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Review

Cancer epigenetics: a perspective on the role of DNA methylation in acquired endocrine resistance

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Abstract

Epigenetic mechanisms, including DNA methylation, are responsible for determining and maintaining cell fate, stably differentiating the various tissues in our bodies. Increasing evidence shows that DNA methylation plays a significant role in cancer, from the silencing of tumor suppressors to the activation of oncogenes and the promotion of metastasis. Recent studies also suggest a role for DNA methylation in drug resistance. This perspective article discusses how DNA methylation may contribute to the development of acquired endocrine resistance, with a focus on breast cancer. In addition, we discuss DNA methylome profiling and how recent developments in this field are shedding new light on the role of epigenetics in endocrine resistance. Hormone ablation is the therapy of choice for hormone-sensitive breast tumors, yet as many as 40% of patients inevitably relapse, and these hormone refractory tumors often have a poor prognosis. Epigenetic studies could provide DNA methylation biomarkers to predict and diagnose acquired resistance in response to treatment. Elucidation of epigenetic mechanisms may also lead to the development of new treatments that specifically target epigenetic abnormalities or vulnerabilities in cancer cells. Expectations must be tempered by the fact that epigenetic mechanisms of endocrine resistance remain poorly understood, and further study is required to better understand how altering epigenetic pathways with therapeutics can promote or inhibit endocrine resistance in different contexts. Going forward, DNA methylome profiling will become increasingly central to epigenetic research, heralding a network-based approach to epigenetics that promises to advance our understanding of the etiology of cancer in ways not previously possible.

Key words Epigenetics, breast cancer, endocrine resistance, methylation, methylome profiling

The landmark discovery of DNA hypo- and hypermethylation in cancer cells in 1983 sparked a series of investigations intended to elucidate how epigenetic changes influence cancer initiation and progression. Since then, a wealth of information has been uncovered linking epigenetic changes to a diverse array of cancer-associated pathways, including silencing of tumor suppressors [1], activation of oncogenes [2], promotion of metastasis [3], and resistance to therapeutic drugs [4-6]. Often defined as the study of heritable change independent of alterations in the gene sequence, epigenetics has made us rethink our understanding of cancer etiology. Under normal circumstances, the assorted modifications to DNA and histones may constitute an "epigenetic code" that dictates and enforces differential cell fate. In cancer the epigenome is often greatly perturbed, resulting in aberrant gene expression patterns that can fuel oncogenesis just as surely as genetic mutations.

Meanwhile, the search continues for biomarkers and drug targets to curb endocrine-resistant breast cancer, a subtype of breast cancer that does not respond to endocrine therapies. Approximately 70% of patients with breast cancer present with tumors that are estrogen receptor-α (ERα)–positive [7], and blockade of estrogen signaling in many cases stabilizes or shrinks these tumors. A variety of anti-estrogens have been developed to interfere with estrogen signaling and combat breast

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cancer, including selective estrogen receptor modulators (SERMs), e.g., tamoxifen; selective estrogen receptor downregulators (SERDs), e.g., fulvestrant; and aromatase inhibitors, e.g., anastrazole. Regardless of the anti-estrogen used, a significant fraction of patients will relapse during or after treatment. As endocrine therapies are generally very well tolerated compared to available alternatives and resistant tumors have a poor prognosis, overcoming or preventing endocrine resistance has been the focus of much study.

This perspective discusses how altered patterns of DNA methylation may contribute to the development of endocrine resistance, with a focus on breast cancer. We briefly review epigenetic fundamentals and their relevance to cancer, then draw a distinction between the clinical phenomenon of intrinsic and acquired resistance, which may be associated with different biological mechanisms. We proceed to discuss evidence for a genomic profile of acquired resistance, and then tackle the interplay between epigenetic regulation and estrogen receptor signaling. In doing so, we focus on distinguishing the mechanisms involved in intrinsic versus acquired resistance, and also on estrogen signaling as both a target and controller of epigenetic regulation. We then present a prospective role for epithelial-mesenchymal transition in endocrine resistance, together with speculation concerning a possible epigenetic mechanism. Finally, we discuss DNA methylome profiling, including techniques and principles, its relevance to the study of endocrine resistance, and future prospects for this budding approach.

Epigenetic Regulation of Gene Expression in Cancer

In general, epigenetic mechanisms regulate the expression of genes and subsequent products (e.g., proteins), rather than altering these products. Epigenetic control is generally thought to alter the accessibility of the DNA to transcriptional machinery. DNA in cells does not exist freely but combines with proteins to form a complex termed chromatin. According to the "beads on a string" model, DNA (the string) in cells is wound around nucleosomes (the beads), which in turn are composed of histone proteins. Current theory is that changes in patterns of DNA methylation and histone modification alter the conformation of chromatin and accessibility of the DNA to transcriptional machinery, either by directly introducing steric hindrance or indirectly causing surrounding chromatin to adopt an "open" or "closed" conformation. In addition, epigenetic modifications may cause individual nucleosomes to shift laterally across the DNA, exposing some areas of DNA for transcription and covering others [8]. While this perspective focuses on DNA methylation due to its high clinical relevance for diagnostics and therapeutics, histone modifications clearly have a role in cancer as well. This topic has recently been reviewed in-depth[9].

DNA methylation patterns and dysregulation in cancer

DNA methylation is a covalent modification that occurs at cytosine nucleotides, in particular at cytosines that precede a guanine (CpGs). The process is catalyzed by DNA methyltransferases (DNMTs), which transfer the methyl group from S-adenosylmethionine to carbon 5 of the target cytosine. Two families of DNMTs have been identified: DNMT1, which predominantly functions in maintenance of DNA methylation during DNA replication, and DNMT3 (including DNMT3a and DNMT3b), which is thought to be primarily responsible for de novo CpG methylation[10]. CpGs are strikingly rare in the genome compared to what would be expected from probabilistic estimates, and outside of transcribed regions, CpGs are generally methylated. Areas of high CpG content, termed CpG islands, are found in approximately 40% of mammalian promoters and, unlike CpGs in the rest of the genome, are usually unmethylated[1]. Studies have shown that the methylation state of CpG islands in promoters can be an important factor controlling gene expression, with heavy methylation blocking gene transcription and sparse methylation permitting it. In addition, evidence suggests that heavy methylation throughout a region of chromatin can mediate long-range silencing that extends even to adjacent unmethylated genes[11].

Precisely controlled DNA methylation is important for imprinting (allele-specific expression of some genes) and cell differentiation in normal cells. In cancer cells, aberrant patterns of DNA methylation are frequently observed. In general, cancer cells feature global hypomethylation and promoter hypermethylation of tumor suppressors. Global hypomethylation has been tied to genomic instability, loss of imprinting, and overexpression of oncogenes [1]. Promoter hypermethylation, namely within CpG islands, leads to epigenetic silencing of the target gene. In contrast to the rest of the genome, tumor suppressor promoters are frequently hypermethylated in tumors, suggesting that epigenetic silencing of tumor suppressors is an effective alternative to loss-of-function mutations.

Pathways to Endocrine Resistance: Intrinsic Versus Acquired

Understanding endocrine resistance requires some knowledge of its origin. A paradigm has arisen based on two patterns of resistance observed clinically in response

to treatment: intrinsic resistance and acquired resistance. Intrinsic resistance reflects preexisting insensitivity of a tumor to a given treatment. ERα-negative tumors, for example, respond poorly to anti-estrogen treatments. Acquired resistance reflects tumor adaptation to treatment over time; the tumor responds initially, but eventually relapses. At the cellular level, acquired resistance can be explained by two hypotheses: selection and adaptation. According to the selection hypothesis, the heterogeneous nature of tumor cell populations leads to selection for cells that are most resistant to treatment; subsequently, this resistant population expands to repopulate the tumor. The adaptation hypothesis holds that treatment induces molecular changes in tumor cells; cells that survive and continue to proliferate have undergone changes that provide a selective advantage. In reality, acquired resistance likely involves a combination of passive and active processes.

Intrinsic resistance and acquired resistance may involve different biological pathways, and thus, a solution for one resistance type may not apply to the other. Resistance to anti-estrogen therapies can be broadly segmented by the biology involved as follows: altered ERα signaling (e.g., altered levels, activity, or function of ERα subtypes or coregulators); activation of alternative growth factor and cytoplasmic signaling pathways (e.g., HER2 or PI3K signaling); dysregulation of cell cycle and survival pathways (e.g., cyclins, MYC, and BCL2); and pharmacological tolerance. This subject has been reviewed in-depth previously [12,13]. Estrogen receptor expression is a standard prognostic factor used to predict response (or conversely, intrinsic resistance) to anti-estrogen therapy. In contrast, acquired resistance seems more complex, with only ~20% of relapses displaying loss of estrogen receptor expression [14]. This perspective will focus on the role that epigenetic mechanisms may play during acquired resistance.

Patterns of Epigenetic Regulation in Endocrine Resistance

Recent studies have implicated epigenetic mechanisms in the development of endocrine-resistant breast cancer. The remainder of this perspective will discuss some themes that have emerged from these studies.

An epigenomic profile of endocrine resistance

The concept of global DNA hypomethylation and promoter hypermethylation in cancer has been well established. A recent study suggests that promoter hypomethylation may predominate during the acquisition of endocrine resistance. Fan *et al*. [15] performed a genome-wide DNA methylation analysis using two cell line models of acquired endocrine resistance. ERα-positive MCF7 cells were cultured long-term with the anti-estrogens tamoxifen or fulvestrant, yielding two endocrine-resistant sublines: MCF7-T and MCF7-F. Methylation analysis revealed that acquisition of endocrine resistance was associated predominantly with global promoter hypomethylation relative to the parental line, although promoter hypermethylation was also observed. Interestingly, this difference did not translate into clear-cut gene expression differences; while MCF7-F showed a tendency towards up-regulation of gene expression, MCF7-T demonstrated a balance of up-regulated and down-regulated genes. Hypomethylation as a mechanism of resistance was further implicated in another study by van Agthoven *et al*. [16] in which pre-treatment of an ERα-positive breast cancer cell line with the DNA methylation inhibitor 5-azacytidine facilitated the acquisition of tamoxifen resistance.

The interplay of epigenetics and ERα signaling

Recent evidence suggests that epigenetic regulation of ERα signaling may play a role in intrinsic resistance in endocrine therapy. ERα-negative tumors respond poorly to anti-estrogens. One hypothesis holds that estrogen signaling is required for the growth of some tumors; therefore, the tumors regress in the absence of estrogen ligand/estrogen receptor–mediated proliferative and survival signals. An alternative hypothesis is that ERα signaling itself can mediate anti-proliferative and apoptotic signaling, particularly in the case of anti-estrogen ligands. Restoration of signaling, in the context of anti-estrogen treatment, would only be beneficial if the second hypothesis holds true, and recent studies suggest this is the case. Treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine and the histone deacetylase inhibitor trichostatin A restored estrogen receptor expression and tamoxifen sensitivity in two ERα-negative breast cancer cell lines [17-19]. The recruitment of corepressor complexes to ERα target genes was shown to play a role in mediating this renewed tamoxifen sensitivity[17]. Although van Agthoven's study[16] suggests treatment of ERα-positive tumors with epigenetic inhibitors may be inadvisable (at least initially), treatment of ERα-negative tumors with epigenetic inhibitors in combination with tamoxifen is a potential alternative to conventional mitotic inhibitor therapy. It remains to be seen whether ERα re-expression affects response to aromatase inhibitors in cancer cells.

Additional evidence suggests that loss of estrogen signaling may actively set tumor cells down the pathway

of acquired resistance. Tamoxifen resistance is frequently associated with HER2 overexpression [13]. Furthermore, estrogen/ERα signaling represses HER2 expression. In contrast, treatment with tamoxifen stimulates HER2 expression in ERα-positive but not -negative cells [20]. A recent study suggests that an epigenetic mechanism could be involved. Transient ablation of ERα by siRNA resulted in durable silencing of progesterone receptor (PR), an ERα target gene and itself an important hormone receptor. Treatment with the DNA methylation inhibitor 5-aza-2'-deoxycytidine was required to re-express PR [21]. Other ERα target genes were also found to be similarly methylated. Similar results were seen by another group, who found that treatment with tamoxifen in particular could initiate epigenetic silencing of ERα-driven transcription [22]. Altogether, these results suggest that temporary loss of ERα signaling can be self-reinforcing and potentially hint at a novel mechanism for loss of repression of estrogen-antagonized genes via epigenetic silencing of estrogen-agonized genes.

ERα itself can participate in epigenetic control as well. ERα binding to estrogen response elements in the genome results in the recruitment of cofactors involved in nucleosome remodeling, including nuclear receptor corepressor 1 (NCOR1) and 2 (NCOR2), steroid receptor coactivator-1 (SRC-1), and amplified in breast cancer 1 (AIB1) [23,24]. Tamoxifen resistance has been associated with dysregulation of these coregulators, which presumably results in reduced repression or inappropriate activation of target genes. Notably, high expression of SRC-1 was only associated with poor prognosis in HER2-overexpressing tumors[25], showcasing yet again the interplay of ERα and HER2 signaling during the acquisition of tamoxifen resistance, with epigenetic mechanisms likely playing a role.

A role for epithelial-mesenchymal transition in endocrine resistance?

The role of epithelial mesenchymal transition (EMT) in drug resistance has been discussed in recent reviews [26,27]. In brief, EMT is a morphological change that occurs in some epithelial tumors. Reflecting the more general theme of loss of differentiation, EMT is often linked to loss of contact inhibition, as well as increased invasiveness and metastasis. EMT has been associated with resistance to a wide variety of chemotherapeutics, including oxaliplatin (DNA crosslinking agent), paclitaxel (mitotic inhibitor), doxorubicin (DNA intercalator), gemcitabine (nucleoside analog), and various tyrosine kinase inhibitors (relevant for EGFR- and HER2-targeted therapies). A recent paper linked EMT to acquired tamoxifen resistance in an MCF7 subline, with EGFR signaling driving phosphorylation of beta-catenin and loss of E-cadherin–mediated cell-to-cell adhesion [28]. In another study, overexpression of the transcription factor Snail in MCF7 cells resulted in EMT. Interestingly, ERα expression was also lost, and further investigation revealed that Snail bound to the ESR1 (ERα) promoter, resulting in deacetylation of H3K9 [29]. Overall, this evidence suggests that EMT could play a role in endocrine resistance, and epigenetic regulation would be a potential mechanism.

Figure 1 presents a hypothetical model summarizing the topics discussed in this perspective. The upper path depicts acquired resistance to endocrine therapy and the lower path depicts intrinsic resistance. Initial evidence for the model primarily comes from preliminary studies of tamoxifen resistance, particularly the association of EMT with acquired resistance. Further study is required to validate the model's general applicability to the broad spectrum of anti-estrogens in clinical use.

DNA Methylome Profiling: A New Frontier

The introduction of high-throughput technologies for genomic profiling has revolutionized genetic research. In the past decade, these technologies have been adapted for epigenetic studies as well, with DNA methylome profiling receiving the most attention by far. DNA methylation has significant advantages over mRNA expression as a biomarker. Unlike mRNA, DNA methylation is stable and can be readily assessed in formalin-fixed paraffin-embedded samples. This stability even extends to circulating DNA in the bloodstream. For example, tumor-specific methylation of glutathione S-transferase P1 (GSTP1) is found in 90% of prostate malignancies and can be detected in a variety of bodily fluids, including blood plasma[30]. The high sensitivity and specificity required to detect and distinguish circulating tumor DNA also indicates the potential of DNA methylation biomarkers for other applications.

Emerging high-throughput methods for analysis of DNA methylation provide distinct advantages over traditional methods. Data can be reanalyzed to answer many different questions. Patterns of DNA methylation can be elucidated among multiple loci, and these often prove to be far more biologically robust than those determined in comparisons of individual loci. In the case of next-generation sequencing-based methods, genome-wide coverage eliminates the bias of having to rely on previous selection of the target sequence(s), allowing for novel and context-specific discoveries. This section will provide a brief overview of selected high-throughput techniques for analysis of DNA methylation, focusing on general principles. Recent findings from DNA methylome profiling studies relevant to the study of endocrine resistance will then be

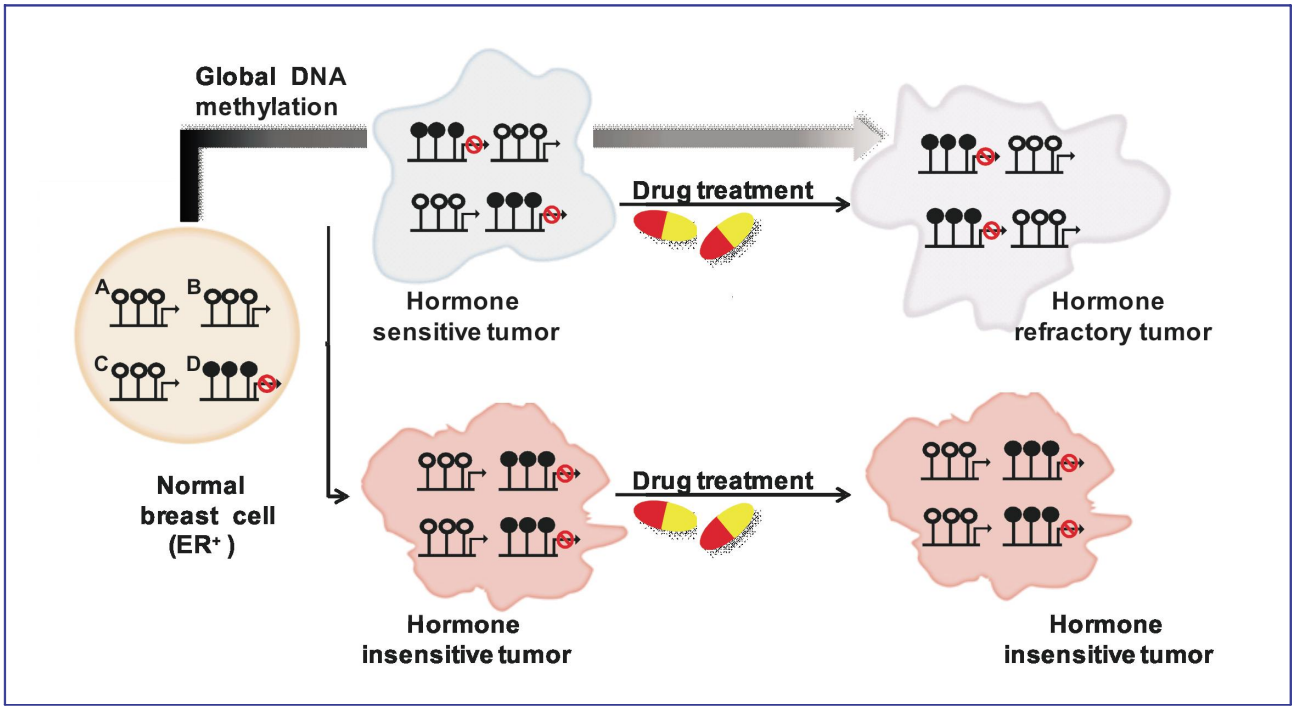

Figure 1. **A proposed model for epigenetic contribution to endocrine-resistant breast cancer.** During tumorigenesis, an estrogen receptor (ER)–positive progenitor can give rise to a hormone-sensitive or hormone-insensitive tumor. DNA promoter hypermethylation may play a role in tumorigenesis, silencing *ESR1* to yield a hormone-insensitive (intrinsically resistant), ER-negative tumor, or alternatively, silencing tumor suppressors to yield a hormone-sensitive tumor. Treatment with anti-estrogens may actively promote the development of endocrine resistance, resulting in promoter hypermethylation (black stalks) and hypomethylation (white stalks). Alternatively, treatment may select for resistant subpopulations with these epigenetic alterations. Dysregulation of estrogen-regulated genes may result in further perturbation of ER signaling. In addition, altered expression of genes involved in cellular differentiation may lead to epithelial-mesenchymal transition, a change associated with increased tumor invasiveness and metastasis. Globally, DNA hypomethylation is observed during tumorigenesis, and promoter hypomethylation may predominate during acquisition of endocrine resistance. Genes A through D represent generic and actual genes: A, generic tumor suppressor (e.g., *CDKN2A*/p16); B, *ESR1* (ER); C, generic estrogen-regulated gene (e.g., *PGR*/progesterone receptor); D, generic epithelial differentiation gene (e.g., *CDH1*/E-cadherin).

discussed. This will be followed by a brief outline of open questions that could be addressed by methylome profiling.

Techniques and principles

Once genomic DNA is isolated from samples of interest, assessment of DNA methylation can be separated into two conceptual steps: fractionation and analysis. The purpose of the fractionation step is to distinguish methylated and unmethylated sequences. Three popular means for achieving fractionation are digestion with methylation-specific restriction enzymes, e.g., differential methylation hybridization[31] and Methyl-seq[32]; sodium bisulfite treatment, e.g., reduced representation bisulfite sequencing (RRBS)[33] and MassArray[34]; and most recently, affinity purification, e.g., methylated DNA immunoprecipitation (MeDIP)[35] and methylated CpG island recovery assay (MIRA)[36]. High-throughput analysis can be segmented into three more categories: loci-specific, e.g., MethyLight[37] and MassArray; array-based, e.g., differential methylation hybridization, HpaII tiny fragment enrichment by ligation-mediated PCR (HELP)[38], comprehensive high-throughput arrays for relative methylation (CHARM)[39], MeDIP, MIRA; and next generation sequencing-based (NGS), e.g., Methyl-seq, MeDIP-seq[40], MIRA-seq, RRBS, bisulfite padlock probe (BSPP)[41]. Loci-specific analysis is generally restricted to a limited number of loci but can be easily extended to a large number of samples. Array-based analysis makes use of predefined arrays with extensive coverage of a given target type, e.g., CpG islands. Analysis based on NGS provides the most thorough assessment and is the sole method that can provide unbiased genome-wide coverage. Recent reviews discuss and compare the general merits and applications of the various methylome profiling methodologies[42,43].

Methylome profiling and endocrine resistance

Several studies have used methylome profiling to investigate the connection between estrogen signaling and genome-wide DNA methylation. Using differential methylation hybridization, Leu *et al*.[21] found that loss of estrogen signaling (enforced by siRNA knockdown of ERα) triggered DNA methylation of ERα target genes in MCF7 cells. Among these methylated targets was progesterone receptor, which was also down-regulated at the RNA level, and could only be re-expressed by restoration of ERα signaling in combination with a DNA methylation inhibitor. Cheng *et al*.[44] utilized MeDIP-chip to study whether abnormal exposure of human breast progenitor cells to high levels of estrogen might lead to cancer-like epigenetic remodeling in epithelial progeny. They found that this exposure, which may be analogous to the effects of endocrine disruptors, resulted in hypermethylation of 0.5% of the CpG islands analyzed, including the promoters of eight tumor suppressors. Fan *et al*.[15] used differential methylation hybridization to directly characterize the effect of anti-estrogen treatment on the methylome and found that promoter hypomethylation predominated over promoter hypermethylation following acquired resistance.

The bright future of methylome profiling

NGS is opening new doors for DNA methylome profiling. In general, NGS-based methods require less sample DNA and provide higher coverage for a relatively low cost compared to array-based methods. Another advantage is that NGS does not limit analysis to the predefined targets on an array, although the fractionation step may still limit the type and extent of the analysis.

The development of DNA methylome profiling technologies will enable studies that were once difficult or impractical. Moving forward, we should soon see large-scale profiling of clinical samples, comparing methylomes of tumors in many contexts. One such context would be anti-estrogen–treated breast cancer patients, in which methylome profiling could explore the association between tumor DNA methylation and treatment response on a genome-wide scale. In addition, the discovery of circulating tumor DNA could provide an opportunity to monitor changes in methylation over time, enabling an unprecedented glimpse into the methylation changes associated with anti-estrogen resistance. One recent study demonstrated the feasibility of such an approach by using circulating tumor DNA, in combination with massively parallel bisulfite sequencing, to assess four loci in 21 serum samples from tumor patients and 21 controls [45]. Increasing availability of methylome profiles from cell lines will also fuel methylation research, allowing a more complete understanding of methylation patterns in widely used model systems.

Conclusions

This perspective highlights exciting new evidence pointing to a role for epigenetics in the evolution of endocrine-resistant tumors. Epigenetic studies could potentially provide DNA methylation biomarkers to predict acquired resistance and could also provide a more accessible and timely means of assessing tumor response to therapy. These biomarkers could also prove useful as traditional prognostic tools for intrinsic resistance. Elucidation of epigenetic mechanisms may also lead to the development of new treatments that specifically target epigenetic abnormalities or vulnerabilities in cancer cells. Examples of two such classes of drugs include DNA demethylating agents and histone deacetylase inhibitors, which have shown promise in the treatment of leukemia and T-cell lymphoma, respectively [1]. A combination of drugs that target epigenetic factors, together with conventional anti-cancer drugs, could prove to be an effective means of preventing the development of or even reversing drug resistance. Epigenetic inhibitors were recently shown to selectively target drug-resistant subpopulations in multiple cancer types and to forestall resistance during co-treatment[46]. This exciting possibility must be weighed against previous evidence that epigenetic inhibitors can also promote endocrine resistance [16]. Further study is required to better understand how altering epigenetic pathways with therapeutics could promote or inhibit endocrine resistance and drug resistance in general.

Rapid advances in sequencing technologies, spurred by the Human Genome Project, are revolutionizing epigenetics. New sequencing-based approaches to assess DNA methylation provide unprecedented coverage with relatively low costs, and costs continue to fall with each passing year. The expected proliferation of genome-wide studies will enable the analysis of perturbed networks of genes rather than single genes in isolation. As we proceed into the genomic age, this network-based approach will be an exciting new frontier that promises to advance our understanding of the etiology of cancer in ways not previously possible.

This is an exciting time for epigenetics research, but it is also a challenging time. With the introduction of sequencing-based technologies also come mounting computational challenges. Going forward, biologists will need to cooperate with computational scientists more than ever to harness the power of these new technologies. These collaborations will allow us to continue to progress in the face of increasingly sophisticated technologies and complex scientific questions.

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