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Tight correlation of 5-hydroxymethylcytosine and Polycomb marks in health and disease

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Modifications to DNA and histone tails represent key epigenetic marks involved in establishing and maintaining cell identity and can be dysregulated in human diseases, including cancer. Two such modifications, tri-methylation of lysine-27 on histone H3 (H3K27me3) mediated by the Polycomb complex and hydroxymethylation of cytosines on DNA, have recently been shown to be dynamically regulated during differentiation. Here, we show that global levels of 5-hydroxymethylcytosine (5hmC) and H3K27me3 are highly correlated across a variety of somatic tissues. In multiple hierarchically organized tissues, both marks showed almost identical cell-by-cell distribution patterns that exhibited a tight association with differentiation. In particular, tissue stem cell compartments were characterized by low levels of both marks, whereas differentiated cell compartments exhibited high levels of 5hmC and H3K27me3. This pattern of correlation between the two marks could be recapitulated in an in vitro model system of induced differentiation in prostate epithelial cells. While the correlation between 5hmC and H3K27me3 levels is also maintained in human cancers, the degree of correlation is reduced. These findings suggest a previously unappreciated link between 5hmC and H3K27me3 regulation that should be explored in future mechanistic studies.

Keywords: 5-hydroxymethylcytosine, DNA methylation, differentiation, cancer, H3K27me3, polycomb, epigenetics

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Introduction

Epigenetic modifications comprise a series of heritable changes in chromatin organization that do not affect the primary DNA sequence. Two of the best-studied classes of epigenetic marks are (1) modifications of the DNA molecule itself, such as methylation of the 5-position of cytosines (5 mC); and (2) posttranslational modifications of histone tails in nucleosomes. The complex interactions between such DNA methylation marks and histone modifications can regulate genome organization and can orchestrate tissue-specific gene expression patterns during development and differentiation.[1] Importantly, many disease states are characterized by alterations of epigenetic marks on DNA and histones; these alterations are often associated with aberrant genome organization and gene expression. Specifically, human cancers almost universally develop dysregulation of epigenetic marks, during both cancer initiation and disease progression.[2]

DNA methylation patterns in human health and disease have been extensively studied over the past four decades.[3,4] In particular, it has recently been shown that methylated cytosines (5mC) in DNA can be further oxidized to 5-hydroxymethylcytosine (5hmC). As a modified base, 5hmC has been detected in mammalian and viral genomes as early as the 1950s using crude fractionation methods.[5] However, the recent discovery that members of the ten-eleven translocated protein family (TET1, TET2, TET3) can enzymatically convert

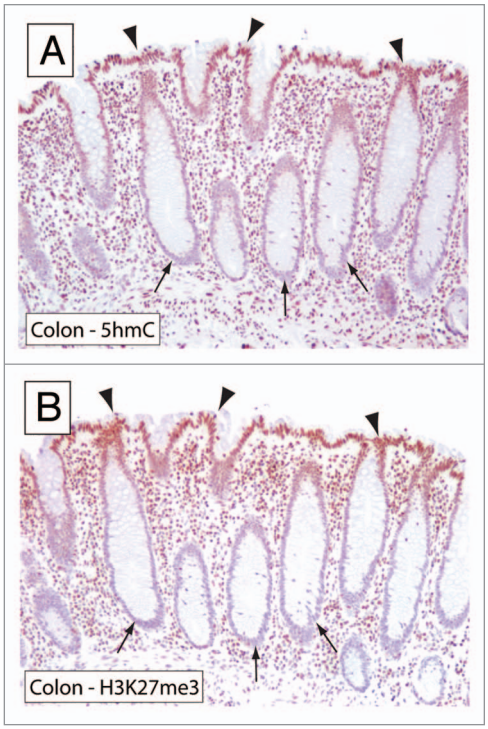

Figure 1. 5hmC and H3K27me3 show highly similar distribution patterns in colonic mucosa. To evaluate the distribution of 5hmC and H3K27me3 in the colonic mucosa, adjacent sections of formalin-fixed paraffin-embedded colonic tissues were stained with 5hmC and H3K27me3-specific antibodies. Note that terminally differentiated epithelial cells toward the lumen of the colon (arrowheads) show strong staining for 5hmC and H3K27me3, whereas cells in the crypts (arrows) show very low levels of 5hmC and H3K27me3.

5mC to 5hmC in a targeted manner has spurred a renewed interest in this DNA modification.[6-8] Previous studies have suggested that 5hmC plays a crucial role in stem cell biology and lineage-specific differentiation.[9,10] In an attempt to further elucidate the biological function of 5hmC, several groups developed novel methodologies to investigate the genome-wide distribution pattern of 5hmC in embryonic stem cells.[11-15] Taken together, these reports showed that 5hmC can be found around the transcriptional start sites of active and repressed gene promoters, suggesting that 5hmC could be involved in transcriptional regulation. Furthermore, 5hmC was enriched at genes whose promoters bear "bivalent" histone 3 lysine 27 trimethylation (H3K27me3) repression marks and histone 3 lysine 4 trimethylation (H3K4me3) activation marks. This "bivalent" chromatin state is thought to poise gene loci for further epigenetic regulation, suggesting that 5hmC could be involved in the transcriptional regulation and fine-tuning of transcriptional output during ES cell differentiation.

More generally, histone H3 lysine 27 trimethylation (H3K27me3) is a well-known histone modification regulated during ES cell differentiation and development. H3K27me3 levels are thought to be regulated by the histone methyltransferase enhancer of zeste 2 (EZH2), as well as the lysine demethylases JMJD3 and UTX.[16] EZH2 is the catalytic subunit of the polycomb repressive complex 2 (PRC2), and trimethylation of H3K27 by PRC2 recruits the PRC1 complex, resulting in gene silencing during ES cell differentiation.[17] H3K27me3 is a polycomb mark usually associated with heterochromatin, and transcriptional repression and is known to be dysregulated in cancer.[18,19]

We have recently demonstrated that global levels of 5hmC are high in terminally differentiated cells in most adult tissues,[20] suggesting a key role of 5hmC in tissue-specific differentiation. Conversely, the stem cell compartments in hierarchically differentiated tissues show greatly reduced 5hmC levels compared with their more differentiated counterparts. This distinct distribution pattern with a clear enrichment for terminally differentiated cells is highly reminiscent of the distribution recently described for H3K27me3.[21] Here we show that 5hmC and H3K27me3 are tightly correlated on a cell-by-cell basis in multiple normal human tissues. Although to a somewhat reduced extent, this association is maintained in human cancers, where both marks are significantly downregulated at a global level. These observations suggest a previously unappreciated link between 5hmC and H3K27me3.

Results and Discussion

Global levels and cellular localization of 5hmC and H3K27me3 are highly correlated. We previously showed that 5hmC global content and global levels of H3K27me3 each track with differentiation in multiple normal tissues in two separate studies.[21] We therefore hypothesized that these marks may be directly correlated with each other in stem and differentiated cell compartments. We began to explore this hypothesis by examining the levels of 5hmC and H3K27me3 in colonic mucosa, which represents a prototypical example of a hierarchically organized epithelial tissue. In the hierarchically organized colonic epithelium, the crypts harbor the stem cell compartment where colonocytes are formed by asymmetric division.[22] Conversely, mature and terminally differentiated colonocytes are localized on the luminal side of the colon. We used previously validated immunohistochemical approaches developed by our group to evaluate the global levels of 5hmC and H3K27me3 in directly adjacent tissue sections from the human colon.[20,21] Interestingly, we observed a high degree of concordance between 5hmC and H3K27me3 levels, in which both marks were observed at high levels in terminally differentiated apical cells, whereas cells at the base of the colonic crypts showed greatly reduced staining of both marks (**Fig. 1**). This observation provided initial evidence for a potential co-regulation of 5hmC and H3K27me3 during differentiation.

To further evaluate the degree of co-localization of 5hmC and H3K27me3 on

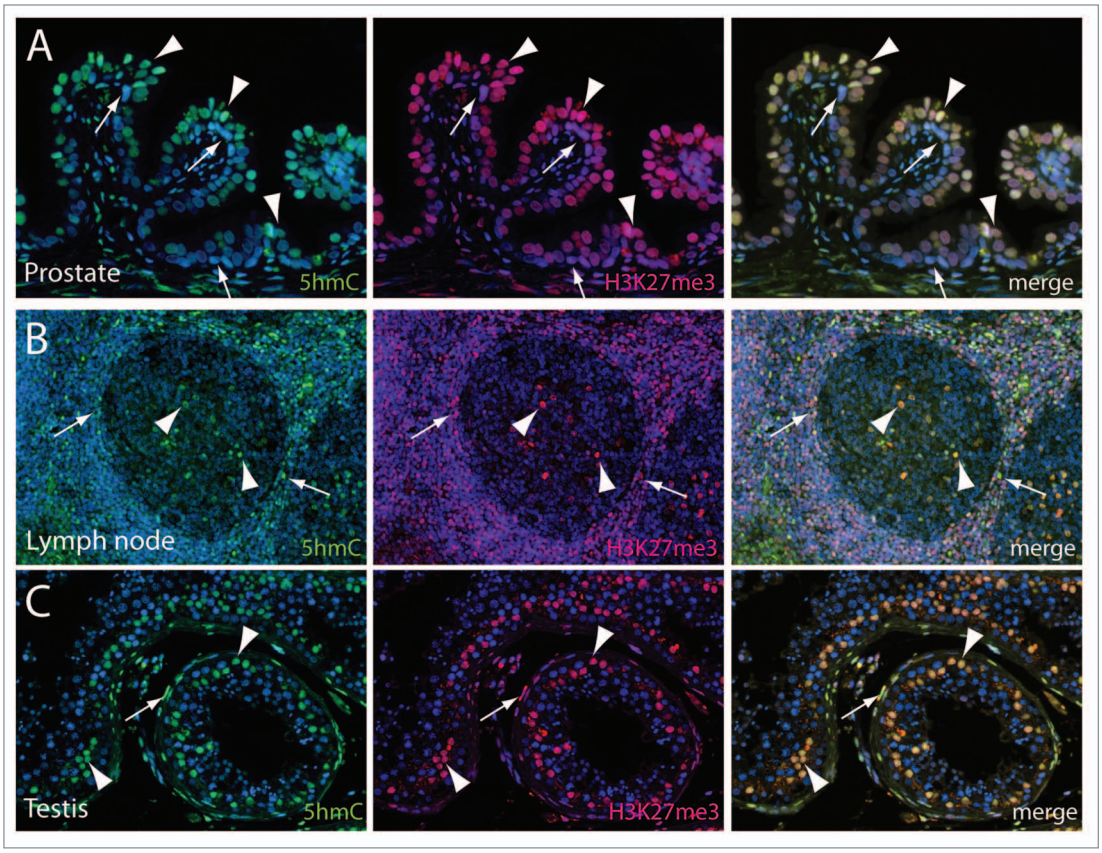

Figure 2. Immunofluorescence double labeling reveals a high degree of co-localization of 5hmC and H3K27me3 in differentiated adult tissues. Tissue sections containing normal prostate epithelium, lymphoid tissue and testis were co-immunolabeled with antibodies specific to 5hmC (shown in green) and H3K27me3 (shown in red). (**A**) In normal prostate epithelium, 5hmC and H3K27me3 are present at high levels in the terminally differentiated luminal cells (arrowheads). Note that basal cells (arrows) exhibit greatly decreased staining intensities for both marks. (**B**) In the activated lymph follicle, 5hmC and H3K27me3 are both present with very high concordance in distinct cell populations in the germinal center (arrowheads) and the marginal zone (arrows). (**C**) In cross sections of the human testis, 5hmC and H3K27me3 are present in Sertoli cells in the seminal tubules (arrowheads) and in stromal cells surrounding the tubuli (arrows), but not in spermatogonia or mature spermatids.

a cell-by-cell level, we used double-label immunofluorescence microscopy to detect both marks in the same tissue section.

Secretory glands of the adult prostate are lined by a two-layer epithelium comprised of a luminal (**Fig. 2A**, arrowheads) and a basal (**Fig. 2A**, arrows) cell compartment. Similar to the colonic mucosa, the basal cell layer harbors the regenerative stem-like compartment, whereas the luminal cells are thought to be terminally differentiated.[23] As shown previously, 5hmC was abundant in the luminal cell compartment but greatly reduced in the basal cell compartment (**Fig. 2A**).[20] Similarly, global H3K27me3 levels were much higher in luminal cells as compared with basal cells of the prostate, suggesting a differentiation-dependent co-regulation of both marks in the prostatic epithelium. It is worth noting that the staining levels for 5hmC showed a slight cell-to-cell variability within the luminal cell compartment, with some cells showing stronger staining intensities than others (**Fig. 2A**). Remarkably, these different staining levels were also reflected in the H3K27me3 staining, indicating a tight correlation of 5hmC and H3K27me3, even at the level of individual cells.

As part of the immune system, lymph follicles are highly organized structures with distinct cellular organization patterns, which enable B-cell proliferation and maturation. The germinal center (**Fig. 2B**, arrowheads), which shows a high density of proliferating B-lymphocytes, is surrounded by the paracortex, which mainly consists of T-cells. Interestingly, the staining distribution of 5hmC and H3K27me3 in such secondary lymph follicles showed a very distinct pattern. The marginal zone (**Fig. 2B**, arrows) of the follicle exhibited a large number of cells with high 5hmC and H3K27me3 levels, whereas cells in the germinal center were mostly negative for both marks (**Fig. 2B**). Only a subset of cells in the germinal center showed high levels of 5hmC and H3K27me3 (**Fig. 2B**, arrowheads). This distinct distribution pattern could suggest that proliferating and maturing B-cells are characterized by low levels of 5hmC and H3K27me3.

Furthermore, in human testis, 5hmC and H3K27me3 showed an almost identical staining pattern (**Fig. 2C**). Both marks were present at high levels in terminally differentiated Sertoli cells within the tubuli seminiferi and in stromal cells surrounding the tubuli. Spermatogonia and mature spermatids, however, were devoid of 5hmC and H3K27me3 staining. In each of these systems, prostate, lymphoid

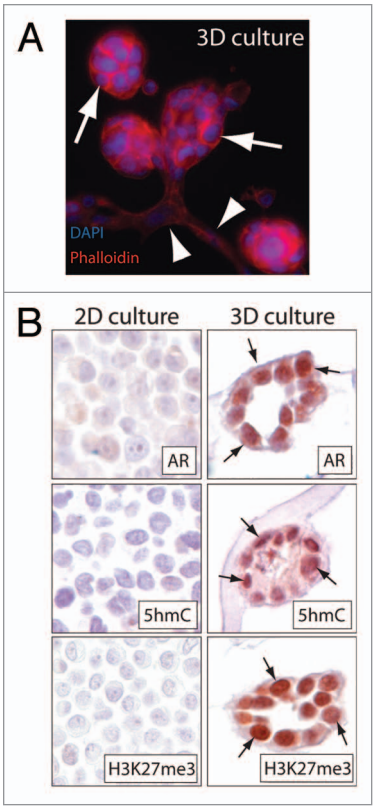

Figure 3. In vitro differentiation of prostate epithelial cells is associated with increased 5hmC and H3K27me3 levels. RWPE-1 prostate epithelial cells showed a basal cell like phenotype characterized by the absence of androgen receptor (AR) expression and low to undetectable levels of 5hmC and H3K27me3 when cultured under standard 2D culturing conditions. (**A**) Introduction of RWPE-1 cells in a 3D matrigel matrix induced formation of highly organized acinar structures (arrows) and ductal branching (arrowheads). This morphological differentiation was associated with an increase in AR levels and accumulation of 5hmC and H3K27me3 (arrows). (**B**) shows representative micrographs of cross sections obtained from 2D cultures and 3D cultures. Note the high level of AR, 5hmC and H3K27me3 staining in the acinar epithelial structures formed in 3D cultures (arrows).

tissue and testis, 5hmC and H3K27me3 levels were highly correlated even at a cell-by-cell level.

5hmC and H3k27me3 levels increase during induced differentiation in vitro. To recapitulate the changes in global 5hmC and H3K27me3 levels in a model system of induced differentiation, we used the normal prostate epithelial line RWPE-1.[24] RWPE-1 cells show a basal like prostate epithelium phenotype characterized by low levels of 5hmC, H3K27me3 and absence/very low expression of the androgen receptor (AR) when cultured under standard cell culture conditions (2D culture) (**Fig. 3**). However, when cultured in a 3D matrix containing laminin and collagen, RWPE-1 cells showed robust glandular differentiation characterized by the formation of branching acini.[25-27] This induced acinar differentiation was associated with an increase of luminal differentiation specific markers such as AR (**Fig. 3B**). Strikingly, 5hmC and H3K27me3 levels were also greatly increased in the acinar structures generated in the 3D culture system, despite being almost undetectable under conventional 2D culture conditions. Therefore this model system allows a partial recapitulation of prostatic epithelial differentiation and highlights the tight association of 5hmC and H3K27me3. Along these lines, it is worth noting that induced differentiation in other model systems has been shown to lead to an accumulation of 5hmC and reduction of 5mC specifically in enhancer and promoter regions, a step that might be required for establishing differentiation specific chromatin states.[28,29]

Global 5hmC and H3K27me3 levels are reduced in solid tumors. Tumor initiation and progression are associated with a wide range of genetic and epigenetic changes.[2,30,31] Changes in DNA methylation patterns are almost universal in human cancers and can be found at early stages during tumor progression. The dysregulation of DNA methylation patterns in cancers often involves both global DNA hypomethylation and gain of methylation marks in CpG islands.[2] We have recently provided the first evidence that global 5hmC levels are greatly reduced in multiple human carcinomas compared with normal tissue.[20] This finding was independently corroborated by a number of groups and expanded to other tumor types, suggesting that the bulk of the neoplastic cells in solid tumors including carcinoma of the breast, prostate, colon, malignant glioma and melanoma show profound decreases in 5hmC levels when compared with their differentiated normal counterparts.[20,32-36] Moreover the loss of 5hmC is far more pronounced than the decrease in 5mC levels observed in many tumor types, suggesting that the reduction in 5hmC occurs independently of reductions in 5mC.[20] This reduction of 5hmC levels appears to be an early event; in some tumors, it occurs at the stage of pre-invasive lesions (Haffner et al. unpublished data). Furthermore, recent evidence suggests that the extent of 5hmC loss in tumor cells tracks with histopathological grade and appears to be a prognostic indicator in glioblastoma multiforme and melanoma.[34,36] The mechanism for this profound loss of 5hmC is currently under investigation, and several hypotheses have been proposed. For instance, in 30% of myeloid malignancies, *TET2* appears to be inactivated by deletion or somatic mutations.[37,38] This genetic inactivation is associated with a decrease in 5hmC levels.[35] Consistent with a "driver" role for these mutations, Tet2-deficient mice show an enlargement of the hematopoietic stem cell compartment and develop myeloproliferative disorders.[39] In solid tumors, however, mutations in *TET* genes are observed less frequently and are therefore not likely to contribute to the almost universally observed decrease of 5hmC. However, loss of 5hmC is frequently accompanied by reduced mRNA expression of *TET1*, *TET2* and *TET3* in a variety of solid tumors.[32] Moreover, recent in vivo data also demonstrate that loss of *TET1* and *TET2* results in increased tumor growth and invasion and a global reduction in 5hmC, suggesting that *TET1* and *TET2* could function as tumor suppressor genes.[36,40] Furthermore, metabolic alterations, such as the generation of TET-inhibitory metabolites like 2-hydroxyglutarate through mutant IDH1 and IDH2, have been recently discussed as potential causes for TET enzyme dysfunction and consequently 5hmC loss in tumors.[41,42]

The role of 5hmC in epigenetic regulation appears to be regulated by a complex network of enzymes.[8,43,44] For instance, recent evidence suggests that 5hmC is likely to be an intermediate in an active de-methylation process in which the first step involves the oxidation of 5mC to 5hmC. It was postulated that 5hmC could get further oxidized to 5-formylcytosine (5fC) or 5-carboxylcytosine (5caC) in a process that involves TET enzymes.[9,43-48] 5fC and 5caC can then get excised by

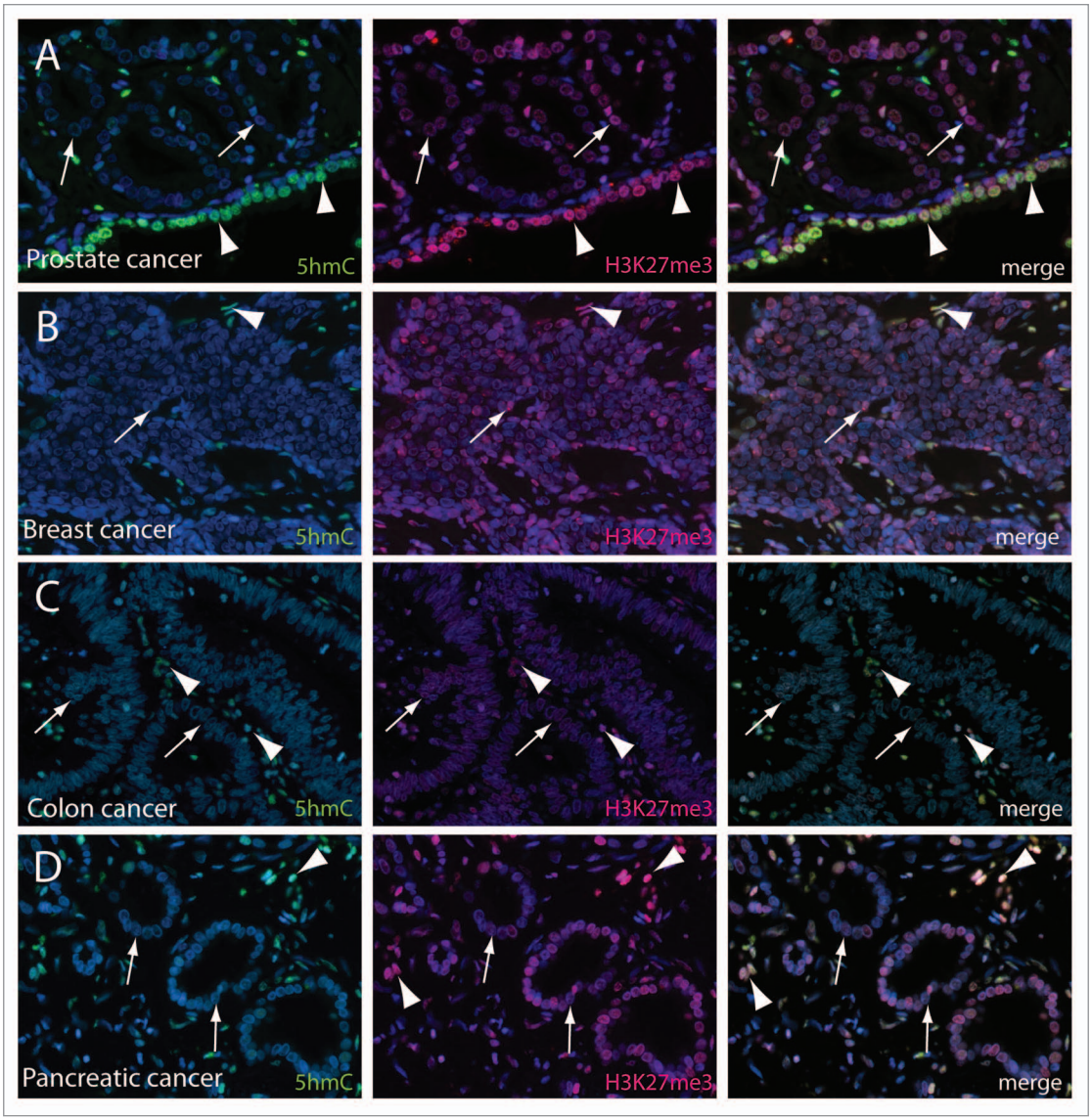

Figure 4. 5hmC and H3K27me3 levels are decreased in cancers. (**A**) Prostate adenocarcinoma (arrows) showed global decreased levels of 5hmC and H3K27me3 as compared to normal prostate luminal cells (arrowheads). Similarly, neoplastic cells in breast (**B**), colon (**C**) and pancreatic adenocarcinoma (**D**) (arrows) were characterized by reduced 5hmC and H3K27me3 staining levels. Tumor associated stromal cells (arrowheads) showed high levels of 5hmC and H3K27me3. Note that the degree of loss between adjacent normal tissue and cancer cells was more pronounced for 5hmC and the correlation of 5hmC and H3K27me3 is less pronounced.

thymine-DNA glycosylase TDG and base excision repair.[47,49] Alternatively, it has been proposed recently that 5hmC could be deaminated by the DNA methyltransferases DNMT3a and DNMT3b and then further repaired in a process involving DNA-glycosylases and base excision repair.[50] The complexity of 5hmC turnover and the potential dynamics of this process present multiple pathways that, if corrupted, could lead to reduced 5hmC levels in tumors.

Similar to 5hmC, global H3K27me3 levels have also been shown to be reduced in breast, ovarian, pancreatic and prostate cancer and low levels of the mark have been associated with worse prognosis.[18,21] Given the tight co-regulation of 5hmC and H3K27me3 in normal tissue, we aimed to further investigate these two epigenetic marks in prostate, breast, colon and pancreatic cancer (**Fig. 4**). In line with previous reports, we observed a profound loss of 5hmC in neoplastic cells, whereas adjacent normal epithelial or stroma cells showed robust staining. H3K27me3 levels appeared generally reduced in cancer samples; however, at close scrutiny, the loss H3K27me3 appeared overall much less pronounced. This was particularly evident in lower-grade lesions (**Fig. 4**). Furthermore, we also observed that single cells within the tumor that showed no detectable 5hmC levels showed strong staining for H3K27me3, suggesting at least a partial uncoupling of the tight correlation of these two marks in cancer cells compared with normal tissues.

Interestingly, high levels of 5hmC and H3K27me3 can for the most part only be found in quiescent, non-proliferating cells. Replicating cells, however, show low levels

of 5hmC and H3K27me3, suggesting that both marks are not actively maintained during replication. This is in agreement with a recent report showing the passive, replication-dependent loss of 5hmC in murine pre-implantation blastomeres.[51] It remains to be shown if passive replication-dependent loss can also explain the 5hmC and H3K27me3 distribution patterns observed in normal adult tissues and the alteration of these patterns in cancers.

In conclusion, we show that global levels of 5hmC and H3K27me3 are tightly co-regulated during hierarchical differentiation in adult tissues. Furthermore, we show that in solid tumors both marks are decreased. Overall these findings suggest that 5hmC and H3K27me3 are linked by a yet unidentified mechanism.

Materials and Methods

Formalin-fixed paraffin-embedded tissue sections were de-paraffinized and then steamed for 40 min in EDTA solution (Zymed) followed by 5 min incubation in 3.5 N HCl. Immunolabeling was performed with rabbit polyclonal anti-5hmC antibodies (1:20K dilution, Active Motif) for 45 min or mouse monoclonal anti-H3K27me3 antibodies (1:75 dilution, ab6002, Abcam) overnight at 4°C as described previously.[21] For detection of androgen receptor (AR), slides were steamed in HTTR buffer for 50 min and incubated with anti-AR rabbit polyclonal antibodies (1:1,000 dilution, N20, Santa Cruz Biotechnologies) for 45 min. Immune complexes were then visualized using the Power Vision+ poly-HRP IHC Kit (ImmunoVision Inc) using 3,3'-diaminobenzidine (Sigma) as a chromogen.

For co-immunolabeling of 5hmC and H3K27me3, antibodies were used at 1:6,000 and 1:75 dilutions, respectively. Immune complexes were visualized with Alexa 488 nm anti-rabbit and Alexa 565 nm anti-mouse (Life Technologies) secondary antibodies. After nuclear counterstaining with DAPI, slides were coverslipped with Prolong (Life Technologies). All slides were imaged on a Nikon 50i epifluorescence microscope. Immunofluorescence images were captured using a CoolsnapEZ digital camera (Photometrics) and the Nikon NIS-Elements (Nikon) software package.

RWPE-1 cells were cultured in KSFM containing 0.2 ng/ml epidermal growth factor (EGF) and 25 mg/ml bovine pituitary extract (Invitrogen). 3D culture experiments were performed as described previously[27,52] and were either stained directly in situ with rhodamine phalloidin (Life Technologies) or embedded in paraffin and processed for immunohistochemistry as described above.

Disclosure of Potential Conflicts of Interest

The terms of the relationship between A.M.D. and Predictive Biosciences, Inc are managed by the Johns Hopkins University in accordance with its conflict-of-interest policies. No potential conflicts of interest were disclosed by any other authors.

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