features of epigenetic mechanisms. In "Metaphor and Mechanism: 'Epigenetic Control Systems' reconsidered" he argued that

> *Differential gene action, masked by systemic properties of differentiated cells, could account in principle for many of the persistent stable states encountered in protists and in developing multicellular organisms. The discussion helped convince some of the most obstinate defenders of the cytoplasm, most notably the European leader of the cytoplasmic faction, Boris Ephrussi*[56].

Nanney described two types of cellular control systems: i. The maintenance of a "library" of specificities" (expressed and unexpressed) accomplished by a template replicating mechanism; ii. The auxiliary mechanisms, involved in determining which specificities are to be expressed in any particular cell. Indeed, although cells share the same genetic material, they may show different phenotypes. So, the expressed specificities are not determined entirely by the DNA present in the nucleus, but epigenetic systems regulate the expression of the genetically determined potentialities. These are expressed in integrated patterns, by which the simultaneity/exclusion of expression reveals that the intercommunication and the metabolic linkage are important features of epigenetic systems. The term "epigenetic" is chosen to emphasize the reliance of these systems on the genetic systems and to underscore their significance in developmental processes. Moreover, the identification of these two systems, despite the difficulties of a clear operational distinction could have avoided confusion discussing cytoplasmic inheritance, developmental alterations, inheritance of acquired characters and genetic recombination.

Noteworthy, Nanney introduced the term "paragenetic" in 1957 and only after the Gif-sur-Yvette conference, upon Pontecorvo's suggestion, he substituted it with the Waddingtonian term "epigenetic"[57]. Also Ephrussi underlined the need to find a specific terminology to avoid misunderstandings. He was impressed by Nanney's proposal at the conference

> *to call 'epigenetic' all the mechanisms that regulate the expression of genetic potentialities [involving functional states of the nucleus], in contradistinction of the truly genetic mechanisms [cytoplasmic] that regulate the maintenance of the structural information*[58].

In addition he suggested to acknowledge the existence of two sorts of hereditary factors – "genetic" and "epigenetic" – according to the source of the information: in the first case, the information is structural, while in the second case, it is based on a sort of "dynamic flux equilibrium"[59]. Since both epigenetic and cytoplasmic mechanisms are sensitive to environmental changes, Ephrussi marked a distinction between truly (classical) genetic changes and epigenetic ones:

> *[…] we must admit that not everything that is inherited is genetic. […] The third and last thing we should avoid is taking for granted that all cytoplasmic effects are going to turn out to be epigenetic and ultimately nuclear*[60].

The American geneticist Joshua Lederberg, addressing to Ephrussi's view, underlined that, at least in English, the term "epigenetic" could be confusing as it was already widely used referring to Waddington's meaning, *i.e.* individual development. Hence, he rather proposed a distinction among "nucleic" and "epinucleic" information. Therefore, by changing the terms, but in agreement with Ephrussi's hypothesis, Lederberg suggested that the "nucleic" information had "the pervasiveness and static precision connoted by genetic", while the "epinucleic" information *regulates the manifestation of nucleic potentialities in the dynamic, temporally responsive functioning of*

actual development[61]. This terminological debate is the mark of a theoretical and disciplinary reflection inside genetics and embryology of the 1950s, which represents the early attempt to frame those studies that could not fit in the traditional field of genetics. In 2001, Lederberg will confirm that *In 1958 epigenetic was already a semantic morass* and will comment*: I knew nothing of methylation in those days; it would be prototypically epinucleic*[62].

Briefly we may claim that Nanney theoretically anticipated the reversible character of the epigenetic control systems. In 1958 and 1959 he foreshadowed two main concepts of nowadays epigenetics, respectively related to the phenotypic differences borne by cells with the same DNA, (the expressed specificities are not entirely determined by the DNA and other devices, the epigenetic systems, regulate the expression of the genetically determined potentialities)[63] and to the reversibility of epigenetic change *an epigenetic change should not result in a permanent loss of information and a return to a previous condition of expression is always theoretically possible*[64]. As already mentioned, Haig considers Nanney the developer of a "tradition" of epigenetics turned on cell differentiation underlining what lies *between genetic and epigenetic causes of changes in cellular phenotype, including the transformation of somatic cells into cancer cells*[65]. Indeed, Nanney in 1989 was invited to a symposium on the "Epigenetics of Cell Transformation and Tumor Development", which discussed similar issues presented four decades before, showing that they still represented open and debated questions[66].

On the other hand, Ephrussi proposed a clear cut distinction of two different sources of hereditary information, while Lederberg supported a very similar concept of the epigenetic changes as interpreted today by the modern molecular biology. This debate together with the different definitions of the term "epigenetics" were the basis of the comprehension of cell differentiation mechanisms and of de-

velopmental processes since the beginning of the 1960s, affecting subsequent experimental studies. The importance of the Gif-sur-Yvette conference and the relevance of this multicenter hidden debate for the development of the novel discipline of epigenetics cannot be overlooked, as Nanney himself stressed decades after:

> *I did not comprehend fully then what was going on at Gif, and I still do not. Periodically I have tried to evaluate what happened. I suspect that events at Gif may be in some way relevant to continuing issues in biology*[67].

From epigenetic regulatory mechanisms to epigenetic heredity: landmarks in the second half of the XX century

Developmental mechanisms and the specific roles of nucleus and cytoplasm have been in the limelight since the 1950s: a renewed interest was sparked by new molecular approaches that made possible to observe gene expression in greater detail, allowing for a deeper connection of experimental embryology, developmental biology and genetics. The progress in understanding gene regulation in development involved several animal and cellular models and raised new questions about cell division and differentiation. Eventually, new insights suggested that specific variations in both somatic and germ cells could be inherited, affecting both progeny cells and progeny organisms, thus the subsequent generations[68].

The British geneticist Mary Frances Lyon played a relevant role in this context. Her studies, since the late 1940s, pioneered researches on the X chromosome inactivation, a fundamental genetic control mechanism that involves the silencing of one of the two X chromosomes in female mammals' cells. Several fundamental discoveries carried out by Lyon and other scientists, spanning from 1953 to 1960, supplied cytological and genetic evidences and opened the path to further Lyon's insights: i) the identification of mice stocks with the typical "variegated" coat color displayed by most of X chromosome linked mutants in heterozygous females, similarly observed in somatic mosaics; ii) the di-

scovery of mice with a single chromosome X (XO) showing a normal phenotype and fertility; iii) the detection of cancer and normal cells of female mice with one condensed (heteropyknotic) X chromosome. This last fact clarified that the so called "Barr body" was very the same condensed and inactive chromosome located in female nuclei conferring the observed mosaic coat color to heterozygous mice[69].

These findings paved the way for Lyon's description of X inactivation mechanism in 1961, first in mice and later in other mammals: they disclosed the notion that a single genetically active X chromosome is required for a normal development of female mice and led Lyon to suggest that the "variegated" phenotype, related to the coat color of heterozygous female mice and defined mosaic, was associated to the inactivation of the X chromosome in the early stages of the embryonic development[70].

At that time little was known about mammalian genetics, and Lyon and other colleagues proposed several alternative explanations of the phenomenon. It was firmly established that the X inactivation was the main regulatory mechanism responsible for the dosage compensation of X-linked genes expression between the sexes in mammals, with cytological evidences in support (the condensed and inactive chromosome was shown). However, a detailed genetic explanation was missing, as well as the answer to the major question concerning the species-specificity of the phenomenon: was it typically murine, human or both?

It turned out that the so-called *lyonization* also happened in our species in normal XX females and when a pathological excess of X chromosomes occurs (for example, in Klinefelter's syndrome with an XXY chromosomal pattern). Evidences and validations emerged in later decades[71], first of all the description of methylation[72] mechanism since the 1970s.

Even though earlier the Japanese geneticists Susumu Ohno and colleagues found that sex chromatin – made up of a single condensed X

chromosome – was present also in man, sex chromosome aneuploidy, such as the XO chromosomal pattern, called scientists' attention on differences between mice and humans: XO female mice showed a normal development and were fertile; conversely, XO human females showed Turner's syndrome characterized by several abnormalities, including small stature and gonadal dysgenesis and infertility. These data suggested that in humans one single chromosome is not enough for a normal female development. M.F. Lyon hypothesized that i) in normal females with two X-chromosomes one of the two is inactivated and forms a sex chromatin body; ii) when there is only one chromosome, as in XO females, it is not inactivated; iii) when X chromosomes exceed, all are inactivated except one (e.g. in XXY males with Klinefelter's syndrome).
Actually, further explanations and validations of the lyonization and its properties – condensation, late replication and lack of transcription – came with knowledge and technological advances between the '70s and 2000, mainly the description of methylation mechanism starting from the mid '70s.
In hindsight, the molecular reinterpretation of Lyon's results eventually turned the inactivation of X chromosome into one of the best example of stably inherited epigenetic modifications:

> *[…] XCI (X chromosome inactivation) is efficient, stable, and somatically heritable, yet genes on the inactive X chromosome (the Xi) retain the ability to function in the next generation. In mammals, XCI was the first recognized epigenetic phenomena […]*[73].

These researches progressively unveiled the role of the differences in gene expressions for cell differentiation and embryonic development. The new kind of observed changes, stably inherited though not within canonical Mendelian rules, superimposed "on the classical genetic system" drew the attention of several scientists' on the underlying mechanisms[74].

In particular, the mechanism of DNA methylation appeared as a tempting explanation of a wide range of phenomena, including X inactivation. Extensive investigations proved that methylation is the major mechanism responsible for the epigenetic regulation of gene expression, though not the only one. It is also now clear that methylation patterns can be inherited through cellular divisions.

> *The real importance of DNA methylation is that it provides the basis for a heritable epigenetic system. This makes it possible to add or subtract information to DNA which may be essential components of development and differentiation. Mistakes or defects in this heritable information may be important in tumour progression and also in ageing*[75].

Methylation research is another focal point in our "multicenter" historical reconstruction of epigenetics. Studies in this field have been pioneered by the Italian scientist Eduardo Scarano between the mid-1960s and the 1970s. His work was originally influenced by the scientists Ernest Borek's and Marvin Gold's findings on methylation in bacteria presented at the Cold Spring Harbour Symposium, "Synthesis and Structures of Macromolecules" (1963)[76]. Scarano and his fellows, among them Maurizio Iaccarino, hypothesized that DNA methylation could vary along development. Iaccarino, recalling to those observations, has pointed out that Scarano's team was the only one to work on methylation in eukaryotes at that time; moreover, he supposed that the methyl groups were some sort of "punctuation of DNA" and that the expression of clusters of genes could be activated or inactivated, anticipating the notion of "CpG islands" (cytosine-phosphate-guanine pairs) — as later called by Adrian Bird (see below) — that is the regions where the methyl groups were located[77].

> *[…] At that time there was no more we could do; we couldn't isolate a sequence of 1000 base pair sequence and find out where CpG groups were located. The only thing we could do at that time and with those methods was that of analyzing if the methylation occurred or not. And it was! What*

> *later it has been determined is that CpGs are organized in clusters; there are many CpGs one near to another and they are responsible of the regulation of gene expression […]*[78].

In order to test a possible correlation between DNA methylation and cell differentiation they studied the DNA methylation in embryos of the sea urchin (*Paracentrosus lividus*) and showed that it occurs during the early stages of the development[79]. Scarano discovered that methylation occurs at the cytosine site on the DNA and that the deamination of cytosine convert it in thymine, therefore leading to alterations of gene activity and of differentiation[80].

He put the control of the gene activity in cellular differentiation and embryonic development at the center of his research. Wondering in which way such processes could be explained in a chemical language by means of the "macromolecules of life", he made the hypothesis that specific enzymes, synthesized during the embryogenesis and the cellular differentiation, are able to modify DNA in a highly specialized manner, so that groups of genes or genomic regions are more prone to transcription than others[81]. As Iaccarino has underlined, since he and Scarano weren't geneticists, they just talked about the regulation of groups of genes and not of epigenetics, as it could easily lead someone to think[82].

The idea — originally suggested by Scarano — that DNA methylation was correlated to the control of gene expression, circulated in the scientific community, though in a limited way[83]. It emerged neatly only in the mid 1970s after some key studies proving that methylation is a fundamental and inheritable epigenetic control system of gene expression during development.

These studies were inspired by some previously described developmental processes such as transdetermination and X chromosome inactivation, in which "superimposed" DNA changes seemed to be involved.

In 1975 the British molecular biologist Robin Holliday with his fellow John Pugh claimed that they want to explore further the hypothesis proposed by Scarano about *certain other base modifications [that] could lead to heritable changes in base sequence and […] could control the activity of adjacent structural genes*[84]. Holliday and Pugh, and independently the American geneticist Arthur Riggs, proposed a model of the DNA modification involving the methylation of the cytosine, which could influence the switching of gene expression (on or off) and could explain several phenomena of cellular differentiation and development, including the X inactivation. According to their hypothesis, specific enzymes act successively, before and after DNA replication, so that the methylation pattern is preserved after the cellular division. More in depth, it was proposed that a sequence specific enzyme methylated a precise region of DNA, and that, after DNA replication, only one filament of the double helix was methylated. In the detailed model proposed by Riggs, if a maintenance methylase was present, it modified also the other filament allowing the inheritance of methylation pattern through cell divisions[85]. Riggs also proposed that methylation could affect the binding of regulatory proteins, as shown later[86]. In 1986 Robin Holliday eventually proposed the term "epimutation" referring to those inheritable and reversible changes affecting groups of cells but not involving any modification of the DNA sequence, as compared to the irreversibility of classical genetic mutation, usually affecting cell lineages[87].

In the same decade, a series of discoveries made clear the link between DNA methylation and gene silencing, in particular in X inactivation[88]. By means of restriction mapping experiments, the British geneticist Adrian Bird later described these CG clusters as *CpG islands* (*C-phosphate-G*), frequently concentrated in transcription control regions of genes[89]. Further, the crucial role of methylation in gene inactivation has been validated.

Conclusions

Since the beginning of XX century, the early successes of the techniques of the newly-established genetics and the subsequent discovery of the "gene" as the specific unit of hereditary transmission, allowed for the taking off of the discipline. Especially thanks to the seminal studies of Morgan's fly group, the traditional questions about the development of the organism acquired a novel focus, shifting towards another level and object of analysis: from the development of the whole organism to genes and their transmission.

Morgan, originally trained as an embryologist, and his group tried to address the issue of a genetic basis of epigenesis and of the development of the embryo but they had to cope with two main problems. Firstly, the intrinsic difficulty in dealing with the complexity of the embryo; secondly, many of the genetic processes were not directly observable: gene activity was hidden, invisible for the technology of the time. The far too complex analysis required to describe the embryo development as a whole (including the shortcomings of a specifically chemical and/or physical approach) and convinced Morgan that a "simpler" and somewhat more limited target was needed: the study of the hidden factors that allow the reciprocal influence of gene and cytoplasm during development. Such shift of focus in Morgan's research program is tactic, and not strategic: it is not the result of an *a priori* elaboration in favor of a gene-centric perspective, rather it stemmed from the need of ordinary scientific practice[90]. Therefore, attempts were made by Morgan's group to ground on genetic evidence (*i.e.* genetic factors) the "micro-environmental" hypothesis, suggesting the *mutual influence* of cytoplasm and nucleus during embryonic development[91]. This should have given birth to a *genetic hypothesis matching with embryological evidences*, as typified by the title of Morgan's book "Embryology and genetics"[92]. Morgan's attempt, though fruitful, was not completely successful, as

highlighted for instance by Boris Ephrussi's disappointment in reading the 1934 essay and his subsequent deep commitment to bridge the gap between embryology and genetics[93].

Indeed, some of the issues raised by the work of Ephrussi have been at stake in many subsequent theoretical discussions, particularly those related to inheritance and raised by cell differentiation involving genetic and epigenetic mechanisms as much as nuclear and cytoplasmic inheritance[94]. In the 1962 lecture "Mendelism and the new genetics"[95], Ephrussi underlined how geneticists hadn't yet provided a satisfactory framework for differentiation and development: while they claimed to have reached the goal of genetics, Ephrussi felt *that they had rather limited it* [96].

The *paradox of cell differentiation, i.e. the problem of the mechanism whereby the descendants of a single cell, all endowed with complete and identical sets of genetic material, acquire widely different and often very stable characteristics*[97] could be solved from the point of view of developmental biology by means of a shift of the unit of analysis: from the gene to the whole cell, taking into account not only the interactions in the nucleus but also those in the cytoplasm. This implied clarifying how the parts that make up the organism integrate each other in more complex structures, rather than making their "complete catalog"[98]. Indeed, classic geneticists had neglected the genetic paradox because they could not show any proof of diverse and stable variations on the same genetic background. Nevertheless, Ephrussi considered some brilliant discoveries, e.g. McClintock's researches on maize and Jacob and Monod's operon model, as the evidence of the involvement of new elements in the genetic mechanisms of differentiation. *I am referring to the demonstration of the existence, in the genome, aside from structural genes which specify the structure of proteins, of a system of gene relays which turn the structural genes on and off, thus regulating gene activity*[99]. Moreover, *The genetic paradox of differentiation cannot be*

solved on paper or on bacteria. It must be solved on somatic cells of higher organisms, for the biochemical and genetic analysis of which we are beginning to have the necessary tools[100].

Ephrussi exploited the major theoretical idea of epigenetics in his attempts to link extranuclear inheritance with the nuclear control systems, thus extending the genetics focus including also the aspects other to the traditional nuclear inheritance[101].

Almost three decades after, Nanney's reconstruction echoes Ephrussi's *genetic paradox*, dubbing it as the *developmental paradox*. Nanney explains that *the belief that 'hereditary' cell variants arise regularly in the course of development, or in the course of cell culture in protists, without modifying the nuclear genetic apparatus*, became the "developmental paradox" which led to the hypothesis of a dual genetic system: *the nuclear hereditary system was assumed to be responsible for the transmission of traits between sexual generations while cellular heredity was regulated by a functionally different system in the cytoplasm […]*[102].

Briefly, classical genetics mostly aimed at explaining the hereditary transmission of characters from one generation to another, and only partially targeted specifically the multiple factors involved in the expression of the individual phenotype and their variation (genetic variability, interactions, complex traits, environmental influences, etc.). In the light of the reconstruction made by Nanney, the "conflict" between genetics and experimental embryology, which has been a leitmotiv of our discussion, may be considered a consequence of a disciplinary strategy.

> *The separation of the problems of genetic transmission from those of genetic expression [Allen, 1975; Sapp, 1988] at the beginning of the century released the new genetics from the immediate requirement to explain the biochemical nature of the gene and of gene function. It also separated the disciplinary practices and languages of embryology and genetics from their common roots in the thinking of 19th century biologists such as Darwin*

> *[1868], Weismann [1891], and E.B. Wilson [1896]. An expression of their alienation was the "Developmental Paradox". How can cells possessing the same genetic components, reliably copied in every mitosis, come to have very different characteristics?*[103].

As we have previously remarked, at the beginning of XX century, the general questions of embryology and genetics were similar, hence, there has been a natural evolution or a strategic consequence, very common in the practice of science, due to the constraints of scientific methods, levels of inquiry and unities of analysis. Therefore, we stress that the disciplinary gap should be understood as the result of an alienation of the common roots of embryology and genetics, further advances in genetics (discovery of DNA and the establishment of the Central Dogma) gave strength to the idea that *Previously stable disciplinary positions were being threatened*, as remarked by Nanney[104]. In the same way Gilbert underlined:

> *Whereas most biologists expected and desired a re-synthesis of these fields, many embryologists actually feared such a re-synthesis. In any merging of these disciplines, they thought, the geneticists would take over. Using rhetoric that reflected the military anxieties of his day, embryologist R.G. Harrison wrote, "Now that the necessity of relating the data of genetics to embryology is generally recognized and the 'Wanderlust' of geneticists is beginning to urge them in our direction, it may not be inappropriate to point out a danger in this threatened invasion*[105].

Our reconstruction of the multicenter development of the epigenetic approach, has allowed us to reconsider this disciplinary gap. We showed that there were many studies already proposing intertwined perspectives (i.e. developmental, biochemical, genetic and molecular) though they did not have Morgan's and Ephrussi's explicit theoretical purpose of bridging embryology and genetics.
Indeed, since then, some geneticists centered their researches on the regulation of gene expression (e.g. Jacob's and Monod's work on

the *lac* gene), which became a more and more relevant field of study. The works carried out between the 1950s and the 1970s through the analysis of animal and cellular models – in particular those led by Lyon, Scarano, Riggs and Holliday – provided key insights in this field, mainly on methylation. Lyon proposed X inactivation as the major regulatory mechanism of the gene expression during development between the two sexes in mammals. X inactivation showed that all cells, although containing the same DNA, exhibit differences in their gene expression: these differences were in turn stably inherited without following the classical genetic laws. Remarkably, Scarano's biochemical background was essential in triggering several hypotheses about the regulation of gene activity, which further emerged in a specific genetic and molecular way[106]. In the mid-1960s, Scarano was the first to study DNA methylation in eukaryotes suggesting a possible link between such chemical modification and cell differentiation. A decade later Holliday and Riggs independently proposed the same model explaining methylation mechanism and how this could be inherited through cell divisions thanks to the activity of specific enzymes. Methylation seemed a tempting explanation of many phenomena, including X inactivation.

From the late 1970s new technologies – such as recombinant DNA – produced two strictly interrelated, and of paramount importance, results. Firstly, the connections between genetics and embryology have been observed from a novel point of view: by studying gene expression during embryonic development. Secondly, another shift of focus occurred: from the observation of the cytoplasmic inheritance to the epigenetic mechanisms and in particular on how epigenetic inheritance is achieved and how it is preserved along cell division process.

Our reconstruction of the history of epigenetics identified three main shifts of focus: i. From the whole embryo to the gene in Morgan's approach; ii. From the gene to the whole cell in Ephrussi's and

Nanney's standpoints; iii. From the cytoplasmic inheritance to the epigenetics mechanisms. Along this path, it is possible to highlight the influence of the specific methods at the various levels of analysis and their correspondent processes: from the whole organism to the gene and then back from the whole cell to the specific underlying mechanisms ruling the gene expression.

Another significant element is how the notion of inheritance has changed as a consequence of the introduction of epigenetic inheritable changes beside the canonical Mendelian inheritance. This is specifically true for biomedical research, where Holliday's research paved the way for the inclusion of "epigenetic" processes in the description of mechanisms involving change in gene expression but not in the DNA sequence.

The first explicit reference to the inheritance of epigenetic defects, unraveling its potential significance for biomedical applications, is attributed to Holliday[107]. From then on, epigenetic processes would be increasingly named to describe those mechanisms which are not linked to the modification of the sequence of the DNA, but to its expression[108]. In Holliday's words, *the classical genetic system* is paralleled by inheritable "epigenetic" mechanisms affecting the final phenotype of a cell: *whatever the controls are, which maintain these specialized phenotypes, they are clearly very stably inherited*[109].

The emphasis on inheritance is the key ingredient for the new flavor of epigenetics in contemporary biomedical sciences. Yet, how far-reaching was the epigenetic change was still debated. In the 1990s the British geneticist John Maynard Smith proposed a "dual inheritance systems" for some eukaryotes: the "familiar inheritance system", depending on the transmission of DNA sequence across generations, and an "epigenetic inheritance system", concerning changes in gene activity but not in the DNA sequence, which governs the various stages of differentiation during ontogeny[110]. Holliday himself provided two different definitions of epigenetics: *1) The study of the changes in gene*

expression, which occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression (only accounting for epigenetic processes within ontogeny) *and 2) Nuclear inheritance which is not based on differences in DNA sequence* (encompassing the transmission across generations). Only if used together these two definitions fully grasped the meaning of epigenetics, in Holliday's view[111]. While Riggs and colleagues extended epigenetics' domain to *the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence*[112]. At the turning of the new millennium, however, the distinction between mitotic or meiotic inheritance was not an issue anymore, and epigenetic inheritance did not need such specification[113]. Eventually, epigenetic mechanisms and inheritance made their way – albeit slowly – into the most common textbooks in molecular biology. Lewin's *Gene IX* included a chapter concerning *epigenetic mechanisms*, but in 2010 the *Lewin's Essential Genes* manual boasted a chapter entitled "Epigenetic effects are inherited". In the latter, it is stated: *Epigenetic inheritance describes the inheritance of different functional states, which epigenetic changes that may have different phenotypic consequences, without any change in the sequence of DNA*[114].

Epigenetics is now fully established as a research field. Moreover, in recent years several pathologies have been ascribed to epigenetic mechanisms, also showing how human body responds to environmental conditions – such as diet and chemical pollution – and how exposure to specific factors may affect physiopathology in later generations (not exposed to the original factor)[115]. The feedback circle between the organisms' genome and the environment, trespassing generation boundaries, opens to a new shift of focus, to be added to three described above, concerning the *dynamic aspects* of epigenetics mechanisms. The genome reacts to the environmental signals, which in turn influence the genetic response and expression in a continuous auto-regulating and dynamic process[116]. The panorama has

changed again, to include a multitude of aspects that historiography has often kept separated, or treated in a linear (sometimes even progressive and "whiggish") fashion. Embryology (as the science of development), genetics and epigenetics – now a trio in the limelight of contemporary life sciences – shall be observed in a common perspective without creating artificial partitions, recognizing that they share a common and inescapable conceptual heredity.

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