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Epigenetic Changes During Cell Transformation

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Abstract

Malignant cancer emerges from normal healthy cells in a multistep process that involves both genetic and epigenetic lesions. Both genetic and environmental inputs participate in driving the epigenetic changes that occur during human carcinogenesis. The pathologic changes seen in DNA methylation and histone posttranslational modifications are complex, deeply intertwined, and act in concert to produce malignant transformation. To better understand the causes and consequences of the pathoepigenetic changes in cancer formation, a variety of experimentally tractable human cell line model systems that accurately reflect the molecular alterations seen in the clinical disease have been developed. Results from studies using these cell line model systems suggest that early critical epigenetic events occur in a stepwise fashion prior to cell immortalization. These epigenetic steps coincide with the cell's transition through well-defined cell proliferation barriers of stasis and telomere dysfunction. Following cell immortalization, stressors, such as environmental toxicants, can induce malignant transformation in a process in which the epigenetic changes occur in a smoother progressive fashion, in contrast to the stark stepwise epigenetic changes seen prior to cell immortalization. It is hoped that developing a clearer understanding of the identity, timing, and consequences of these epigenetic lesions will prove useful in future clinical applications that range from early disease detection to therapeutic intervention in malignant cancer.

9.1 Introduction

Malignant cancer cells arise from normal cells via a multistep process that involves both genetic and epigenetic change. Similar to genetic lesions, epigenetic lesions can be diverse in nature, serving to alter the structure and function of the genome thereby participating in a cell's acquisition of limitless uncontrolled growth and the phenotypic hallmarks of the malignant cancer cell. In general, the degree of epigenetic difference between cancer cells and normal cells greatly exceeds the epigenetic differences that are seen between normal cells of different phenotypes and even different germ layers (e.g., fibroblasts and epithelial cells). Since epigenetic mechanisms are a primary determinant governing normal cell identity, this comparison underscores how epigenetically different cancer cells are from normal cells. Mutation and altered expression of proteins involved in the writing or reading of the epigenetic code are two mechanisms that help produce aberrant epigenetic changes seen in not only cancer, but other human diseases as well. The complexity and the frequency of the epigenetic changes seen in cancer cells, however, seem to defy explanations that rely on a single event. Instead, it appears that pathologic epigenetic change during carcinogenesis results from myriad genetic mutations and environmental inputs which perturb the manifold nodes of epigenetic regulation.

Environmental inputs acting on the epigenetic nodes are highly variable and can include contributions from both physiologic and xenobiotic sources such as hormonal status; microenvironmental milieu; nutritional, metabolic, or oxidative state; and toxicant and

therapeutic drug exposures. Since the epigenetic state is important in governing cell identity, cellular nodes of epigenetic control acted upon by stimuli will show some variation between different cell types, suggesting that environmental inputs may show cell type selectivity, as well as display activity towards a broad array of cell types. Once these epigenetic changes are “fixed” into the chromatin, they can be vertically transmitted through cell generations. The inherent plasticity of the epigenetic control systems coupled to the cancer cell's limitless replicative potential provides the ability to generate extraordinary phenotypic diversity and rapidly respond to changing environmental stimuli and stresses.

Chromatin is rich in epigenetic marks, and these marks participate in the regulation and control of likely most or all genomic functions. The primary epigenetic mark found on DNA, 5-methylcytosine, is produced via the enzymatic methylation of the C5 position of cytosine through the action of multiple specialized DNA methyltransferases. The patterns and levels of DNA methylation across the genome have been mapped for a variety of normal and cancer cells, with cancer cells showing complex and extensive patterns of DNA methylation derangements. These DNA methylation derangements either participate in or reflect a number of different genomic processes, with its role in the regulation of gene expression being the best understood. Other C5 cytosine modifications have been identified recently, such as 5-hydroxymethylcytosine. It appears that these newly identified modifications are a result of an active DNA demethylation process and it is likely that these DNA epigenetic marks will prove biologically important; however, it has not yet been elucidated how these marks change and participate in the process of malignant transformation.

Posttranslational histone modifications are an additional layer of epigenetic control altered during human carcinogenesis. These posttranslational modifications include acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation, and over 40 different amino acid residues in histones are currently known to undergo one or more of these modifications, especially in histones H3 and H4. Similar to DNA methylation, the histone posttranslational marks participate in a number of different genomic processes. Some histone marks are highly predictive of gene promoter location and transcriptional activity, such as histone H3K4 trimethylation and histone H3 and H4 lysine acetylation, and these modifications show strong negative correlations with DNA methylation levels in a typical genomic region. Other posttranslational histone modifications are linked to a transcriptionally repressed state and display positive correlations with DNA methylation levels, such as H3K9 methylation repressive marks. Still other histone marks, such as H3K27 trimethylation, are closely linked to transcriptional repression, preferentially target developmentally regulated genes and largely appears to be a repressive epigenetic control system that operates independently of the repressive DNA methylation system. Overall, a number of in vitro studies have provided clear mechanistic links between DNA methylation and histone modification state indicating that the control of the DNA methylation and histone modification patterns are deeply intertwined. As such, it is not surprising that, similar to DNA methylation, the normal levels and patterns of histone posttranslational modifications become compromised in human cancer cells.

In a clinical setting, the multistep nature of epithelial cell malignant transformation manifests as hyperplasia, dysplasia, benign tumor, carcinoma in situ, and finally frank malignancy and metastases; analogous pathologic progressions can be seen in some hematologic pathologies, as well, and may very well exist for most or all human cancers. Analysis of clinical specimens has shown that epigenetic aberrations are seen in the earliest stages of this multistep process, although obtaining quantitative information-rich epigenetic data from minute clinical specimens creates unique technical challenges that have slowed the ability to identify pathoepigenetic events that directly translate to clinical impact with

respect to the detection, prognostication, treatment, and management of human cancer. For example, technical limitations such as specimen size and quality have hindered success in analyzing the posttranslational modification state of histones in clinical specimens. With respect to DNA methylation analysis, quantitative high resolution approaches for the analysis of the minute clinical cancer specimens typically available have been available for over 20 years in the form of bisulfite sequencing [1, 2], and today comprehensive DNA methylome sequencing approaches have emerged and should attain wide availability over the next few years [3, 4]. In the translational science arena, there are a few early applications where the results indicate DNA methylation analysis may be a useful tool in predicting response to cancer therapy [5, 6]. Results such as these should provide significant optimism and encouragement to investigators that epigenetic analysis will prove useful in the areas of prediction, detection, prognostication, as well as treatment of cancer. While significant progress has been made in understanding the causes, consequences, and temporal sequence of pathologic epigenetic events in cancer, their utility on the clinical management of cancer is largely a promissory note with their potential not yet fully realized.

9.2 Laboratory Model Systems of Cell Transformation

To better discover and understand the pathoepigenetic events that mechanistically participate in the conversion of a normal cell to a malignant cell, there is value in using experimentally tractable model systems that faithfully reflect the *in vivo* process. To this end, a variety of useful and complementary *in vitro* human cell line and animal model systems have been developed that recapitulate aspects of clinical multistep carcinogenesis and that allow for detailed analysis of epigenetic/epigenomic events as they unfold during the transformation from the normal to the malignant phenotype. These models have a number of advantages as laboratory tools—certainly the most important being that the genetic and epigenetic changes present in them accurately reflect the known (epi)genetic etiology of the clinical form of the disease, thereby providing a solid platform for the discovery and dissection of new epigenetic events relevant to clinical cancer. These cell line systems also allow for the production of pure and reproducible populations of cells that can be fairly easily generated in large number and at relatively low costs. In our experience, the epigenetic state of the cell line models we have employed does not vary to a significant extent when grown under appropriate and consistent conditions. We routinely verify cell line identity using STR profiling using 13 CODIS markers; reference DNA fingerprinting data for most of the widely used cell lines are available from cell line collections such as the ATCC or from the investigators who developed the models [7, 8].

A majority of the human cell culture model systems that have been developed perhaps best address the final step(s) of malignant human cancer, specifically the steps that follow cell immortalization. Since immortalization through telomerase activation may be a rate limiting step in human carcinogenesis, these models may not be best suited for the identification of the earliest epigenetic events in carcinogenesis. Cell model systems that adequately address the earliest steps in human carcinogenesis, prior to cell immortalization, are more limited. These are discussed later in the chapter. As is always the case, each model system used to evaluate the steps from normal finite life span cell to immortal malignant cancer cell has distinct qualities and limitations. Together, these laboratory models allow for the molecular dissection of epigenetic dysfunction during the pathologic process and help provide new insights that can be used to develop approaches to better detect, prognosticate, treat, and manage the myriad human cancers.

9.3 Immortalization to Malignant Transformation

Cell line systems that model the epigenetic events that occur following epithelial cell immortalization are widespread and provide useful tools to study malignant transformation (meant here as the *in vitro* assessments of anchorage independent growth and tumor forming ability in immunocompromised mice). These immortalized cell line model systems have generally overcome normal cell proliferation barriers either by (1) direct immortalization of primary cell strains through overexpression of hTERT, (2) selective genetic strategies that inactivate the p16/Rb and p53 pathways, frequently via viral approaches, or (3) establishing cell lines from cultured pathologic specimens that are already immortal, but not fully malignant. A variety of immortalized variants of different epithelial cell models have been generated and examples include, but are not limited to, prostate epithelial cells immortalized by HPV18 (RWPE), bronchial epithelial cells immortalized with SV40 (HBE16, BEAS-2B), keratinocytes that arose spontaneously in culture from primary cells (HaCAT), breast epithelial cells derived from diseased tissue (MCF10A) or non-diseased healthy tissue (HMEC), and urinary bladder cells immortalized with hTERT or SV40 (UROtsa) [9–18]. Although some approaches used to immortalize cells are not themselves etiologic agents involved in clinical human carcinogenesis (e.g., viral inactivation of p53 or the genetic introduction of hTERT), they do provide reproducible approaches that target proteins and pathways known to be critical to the human tumor cell phenotype.

These immortalized cell line systems should not be considered normal cells; however, since they have had perhaps the most dramatic phenotypic shift possible—acquisition of limitless replicative potential. In addition, these cells have often also acquired genetic abnormalities (e.g., deletions, translocations, aneuploidy). It is highly likely that these immortalized cells have undergone changes in the epigenetic state, if compared to its normal finite life span counterpart, although detailed studies to this end are limited. Indeed, the p53 inactivation strategies used in immortalization strategies may instigate epigenetic change itself. Following a cellular stress, activated p53 binds to DNA in a sequence-specific manner while also recruiting coactivators or corepressors to participate in transcriptional regulation. Thus, loss of p53 binding and coactivator/corepressor recruitment may produce long-term epigenetic changes at p53 target loci disrupting their normal transcriptional regulation and altering attendant cellular phenotypes [19–21]. As such, these immortalized models likely provide more limited information regarding the nature of the epigenetic changes that may occur early in multistep carcinogenesis and prior to immortalization. Overall, these models have proven useful in identifying novel epigenetic changes, the molecular mechanisms responsible for these epigenetic changes, and the genetic and/or environmental events that provoke the epigenetic changes.

9.4 Epigenetic Remodeling by Environmental Arsenicals

Our laboratory has been interested in the effect that environmental arsenicals has on the epigenetic state. Arsenic is a widespread environmental toxicant that exists as a number of different molecular species and ranks as the 20th most common element in the earth's crust. Humans may be exposed to arsenicals to varying degrees through water, air, soil, and food. Arsenic may also be the world's most well recognized poison. Acute high dose exposure to arsenic has been used repeatedly throughout history for murder by intentional poisoning and has earned the moniker, “Poison of Kings and King of Poisons [22].” In contrast, various forms of arsenic have also been used for centuries to treat a wide range of illnesses, including syphilis, malaria, asthma, chorea, eczema, psoriasis, and cancer [23]. Today, one molecular species of arsenic, arsenic trioxide (As_2O_3) is an FDA-approved therapy to treat acute promyelocytic leukemia and also shows promising anticancer activity in laboratory models of other human cancers [24–26]. In the most common setting, however, that of

chronic low dose, environmental exposures, arsenicals are associated with a number of human maladies, among them cancer, neurologic disorders, cardiovascular disease, developmental abnormalities, and diabetes [27–30].

Of all the pathologic effects associated with long-term arsenic exposure, cancer is the most widely studied. A number of epidemiological studies have convincingly linked human arsenic exposure with various cancers, especially cancers of the lung, urinary tract, and skin [31]. Arsenicals are classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC); however, a precise mechanism of arsenical action remains wanting. A few observations suggest that epigenetic remodeling may be important in arsenical-associated cancers. Arsenicals do not appear to cause point mutations and on their own are unable to cause cancer in standard animal assays or immortalize primary human epithelial cells [32, 33]. However, earlier studies showed arsenicals can change DNA methylation levels [34], and long-term nontoxic exposure to arsenicals has been sufficient to reproducibly induce malignant transformation in a variety of immortalized nonmalignant human epithelial cells derived from tissues with known arsenical sensitivity. Examples of cell line models that have been malignantly transformed by arsenicals include HaCaT, BEAS-2B, RWPE, and UROtsa [35–39].

Human transitional carcinoma of the bladder arises from the transformation of urinary bladder epithelial cells, and those tumors that progress clinically to a malignant phenotype generally demonstrate genetic inactivation of the p16/Rb and p53 pathways [40]. In vitro, benign immortalized urothelial cell lines that resemble the earlier stages of clinical bladder cancer can be reproducibly generated from finite life span urothelial cell strains via genetic manipulations that target these pathways for inactivation. In our studies of epigenetic changes that occur during the transition from a benign immortal cell to a malignant cancer cell, we have used the immortalized, non-tumorigenic human urothelial cell line, UROtsa, generated from the urothelial cells of a young female donor and immortalized using a temperature sensitive SV40 large-T antigen construct [14]. Further evaluation of these cells has revealed hypodiploidy, genetic deletion of a small region of chromosome 9 that contains p16, and hTERT expression (unpublished observations).

Malignant transformation of UROtsa cells using long-term nontoxic exposures to environmental toxicants such as arsenic has been successfully performed by multiple independent laboratories [36, 39]. The phenotypic manifestations of the malignant conversion process can first be detected in these cells at approximately 12 weeks of exposure at a faster growth rate. With increased exposure time, the ability to form colonies in an anchorage independent fashion occurs, and finally arsenic-exposed UROtsa cells acquire the ability to form tumors in immunocompromised mice. Interestingly, the arsenical-induced malignant phenotype is stable, as removal of the toxicant for at least 6 months has not led to the reversion to a more benign phenotype (Fig. 9.1).

Broad epigenetic changes begin to rise in UROtsa cells during exposure to arsenic at concentrations seen in real-world situations, such as can be found in drinking water from wells (5–10 ppb). We examined epigenetic changes in a genome-wide and temporal manner using histone modification-specific chromatin and 5-methylcytosine-specific immunoprecipitations coupled to two-color DNA microarray analysis. We found global changes emerging around 12 weeks after initial exposure. These epigenetic changes appear progressive—the degree of epigenetic change increases at the individual targets with time. The epigenetic changes also are stable—after malignant transformation, the toxicant can be removed, but the malignant phenotype as well as the epigenetic changes remains. Some of the epigenetic changes identified were in genes overtly relevant to the malignant phenotype and have functional roles in cancer in general, and bladder cancer in particular [41], while

the roles for most of the changes seen remain enigmatic. It appears unlikely that the observed epigenetic changes seen in UROtsa following arsenical exposure are simply due to the outgrowth or simple selection of a preexisting clone, since the arsenical-transformed cells grow significantly faster (~35%) than the nonmalignant parental UROtsa cell line. Rather, it seems possible that (epi)genetic alterations may arise during and as a result of arsenic exposure, and given enough time (cell divisions), which is provided by the cell immortality, and optimal growth conditions, a faster growing, more malignant population of cells emerges, which are then selected for based on their growth characteristics.

Probing the DNA methylation profile of the arsenical transformed UROtsa cells and comparing them to the non-transformed immortal parental cells revealed that ~3% of the assessed regions were hypermethylated, while ~1% were hypomethylated. The hypermethylation events occurred mostly within gene promoters, whereas the hypomethylation events were more prevalent in repetitive elements spread throughout the genome [42], consistent with what is well established for human cancers. We attempted to assess whether the DNA methylation changes acquired during malignant transformation were specifically or randomly distributed in the genome by analyzing two different arsenical-transformed UROtsa cell lines, created in two different laboratories using two different arsenicals (i.e., sodium arsenite and monomethyl arsenous acid). A statistical analysis of the numerical size of the overlap of aberrantly DNA methylated promoters between these two cell lines indicates that the DNA methylation changes seen are nonrandom and suggest that common epigenetic changes occur in association with arsenical malignant transformation.

The types of DNA methylation changes observed during the arsenical-mediated malignant transformation can be roughly divided into two groups, focal and long range. Focal DNA methylation events refer to DNA differentially methylated regions that cover a single gene promoter and are typically 1 kb in size. These types of aberrant DNA hypermethylation events seem to predominate and are closely linked to the silencing of a large number of tumor suppressor genes. In the UROtsa malignant transformation model, several potential tumor suppressor genes were found to be hypermethylated such as DBCCR1 (deleted in bladder cancer chromosome region candidate1); its relevance to bladder cancer having been previously ascertained [41]. Overall, the DNA hypermethylation changes were correlated to corresponding losses in the permissive histone modification marks of histone acetylation and H3K4 methylation and loss of gene expression, although as is often the case, apparent exceptions to the general rules could also be detected.

The DNA differentially methylated regions that cover much larger contiguous regions, along with corresponding changes in histone modifications, are linked to chromatin remodeling of more extended regions of the genome in a process termed long-range epigenetic silencing [43]. This type of epigenetic lesion has been found in a number of human cancer cell lines as well as clinical tumor specimens, suggesting that this type of coordinate epigenetic regulation over large regions may be a common and important event in cancer [43–46]. Interestingly, it appears that the gain of aberrant agglomerative DNA methylation changes and associated long-range epigenetic silencing can be observed over the time course of arsenical-mediated transformation of UROtsa from a benign to a malignant phenotype. Recent studies in the laboratory indicate that the PCDH and HOXC gene clusters undergo extensive aberrant DNA and that these epigenetic lesions are also found in malignant human bladder cancer specimens. Overall, these results suggest that the UROtsa malignant transformation model may be a laboratory tool to discern the molecular underpinnings responsible for long-range epigenetic silencing and identifies a significant environmental toxicant as a possible etiologic agent of this pathologic epigenetic lesion.

In an initial measure evaluating the commonality of the epigenetic change in arsenical-induced malignant transformation, we sought other human epithelial cell line models of arsenical-mediated malignant transformation. The immortalized human prostate epithelial cell line RWPE-1 was shown to undergo genomic hypomethylation after chronic exposure to AsIII [47, 48], and we have made preliminary comparisons between this model and the UROtsa model. We have found a significant overlap in gene promoters targeted for aberrant DNA methylation in both the UROtsa and RWPE models of arsenical-mediated malignant transformation that is beyond what is expected by random chance. These results suggest that a common ground of epigenetic change occurs in these laboratory models of arsenical exposure and suggests that they may be useful to help identify new epigenetically targeted genes important to malignant transformation and the cellular processes responsible for these epigenetic changes.

Epigenetic regulation resides at a nexus of gene–environment interactions. Together these results suggest that environmental arsenicals may exert their carcinogenic activity by eliciting epigenetic change thereby acting as an epimutagen, an agent whose exposure induces stable and heritable changes to the epigenetic state. The epigenetic changes seen are linked to gene expression changes and coincide with the advent of an increasingly malignant phenotype. Furthermore, results from epigenome-wide analysis suggest that common regions are epigenetically targeted during arsenical-mediated malignant transformation. Importantly, the DNA methylation changes seen in the laboratory models are consistent with what is seen in the relevant *in vivo* correlates—clinical cancer specimens. These experimentally tractable systems provide a unique opportunity to better discern the causes and consequences of epigenetic change in arsenical-associated cancers.

9.5 Epigenetic Models of Finite Life span to Immortalization (and Beyond)

A cell model we have found particularly useful to study the epigenetics of cell transformation is the human mammary epithelial cell (HMEC) model system developed by Dr. Martha Stampfer during the past 30 years [9, 44, 49, 50]. The utility of this model system for the examination of the early molecular events in human breast carcinogenesis has been demonstrated in a number of studies, both with respect to genetic and epigenetic events [49–53]. In our estimation this isogenic cell model system offers a number of benefits and allows for the temporal analysis of molecular events that occur during the transitions from finite life span through immortalization and on to malignant transformation. This model also allows one to study the effects that directed genetic changes and environmental stressors can have on the epigenetic state.

In this model system, cultured finite life span HMEC must overcome two distinct proliferation barriers in order to achieve immortality and ultimately acquire a malignant phenotype. The first proliferation barrier is termed stasis or stress-induced senescence and is mediated by the Rb protein, characterized by elevated levels of p16INK4A. This first barrier, stasis, has been overcome or bypassed in cultured HMEC by various means, such as exposure to benzo(a)pyrene. The resultant post-stasis cells commonly show p16 inactivation by gene mutation or promoter hypermethylation [50, 54]. Loss of p16 expression due to silencing or mutation is also a common event during *in vivo* human breast cell transformation [55]. When grown in a serum-free medium, rare HMEC will “spontaneously” silence p16, generating a type of post-stasis HMEC population that has been called post-selection [9, 54]. HMEC that escape the stasis barrier can continue to proliferate for dozens of additional population doublings before encountering a second more stringent proliferation barrier resulting from critically shortened telomeres [49, 56]. When approaching the telomere dysfunction barrier, HMEC exhibit increased chromosomal instability and a DNA damage response. Rare cells that gain telomerase expression may escape this barrier and

become immortal, whereby HMEC activates telomerase by as yet undefined, and potentially novel, epigenetic mechanisms. In addition, HMEC systems can acquire immortality through genetic perturbations. For example, under appropriate circumstances direct genetic introduction of constructs that express CMYC, or ZNF217, hTERT can promote HMEC immortalization [57, 58]. Nondirected mutagenesis can also promote HMEC immortalization, as evidenced by the effects of the complete carcinogen benzo(a)pyrene on HMEC. This limitless replicative potential allows for the acquisition and accumulation of additional epigenetic and genetic events that promote the development of additional malignant properties [50, 59–61].

We have used this HMEC model system to begin to develop a timeline of the DNA methylation changes that occurs over the course of multistep breast carcinogenesis, with a particular interest on the earliest stages of the process. Figure 9.2 shows a generalized view of cells we have analyzed, their temporal position in relation to the cellular proliferation barriers, the approximate clinical correlates, and the timing of DNA methylation changes. This figure is an example and not an exhaustive or detailed review of the HMEC strains and cell lines or the multiple treatments and exposures used to create them, and for a more detailed view one can see [62] or visit <http://hmec.lbl.gov/mindex.html>. In our initial studies using this model system, DNA methylation state was determined using 5-methylcytosine antibody immunoprecipitations (MeDIP) coupled to two-color hybridization on a custom 13,500 element human gene promoter microarray and verified using the orthogonal technology of mass spectrometric analysis using Sequenom MassArray [63].

Overall, in this model we observed a stepwise progression of DNA methylation changes with each step coinciding with overcoming a cellular proliferation barrier [62]. In HMEC that overcame stasis produced by stress-inducing serum-free medium, we found, in addition to p16 methylation, hundreds of other differentially methylated regions in the post-stasis cells when compared to pre-stasis cells, representing approximately 2% of all gene promoters on the microarray. These DNA methylation events were both of the focal and long-range variety. Considering that probably 5–10% of gene promoters in malignant cancer cells show aberrant DNA methylation, a considerable number of DNA methylation changes may occur very early in multistep breast carcinogenesis, and these changes are coincident with overcoming the critical Rb/p16 cell proliferation barrier. Since a majority of the DNA methylation changes seen in the transition of HMEC from pre-stasis to post-stasis in this setting are also seen in malignant breast cancer cell lines and tumor specimens, this transition through the stasis proliferation barrier may represent a critical early event in some pathways of human breast carcinogenesis.

It is worth noting here that current commercial sources of HMEC appear to be of this post-stasis (or post-selection or variant) stage, since these HMEC are produced via the process described above—post-stasis cells that emerge from serum-free media induced stress. As such, the commercially available HMEC may have not only undergone p16 DNA methylation, but are likely to have also acquired hundreds of additional aberrant DNA methylation events [62]. As such, caution should be exercised when evaluating the epigenetic state of primary epithelial cells and considering what is epigenetically “normal.”

HMEC that become post-stasis following exposure to the genotoxin and complete carcinogen benzo(a)pyrene showed more than an order of magnitude reduction in DNA differentially methylated regions when compared to the DNA methylation changes induced by stressful serum-free growth conditions. Similarly, HMEC that became post-stasis following genetic knockout of p16 using p16-targeted shRNA have very few DNA methylation changes, underscoring the functional importance of p16 in the first growth barrier. The few DNA methylation changes seen in the benzo(a)pyrene and p16 shRNA-

treated cell lines suggest that different pathways through the stasis barrier will have distinct effects on the epigenetic state.

A second step of epigenetic change occurs when telomere dysfunction is overcome and cells acquire immortality. Regardless of the mechanism by which cells pass through telomere dysfunction, hundreds of DNA methylation changes occur. Similar to the DNA methylation changes acquired during the pre-stasis to post-stasis transition, changes that occur during the transition from finite life span to immortal can be focal (1 kb) and limited to a single gene or the changes can represent examples of long-range epigenetic silencing and cover extended regions of the genome [64].

These changes seen in the premalignant stages represented by the HMEC model show significant overlap to the DNA methylation changes seen in other human breast cancer cell lines and clinical tumor specimens. Overall, results from the studies using the HMEC model indicate that epigenetic changes occur in a stepwise fashion at critical junctions in the path to cell immortality. These results are consistent with an epigenetic progenitor model where epigenetic changes may occur early, in a stepwise fashion, can precede genetic mutation and allow for an expansion of epigenetically compromised population of cells. The large number of genes affected by epigenetic changes during the transitions through proliferation barriers can provide a foundation for the phenotypic variability and biologic heterogeneity often seen in clinical disease. The DNA methylation changes identified can potentially provide a bank of epigenetic biomarkers for assessing breast cancer risk in premalignant lesions and provide targets for therapeutic interventions.

9.6 Conclusion

In summary, complex and intertwined epigenetic changes occur during multistep carcinogenesis. These changes may be viewed as epigenetic lesions and exist in the genome in a number of forms, from focal to long range. The scope of the epigenetic lesions is likely due to multiple distinct inputs: genetic, such as mutations to chromatin modifier genes; physiologic, such as hormonal and nutritional state; and environmental, such as toxicant exposures. Experimentally tractable laboratory model systems that accurately reflect clinical cancer have been developed and allow for investigations into the causes and consequences of epigenetic change during cell transformation. Results from these systems suggest that early critical epigenetic events occur prior to cell immortalization and coincide with the transition through well-defined barriers of cell proliferation. Following immortalization, laboratory models suggest that cells can be induced towards malignancy by a variety of stimuli, and that the epigenetic changes arise in a seemingly more progressive smoother fashion, as opposed to the stark stepwise events prior to immortalization. It is hoped that developing a clearer understanding of the identity, timing, and consequences of these epigenetic lesions will prove useful in future clinical applications that range from early disease detection to therapeutic intervention in malignant cancer.

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Fig. 9.1.

UROtsa cell line model of malignant transformation. The immortalized urothelial cell line UROtsa was exposed to arsenicals for periods of up to a year. Arsenical exposed cells were probed at various time points for markers of malignant transformation. After 3 months there was a significant increase in proliferation rate, after 6 months a significant increase in anchorage independent growth, and after 12 months, arsenic exposed cells formed tumors in immune compromised mice [36, 39]. Progressive epigenetic changes occur during this transition from a benign immortal to malignant phenotype

Fig. 9.2.

Schematic representation of breast cancer progression and the timing of the underlying DNA methylation changes, with connections between the in vitro HMEC model system and clinical progression based on earlier work [51, 56, 65]. *Top*, the clinical correlates of the HMEC system in relation to the temporal position of the two epithelial cell proliferation barriers of stasis and telomere dysfunction that divides the timeline into pre-stasis, post-stasis, immortal, and malignant epithelial cells. *Middle*, a very simplified view and two examples of HMEC culture models, and the treatment or genetic manipulations used to generate these models. *Bottom*, the timeline of DNA methylation changes identified during the passage of finite life span HMEC through stasis, telomere dysfunction, and culminating in a malignant phenotype. *Arrows* on the DNA methylation changes *curve* show the time points analyzed for DNA methylation state