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Essay

Epigenetics, cellular memory and gene regulation

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The field described as 'epigenetics' has captured the imagination of scientists and the lay public. Advances in our understanding of chromatin and gene regulatory mechanisms have had impact on drug development, fueling excitement in the lay public about the prospects of applying this knowledge to address health issues. However, when describing these scientific advances as 'epigenetic', we encounter the problem that this term means different things to different people, starting within the scientific community and amplified in the popular press. To help researchers understand some of the misconceptions in the field and to communicate the science accurately to each other and the lay audience, here we review the basis for many of the assumptions made about what are currently referred to as epigenetic processes.

The substantial current interest in what is often called 'epigenetics' is driven by multiple influences. These include the idea that this research area aims to understand how cell fate is maintained and reprogrammed, or how the environment can influence the genome, or how non-genetic information can be passed on to the next generation. When the excitement of research advances in these areas reaches the lay public, epigenetics is often described as a mysterious second 'code' that determines which genes are activated in particular cell types. Popular misconceptions of epigenetics have in some cases led to pseudo-scientific speculations of nurture shaping nature, Lamarckian evolution, and even how meditation can cause biological transformations through epigenetic mechanisms. In this essay we discuss how enthusiasm for epigenetics among researchers has led to a number of problems, including confusion about the meaning of the word epigenetics itself, and a temptation to refer to any molecules that have been implicated in epigenetic events as epigenetic regulators. Ironically, this scattershot definition often leaves out transcription factors, which actually have many of the required properties of a regulator of cellular memory or a mediator of environmental influences. In each of the following sections, we address various epigenetic processes, with the goal of stimulating a critical reappraisal of some prevalent assumptions.

Definitions matter: the meaning of epigenetics

The word 'epigenesis' was coined by Aristotle to describe the elaboration of a diversity of parts from an undifferentiated egg, in opposition to the theory of preformation. In the 1940s, the term was resurrected in its adjectival form by Conrad Waddington to describe his idea of an 'epigenetic landscape', a way of illustrating how genes influence cell fate decisions, a foundation for the field we today call developmental genetics [1]. In the 1970s, Robin Holliday, prompted by insights into the molecular heritability of DNA methylation, resurrected the idea of the epigenetic landscape with a focus on how cells remain canalized, or how they maintain memory of the state of their parent cell post-mitotically [2].

As such, the term 'epigenetics' has solid roots in describing how a cell retains memory of past states and perturbations. The concept of epigenetics is perhaps best illustrated with a familiar example. The large colored patches on the fur of a calico cat represent alternative alleles of a coat color gene on the X chromosome (Figure 1). Patches are either orange or black, which reflects the fact that one X chromosome is inactivated in orange fur cells and the other X chromosome is inactivated in black fur cells, an all-or-none distinction between active and silent states of a gene. The patches are large clones of cells resulting from inactivation events during blastula development that are inherited through multiple rounds of cell division — what has been termed 'cellular memory' [3,4]. Because both X chromosomes are needed for this phenotype to reveal itself, and the X chromosomes need to be heterozygous for two coat color alleles, calico cats are nearly always female, and the phenomenon provides a glimpse into a normal epigenetic process that represents the memory of an event occurring during early female development. Other epigenetic phenomena include position-effect variegation, paramutation, genomic imprinting and silencing of the paternal set of chromosomes in mealybugs [5]. What these phenomena all have in common is cellular memory — the maintenance of an activity state of a gene once it is triggered by stress, a change in gene expression, a developmental signal, an environmental cue, or the production of a small RNA. Cellular memory is central to development of multicellular plants and animals. For example, in *Drosophila*, transcription factors expressed in the blastoderm of the embryo initiate a developmental program, but the program persists throughout the lifetime of the fly, even though the initiating transcription factors are expressed only at the blastoderm stage [3]. More generally, we might think of epigenetics as self-perpetuation of a transient event or signal [6], to distinguish it from gene regulation, signalling and other cellular processes that might or might not mediate cellular memory.

This broad definition of epigenetics encompasses phenomena as diverse as yeast prions, which template protein folding states to perpetuate a cellular phenotype [7], and human neocentromeres, which, once formed on non-centromeric DNA, perpetuate themselves by hijacking the segregation apparatus [8]. However, this definition excludes infectious processes that require the continued presence of the pathogen, and some other templating processes, such as RNA interference, which requires the continued presence of the RNA. Ultimately, to elucidate the epigenetic phenomenon of cellular memory during development, we need to understand

how the activity state of a gene is reproduced in daughter chromatids following replication of its DNA.

Emerging ambiguity in the use of the word 'epigenetics'

In the quest to understand the molecular mediators of cellular memory, the idea of epigenetic regulatory processes was linked to the position effect variegation studies of Herman Muller in 1930, and to the work of Edward Lewis in the 1970s in *Drosophila*, showing that Polycomb is an important molecular mediator of cellular memory. Insights into the mechanisms of X chromosome inactivation and genomic imprinting began to associate molecular processes like DNA methylation, certain histone modifications and Polycomb effects to the selective silencing on one X chromosome or one parental allele [5]. These kinds of molecular processes therefore became strongly identified with epigenetic (cellular memory) outcomes.

With the development of genome-wide assays to study these presumed regulators of epigenetic events, 'epigenomic' studies became commonplace, looking for influences upon gene regulation occurring at loci not undergoing genomic imprinting or X inactivation. What was being sought was an influence on gene regulation that could reflect environmental or other influences, potentially gaining an insight into disease and other phenotypes. The word epigenetic was retrofitted with a new definition, derived from 'epi' meaning 'above' or 'upon' and 'genetic' meaning the information encoded in DNA sequence [1]. This definition has filled a void, but in reality offers no distinction from generic transcriptional regulatory mechanisms, with everything from chromatin states to covalent modifications of RNA now being referred to as epigenetic. Other phenomena, such as transgenerational inheritance, also began to be called epigenetic as the term permeated popular culture [9]. Given this muddle, it is no wonder that scientists are confused by the use of the term, and the lay public even more so.

Transcription factors and cellular memory

Transcription factors occupy the apex of gene regulatory networks, capable of driving reprogramming of differentiated cells to assume alternative cell fates [10]. The sequence-specificity of transcription factors solves the problem of how epigenetic phenomena can be limited to particular loci for differential gene expression to occur during development. Therefore, transcription factors provide attractive candidates for transmitting cellular memory, such as by binding and activating expression of their own genes [6]. For example, if an extra-cellular signal stimulates the activation of a transcription factor, which then activates a series of other genes, including the gene encoding the transcription factor itself, the original signal can be withdrawn and the new set of regulatory signals can sustain their activities by means of this new feedback loop. This kind of mechanism is well worked out in simple systems like phage lambda but also appears to act in higher eukaryotes. However, such a simple positive feedback loop does not account for the orange and black patches of the calico cat, because in each cell both the active and the inactive X chromosomes are exposed to the same transcription factor concentration, but only one stays active. Transcription factors may be necessary for cellular memory but they might not always be sufficient, prompting us to explore other possible molecular mediators.

Figure 1. An example of cellular memory.
The calico cat displays features of epigenetic inheritance. Her orange and black patches result from random inactivation of one or the other X chromosome, a mechanism for equalizing the dosage of X-chromosome genes between females and males. The white background is a consequence of a separate autosomal mutation that prevents pigment cell migration in the hair. (Photo: Nzrst1jx, Wikimedia Commons.)

DNA methylation and cellular memory

DNA methylation is an obvious candidate for transmitting epigenetic information because, unlike DNA-associated proteins, covalent modifications stay on the DNA as the replicative helicase unwinds the two strands from one another. Furthermore, there is a simple mechanism whereby methylation of a CG dinucleotide can be faithfully inherited by the maintenance cytosine DNA methyltransferase Dnmt1, which converts meCG/GC hemi-methylated dinucleotides to meCG/GCme symmetrically methylated dinucleotides post-replication. Indeed, transmission of cellular memory is attributable to DNA methylation in some cases, for example in imprinting in the endosperm of flowering plants [5]. However, DNA methylation has not been found to transmit cellular memory in most cases that have been examined [11]. Moreover, many organisms that display cellular memory, such as *Drosophila*, lack maintenance DNA methylation [5]. DNA methylation, therefore, can be said to have a role in mediating memory of cellular states in only a subset of organisms.

Histone modifications and cellular memory

Most histone modifications are continually maintained by localized

transcription factors. Nucleosomes that package the genome unravel as the replication fork passes through, and histone octamers are rapidly reformed on the leading and lagging strands. Histone chaperones, including the MCM2 subunit of the replicative helicase, bind histones H3 and H4 and efficiently redeposit them together with H2A and H2B to form new octameric nucleosomes, while the Caf1 complex assembles newly synthesized histones into nucleosomes to fill in the gaps [12]. Recycled histones retain their modifications during this process, but newly deposited histones are only slowly modified [13]. As some histone modification patterns can be very stable, it is likely that newly assembled nucleosomes can acquire the same marks as recycled nucleosomes nearby, and several models for the involvement of histone modifying enzymes in cellular memory have been proposed [12]. For example, the Polycomb Repressive Complex 2 (PRC2) can mono-, di- and tri-methylate histone H3 on lysine-27, and in the absence of the enzyme, H3K27me3 becomes gradually diluted over the course of multiple cell cycles, indicating efficient recycling behind the replication fork [14]. A popular model proposes that binding of the PRC2 complex on an old nucleosome tethers it such that it can methylate nearby nucleosomes post-replication. Another model for self-propagation of H3K27 methylation is based on sequence differences between canonical histone H3 laid down behind the replication fork and the H3.3 histone variant that accumulates at interphase [15]. However, newly deposited nucleosomes become fully trimethylated on H3K27 in mammalian cells over the course of multiple cell generations [13], which is inconsistent with either of these models maintaining H3K27me3 directly behind the replication fork over the entire genome. If it can be shown that histone modifications can self-propagate from parent to daughter cell at the same genomic locations, only then can we consider histone modifications to be epigenetic.

Even if this were demonstrated to be the case, the direct relevance of histone modifications to cellular memory is uncertain, challenged by genetic experiments showing that the classical cellular memory modules that drive *Drosophila* development are regulatory elements as small as 138 bp [3], loci that are depleted of nucleosomes but are bound by clusters of transcription factors. Histones with specific patterns of modifications can be thought of as physically flanking and associated with these critical elements that are characterized by being bound by transcription factors. One possibility among many is that compaction of nucleosomes mediated by the recruitment of the PRC1 complex [3], which specifically binds to H3K27me3, interferes with the activity of the clustered adjacent transcription factors.

Histone modifications and the regulation of gene expression

Histone modifications are key components of the machinery by which localized transcription factors promote the binding of additional transcriptional regulators. The discovery of histone acetylation and methylation and the proposal that they are important for gene activation dates back to the 1960s. However, it was not until the mid-1990s that the first genetic evidence for gene regulation by histone modification was described [5]. Specifically, the Gcn5 component of the yeast SAGA complex, a genetically defined coactivator of gene expression, was shown to be a histone acetyltransferase (HAT), although later work showed that eliminating the HAT activity without otherwise disrupting Gcn5 had only a very slight effect on global gene expression [16]. Histone tail acetylation is associated with decompacted chromatin, and decompaction is a feature of high-level gene expression. However, there are as many active genes enriched for HATs as enriched for histone deacetylases (HDACs), while HDACs, surprisingly, are not enriched at silenced genes [17]. These observations suggest that dynamic histone acetylation/deacetylation cycles may facilitate gene expression by transiently decompacting nucleosomes to allow RNA polymerases to pass through [18], but the evidence does not support the original proposal that they are key regulators of gene activation [4].

Methylation of H3K4, H3K36 and H3K79 and the incorporation of the histone variants H2A.Z and H3.3 are prominent marks of active transcription [18]. As the modified nucleosomes are found over gene bodies, it is likely that their roles are to facilitate RNA Polymerase II (RNAPII) transit. This might account for the weak effects on gene expression when residues subject to modification are mutated in yeast, because initiation of transcription, not elongation, is usually rate-limiting for RNA production. Therefore, although histone modifications are involved in regulating gene expression, their roles appear to be secondary to other components of the genomic landscape. These include transcription factors, which specify activation and repression and act upstream of RNAPII, which is regulated in turn by phosphorylation of its carboxy-terminal domain (CTD) and by proteins bound to the CTD that modulate transcriptional elongation. Other components that are directly or indirectly recruited by transcription factors are chromatin remodelers, which are DNA translocases that mobilize nucleosomes and reposition them to create nucleosome-depleted regions where transcription factors bind and RNAPII initiates.

Many functional properties of histone modifications are inferred from associating their patterns with transcriptional states of loci nearby. A robust example of a histone modification with clear functional properties is provided by the random inactivation of the X chromosome, the process that results in the orange and black patches on the calico cat (Figure 1). The *Xist* long non-coding RNA that targets one of the two X chromosomes for X-inactivation recruits the PRC2 complex, which trimethylates H3K27 and is a central component of developmental silencing [3]. This illustrates how a single histone modification (H3K27me3) is involved in repressing gene expression.

An epigenetic code?

As each of the histone proteins can be modified with different post-translational modifications at different amino acids, the reasonable question that arose was whether these modifications in different combinations

constituted a form of information, or a histone code [4]. According to the histone code hypothesis, combinations of modifications dictate downstream biological effects. However, given that the role of histone modifications might be likened to cogs in a machine [18], perhaps an updated statement would be that histone modifications *influence* downstream events. Contrary to expectation [4], histone modifications that are characteristic of promoters (e.g. H3K4me3 and H3K9ac) and enhancers (e.g. H3K4me1 and H3K27ac) are conspicuously *absent* from transcribed genes that are differentially regulated during *Drosophila* development [19]. These observations suggest that any influence that these landmark histone modifications have on active gene expression is unrelated to cellular memory during development. We then have the question of whether histone modifications act in combinations to achieve a complex read-out of gene regulatory information. However, there is little evidence that modifications act in anything other than an additive manner [18]. Arguably the most important histone modifications involved in genetic processes are those associated with silencing: H3K9me3, which is needed for compacting pericentric regions of chromosomes, and H3K27me3, which is essential for developmental silencing. Yet these modifications do not appear to work directly in combination with other histone modifications. Given the evidence that histone modifications do not act combinatorially in influencing downstream developmental events, it would appear that the epigenetic code is a metaphor that has not stood the test of time.

'Epigenetic' drugs

Drugs that interfere with cellular memory have, in principle, the potential of reversing the distinctive cellular phenotype in cancer, a prospect that has driven development of small molecule drugs targeted to DNA and histone modification enzymes and other chromatin regulators. Deoxycytidine analogs act by preventing release of a DNA methyltransferase when it is in a covalent intermediate with DNA, thus depleting the enzyme when given at high doses [20]. However, low doses have been found to be effective in the treatment of myelodysplastic syndrome, and recent studies have implicated the interferon response in the therapeutic action of these inhibitors [21]. A plethora of broadly acting HAT and HDAC inhibitors have been used in the clinic to treat cancer and other diseases [5]. However, whether their effects are due to the hyperacetylation or deacetylation of histones that results from treatment has not been established. HATs, HDACs and other histone-modifying enzymes were given these names because histones have traditionally been the substrates used in enzyme assays, but it is notable that nearly all of them have non-histone substrates as well. For example, the HAT p300/CBP acetylates both histones and the p53 transcription factor and up-regulates a p53 positive feedback loop [22], which complicates interpretation of the anti-cancer therapeutic action of HAT and HDAC inhibitors. As histones and p53 are among the most studied of all proteins, the number of non-histone substrates for these enzyme classes is likely to be very large.

Even if the therapeutic effect of HDAC inhibitors comes from their action on histones, this might not be the result of a chromatin change *per se*, because the global retention of acetyl groups on histones caused by HDAC inhibition has the metabolic effect of lowering the intracellular pH [23]. Small molecule inhibitors that interfere with binding of proteins to acetylated histone lysines also inhibit binding to key transcription factors, including p53 [24], so it should not be assumed that these inhibitors act by altering the properties of nucleosomes. Other small molecule drugs in clinical trials target specific histone methyltransferases and demethylases, and it seems likely that at least those that inhibit H3K27 methylation act by interfering with cellular memory and causing cancer cells to change their fate. However, it is also possible that many drugs intended to reverse the cancer phenotype act non-specifically to damage chromatin in rapidly dividing cells. Indeed, there may be no clear distinction between so-called epigenetic drugs and traditional anti-cancer agents, with the example of doxorubicin and other anthracycline compounds widely used in the clinic for four decades known to cause massive nucleosome eviction [25]. Referring to a drug as epigenetic in its actions is therefore often based on assumptions that may not be valid. What is more important is whether the drug succeeds in treating patients, regardless of whether its mechanism of action involves cellular memory, gene regulation or genomic stability.

Interpreting DNA methylation differences between individuals

DNA methylation is often studied as representative of epigenetic perturbations that distinguish individuals with a phenotype from control subjects. The hypothesis being tested is that the same cell type from these two groups of individuals will differ measurably in DNA methylation levels at specific loci, with the assumption that these changes influence expression of genes nearby. These studies are increasing rapidly in number and scope [26] and yielding what appear to be insights into a range of phenotypes. While this has generated optimism, we now appreciate that DNA methylation differences between individuals can reflect cell subcomposition differences, differences in transcription through the locus and DNA sequence polymorphism, estimated to account for 22–80% of DNA methylation variability. A positive result from one of these epigenome-wide association studies (EWAS) can therefore be interpreted as having tested the starting hypothesis only if the subcomposition, transcription, and genotypes of the cells studied have been excluded as confounding effects.

Can DNA or histone marks transmit epigenetic information across generations?

A major question in the field of epigenetics is whether the mechanisms for persistence of cellular memory through mitosis can also mediate memory of past events across generations. Misconceptions abound [9]. For example, the popular press has touted as epigenetic the evidence that the children of men exposed to the 1944–1945 Dutch 'Hongerwinter' during gestation tended to be overweight like their fathers. But this transgenerational interpretation

overlooks the researchers' own explanation, that parents control the food and influence the eating and exercise habits of their children. In model organisms, however, transgenerational mechanisms can be experimentally tested. In nematodes, histones are retained in germ cells, and di-methylation of H3K4 has been shown to persist through multiple organismal generations and affect the balance between fertility and longevity. In mammals, the majority of transcription factors and histones are lost during sperm maturation and DNA methylation is erased between generations [27], so that other mechanisms need to be considered. For example, small RNAs produced in pollen vegetative nuclei are thought to mediate silencing of transposons in adjacent sperm nuclei [9]. In mice, tRNA fragments produced in the epididymis are transferred to sperm as it matures [28]. As small RNAs in other contexts are known to mediate sequence-specific interactions with the genome and can pass between cells via exosomes and the vasculature, it seems at least plausible that they might also mediate transgenerational effects without any involvement of histone modifications or other chromatin features. It is as yet unclear how these small RNAs influence differentiated somatic cells and the germ line of the next generation, as mammals lack the RNA-dependent RNA polymerases that allow small RNAs to be propagated directly through cell division. For example, when induced by starvation, such a small RNA amplification system in nematodes targets nutritional genes to mediate transgenerational inheritance of longevity [29]. So although transgenerational inheritance mediated by transcription factors, nucleosomes, DNA methylation or small RNAs is an attractive concept, the molecular mechanisms that could mediate these processes in mammals are unclear.

Conclusions

There is much excitement about the potential of epigenetics to explain aspects of phenotypic variability, disease risks and the potential for therapeutic interventions. This excitement has transmitted itself to non-scientists, with ideas like stresses in one generation being transmitted to descendants catching the public imagination. This essay serves to challenge some widely held preconceptions that may be misleading, allowing us to communicate more effectively within the scientific community and more accurately with the general public.

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