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Review

Epigenetics

A Historical Overview

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ABSTRACT

In the first half of the twentieth century, developmental biology and genetics were separate disciplines. The word epigenetics was coined by Waddington to link the two fields. Epigenetics could be broadly defined as the sum of all those mechanisms necessary for the unfolding of the genetic programme for development. Several decades later specific mechanisms were proposed in which information was superimposed on DNA sequences. In particular, it was suggested that 5-methyl cytosine had a role in controlling gene expression, and also that the pattern of methylation was heritable. These predictions are now supported by a large body of evidence which shows that methylation is strongly associated with gene silencing in a variety of biological contexts. There are now also many examples of epigenetic inheritance through the germ line There are several other important epigenetic mechanisms involving chromatin and histone modifications, and also the expanding field of regulatory RNAs. The human epigenome project will unravel the pattern of DNA methylation in different tissues, and will this determine whether the regulation of gene expression is at the level of DNA or chromatin, or both.

INHERITANCE AND DEVELOPMENT

In the nineteenth century the leading biologists considered inheritance and development to be one and the same problem. The genius of Gregor Mendel was to realize, and then to demonstrate, that inheritance could be studied on its own, without including development. In a scholarly review and discussion of nearly 70 pages, Sandler and Sandler[1] explain that this was the major reason why Mendel's work was ignored by the leading biologists of his day. When it was finally re-discovered thirty five years later, the science of genetics subsequently flourished. Again, the problem of development was sidelined, and it is remarkable that one of the pioneers of the new genetics, Thomas Hunt Morgan, was by background an embryologist, but his laboratory did not study *Drosophila* development. It was only in his books that he re-visited embryology.

Whilst the science of genetics was making rapid progress, embryologists and developmental biologists were using methods and procedures that took little account of genes and gene action. Towards the middle of the twentieth century, there were a few leading biologists who realized that genetics and developmental biology were indeed related and should eventually come together in a common discipline. One was Conrad Waddington, who was knowledgeable in both fields of research. He took the Greek word epigenesis, a theory of development which proposed that the early embryo was undifferentiated, and changed it to epigenetics.[2] He was the Buchanan Professor of Genetics at Edinburgh University, and he also set up an Epigenetics Research Unit supported by the Medical Research Council for some years. Epigenetics could be broadly defined as the unfolding of the genetic program for development, but to Waddington, epigenetics was not very different from embryology. For example, his book *The Epigenetics of Birds* is largely an account of the development of the chick.[3] He also coined the term epigenotype, which was defined as "The total developmental system consisting of interrelated developmental pathways through which the adult form of the an organism is realized."[2] This is so broad that it is not very useful, and I will return to a more specific definition of the epigenotype later on.

The another leading biologist interested in both genetics and development was Ernst Hadorn in Zurich. Many of his studies were on mutations that affect *Drosophila* development, and he also wrote a book *Developmental Genetics and Lethal Factors*.[4] He also worked for many years on the remarkable properties of the imaginal discs of *Drosophila*. These are regions of embryonic tissue that are present in fly larvae. Each disc will later

develop into a specific adult structure: two for each wing, two for antennae, and so on. The disc cells are completely undifferentiated, but it can be said that they are determined to differentiate later on. Hadorn and his colleagues grew disc tissue in the abdomen of adult flies, and passaged it from fly to fly. When the disc tissue was treated with the hormone ecdysone, it differentiated into the appropriate adult structure. In other words, the determined state was heritable, sometimes for hundreds of cell divisions. However, from time to time the disc changed from one determined state to another, for example from a leg to a wing. This event was called transdetermination, and in innumerable studies it was shown that transdetermination followed certain pathways. For example, disc A could change into disc B, and B to C, but A never changed directly into C. This remarkable experimental system (reviewed in ref. 5) has not been exploited in modern experimental studies. Everything that is known about it comes from Hadorn's laboratory years ago.

Waddington and Hadorn were not the only important biologists who wanted to make connections between genetics and development. Another was Richard Goldschmidt, but his views were quite controversial (see ref. 6). Others, such as Julian Huxley[7] and J.B.S. Haldane, certainly understood the importance of the relationship, but the latter was particularly interested in the the biochemistry of gene activity. In this area there had been the early insights of Garrod, who realized that some inherited defects in man blocked specific steps in metabolic pathways.[8] This interpretation was ignored for many years, until Haldane became involved in the genetics of pigment formation in plants, and Ephrussi and Beadle attempted similar studies in *Drosophila*. Finally, Beadle and Tatum started to isolate biochemical mutants and their effects on metabolic pathways in Neurospora. Their work was very successful and culminated in the concept of one gene-one enzyme,[9] which was eventually verified in the 1950s. However, it was independent of studies of development.

After Waddington, there was spasmodic discussion of epigenetics by several scientists; much of this was reviewed by Nanney[10] and much more recently by Haig.[11] Some of the examples related to cytoplasmic inheritance, the phenotypes of cultured mammalian cells, or cancer cells. In general, observations that were not easily interpreted in genetic terms but had a heritable component, were liable to be labeled epigenetic. However, each author had his own idea of the meaning or definition of epigenetics. and no specific mechanisms were proposed. This was also true of the earlier work of Waddington, although he did introduce important new concepts such as canalization.[12]

THE NEED FOR EPIGENETIC MECHANISMS

The importance of the work of Waddington and Hadorn was to relate genes and gene action to development, in an environment in which most geneticists and most developmental biologists were not communicating with each other. As time went on, it became apparent that there were certain fundamental features of development that demanded explanation. One was the fact that differentiated cells, such as fibroblasts or lymphocytes, stably maintain their phenotypes through cell division. This means that some specialized genes which determine the phenotype of differentiated cells are permanently turned on, and other genes—active in some other cell type—are permanently turned off. These controls are heritable, just as the determined state of *Drososphila* disc cells are heritable. Traditionally, inheritance refers to the transmission of genes from generation to generation, but it was now realized that there is also mitotic inheritance in somatic cells of higher organisms. Of course, such inheritance had long been studied in yeasts and fungi, and then in cultured mammalian cells, but it had rarely been spelled out that it also regularly occurred in vivo, that is, in the normal somatic cells of higher organisms with specialized phenotypes. Another feature of higher organisms is the stem cell. Here an undifferentiated cell divides to produce a differentiated cell, and another undifferentiated stem cell. In the case of bone marrow stem cells, a variety of blood cell types are produced. In this situation there are clearly switches in gene activity associated with cell division. A third example is the X chromosome of female eutherian mammals. Early in development one X chromosome is randomly inactivated in every cell, whilst the other remains active. These two chromosome have almost identical DNA sequences, and they reside in a common nucleoplasm and cytoplasm, so the differences in gene activity are intrinsic to the chromosomes themselves. It is evident that there is a switch mechanism early in development, the result of which is the inactivity of one chromosome and the activity of the other. The switch is random and once made it is permanent. This example therefore embodies both a switch in gene activities and also its subsequent heritability.

The first suggestion that DNA methylation (or demethylation) might have an important biological role was made by Griffith and Mahler, who proposed in 1969 that it could provide a basis for long term memory in the brain.[13] In 1975 two papers were published which outlined a molecular model for the switching of gene activities, and also the heritability of gene activity or inactivity. It was based on the enzymic methylation of cytosine in DNA, which can also be referred to as DNA modification. The proposals by Riggs[14] and Holliday and Pugh[15] were very similar, but were made completely independently of each other. The suggestion was that DNA methylation could have strong effects on gene expression, and that changes in DNA methylation might therefore explain the switching on and off of genes during development. The enzyme(s) methylating a particular region of DNA would be sequence specific, or interact with another protein that was sequence specific. It was also proposed that the pattern of methylation could be heritable, if there was an enzyme called a maintenance methylase that recognized hemimethylated DNA soon after replication, but did not act on unmethylated DNA. This provides a mechanism for the heritability of the methylated and non-methylated state of DNA, and therefore for the heritability of a given pattern of gene activities. The issue of X chromosome inactivation was addressed particularly by Riggs. There might be an initial methylation that was immediately shut off, so that only one chromosome is marked. There would also have to be a spreading mechanism which inactivated the whole chromosome. Since it was much easier to envisage a processive methylating enzyme than the reverse, this implies that methylation of DNA is associated with gene inactivity. This can also explain the inactivation or silencing of autosomal DNA in several cases of X-autosome translocations.

There was also the possibility that developmental clocks might be important in unfolding the genetic program for development. This would be a mechanism that counts a specific number of cell divisions before a given gene or genes is activated or inactivated, and several molecular models were discussed.[15] Although there is scattered evidence for developmental clocks, it is not a commonly discussed topic, and only time will tell whether they are a significant component of development. As well as DNA methylation, there was also the possibility that specific base changes might occur, for example, the enzymic deamination of 5-methyl cytidine to form thymidine, and thus the substitution of an G-C base pair by a A-T base pair, a mechanism

that had previously been proposed by Scarano.[16] The existence of the enzyme cytidine deaminase which converts cytosine to uracil in DNA is now very well documented in the immune system and also in pluripotent cells.[17,18]

A third paper on DNA methylation by Sager and Kitchin also appeared in 1975, which proposed that there are enzymes in eukaryotic organisms that restrict unmodified DNA.[19] They explored the possibility that the many known examples of chromosome elimination or silencing might involve such a mechanism. It also became apparent that changes in DNA methylation might be important in tumor progression.[20,21] There was much accumulating evidence that changes in gene expression in cancer cells was due to mutation, but if the methylation model was correct, then aberrant changes in the distribution of 5-methyl cytosine in cancer cells could also result in changes in gene expression. The word epigenetics was not used in any of the 1975 papers on DNA methylation and gene expression, possibly because it had previously been used in several quite different contexts and remained undefined.[10,11]

EVIDENCE RELATING DNA METHYLATION TO GENE EXPRESSION

In 1975 when the DNA methylation models were proposed, there was no experimental evidence to support them. Nor did the models predict that specific DNA methylation would be associated with the activity or inactivity of genes. However, the spreading model for X chromosome inactivation did propose that methylation was the basis for such inactivation. With the cloning and sequencing of DNA, means were discovered for screening DNA methylation in specific DNA sequences. There were restriction enzymes which recognize and cut unmethylated sequences of DNA (usually four or six bases). In some cases there were two restriction enzymes which recognized the same base sequences, but only one of them would cut this sequence when it was methylated. This pair of enzymes were called isoschizomers, and examples were Hpa II and Msp I. Both cut DNA at GCGC sites, but only Msp cuts this sequence if the internal C is methylated. Using Southern blots it became possible to determine whether a given sequence containing a GCGC site was methylated or not. It was soon discovered that many genes with methylated promoter regions were inactive, and also that the corresponding active gene was unmethylated. This early work was reviewed by Doerfler.[22,23] A limitation of the method is that it detects only a subset of possible methylation sites, usually about 10%. Later on a more powerful method was introduced which can detect all methylated and non-methylated cytosine sites in a given stretch of DNA (see below).

Other evidence for the significance of DNA methylation came from the use of the nucleoside analogue 5-azacytidine. This is incorporated into DNA, inactivates DNA methyl transferase and thereby demethylates DNA. It was shown in many contexts that azacytidine reactivates silent genes, often at very high frequency (reviewed in ref. 24). This included the reactivation of genes on the inactive X chromosome. It had been shown that strains could be isolated in cultured mammalian cells which had biochemical deficiencies. Originally it was thought that these were mutations, but it now became apparent that they were often genes silenced by methylation, reactivable by 5-azacytidine.[25]

DEFINITIONS OF EPIGENETICS

Waddington did not use a specific definition for epigenetics. What he had in mind was: "All those events which lead to the unfolding of the genetic program for development." There is nothing wrong with that, except that it is not very specific. By the mid-1980s it was clear that there was a new type of inheritance, not based on changes in DNA sequence. In 1987 I wrote a paper "The inheritance of epigenetic defects."[26] In this I re-visited Waddington's use of the term, and I applied it to situations where changes in DNA methylation also changed gene activity. Possible epigenetic changes in cancer and also in ageing were discussed, and it was also suggested that some transgenerational effects that could not easily be explained by Mendelian genetics, might sometimes be due to the transmission of DNA methylation, or lack of it, through the germ line. It was also possible that some epigenetic defects might be recognized and repaired by genetic recombination at meiosis. At this time the word epimutation was introduced to describe heritable changes in genes which were not due to changes in DNA sequence. It has been suggested that this 1987 publication "was the critical paper that lit the fuse for the explosion in use of 'epigenetic' in the 1990s".[11]

Genomic imprinting in mammals had by now been discovered, and it was apparent that this was due to information superimposed on DNA that could be reversed at meiosis or during gametogenesis. New definitions of epigenetics were needed, and two were suggested in 1994: 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, and 2) Nuclear inheritance which is not based on changes in DNA sequence.[27] The first definition is quite broad, which can include DNA methylation, but also a number of other mechanisms. The second definition includes imprinting and many other documented cases of epigenetic inheritance. It excludes cytoplasmic events, but they can be included in the first definition. Both definitions are in fact incomplete, but they seem to cover most known epigenetic processes. They do not include development itself, and for that we can use Waddington's general definition, which will become more specific as new information accumulates in the future.

DIFFERENCES BETWEEN GENETIC AND EPIGENETIC SYSTEMS

Much less is known about the epigenetic inheritance system than traditional genetics. Genetics is based on cell lineages and clonal inheritance. Gametogenesis produces single haploid cells that fuse to form a diploid zygote. The organism thus starts as a single cell, and ends up as a clone of cells. If a mutation or chromosomal change occurs in a somatic cell, then all its descendents would be expected to have the same genotype. In contrast, epigenetic changes often occur in groups of cells, for example, the induction of muscle tissue in mesoderm cells. This is due to a specific signal which impinges on a group of cells with the same receptor. Some epigenetic events are clonal, and X chromosome inactivation is an excellent example. Genetic changes are stable and rarely reversed, whereas epigenetic changes are often reversed. A good example of that is genomic imprinting, where the changes imposed on DNA sequences may be lost during development, or if they persist, are erased and re-set during gametogenesis. Environmental influences do not change the genotype (leaving aside mutagens), and there is no inheritance of acquired characteristics. Epigenetics is quite different, because normal development depends on communication between cells. Thus, a hormone, morphogen or growth factor may induce an epigenetic change that

may be heritable. This means that the environment of a cell may be all important in determining its properties or its fate in the developing organism. In this sense, epigenetics encompasses Lamarckian inheritance.

Maynard Smith[28] introduced the term dual inheritance, by which he meant that there is classical inheritance based on changes in DNA sequence, and also epigenetic inheritance which is not based on changes in DNA sequence. He was responding to the proposals by Jablonka and Lamb[29,30] that epigenetic inheritance in the germ line might introduce the possibility that environmental influences which induce phenotypic changes could become heritable. There are now many well documented examples of transgenerational effects, presumed to have an epigenetic basis.[31-38] Dual inheritance has also been demonstrated in experiments with cultured mammalian cells.[39] In some cases, what had long been thought to be a classical mutation has been shown to be due to a heritable change in DNA methylation, and a good example of that is a well known change in floral symmetry,[40] which can now be labeled as an epimutation. Transgenerational epigenetic inheritance and related topics have been recently reviewed by Jablonka and Lamb.[41]

It is well established that DNA methylation is involved in genomic imprinting, but the biological reasons for the existence of imprinting remain a matter for debate. (reviewed in ref. 42). One interesting possibility arises from the fact that imprinting results in haploid gene activity, because one of the gametes has an inactive gene. It may be important in early development to have single copies of single genes, particularly if a switch in gene activity takes place prior to or during division. Switching two copies has more than one consequence, but switching one simply leads to a plus and minus situation.[43] A challenge for the future is the unravelling the specificity of genomic re-programming when the germ cells and fertilized egg are formed. Little is yet known about this, although it is established that there are massive changes in DNA methylation at this time and also in early development. These are global changes, whereas information is needed about specific changes, as have been established in the case of imprinting.

OTHER EPIGENETIC MECHANISMS

Chromatin structure and gene expression has become an intensively active field of research. Chromatin can be in the open form that allows access of the machinery for transcription, and a closed form which does not allow transcription. The modification of histones, particularly acetylation and methylation, play a crucial role in this change, and many believe that it is this switch, rather than DNA methylation, which is the more important (reviewed in ref. 44). However, it is not at all obvious how chromatin configurations can be stably inherited. The evidence that DNA methylation can provide a primary switch is very strong,[45] and one likely possibility is that the presence of such methylation triggers the changes that lead to the closed chromatin configuration.

The role of RNA in epigenetic events has become increasingly important. The alternative splicing of gene transcripts can be regarded as an epigenetic mechanism This can produce many isoforms of a given protein that have subtly different properties, and distinct cell types are likely to have specific isoforms. The specificity of splicing events remains a problem, which might be solved if there were small RNA molecules that hybridized across splice junctions.[46] Another prediction is that there are large RNA molecules in the egg or early embryo that have an essential spatial, positional or structural role.[47] This could be essential for the correct 3-dimensional distribution of proteins. If substantiated, this can also be regarded as an epigenetic mechanism. It is now evident that there are a huge number of small regulatory RNA molecules in cells (reviewed in refs. 48–50), and their activities comprise new epigenetic controls. An exciting possibility is that some of these molecules can transmit signals by moving from one cell to another.

The DNA sequence remains constant in most somatic cells, but there is a special epigenetic mechanism in cells of the immune system that can join one constant and one variable sequence, from a pool of such sequences in the whole region, to form a particular genotype that is clonally inherited. Another mechanism to generate antibody variability depends on enzymes that can deaminate cytosine to uracil, or 5methyl cytosine to thymine.[17] This is in effect a mutation, but induced by an enzyme. It could be argued that such a mutation is not an epigenetic change, but it is certainly the result of a protein-DNA interaction and in this respect is epigenetic.[18]

THE GENOME, THE EPIGENOME AND EPIGENOTYPES

In the sequence of the human genome there are just four bases, yet with cytosine in methylated or non-methylated form, there are five, and there is the possibility of six.[51] The epigenome project sets out to determine the pattern of cytosine methylation in a variety of cell types.[52] This depends on the bisulphite sequencing technique introduced in 1992.[53] Since then the technique has been greatly improved, but the underlying chemistry remains the same. It relies on the fact that bisulphite can deaminate cytosine to uracil under conditions in which 5-methyl cytosine is not deaminated. Thus when bisulphite-treated DNA is amplified and sequenced, all the 5-methyl cytosine residues remain as cytosine, but the non-methylated cytosines have become thymines. This technique has been applied in a large number of contexts, and particularly to demonstrate the methylation of many inactive tumor suppressor genes in cancer cells.[54]

The epigenome project will take a long time to complete; nevertheless along the way, we can expect that interesting information will be continually uncovered. We might expect that some regions of the DNA will have the same, or a very similar pattern of methylation in all cell types. These sequences will include many repetitive or transposable elements which have entered the genome at some time and have been silenced by DNA methylation. Much more interesting information will come from specialized genes that are active in one cell type and inactive in another. The importance of DNA methylation in determining the cell phenotype will then be revealed. In the epigenome project, a new terminology will be necessary to classify differences in DNA methylation between cell types.

This introduces the concept of the epigenotype. It has been suggested that the epigenotype is the actual pattern of gene activity in a specialized cell type.[55] These cells are said to have household enzymes and proteins, necessary for normal metabolism in all cell types, and also luxury proteins which have specialized functions. The epigenotype includes all those genes necessary for both household and luxury functions, and also those that are silent or repressed in a given cell type. Thus, fibroblasts and lymphocytes have the same genotype, inherited from the fertilized egg, but they have very different epigenotypes. Of course, as in the case of genotypes, any terminology may apply just to one gene or a subset of genes.

CONCLUSIONS

This overview began with a brief historical account of genetics and developmental biology, and how they diverged for a major part of the twentieth century. Epigenetics is the field that attempted to unite them, and provide new insights into the mechanisms for unfolding the genetic program for development. In the last two decades of the twentieth century much progress has been made on the relationship between DNA methylation and gene expression in a variety of biological contexts, and the experimental study of epigenetics was established. The field has now widened to include another of other mechanisms, especially those involving RNA. Many new insights into the mechanisms for development will be gained in this century.

The sequencing of the human genome is being followed by the epigenome project, which will eventually unravel the significance of DNA methylation in the control of specialized gene functions. It will become apparent whether the primary controls are at the DNA or at the chromatin level. In either case, the nature of the continual interactions between proteins and DNA will further advance the field of epigenetics, and illuminate current problems, such as the re-programming of the genome which initiates the normal processes of development.

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