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DNA methylation and breast cancer

Moshe Szyf[a,*], Pouya Pakneshan[b], Shafaat A. Rabbani[b]

[a]*Department of Pharmacology and Therapeutics, McGill University, 3655 Sir William Osier Promenade, Montreal, Canada PQ H3G 1Y6*
[b]*Departments of Medicine and Oncology, McGill University Health Center, Montreal, Canada*

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Abstract

DNA methylation and chromatin structure patterns are tightly linked components of the epigenome, which regulate gene expression programming. Two contradictory changes in DNA methylation patterns are observed in breast cancer; regional hypermethylation of specific genes and global hypomethylation. It is proposed here that independent mechanisms are responsible for these alterations in DNA methylation patterns and that these alterations deregulate two different processes in breast cancer. Regional hypermethylation is brought about by specific regional changes in chromatin structure, whereas global demethylation is caused by a general increase in demethylation activity. Hypermethylation silences growth regulatory genes resulting in uncontrolled growth whereas hypomethylation leads to activation of genes required for metastasis. DNA methylation inhibitors activate silenced tumor suppressor genes resulting in arrest of tumor growth and are now being tested as candidate anticancer drugs. Demethylation inhibitors are proposed here to be potential novel candidate antimetastatic agents, which would bring about methylation and silencing of metastatic genes. Future therapeutic application of either methylation or demethylation inhibitors in cancer therapy would require understanding of the relative role of these processes in the evolution of cancer.
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1. Introduction

Human breast cancer initiates either as a less aggressive hormone (estrogen) dependent type which gradually progresses to a highly invasive hormone independent phenotype [1] or as a subset of growth factor negative cells which emerges in the primary tumor [2,3]. At its site of origin, tumor cells continue to grow and invade into the tumor surrounding extracellular matrix (ECM) by producing a variety of proteolytic enzymes [4,5]. Those most commonly implicated in this processes are urokinase type plasminogen activator (uPA) [6,7] and matrix metalloproteinases (MMPs) [8,9], which can promote tumor cell invasion into blood vessels and spread of tumor cells to distant organs. A number of metastasis suppressor genes, which are silenced in metastasis also received considerable attention in the last years [10,11].

Since metastasis involves the coordinate expression and repression of multiple genes, there must be common mechanism(s) responsible for coordinating these changes. A nodal issue in breast cancer biology and therapy, is understanding the mechanisms involved in the programs orchestrating the induction of multiple genes required first for cell growth and later for metastasis. This review will discuss the potential role of DNA methylation in controlling gene expression programs required for both deregulated growth and metastasis and propose that different facets of the DNA methylation machinery are involved in these processes. Silencing tumor suppressor and metastasis inhibitor genes involves regional hypermethylation whereas activation of pro-metastatic genes is associated with global hypomethylation. Understanding the differential roles of regional hypermethylation and global hypomethylation in breast cancer will fuel novel therapeutic approaches to breast cancer therapy.

Abbreviations: DNMT, DNA methyltransferase; HDAC, histone deacetylase; HAT, histone acetyltransferases; SAM, *S*-adenosyl-L-methionine; 5-aza-CdR, 5-aza-2′-deoxycytidine

* Corresponding author. Tel.: +1 514 398 7107; fax: +1 514 398 6690.
E-mail address: moshe.szyf@mcgill.ca (M. Szyf).

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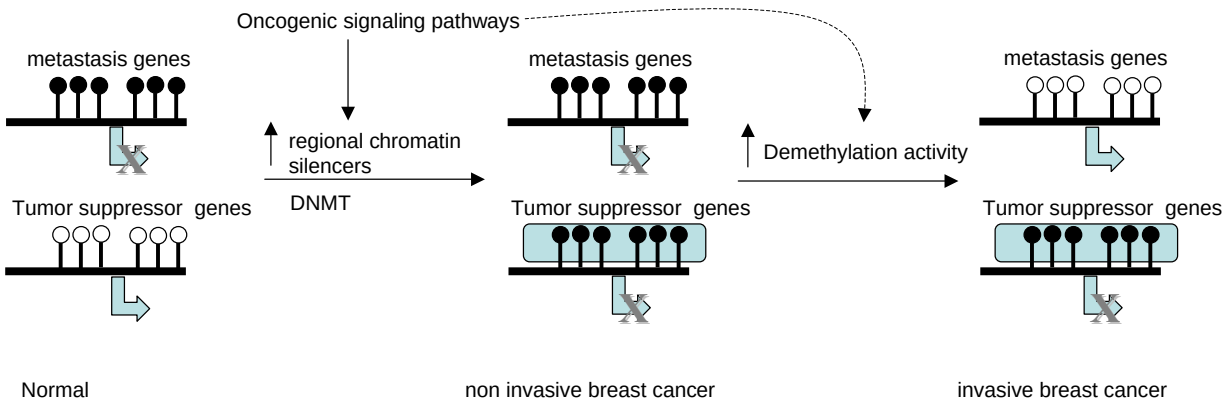

Fig. 1. Hypermethylation and hypomethylation in breast cancer. In normal epithelial breast cells tumor suppressor genes are active (transcription indicated by horizontal arrow) and unmethylated (open circles indicate unmethylated CGs). Metastatic genes are methylated and inactive (closed circles). Activation of oncogenic pathways leads to interaction of specific repressors with certain tumor suppressors leading to chromatin inactivation and DNA methylation (closed circles). At a more advanced stage the same or additional oncogenic pathways lead to induction of demethylase activity resulting in global demethylation as well as demethylation and activation of genes required for metastasis leading to a highly metastatic phenotype. Tumor suppressor genes are protected from demethylation by regional repressors maintaining an inactive chromatin structure.

2. Chromatin structure and DNA methylation are tightly linked and involved in controlling programs of gene expression

Both cancerous growth and metastasis involve coordinated silencing and activation of multiple genes. Programs of gene expression are controlled by the epigenome, which is comprised of the pattern of modification of histones [12,13] and the pattern of distribution of methyl residues at the 5′ position of cytosine rings in the dinucleotide sequence CG [14,15]. Active genes are associated with open hyperacetylated chromatin, which is hypomethylated at some residues such as K9 and K27 of the H3-histone tail and di- and tri-methylated at K4 [12,13]. Regulatory regions of active genes associated with such chromatin structures are hypomethylated [15]. This tight regulation of chromatin structure and DNA methylation might provide a cue as to the functional role of DNA methylation and as to the mechanisms responsible for generating DNA methylation patterns.

As gene expression programs vary from cell type to cell type, the epigenome is unique for each cell type. Whereas the chromatin is associated with the genome, the methyl groups are part of its covalent chemical structure. Thus the pattern of methylation of DNA in a specific tissue is a stable mark of its identity and its unique gene expression program in contrast to chromatin, which is disrupted once the DNA is extracted and is hard to preserve in clinical samples. Profiling DNA methylation patterns provides therefore a stable image of the living genome. Since primary breast cancer and metastatic breast cancer execute different gene expression programs it is not surprising that they reveal distinct DNA methylation profiles [16–18]. A number of high throughput DNA mapping techniques were recently utilized to profile DNA methylation patterns of breast cancer and to obtain methylation signatures of distinct clinical stages.

A causal link between DNA methylation and chromatin structure has been established. It is widely accepted that methylated DNA binding proteins such as MeCP2 target methylated DNA. MeCP2 recruits co-repressor complexes and histone deacetylases and histone methyltransferases to the methylated gene precipitating an inactive chromatin structure [19–21]. This model implying that DNA methylation is primary and dominant over chromatin structure is supported by the observation that the histone deacetylase (HDAC) inhibitor trichostatin A which causes hyperacetylation and activation of chromatin can activate tumor suppressors silenced by methylation only following DNA methylation inhibition [22]. However recent data suggests that chromatin structure might also define DNA methylation patterns as discussed below.

Another important issue is whether the DNA methylation pattern is reversible and is determined by a balance of methylating and demethylating enzymes or whether it is determined solely by the activity of DNA methyltransferases (DNMT). DNA methyltransferases catalyze the transfer of a methyl residue from the methyl donor *S*-adenosyl-methionine (SAM) to the 5 position of the cytosine ring. The main methylated sequence in vertebrate DNA is the dinucleotide CG. The classic model is that the pattern of methylation is fashioned during development by de novo DNMTs and demethylases and is thereafter maintained by the maintenance DNA methyltransferase DNMT1, which accurately replicates the DNA methylation pattern during replication [23]. Such a model assumes that the DNA methylation is fixed and solely determined by the DNA methylation pattern of the parental strand of DNA. Understanding how the methylation pattern is maintained is obviously critical for understanding the aberrant changes in DNA methylation in breast cancer and for devising therapeutic approaches, which target DNA methylation [24].

Recent data challenges some of these basic concepts on the relation between chromatin and the DNA methylation

pattern and the manner by which the pattern is replicated and maintained [25,26]. First, activation of chromatin with histone deacetylase inhibitor was shown to bring about active replication-independent DNA demethylation [27]. We therefore proposed that the state of histone acetylation gates demethylase accessibility. Second, inactivation of chromatin structure was shown to precede de novo methylation of the p16 tumor suppressor gene in colorectal cancer cell lines bearing knock out mutations in DNA methyltransferase genes suggesting that an inactive chromatin structure brings about de novo methylation [28]. Taken together these and other results suggest that the pattern of methylation is dynamic, reversible and dictated by chromatin structure. Third, de novo methyltransferases such as *DNMT3A* and *DNMT3B* were shown to be required for maintenance DNA methyltransferase suggesting that replication of the DNA methylation pattern is not dictated solely by the DNA methylation pattern of the parental strand but requires ''de novo'' methylation to be maintained [29–31]. We therefore proposed that a balance of methylation and demethylation dynamically maintains the state of DNA methylation in somatic cells [25,26]. The state of activity of chromatin structure determines the balance between DNA methylation and demethylation by recruiting DNMTs or gating the accessibility to demethylases. This equilibrium is disrupted when there is a change in chromatin structure. This view of the DNA methylation pattern has implications on our understanding of how DNA methylation patterns are disrupted in cancer and DNA methylation targeted therapy. Thus, it is proposed that a bilateral relation exists between DNA methylation and chromatin structure [25,26]. Methylation precipitates inactive chromatin structure whereas chromatin structure can direct the DNA methylation pattern.

3. Two conflicting aberrations of DNA methylation in cancer

Since cancer involves multiple changes in gene expression programming, it stands to reason that such a change involves a global mechanism of gene expression regulation such as DNA methylation. Indeed it is well established that two kinds of changes in the DNA methylation pattern occur in breast cancer, regional hypermethylation of certain genes [32] and global hypomethylation [33,34]. This paradoxical coexistence of a global decrease in methylation with regional hypermethylation implies that independent and different processes are responsible for hypomethylation and hypermethylation. We have therefore suggested that global hypomethylation is caused by a global increase in demethylase activity whereas regional hypermethylation results from local changes in chromatin structure, which prevent access to the abundant demethylase(s) [25,26] (Fig. 1). It is clearly critical to determine whether both regional hypermethylation and global hypomethylation play a causal role in breast cancer. If both processes were important would it be possible to inhibit cancer by inhibiting one of these processes without affecting the other process?

4. Hypermethylation of genes in breast cancer, role in silencing tumor suppressor genes

The phenomenon of regional DNA hypermethylation and silencing of tumor suppressor genes in cancer has been the focus of attention in the last decade. A significant amount of data has established a list of genes hypermethylated in cancer and recently whole genome approaches have identified methylation signatures of breast cancer cells [16–18]. These methylation signatures, which are the unique combination of methylated CpG islands in a cancer cell were correlated with breast cancer stage and have been proposed to be a diagnostic marker of breast cancer cells. In addition to their diagnostic value in breast cancer it is clear from the repertoire of methylated genes that silencing of these genes by DNA methylation plays a role in the transformation process (Table 1). Amongst the methylated genes are tumor suppressor genes such as p16 whose methylation is proposed to silence this gene and override cell growth regulatory signals [35,36]. p16 methylation in DNA prepared from plasma of breast cancer patients was associated with nodal metastasis [37]. Another group of methylated genes in breast cancer is composed of damage response genes such as BRCA1 [38,39], which is also mutated in familial breast cancer and mismatch repair genes hMLH1 and HMSH2 [40]. Disruption of repair genes might increase sporadic mutations frequency, a hallmark of cancer cells. Steroid receptor genes family members such as the estrogen receptor [41] and retinoic acid beta 2 (*RARβ2*) receptor are methylated and silenced in a fraction of breast cancers [42,43]. Interaction of *RARβ2* receptor with retinoic acid might have an antiproliferative effect and its silencing confers a selective advantage on advanced breast cancer cells. Cell adhesion and cell surface molecules such as E-cadherin [44] and inhibitors of proteases such as TIMP-3 [45] whose silencing might promote metastasis are also found to be methylated in breast cancer.

DNA methyltransferase inhibitors, such as the nucleoside analogue 5-aza-deoxy-cytidine (5-aza-CdR) as well as antisense oligonucleotides targeting *DNMT1*, activated methylated genes in cancer supporting the hypothesis that DNA methylation plays a causal role in silencing of these genes in other cancers as well as breast cancer cells. For example, estrogen receptor (ER) in receptor negative breast cancer cells MDA-MB-231 was demethylated and activated by either 5-aza-CdR or 5-aza-cytidine treatment [46], and 5-aza-CdR demethylated and activated the RARβ2 in receptor negative beast cancer cells [47]. A DNMT1 antisense oligonucleotide activated ER in ER-breast cancer

Table 1
Examples of aberrations in DNA methylation in breast cancer

Methylated genes in breast cancer
Estrogen receptor [41]
p16 [35]
Progesterone receptor [108]
Methyl-sensitive fingerprints HBC-a, HBC-2 [16]
HIC-1 [109]
TIMP-3 [45]
30 CpG loci identified by differential methylation hybridization arrays (5–14% of normal control [17])
WT-1 gene [110]
Gelsolin [111]
600 out of 45,000 CG islands methylated in a tumor specific manner (RLGS) [112]
E-cadherin [44]
BRCA-1 [38,39]
Retinoic acid β 2 receptor [42,43]
CpG island arrays epigenetic signatures [90]
14-3-3 sigma [113]
APC (adenomatous polyposis coli) [114]
Reduced folate carrier gene in methotrexate resistant cancer cells [115]
Retinoic acid α 2 receptor in breast cancer cell line [116]
HMSH2 and hMLH1 [40]
DUTT1 member of NCAm receptor family [117]
Expressed CpG island sequence tag microarray revealed 30 methylated CpG islands in a breast cancer cell line [118]
LOT1 tumor suppressor [119]
Candidate 3p21.3 suppressor gene BLU in breast cancer cell lines [120]
Guanine-nucleotide-binding protein alpha-11 (GNA11) [121]
Monocarboxylate transporter 1 (MCT1) [121]
Methyl CpG binding proteins identified CpG islands [122]
463 methylation target array CpG islands [18]
DAP-K1 death associated protein kinase [123,124]
RASSF1A [125]
HIN-1 [126]
CyclinD2 [126]
NORE1 homologue of RASSF1 [127]
Global and specific genes hypomethylation in breast cancer
Line1 repetitive sequences [56]
Global hypomethylation [55]
Hypomethylation of pericentromeric DNA [34]
Global hypomethylation correlates with stage of disease and invasiveness [54]
DNA hypomethylation, correlation with chromosomal instability in breast cancer lines [72]
Global hypomethylation in both hereditary and sporadic cancer [128]
MAGE [61]
SYNUCLEIN γ [65]
uPA [63]
DNA methyltransferase deregulation
Disruption of cell cycle regulation of DNMT1 [67]
DNMT3B overexpression [66]

cells although this activation did not involve a change in DNA methylation [48]. 5-aza-CdR also induced cell arrest in breast cancer cell lines and it was proposed that this was brought about by demethylation and activation of genes, which suppress tumor growth such as RARβ2 [47]. However, two critical questions remain unresolved. First, although it is clear that a number of tumor suppressor genes are methylated and silenced in cancer, it is still possible that DNA methylation is not the primary event that triggered the silencing of these genes as discussed below. 5-aza-CdR might be activating the genes by a different mechanism and demethylation might be a consequence rather than the cause of gene activation. Similarly, even if DNA methylation inhibitors such as 5-aza-CdR simultaneously inhibit DNA methylation, DNMT1 activity and also cause cell arrest it is still possible that DNMT1 is involved in cell growth by methylation-independent mechanisms. Resolving of these issues is critical for taking advantage of the therapeutic potential of DNA methyltransferase inhibitors [24].

4.1. Therapeutic implication of hypermethylation of genes in cancer

Since it is well established that inhibitors of DNA methylation can induce tumor suppressor gene expression, it was proposed that DNA methylation inhibitors might serve as anticancer agents. The induction of ER [46] and RARβ2 [47] in ER and RARβ2 negative cells by a demethylating agent raises also the prospect of combination therapy of a demethylating agent with either an estrogen receptor antagonists or a retinoid in receptor negative tumors.

Two classes of DNA methyltransferase inhibitors are now in clinical trials. First, the nucleoside analog 5-aza-CdR, which is incorporated into DNA during replication following its phosphorylation to the trinucleotide form and traps the DNA methyltransferase as it moves along with the replication fork. As the replication fork is progressing, nascent DNA is synthesized in the absence of DNA methylation [49]. A second inhibitor is an antisense oligonucleotide inhibitor of DNMT1, which knocks down DNMT1 protein levels [50]. The two agents have somewhat different effects on DNA replication and DNA methylation reflecting their different mechanisms of action. Knock down of DNMT1 induces some tumor suppressor genes and inhibits DNA replication by a methylation-independent mechanism [51–53]. This effect on tumor suppressor expression is probably mediated by the protein–protein interactions of DNMT1. Similarly, it has recently been shown that antisense DNMT1 knockdown in ER-breast cancer cells induces ER expression without causing a change in DNA methylation supporting the hypothesis that DNMT1 regulates genes required for the transformed state by methylation-independent mechanisms [48]. The inhibition of replication caused by DNMT1 knock down limits the extent of DNA methylation inhibition since nascent unmethylated DNA is not synthesized in the absence of DNMT1 [52]. These data points out to the possibility of targeting DNMT and its cell growth and gene regulatory functions without causing global demethylation [24]. This is important since global demethylation might promote tumorigenesis as discussed below.

5. Global hypomehthylation coexists with hypermethylation in breast cancer

Although hypermethylation of tumor suppressor genes has been the focus of attention in the field, it has been well documented that DNA from breast cancer as well as other cancers is globally hypomethylated [54,55]. The extent of global hypomethylation was found in one study, which measured in vitro methylation of genomic DNA by a CG methylase as an indicator of genomic hypomethylation, to be correlated with the histological grade and malignancy [54]. An earlier study using methylation sensitive restriction enzymes found global hypomethylation to characterize breast cancers but no clear correlation was found with clinical stage [55]. This difference might reflect the difference in sensitivity of the two assays. Repetitive sequences [56] and satellite DNA sequences [34] are hypomethylated in breast cancer. Different members of the melanoma associated cancer/testis antigens *MAGE*, which were shown to be methylated and silenced in adult tissues and hypomethylated in multiple tumors are also expressed in breast cancer cells [57–60]. Their expression was shown to be associated with poorly differentiated stage in invasive ductal breast cancers [61]. Unique genes such as *uPA* [62,63] and Breast Cancer Specific Gene *1-SYNUCLEIN* γ [64,65] were also shown to be hypomethylated in breast cancer. This data points out to the possibility that there is a general defect in the DNA methylation machinery in cancer cells. There is no reduction of DNA methyltransferase activity in cancer cells [66], on the contrary upregulation of expression of DNA methyltransferase in cancers during G1 phase of the cell cycle was reported in ER-breast cancer cells [67]. We therefore proposed that global demethylation in cancer might be caused by an excess of demethylation activity [24] (Fig. 1).

This obviously leaves open the question of how could tumor suppressors be hypermethylated in an environment that is markedly demethylating. Recent data suggests that inactivation of the chromatin structure at the tumor suppressor *p16* gene comes about even in a colorectal cancer cell line bearing a homozygous knock out of three DNMTs DNMT 1, 3A and 3B [28], suggesting that inactivation of chromatin is the primary event in silencing of *p16*. The inactivated p16 is slowly hypermethylated after chromatin structure inactivation [28] by residual DNMT activity suggesting that regional hypermethylation is a consequence of chromatin inactivation. Although it is yet unknown which factor recruits repressor complexes to p16 promoter and how this leads to DNA methylation, an oncogenic transcriptional repressor such as the leukemia-promoting PML-RAR fusion protein was shown to recruit histone deacetylases HDACs and DNA methyltransferases to target promoters [68]. It is also possible that localized chromatin inactivation blocks access to demethylases and that regional chromatin structure inactivation is responsible for maintenance of regional hypermethylation even when high levels of demethylase are present in the cell. In accordance with this hypothesis it has been shown that the InHAT complex which inhibits histone acetyltransferases can block active demethylation of ectopically methylated DNA [69], thus any local change in chromatin structure such as histone deacetylation could result in local inhibition of demethylase activity. Thus, regional hypermethylation is directed by a different enzymatic process than global demethylation and could therefore coexist with global hypomethylation (for model see Fig. 1).

6. Possible roles of global hypomethylation in breast cancer

There is genetic support for a causal role for DNA hypomethylation in some cancers since mice bearing a hypomorphic *dnmt1* allele develop aggressive T lymphomas [70]. Three possible roles for hypomethylation in breast cancer were previously proposed. First, a global reduction in methylation was shown to increase chromosomal instability in *dnmt1* knock out mice [71]. A study in breast cancer cell lines correlated chromosomal instability events with the extent of global hypomethylation and expression of MBD2/demethylase [72]. It was also shown that global hypomethylation correlates with the extent of satellite 2 demethylation, which is located adjacent to the centromeres of chromosomes 1 and 16 [34]. Rearrangements in these regions are frequent in breast cancer. Rearrangements in these regions and hypomethylation of satellite 2 DNA occur also in patients with ICF (immunodeficiency, centromeric region instability, and facial anomalies) supporting the potential involvement of global hypomethylation in chromosomal instability [73]. Second, hypomethylation could be possibly involved in activation of tumor promoting genes. Although several studies have suggested this possibility [74–76] there are no clear examples of proto-oncogenes induced by demethylation in either breast cancer or other tumors. Third, hypomethylation could activate the expression of pro-metastatic genes. A number of genes required for metastasis such as *HEPARANASE* gene which encodes a protein which degrades heparan sulfate proteoglycans and is preferentially expressed in neoplastic tissues and contributes to tumor metastasis [77] or *S100A4* [78] a calcium binding protein which is highly expressed in metastatic pancreatic tumors and not in normal tissues, are hypomethylated in metastatic cancer and induced by the hypomethylating drug 5-aza-CdR in non-expressing cells. Two genes clearly involved in metastasis in breast cancer were shown to be regulated by DNA methylation uPA [62,63] and *SYNUCLEIN* γ [64,65]. uPA is a serine protease which is implicated in breast cancer metastasis and invasion. uPA gene promoter is unmethylated and highly active in invasive breast carcinoma cancer cell lines such as MDA-MB-231 and methylated and

inactive in non-invasive cancer cell lines such as MCF-7 and normal breast cells [63,79]. The causal role of methylation in suppressing uPA expression in non-invasive breast cancer was demonstrated by showing that the DNA methylation inhibitor 5-aza-CdR induces demethylation and expression of uPA and invasiveness of non-invasive MCF-7 breast cancer cell line [63,79]. Similarly, 5-azaCdR treatment of MCF-7 cells results in induction of *SYNUCLEIN* γ [64,,65]. These data suggest that hypomethylation might be responsible for the coordinate induction of a number of genes involved in metastasis since additional genes known to be regulated by methylation were shown to be activated in metastatic breast cancer cells.

Other potential examples of hypomethylation are the different members of the MAGE gene family, which are methylated and silenced in all normal tissues and are hypomethylated and expressed in multiple tumors and male germ cells [59]. MAGE antigens are activated by 5-aza-CdR in a number of non-expressing tumor cell lines [57] and their expression is correlated with global hypomethylation [58]. MAGE antigens were implicated in metastasis of a number of tumors [80] and are also expressed in metastatic breast cancer [61,81]. The expression of MAGE antigens is a marker of poor prognosis, is associated with poorly differentiated invasive ductal carcinoma [61] and is detected in breast cancer patient's sentinel lymph nodes [82] and correlates with the stage of the disease [83]. Although hypomethylation of MAGE genes was not directly demonstrated in metastatic breast cancer it is highly probable, based on what is known from other tumors, that *MAGE* gene expression is strongly associated with hypomethylation.

Another gene whose expression was demonstrated to be directly implicated in metastasis in breast cancer is the calcium binding proteins S100A4 [84]. Expression of S100A4 is highly correlated with reduction in survival in breast cancer patients [85]. Expression of S100A4 was correlated with the metastatic state of breast cancer cells and ectopic expression of S100A4 caused enhanced invasiveness and mobility but no change in cell growth parameters of non invasive breast cancer cell line MCF-7 [86]. S100A4 is hypomethylated and expressed in poorly differentiated pancreatic ductal adenocarcinoma [78] suggesting a role for hypomethylation in induction of this gene in metastatic cells. There is data suggesting that hypomethylation might correlate with the expression of S100 cluster in breast cancer as well [87]. The hypomethylating drug 5-aza-CdR induces S100A4 expression in colon adenocarcinoma cell lines [88] supporting a causal role for DNA methylation in regulating the expression of this gene. Although there is no direct evidence in breast cancer that S1000A4 is regulated by DNA methylation, it is probable that it is regulated by methylation in breast cancer similarly to the situation in other tumor types.

We would like to suggest that regional hypermethylation and global hypomethylation are involved with different stages of breast cancer. Regional hypermethylation is required for bypassing cell growth regulatory arrest signals, which is required at the initial stages of transformation. Global hypomethylation, which involves the hypomethylation of a heterogeneous set of genes in different tumors, is proposed to be required for metastasis and invasion. Global hypomethylation might be triggered by a global increase in the demethylation capacity of the cancer cells (Fig. 1). We have previously shown that ectopic expression of *Ha-ras* induced increased demethylation activity in embryonal carcinoma P19 cells [89]. It is possible that oncogenic programs such as the one triggered by RAS would lead to increased demethylation capacity by increasing demethylase levels. The identity of the demethylase involved in this process and how it is regulated by oncogenic signals remains to be determined. However, the widespread appearance of global hypomethylation in cancer and the emerging evidence that it is correlated with increased metastatic potential support the hypothesis that global hypomethylation is an integral component of the program leading to metastasis. Since the transition of breast cancer into a metastatic state requires the expression of multiple genes, global hypomethylation is a reasonable mechanism to coordinate such a change in gene expression program. The demethylation machinery might therefore be a prime target for antimetastatic drugs since inhibition of this machinery should coordinately shut down a number of proteins driving metastasis simultaneously. This approach has clear advantage over drugs targeting single metastatic protein because of the potential redundancy in the array of genes required for metastasis (Figs. 1 and 2).

It is important to note however that all the data supporting the presence of high demethylase activity in metastasizing breast cancer cells is derived from cell culture data as discussed above. We do not know whether similar activities exist in tumors in vivo and whether they are increased in tumors relative to normal breast tissue. Future experiments are needed to characterize the identity of the demethylase responsible for global hypomethylation in general and genes involved in metastasis in particular in tumors.

7. Therapeutic and diagnostic implications of global hypomethylation in breast cancer

Hypermethylation has received most of the attention in recent years both as far as diagnostics and therapeutics are concerned. There has been an important attempt to utilize whole genome screening technologies of methylated CG islands such as CG island arrays [90] or methylation microarrays [91] to classify and stage breast cancer by their methylation signature. Methylation arrays might prove to be advantageous over expression arrays since DNA is more stable easily stored and could be collected

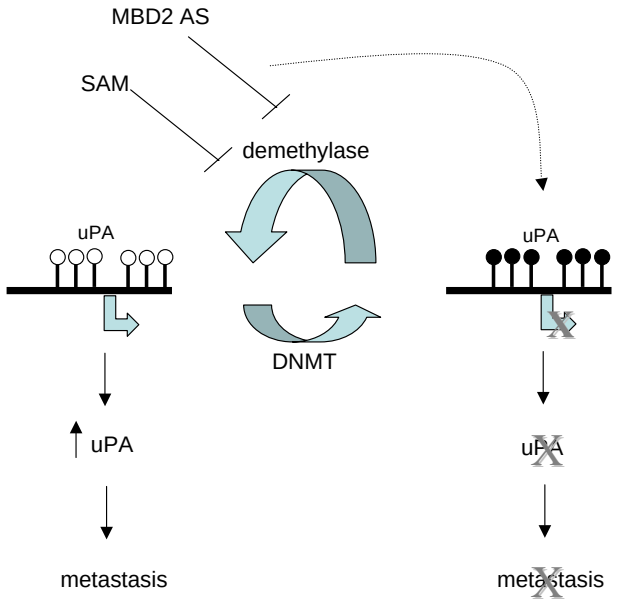

Fig. 2. Inhibition of demethylation leads to silencing of genes involved in metastasis in highly invasive breast cancer cells. In highly metastatic breast cancer cells MDA-MB-231 the gene encoding uPA is unmethylated and highly active. Expression of uPA promotes metastasis. The hypomethylated state of the gene is maintained by the highly active demethylation activity. Inhibition of demethylase with either SAM or an antisense knock down of MBD2 shifts the equilibrium toward methylation (DNMT-DNA methyltransferase) resulting in methylation and silencing of uPA and inhibition of metastasis [129].

from any quality of biopsy. The methylation-based assay is independent of the total amount of starting material since it measures the ratio of methylated to unmethylated CG islands rather than the amount of total signal. Global hypomethylation could potentially be as important in diagnostics since it does not require the knowledge of specific hypomethylated sequences. Antibodies against 5-methy-cytosine could measure global levels of DNA methylation either in situ [92] or in dot blotted DNA samples [93]. The potential correlation of global hypomethylation with metastatic potential might position immunohistochemistry-based detection of globally hypomethylated cells as a powerful and early diagnostic marker for metastatic breast cancer. A study in colorectal cancer has recently demonstrated excellent correlation between global hypomethylation as measured using anti 5-methylcytosine antibodies and lack of chemoprevention of colon cancer [93]. It will also be important to utilize whole genome approaches that were previously used to define methylation signatures of breast cancer to delineate the hypomethylation signature of metastatic breast cancer. Such a signature could probably be used for staging breast cancer using methylation microarray methodology [91].

DNA methylation inhibitors are currently tested as candidate anticancer agents since they bring about demethylation and activation of tumor suppressor genes [94]. If however global hypomethylation plays a causal role in metastatic cancer as suggested here, extreme care should be exercised in using DNA methylation inhibitors in anticancer therapy [24]. DNA methylation inhibitors might also promote metastasis by demethylating pro-metastatic genes in addition to their activation of tumor suppressors. In support of this concern treatment of the nonmetastatic breast cancer cell line MCF-7 5-with the DNA methylation inhibitor 5-aza-CdR was shown to activate a protease implicated in metastasis uPA and to increase invasiveness in vitro and metastasis in vivo [95]. Since DNMT1 has both methylation-dependent and independent functions in cellular transformation as discussed above, inhibitors of DNMT1, which target the methylation-independent functions of DNMT1 might be an alternative option in anticancer therapy [24]. These inhibitors might suppress growth without causing hypomethylation and stimulation of metastasis.

The second implication of global hypomethylation in cancer is the potential for inhibiting or preventing metastasis by inhibiting demethylation. There is extensive data from animal and some human data that suggests that global hypomethylation might be modulated by the dietary intake of folates which are required for the synthesis of *S*-adenosyl-L-methionine (SAM) the methyl donor of a large number of different methylation reactions in the cell including DNA methylation [96]. It was shown in animal studies that low methyl diets could bring about global hypomethylation of DNA and precipitate liver cancer [97,98]. Human studies suggest that low folate intake combined with high alcohol intake could result in global hypomethylation and increased risk of colorectal cancer [96]. There is also some evidence that folate metabolism and the resultant supply of methyl moieties might play a role in breast cancer since pre-menopausal women with the polymorphism in the methylene-tetrahydrofolate reductase (MTHFR), an enzyme required for the synthesis of methionine a precusor of SAM, had a three-fold increased breast cancer risk [99]. SAM was shown to be chemoprotective for liver cancer in chemically induced rat liver cancer [100]. Although it was originally believed that SAM acts by stimulating DNA methyltransferase we have recently shown that SAM inhibits active demethylation in vitro and in living cells [101]. Thus SAM might be used as an inhibitor of demethylation.

We therefore tested the hypothesis that inhibition of hypomethylation with SAM would inhibit metastasis and invasiveness of a metastatic breast cancer cell line MDA-MB-231 by causing hypermethylation and silencing of pro-metastatic genes such as uPA. Our data supported this hypothesis and illustrated that SAM treatment at the 0.1–1 mM concentration range of MDA-MB-231 cells for 6 days lead to a dose dependent inhibition of uPA expression, inhibition of the invasiveness of MDA-MB-231 cells in a Boyden chamber invasion assay and inhibition of metastasis of the cells to lung, kidney, liver and spleen when injected in vivo. While the uPA promoter is fully hypomethylated in controlled cells it becomes methylated in SAM treated cells as determined by methylation specific PCR [129]. We have

previously suggested that the methylated binding protein 2 MBD2 could act as a DNA demethylase [102,103]. We also showed that knock down of MBD2 by antisense RNA inhibited active demethylation induced by valproic acid [104]. In vivo treatment with either an MBD2 antisense mRNA or oligonucleotide inhibits the growth of human tumor cell lines implanted in mice [105–107]. Our unpublished data showed that antisense oligonucleotide knockdown of MBD2 in MDA-MB-231 cells also resulted in methylation and inhibition of uPA expression and inhibition of invasiveness in vitro and metastasis in vivo. Interestingly a transient treatment with either SAM or MBD2 antisense resulted in a stable inhibition of uPA expression in the implanted tumor in vivo up to two months after implantation [129]. This is consistent with methylation being the mechanism silencing uPA, since methylation is considered a stable silencing of transcription (Fig. 2).

8. Conclusions

It is becoming clear that both hypermethylation and hypomethylation contribute to breast cancer and are therefore candidate targets for intervention in anticancer therapy. Hypomethylation and hypermethylation are caused by independent processes and appear to be critical for different stages of cancer. Differentiating the relative roles of these two processes is critical for designing strategies that would inhibit cancer and avoid enhancing the reverse methylation process. The data discussed here points out to the possibility of using inhibitors of demethylation as a new approach to metastasis. Since hypomethylation appears to regulate multiple genes involved in tumorigenesis, inhibition of demethylation could result in abolishing multiple components of the metastasis program. There is therefore a need to identify and develop demethylation inhibitors since the only available small molecule SAM might target multiple methylation mechanisms and is highly unstable in vivo. Future studies are needed to define the mechanisms leading to upregulation of demethylation in cancer and characterizing the molecular targets of global hypomethylation.

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