Published in final edited form as:

Epigenomics. 2011 April 1; 3(2): 145–155.

Cancer cells' epigenetic composition and predisposition to histone deacetylase inhibitor sensitization

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Abstract

Normal cells are up to ten times more resistant to histone deacetylase inhibitors (HDACis)-induced cell death compared with transformed cells. The molecular processes underlying this selectivity for cancer cells are still not well understood. Although a differential response to oxidative stress and capacity to repair damaged DNA have been described in some systems, these cannot fully account for the sensitivity of cancer cells to HDACis since the heterogeneity of cancer cells prompts differential sensitivities to reactive oxygen species and generates a panoply of defective DNA repair mechanisms within given histologies, cancer cell lines and tumor xenografts. It seems also unlikely that the influence of HDACis on cancer treatments reside primarily on gene transcription, since gene-expression profiling aimed at defining correlation with response to HDACis in cancer cells indicates that less than 5% to approximately 20% of transcribed genes are altered by HDACis treatment. Moreover, the altered genes vary from cell line to cell line and between different HDACis. Therefore, no consistent picture of a target(s) or pathway(s) modulated by HDACis has emerged. One consistent parameter that has however been observed in peripheral blood mononuclear cells of patients treated with HDACi is the accumulation of acetylated histones. Because one of the primary functions of histone acetylation is to increase chromatin accessibility, this article will explore the possibility that intrinsic molecular and structural characteristics of cancer cells provide a selective advantage for HDACis sensitivity.

Keywords

cancer cell morphology; chromatin; epigenetic; HDACi; histone deacetylase inhibitors

Cancer cell predisposition: morphology

As early as 1860, abnormal structural morphology of unstained cancer cells obtained from the sputum of a patient with cancer of the pharynx was described [1]. The cancer cells had different nuclear sizes and cellular shapes compared with the normal cells. To this day, cancer is still often diagnosed by an abnormal cellular and nuclear morphology [1]. The Pap test, for example, which uses a staining technique developed by George Papanicolaou, is still the main test used worldwide to detect precancerous and cancerous cells of the cervix. The early detection of a broad range of abnormal cytoplasmic and nuclear structural features [2] has reduced cervical cancer death rates by 74% between 1955 and 1992 in the USA

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Financial & competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

alone (American Cancer Society). Increasing abnormalities of nuclear morphology are reliable markers correlating with colon, thyroid, endometrium, lung, prostate and breast cancer progression [3]. The progressive distortion of nuclear structures is thus a relatively generalized phenomenon in neoplasia [4]. Abnormal nuclear morphology associated with malignancy include an increased size of the chromatin granules and chromatin clumping, sharp angularity of large nucleolar and/or chromocenter borders with pointed projection (spicule), irregular parachromatin clearing, uneven thickness of chromatin at the nuclear border and cell-to-cell variability of nuclear size and shape [5]. Although some of these alterations can be characteristic of a given tumor type and used for diagnostic staging, they are most likely related to the altered functional properties of cancer cells. While it is still difficult to understand the relationship between nuclear morphology and the malignant phenotype, insights can be gleaned from the resulting structural changes and their possible effects on drugs targeting DNA or enzymes acting on the DNA.

The 3D spatial arrangements of the nucleus are in part maintained by complex attachments to the nuclear matrix which is essentially devoid of histones and lipids [4]. The nuclear matrix is a dynamic fibrous network of proteins, RNA and polysaccharides supporting specific subcomponents such as a pore complex, lamina, residual nucleoli and internal ribonucleoprotein particles [4]. The regular and elliptical shape of most normal cells nuclei is believed to be dictated by the lamina. The lamina is located at the inter-face between the inner nuclear membrane and chromatin. This layer is composed of proteins; lamin A/C and lamin B and their associated proteins [6]. Therefore, the often absent or defective lamina proteins in cancer cells likely contribute to the irregular contour of cancer cells nuclei [1]. Because the nuclear matrix participates in spatial organization of DNA sequences in the nucleus, much of the cellular metabolism such as DNA, RNA and protein synthesis is affected by changes in cellular shapes [7–9]. In fact, more than three decades ago the role of cell shape in growth control was recognized [10], and shortly thereafter the loss of metabolic control was correlated to increasing transformed phenotype [11]. Although the development of cancer is clearly associated with distorted nuclear structures [4], the relationship between abnormal nuclear morphology and molecular genetic alterations is still ill defined [3]. The cellular events leading to DNA reorganization in cancer cells include gene amplification, sister chromatid exchange, a series of different subtypes of rearrangements such as translocation and a variety of deletions. Each of these events perturbs the normal order of DNA sequence arrangements and may be the triggering point to initiate carcinogenesis. Any reorganization of the cellular DNA that can confer a growth advantage is susceptible to cause hyperplasia or tumor formation [1]. Chromatin DNA in normal cells is attached at several points to the inner surface of the nuclear lamina (Figure 1) [12]. This layer of peripheral chromatin associated with the lamina is transcriptionally silent [1]. Chromatin DNA is also organized in loop domains that are formed by periodic attachment of chromatin at their base to DNA sequences called matrix attachment regions (MARs) to the nuclear matrix which topologically constrain the DNA (Figure 2A) [13]. Alteration in proteins that binds to MAR can have devastating consequences on cellular functions. For example, knocking out the MAR-binding protein SATB1 affects the position of genes that usually bind SATB1, moving them from chromatin loop bases to more peripheral positions. As a consequence, SATB1 null mice die by 3 weeks after birth [14]. This exemplifies how vulnerable cells are to alteration of higher order chromatin structure and the effect that the loss of a single MAR-binding protein can have. In cancer cells when the nuclear chromatin organization is distorted, genes could also become accessible to the transcriptional machinery and contribute to transformation [1]. Accessibility to anticancer drugs targeting the DNA or enzymes acting on the DNA is likely to be modified as well.

In normal cells, each chromosome occupies a discrete region of the nucleus better known as chromosome territories (CT) (Figure 2C) [1,15]. Although the positioning of CT is cell type

specific, alterations in CT positioning observed in tumor cells do not require karyotypes changes [16]. It is thus believed that smaller chromosomal subregions or even individual genes could regulate CT positioning with chromatin. Alterations in CT positioning could affect the rate of the chromosomal translocations that arise from illegitimate DNA end joining of closely positioned loci [17], which could contribute to transformation. When CT are moved around, the compaction of chromatin domain is likely to be altered as well and the resulting chromatin texture changed. Change in chromatin texture is one of the most important criteria for cancer cell diagnosis. Chromatin coarsening (increased heterochromatin) and exaggerated open chromatin (loss of heterochromatin aggregates) are two significant changes observed in chromatin texture. Chromatin coarsening rarely occurs in normal cells but can be induced by expression of the activated GTPase v-Ha-ras Harvey rat sarcoma viral oncogene homolog *HRAS* [18] in a variety of cell types [1]. While chromatin coarsening is not associated with cell proliferation it is closely associated with metastatic potential [19]. The mitogen signaling pathway is apparently dispensable for the *HRAS*-induced morphologic alterations since neither the mitogen-activated protein kinase kinase (MAPKK) [20] nor c-Myc could produce chromatin coarsening [19]. Heterochromatin is usually associated with transcriptionally silent chromatin considered to be inaccessible to the transcriptional machinery [21]. Interestingly, transfection of the *Ras* oncogene in NIH3T3 cells increased the sensitivity of bulk chromatin to micrococcal nuclease digestion, hence supposedly increased chromatin accessibility [22]. It thus appears that the relationships between the Ras-induced coarsening of chromatin texture and chromatin accessibility is rather complex. Although this apparent discrepancy is still not clearly understood there are several possibilities that could explain this effect. For example, even though no clear differences have been observed in the global levels of histone H1 or H1 phosphorylation between transformed and untransformed cells [19], it is possible that Ras affected the distribution of H1 away from the linker DNA as well as disrupted the nuclear matrix proteins. This could increase micrococcal nuclease digestion at the linker DNA while at the same time produce a coarsening of chromatin texture. Changes in chromatin texture are also triggered by the expression of the oncogene tyrosine kinase rearranged in transformation/papillary thyroid carcinomas (*RET/PTC*) in thyroid cells [23]. Expression of *RET/PTC* is associated with the loss of heterochromatin aggregates, which results in exaggerated open chromatin structure and distorted nuclear shape. Loss of heterochromatin aggregates is not unique to thyroid cells and has been reported in many other human tumors [24]. Therefore, irrespective of what causes the changes in the nuclear structure, chromatin conformation and accessibility is likely to differ between normal and cancer cells. The mechanisms by which oncogenes such as *HRAS* and *RET/PTC* affect chromatin structure are not well understood but could possibly involve epigenetic modulations of chromatin such as post-translational modifications (PTMs) of histones, methylation of DNA or chromatin remodeling.

Epigenetic modifications affecting chromatin compaction

The most common epigenetic modifications that occur in cancer cells are increased methylation of CpG islands within gene promoter regions and deacetylation and/or methylation of histone proteins [25]. DNA methylation, while an important epigenetic mark for gene silencing, is probably not affecting the overall nuclear morphology *per se* since no changes in global DNA methylation or methylation at a specific gene (ornithine decarboxylase) were observed in *H-Ras* transformed cells compared with untransformed cells [19]. Alterations in chromatin structure can however be achieved by incorporation of histone variants by histone chaperones, chromatin remodelers that can mobilize nucleosomes or facilitate the removal of core histones and by PTMs at specific residues in the histone tails [26]. The impact that histone PTMs have on the structural status of chromatin is most likely owing to the intricate interactions that occur between the histones

and the DNA at each layer of chromatin compaction. The core histones are organized into nucleosomes composed of a tetramer of histones (H3-H4)₂ and two H2A-H2B dimers localized on each side of the tetramer. Stretches of 146 bp DNA are wrapped around these histone octamers to constitute the structural repeat units of chromatin known as the nucleosomes. Histone H1 further compacts the chromatin into a higher structure by binding linker DNA between each nucleosome and stabilizes this complex structure [21]. This tightly organized hierarchy of DNA compaction results in the high level of packaging necessary to fold 2 m of DNA into a condensed nucleus of 5–10 µm in diameter. The core histones have positively (basic) charged N-terminal tails that protrude outside the bead like shaped nucleosomes and form electrostatic interactions with the negatively charged phosphodiester backbone of the DNA (Figure 2B) [27]. These relatively easily accessible tails are subjected to a variety of PTMs including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, proline isomerization and ADP ribosylation [25]. The presence or absence of a particular sequence or combination of any of these PTMs can increase or decrease to some extent protein–DNA interactions, and consequently, affect chromatin accessibility at a particular locus. Histone acetylation occurs in all eukaryotes and is the most important modification from a quantitative point of view. Acetylation and methylation are however the two histone PTMs clinically associated with pathological epigenetic disruptions in cancer cells [25]. More specifically, loss of histone acetylation and methylation at specific residues on core histone H3 and H4 are common markers of tumor cells [28,29]. Acetylation of the core histones has been associated with a looser more open chromatin structure that facilitates accessibility to transcription, replication and repair machinery [30]. Histone H4 possess a total of 27 positive charges and eight negative charges at physiological pH for a net charge of approximately +19. The loss of only three positive charges has an important effect on histone DNA interaction [31]. In fact, high levels of histone acetylation improve chromatin solubility which implies that the higher order chromatin structure is less stable [21]. Although constitutive hyperacetylation of the core histones does not reduce the association of histone H1 with bulk chromatin it inhibits complete condensation of interphase chromatin [21]. Therefore, levels of histone acetylation can directly impact chromatin accessibility at specific loci as well as affecting the overall structure of higher order chromatin conformation.

Histone acetylation is mediated by the histone acetyltransferases (HATs) and the reversible reaction, removal of acetyl groups, is catalysed by the histone deacetylases (HDACs). Deacetylation of the core histone neutralize the positive charges on the histone N-termini and thus results in a repressed (compacted) chromatin structure that is inhibitory to most cellular processes [30]. In cancer cells, acetylation of K16 on histone H4 (H4-K16Ac) is markedly reduced [28,32]. Studies performed with reconstituted nucleosomes homogenously acetylated at K16 on histone H4 have shown that this single modification prevents the formation of higher order chromatin structure and contributes to regions of decondensed chromatin [33]. This would suggest that some chromatin regions of cancer cells may be more repressed than normal cells and some normal cells chromatin domains may be more decondensed than in cancer cells. H4-K16 is acetylated by a variety of HATs and deacetylated by a number of HDACs that have been shown to be defective in cancers [25,34–36]. The overall effect of H4-K16Ac modifications may be dysregulation of heterochromatin which is likely to increase cancer susceptibility. Loss of trimethylated Lys20 residues of histone H4 (H4-K20me3) is another important epigenetic mark that has been associated with cancer progression [28]. Histone H4 contains 5 lysine residues in its N-terminal tail but K20 is the only lysine residue methylated in mammalian cells [37]. Methylated histone H4K20 has been related to condensed and silent chromatin [38,39]. Loss of H4-K16Ac and K4-K20me3 appear early in tumor progression and could contribute to the overall morphological changes associated with neoplasia. Methyl groups are however relatively small and do not neutralize the positive charges of lysine and arginine residues

[40]. Methylation alone is thus not expected to affect chromatin structure significantly. It is thought that methylation of histones contributes to the histone code by creating binding sites for regulatory proteins. The heterochromatin protein 1 (HP1) is such a protein that binds trimethylated lysine 9 on histone H3 (H3-K9me3). H3-K9me3 is required for the induction of H4-K20me3 and the sequential methylation of these histones may provide a mechanism to establish H3-K9 and H4-K20 trimethylation at pericentric heterochromatin [39]. HP-1 also interacts with the nucleoplasmic domain of the lamin B receptors and is therefore an important player in maintaining the peripheral chromatin transcriptionally silent. Alterations in histone methylation status could thus disrupt the equilibrium of anchoring proteins and affect cellular morphology. In fact, low levels of HP1 α correlates with highly invasive and metastatic breast cancer [41]. HP1 α is a mobile protein that could also affect cellular morphology by relocating from heterochromatin to euchromatin [42]. Therefore, regardless of the cause of the profound morphological changes occurring in cancer chromatin, the redistribution of the chromatin texture is likely to differentially affect the accessibility of cellular machineries such as repair and transcription and the efficiency of anticancer drugs targeting chromatin DNA.

Chromatin of normal & cancer cells are differentially affected by histone deacetylase inhibitors

The packaging of DNA into nucleosomal structures is known to restrict DNA accessibility to cellular proteins and to inhibit most cellular processes. Even so, this has not been exploited as a potential limiting factor for the killing efficiency of anticancer drugs targeting DNA or enzymes acting on the DNA. Nonetheless, nucleosomes are known to prevent the accessibility of topoisomerase (Topo) enzymes to DNA and incite it to act at the linker regions [43]. Indications that chromatin modulation could impact on Topo activity were provided by earlier studies on the minor groove binder distamycin [44]. This antibiotic was shown to increase Topo2 cleavage by a possible redistribution of the histone H1. Cleavage activity was stimulated by approximately tenfold by distamycin [44]. The high mobility group I/Y (HMG-I/Y^{HMGAI}) protein also increases chromatin accessibility to Topo2 [45]. Interestingly, the predicted secondary structure of HMG-I/Y^{HMGAI} DNA binding domain is similar to distamycin [46,47]. Early comparative studies of computer simulations model building (Corey–Pauling–Koltun molecular models) between HMG-I/Y^{HMGAI} DNA binding domain and the crystallographic analyses of distamycin indicated that both molecules have relatively planar backbone and a similar type of arc or crescent shape in addition to containing a number of potential hydrogen bonding donor amine groups on their concave edges [46,47]. Both distamycin and HMG-I/Y^{HMGAI} preferentially bind to A–T rich DNA stretches via highly positively charged segments called AT-hooks [46].

Pretreatment of cancer cells with histone deacetylase inhibitors (HDACis), increase the killing efficiency of a variety of anticancer drugs including drugs that are targeting the DNA or enzymes acting on the DNA such as the Topo enzymes [48,49]. Interestingly, this sensitization is specific for cancer cells since it is not observed in normal cells [48,49]. As mentioned earlier, normal cells are rather insensitive to HDACi-induced cell death compared with transformed cells [50]. Several mechanisms have been proposed to explain this preferential selectivity toward cancer cells but none can fully account for this rather generalized phenomenon that crosses multiple cell lines and tumor types irrespective of cell-line specific control of gene transcription. For example, the HDACi vorinostat (SAHA) has been shown to decrease expression of thioredoxin (TRX), a reactive oxygen species (ROS) scavenger, in transformed but not normal cells [51]. While this effect probably contributes to the differential selectivity of HDACis for some cancer cells, it does not account for the differential sensitivities to ROS that exists within given histologies, cancer cell lines and xenografts [49]. Nonetheless, downregulation of TRX could provide a favorable

environment for HDACi sensitization since ROS can increase HDAC activities and hence generate an amplification process for preferentially targeting cancer cells [50–55]. Aberrant HDAC activity has been observed in many types of cancer but the mechanisms that lead to HDAC activation in tumor cells are not well understood. Both transcriptional as well as post-transcriptional mechanisms have been reported [54,55]. It is also known that HDACs can modulate the expression of a variety of genes. However, the limited range of genes affected by HDACs and the wide variability of genes affected from cell line to cell line and between different HDACs indicate that HDACs primarily influence on cancer treatments is probably not through gene transcription. Another potential selectivity mechanism was recently proposed where the capacity of normal but not cancer cells to repair HDACi-induced DNA damage was believed to provide a preferential selectivity criterion [56]. However, a large number of anticancer drugs do damage DNA that is repaired by normal but not cancer cells and do not show this preferential selectivity. Moreover, the extent of clinically induced DNA damage that remains unrepaired with HDACs treatment is not clear. What has been clinically consistent though is the accumulation of acetylated histones in peripheral blood mononuclear cells of patients treated with a variety of HDACs [49,57,58]. What is also consistent in cancer progression is the development of an altered and distorted cellular and nuclear morphology. It is therefore possible that altered chromatin structure in cancer cells increases the accessibility to the histone tails and allow a more profound distribution of histone PTMs affecting accessibility to drug targeting DNA or enzymes acting on the DNA. Such a broad mechanism would not be HDACi specific or cancer cell type specific but would provide HDACi selectivity toward cancer cells.

Indications that HDACs affect the accessibility of normal and cancer cells differently are demonstrated in Figure 3, where cleavage at Topo2 targeted sites are measured by a PCR-Stop assay. The basic principle of the PCR-Stop assay is that a DNA polymerase encountering a break or an adduct will fall off the template or stall. Consequently, the amount of amplified DNA is inversely proportional to the levels of DNA damage. Topo2 poisons have been shown to induce prominent DNA cleavage in the P1/P2 promoter region of the *c-myc* gene [59]. Here, we optimized the original PCR-Stop parameters to the sensitivity of real time (RT)-quantitative PCR (qPCR) to evaluate the amount of DNA cleavage produced at a drug targeted site. The data shown in Figure 3B indicates that pretreating colon carcinoma (RKO) cells with a HDACi before exposure to a Topo2 inhibitor (VP-16) produced more DNA breaks (57% breaks; 43% amplification) than treating the cells in the opposite order (37% breaks; 63% amplification). In contrast to cancer cells, the nontransformed FHs Int-74 cells are not sensitized to VP-16 by HDACi pretreatment (Figure 3A). These data are in good agreement with previous survival assays showing preferential HDACi sensitization of cancer cells [48]. Validation of the PCR-Stop-RT-qPCR data as a measure of DNA strand breaks was provided by γ H2AX [60]. Figure 3D indicates that there is almost three times as much γ H2AX when RKO cells are pretreated with HDACi than when they are treated with VP-16 first followed by HDACi. Conversely, levels of γ H2AX remain relatively stable in untransformed cells (Figure 3C). As mentioned above chromatin structure restricts accessibility of the Topo enzymes and incites it to cleave primarily linker DNA. The nuclear Topo enzymes are responsible for regulating and maintaining DNA topology. By breaking and religating complementary DNA strands they reduce torsional stress encountered during cellular metabolism, such as in transcription and replication. The Topo enzymes are thus important for proliferating cells including cancer cells.

The catalytic activity of Topo can be divided into at least four steps:

- Binding of the enzyme to DNA;
- Cleavage of one (Topo1) or two (Topo2) strand(s) of DNA [61];

- Passage of the strand(s) through a nick (Topo1) or a gap (Topo2);
- Ligation of the cleaved DNA strand.

A number of anticancer agents can inhibit the reversible Topo–DNA interaction, and thus, convert the endogenous enzyme into a cellular poison by locking the enzyme on the DNA. Topo inhibitors have increased cellular sensitivity during the replicative phase (S-phase) of the cell cycle where the Topo enzymes are highly active [62]. Given this increased activity during the S-phase, it is believed that ongoing DNA synthesis is involved in the cellular killing by the Topo inhibitors. However, our data support the possibility that the looser chromatin structure generated by pretreatment of the cancer cells with HDACi facilitate Topo enzymes accessibility to chromatin DNA and consequently increase the trapping of the Topo–DNA complexes by VP-16. It is also of interest to note that the Topo2 enzyme is one of the proteins that the nuclear matrix shares with the chromosome scaffold [13,63]. Alteration in cancer cells nuclear morphology is thus also likely to contribute to the differential HDACi induced sensitivity to Topo inhibitors.

Another indication of the effect of HDACi on chromatin structure is provided by the increased UV sensitivity of cells pretreated with HDACis [64]. The formation of UV-induced DNA adducts is not random on the chromatin but is directly influenced by the nucleosomal structures. The 6–4 photoproducts are predominantly formed in linker DNA while cyclobutane pyrimidine dimers occur at sites where the DNA minor grooves face the outside of the nucleosome cores [65]. Pretreating cancer cells with HDACi increased UV killing efficiency by more than threefold [64]. This treatment also increased the formation of cyclobutane pyrimidine dimers and therefore apoptosis. Treating the cells in the reverse order had no more effect than UV exposure alone. Therefore, the HDACi mediated sensitization to anticancer drugs or other genotoxic agents such as UV radiation apparently requires a primed (open) chromatin structure since modulation of the chromatin structure after exposure to DNA damaging agents has no synergistic effects.

HDACis & cell differentiation

Although a connection between histone acetylation and cell differentiation has long been known [21], relatively little effort has been directed at clinically exploiting this effect. HDACis can induce maturation of a variety of human solid and hematopoietic tumor cell lines [66–68], thus restricting cell transformation. The mechanisms underlying HDACi mediated cell differentiation are still poorly understood but cell type specific gene re-expressions have been described [69,70]. Interestingly though, the unscheduled expression of a single transcription factor has been shown to be sufficient to reprogram a mature cell into a different cell type [71]. It has been found that a panel of transcription factors containing Myc, Oct4, Sox2 and Klf4 can convert a terminally differentiated cell into a pluripotent state cell [71]. This exemplifies the plasticity of the differentiation process. Moreover, genome-wide analysis of DNA methylation patterns recently indicated that 25% of the methylation occurs outside the CpGs islands and localized on CpAs instead [72]. CpAs methylations are only found in stem cells undergoing differentiation [72]. It is believed that one epigenetic alteration can affect others and that crosstalks between DNA methylation and histone acetylation are dysregulated in cancer cells [66]. Intriguingly, the HDACi-induced differentiation is reversible [73]. This may be a reason why clinical applications for this HDACis property have not been explored with the same intensity as the cytotoxic properties of HDACis. It remains to be determined whether the effect of HDACi-induced differentiation is influenced by the alterations in chromatin structure or if PTMs induced by HDACis trigger a chain reactions leading to cell differentiation.

Future perspective

Preferentially targeting cancer cells while sparing normal cells as much as possible still remains one of the most challenging aspects of cancer therapies. Studies on HDACis are teaching us new approaches to reach this goal. For instance, using the maximum tolerated dose may not be the best route to take when using HDACis, especially in drug combinations. Reports of patients doing as well, if not better, with much reduced HDACis doses than the maximum tolerated dose are starting to emerge and more are likely to come in the years to come. The clinical benefits of a panspecific low-affinity HDACi such as vorinostat is another important lesson for us to learn on drug binding affinity. Can combining an 'on target', 'off target' binder to a higher specificity drug be sufficient to reduce toxicity while maintaining clinical benefits? Low-affinity drugs are routinely being discarded or overlooked on the basis that they are predicted to lack specificity and generate numerous side effects. We may have to rethink this approach based on what we see with vorinostat and based on what is happening naturally in our bodies. For example, glucocorticoid hormones are released in a pulsatile manner rather than being continuously secreted. Although these hormones are highly specific for their receptors, this delivery system is most likely producing an environment where not all the targeted receptors are saturated at the same time. This may be a reason why none of the synthetic hormones, which are typically designed for high affinity binding, work as well as the natural drug. Low drug affinity such as vorinostat may thus be mimicking a pulsatile release of drug by saturating some of the targets, but not all, at any given time. Low dose panspecific effects of HDACis are consistent with the concept that discreet but targeted chromatin modulation may be at the basis of HDACis selectivity for cancer cells. It would not be surprising to find that HDACis treatments affect different histone variants and histone PTM in normal and cancer cells but that the overall patterns of the response always favors chromatin accessibility in cancer cells. This would indicate that the intrinsic structural characteristic of cancer cells predispose them to HDACis sensitivity. The questions that remain to be addressed include: what causes these structural changes and would re-establishing a 'normal' chromatin structure be sufficient to counter cancer progression? It is our hope that a better understanding of the mechanisms underlying HDACis selectivity for cancer cells will contribute to answer these questions in the not too distant future.

Executive summary

Cancer cell predisposition: morphology

- To this day, cancer is still often diagnosed by an abnormal cellular and nuclear morphology.
- Progressive distortion of nuclear structures is a relatively generalized phenomenon in neoplasia.
- The regular shape of most normal cells nuclei is believed to be dictated by the lamina that is why the often absent or defective lamina proteins in cancer cells is likely to contribute to the irregular contour of cancer cells nuclei.
- Because the chromatin DNA in normal cells is attached at several points to the inner surface of the nuclear lamina, its distortion in cancer cells is affecting chromosome territories (CT) positioning. Alterations in CT are likely to affect the rate of chromosomal translocations that arise from illegitimate DNA end joining of closely positioned loci which could contribute to transformation.
- Change in chromatin texture is one of the most important criteria for cancer cells diagnosis.

- Chromatin coarsening (increase heterochromatin) is rarely occurring in normal cells and while not associated with cell proliferation it is closely associated with metastatic potential.

Epigenetic modifications affecting chromatin compaction

- The most common epigenetic modifications that occur in cancer cells are increased methylation of CpG islands within gene promoter regions and deacetylation and or methylation of histone proteins.
- DNA methylation while an important epigenetic mark for gene silencing, is probably not affecting the overall nuclear morphology *per se*.
- Acetylation and methylation are two histone post translational modifications (PTMs) clinically associated with pathological epigenetic disruptions in cancer cells.
- Levels of histone acetylation can directly impact chromatin accessibility at specific loci as well as affecting the overall structure of higher order chromatin conformation.
- In cancer cells, acetylation of K16 on histone H4 (H4-K16Ac) is markedly reduced, this modification prevents the formation of higher order chromatin structure and contributes to regions of decondensed chromatin.
- Loss of H4-K16Ac and K4-K20me3 appear early in tumor progression and could contribute to the overall morphological changes associated with neoplasia.

Chromatin of normal & cancer is differentially affected by histone deacetylase inhibitors

- Nucleosomes are known to prevent the accessibility of topoisomerase (Topo) enzymes to DNA and incite it to act at the linker regions.
- Several mechanisms have been proposed to explain histone deacetylase inhibitors (HDACis) preferential selectivity toward cancer cells but none can fully account for this rather generalized phenomenon.
- Accumulation of acetylated histones in peripheral blood mononuclear cells of patients treated with a variety of HDACis is clinically consistent.
- Development of an altered and distorted cellular and nuclear morphology is consistent with cancer progression.
- Altered chromatin structure in cancer cells may increase the accessibility to the histone tails and allow a more profound distribution of histone PTMs affecting accessibility to drug targeting DNA. Such a broad mechanism would not be HDACi specific or cancer cell type specific but would provide selectivity toward cancer cells.

HDACis & cell differentiation

- HDACis can induce maturation of a variety of human solid and hematopoietic tumor cell lines.
- CpGs methylations are only found in stem cells undergoing differentiation.
- HDACi-induced differentiation is reversible.
- It remains to be determined whether the effect of HDACis-induced differentiation is influenced by the alterations in chromatin structure or if

PTMs induced by HDACs trigger a chain of reactions leading to cell differentiation.

Acknowledgments

The authors received financial support from NIH/NCI, RO1 1CA116491-01 (FC).

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- of interest
- ▪ of considerable interest

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Figure 1. Structural alterations of the cancer cells

Cancer progression is a multistep process. The structures of virtually all subcomponents of the nuclear and cellular matrix are altered in cancer cells. Only structures discussed in the text are shown. **(A)** In normal cells, the nuclear boundaries are dictated by the lamina (dark blue), a layer composed of proteins (lamins and associated proteins). The cytoplasmic face of the lamina is connected to the double membrane nuclear envelope while the inner surface in most cells is connected to heterochromatin (purple). The nucleoli (blue) are also surrounded by heterochromatic domains. Normal cell nuclei usually contain 1–3 nucleoli. The dynamic interactions of the different structural components provide the framework for the overall cellular shape. The nuclear matrix (internal network of nuclear matrix proteins indicated by fine black lines) is connected to the cytomatrix (microtubules, microfilaments and intermediate filaments; fine blue lines). Using the membrane matrix as an anchoring structure, the cytomatrix is in turn attached to the extracellular matrix composed of collagens, fibronectins, proteoglycans and laminins. **(B)** Some of the structural changes occurring during the transformation process include irregular nuclei shapes, appearance of coarse heterochromatin aggregations (purple) and enlarged nucleoli. In some cell types, a perinucleolar compartment (green) can also be observed. The overall cellular shape is also distorted owing to alterations of cytomatrix components. **(C)** As a cell progresses through

carcinogenesis its motility is induced by oncogenes acting on the periphery of the cell which is likely to favor metastasis.

Figure 2. Chromatin organization

(A) DNA is wrapped twice around each core histone octamers (blue) composing the nucleosomes. Stretches of linker DNA separate each nucleosome particle to constitute the bead on a string-like structure. Linker histone H1 further compacts the chromatin into a higher structure (30-nm fibers) by binding linker DNA between each nucleosome and stabilizes this complex structure. Chromatin loop domains are formed by periodic attachment of chromatin at their base to MARs to the nuclear matrix which topologically constrain the DNA. Alteration in proteins that bind to MAR (green and red ovals) can have devastating consequences on cellular functions. **(B)** The core histones have positively (basic) charged N-terminal tails (orange circles) that protrude outside the nucleosomes and form electrostatic interactions with the negatively charged phosphodiester backbone of the DNA (purple tube). These relatively easily accessible tails are subjected to a variety of post-translational modifications including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, proline isomerization and ADP ribosylation. Inhibition of histone deacetylase results in increased histone acetylation which loosens up the histone–DNA interaction and facilitates accessibility to chromatin DNA. **(C)** In normal cells, each chromosome occupies a discrete region of the nucleus better known as chromosome territories. A map of all chromosome territories found in a human male fibroblast nuclei is shown.

HDACi: Histone deacetylase inhibitor; MAR: Matrix attachment region.

Figure 3. Pretreatment with histone deacetylase inhibitors increases the amount of VP-16-induced DNA strand breaks at Topo2 targeted sites in cancer but not untransformed cells (A) PCR-stop-RT qPCR amplification of *c-myc* P1/P2 promoter from FHs Int-74 cells expressed as a percentage of amplification of treated samples over the untreated samples by DCT method. TSA/VP-16: FHs Int-74 cells were treated first with TSA (10 ng/ml) for 4 h followed by VP-16 (25 μ M) for 1 h. Following incubation, genomic DNA was isolated and subjected to RT-qPCR; VP-16/TSA: cells were treated in the reverse order; VP-16 followed by TSA. Untreated: FHs Int-74 cells that were treated with neither of the drugs were used as a control. (B) Same as (A) except that the assay was performed with RKO cells. (C & D) Protein chip array results for γ H2AX levels in untransformed (FHs Int-74) and colon cancer (RKO) cells treated with the indicated drug combination. qPCR: Quantitative PCR; RFI: Referenced fluorescence intensity; RT: Real time; TSA: Trichostatin A.