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Review

The methylation landscape of tumour metastasis

Alicia Cock-Rada* and Jonathan B. Weitzman†[1]

*Unidad de Genética Médica, Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia, and †Univ. Paris Diderot, Sorbonne Paris Cité, UMR 7216 CNRS Epigenetics and Cell Fate, Paris 75013, France

The metastatic cascade which leads to the death of cancer patients results from a multi-step process of tumour progression caused by genetic and epigenetic alterations in key regulatory molecules. It is, therefore, crucial to improve our understanding of the regulation of genes controlling the metastatic process to identify predictive biomarkers and to develop more effective therapies to treat advanced disease. The study of epigenetic mechanisms of gene regulation offers a novel approach for innovative diagnosis and treatment of cancer patients. Recent discoveries provide compelling evidence that the methylation landscape (changes in both DNA methylation and histone post-translational modifications) is profoundly altered in cancer cells and contributes to the altered expression of genes regulating tumour phenotypes. However, the impact of methylation events specifically on the advanced metastatic process is poorly understood compared with the initial oncogenic events. Moreover, the characterisation of a large number of histone-modifying enzymes has revealed their active roles in cancer progression, via the regulation of specific target genes controlling different metastatic phenotypes. Here, we discuss two main methylating events (DNA methylation and histone-tail methylation) involved in oncogenesis and metastasis formation. The potential reversibility of these molecular events makes them promising biomarkers of metastatic potential and potential therapeutic targets.

EPIGENETICS: above and beyond the genome

The field of epigenetics is evolving at a rapid pace, with a continuous discovery of new molecular players that actively participate in the determination and maintenance of diverse cellular phenotypes. Epigenetic mechanisms involve modifications 'above' or 'on top' of the genome, (*επί* in Greek) which regulate gene expression without altering the DNA sequence (*i.e.* independently of mutations) and are inherited by transmission through cell division (Bonasio et al., 2010). As the study of cancer epigenetics grows in intensity, we are beginning to unveil the complex epigenetic landscape of cancer cells and the potential interplay between different levels of epigenetic control of chromatin states and gene expression (Baylin and Jones, 2011).

An exciting clinical implication of these findings is that the manipulation of epigenetic mechanisms might be exploited to reverse the cancer cell phenotype and eliminate cancer cells. Recent progress in epigenetic cancer therapy suggests that drugs which inhibit methylation events may offer therapeutic value. For example, two inhibitors of DNA methylation, 5-azacytidine (5-Aza-CR) and 5-Aza-2′-deoxycytidine (5-Aza-CdR), have been used to treat myelodysplastic syndrome; and two histone deacetylation (HDAC) inhibitors, romidepsin and vorinostat, are used to treat cutaneous T-cell lymphoma (Kaminskas et al., 2005; Kelly et al., 2010). The major disadvantage of these drugs is that their effects can be transient and they induce systemic toxicity by affecting both cancer and non-cancer cells (Herman and Baylin, 2003; Yoo and Jones, 2006). Other drugs with less toxicity and enhanced stability

[1]To whom correspondence should be addressed (email jonathan.weitzman@univ-paris-diderot.fr)
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Abbreviations used: 5-Aza-CdR, 5-Aza-2′-deoxycytidine; 5-Aza-CR, 5-azacytidine; 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; CpGI, CpG island; DNMT, DNA methyl transferase; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; ESCs, embryonic stem cells; HDAC, histone deacetylase; HDM, histone demethylases; HKM, histone lysine methylation; HMT, histone methyl transferase; MAO, monoamine oxidase; miRNA, microRNA; MMP, matrix metalloproteinase; TSG, tumour-suppressor gene.

and efficacy are currently under study in clinical trials for treatments of solid tumours and other haematological malignancies (ClinicalTrials.gov) (Yoo and Jones, 2006).

Much of our current knowledge about epigenetic mechanisms in cancer cells has focused on initial tumourigenic events. In contrast, relatively little is known about the role of epigenetic events in cancer progression and advanced disease. The development of improved epigenetic cancer therapies will require better understanding of tissue-specific differences in the epigenomic landscape and the order of epigenetic events which contribute to disease progression. Here, we focus on the role of two methylation phenomena (DNA and histone methylation) in cancer progression and metastasis formation. We discuss evidence for the contribution of aberrant DNA methylation and histone H3 lysine methylation patterns to the metastatic phenotype. Although we focus on three particular histone lysine methylation (HKM) modifications (H3K27, H3K9 and H3K4), which are well known to play a role in transcriptional regulation, it is important to consider that other cancer-related modifications, such as H3K36 or histone arginine methylation, could also play a role in the metastatic phenotype. Finally, we highlight several histone-modifying enzymes that could serve as biomarkers to predict metastatic potential and could represent potential targets for preventing or curing metastatic disease.

The dynamics of DNA methylation

DNA methylation constitutes an important mechanism for silencing gene expression in physiological and pathological conditions (Portela and Esteller, 2010). It influences the cellular chromatin state and regulates gene-expression patterns which can be maintained in subsequent generations (Bird, 2002). DNA methylation provides a stable mechanism for gene silencing by preventing or promoting the recruitment of regulatory elements to the DNA or by providing binding sites for methyl-binding proteins which recruit histone-modifying enzymes to induce a repressive chromatin state (Sharma et al., 2010a). DNA methylation plays a crucial role during development in the inactivation of imprinted genes and in X chromosome inactivation (Riggs, 1975). It also regulates the expression of germline-specific genes or tissue-specific genes in somatic cells (Illingworth et al., 2008). *De novo* methylation is established by the DNA methyl-transferases A and B (DNMT3A and DNMT3B) and is maintained and propagated during replication by DNMT1 (Okano et al., 1999). In mammals, most DNA methylation occurs at cytosine residues in CpG dinucleotides. Genomic regions rich in repetitive sequences (such as centromeres and transposon elements) are normally highly methylated to maintain genomic stability. CpGs are often clustered in 'CpG islands' (CpGIs), generally located around gene promoters (Bird, 2002). Around 60% of human promoters have CpGIs, including many housekeeping genes and tissue-specific genes (Gardiner-Garden and Frommer, 1987; Wang and Leung, 2004). Most CpGIs are not normally methylated, in contrast to scattered CpGs which are mainly methylated (Bird, 2002). 'CpGI shores' with lower CpG content are often located in promoter regions or in the gene body within 2 kb of the transcription start site (Irizarry et al., 2009) and their methylation status also has a strong inverse correlation with gene expression. Recent findings suggest that non-CpG DNA methylation is a feature of undifferentiated embryonic stem cells (ESCs) (Lister et al., 2009).

DNA methylation status may be more dynamic than that originally proposed, but the details of a demethylation process and its role in gene regulation are still unclear (Zhu, 2009; Wu and Zhang, 2010). Recent findings suggest that a mechanism of active demethylation is initiated by the TET (Ten-eleven translocation) family of enzymes (Tet1, Tet2 and Tet3). These 2-oxoglutarate-/Fe(II)-dependent oxygenases can catalyse the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (Tahiliani et al., 2009; Ito et al., 2010). However, the mechanisms that convert 5hmC into unmethylated cytosine are not well elucidated. It has been proposed that oxidation is followed by deamination by the AID/APOBEC family of deaminases to generate a G/T mismatch, which is repaired by a base excision repair pathway (Wu and Zhang, 2010; Guo et al., 2011). Recent studies revealed roles for the Tet enzymes during development via the regulation of global or locus-specific demethylation processes. For example, Tet1 directly contributes to ESC maintenance and inner cell mass cell specification by preventing hypermethylation and repression of the Nanog promoter (Ito et al., 2010). Tet2 mutations favour the development of myeloid malignancies by altering global

hydroxymethylation patterns that impair normal myeloid differentiation (Ko et al., 2010). These novel functions of the Tet enzymes in the maintenance of the balance of 5mC and 5hmC in stem cell renewal and cell differentiation could be relevant to their role in cancer cell phenotypes.

Histone methylation and chromatin states

Histone modifications play key roles in establishing and maintaining cellular identities, by inducing chromatin conformational changes that regulate the accessibility of DNA during gene transcription, replication and DNA repair processes (Chi et al., 2010). The histone N-terminal tail protruding from the nucleosomes can be modified at different residues by methylation, acetylation, phosphorylation, sumoylation and ubiquitylation (Zhang and Reinberg, 2001; Kouzarides, 2007). The combination of events, depending on which residues are altered by which modifications, leads to changes in the chromatin configuration; chromatin can adopt a condensed states which is inaccessible to the transcription machinery (heterochromatin) or a relaxed and transcriptionally active state (euchromatin) (Jenuwein and Allis, 2001). Histone-tail modifications not only alter the chromatin configuration, but also serve as docking sites for regulatory proteins (Martin and Zhang, 2005; Chi et al., 2010). Certain histone signatures are typical of transcriptional states; active genes are associated with histone acetylation and methylation of selective H3 lysine residues (H3K4, H3K36 and H3K79), whereas repressed genes are commonly marked by H3K9, H3K27 and H4K20 methylation (Jenuwein and Allis, 2001). Interestingly, ESCs have 'bivalent' chromatin domains consisting of repressive and activating histone marks on developmental genes, allowing phenotypic plasticity before committing to a specific cell fate (Bernstein et al., 2006). These marks are maintained by the repressive Polycomb Group (PcG) proteins and the activating Trithorax Group. Upon differentiation, ESCs loose the bivalent marks and acquire a less dynamic chromatin structure which is maintained throughout subsequent cellular divisions (Bernstein et al., 2006).

Histone lysine residues can have four states of methylation: unmethylated, mono-methylated (me1), di-methylated (me2) and tri-methylated (me3) (Kouzarides, 2007). HKM does not alter the lysine charge, but changes the chromatin structure and regulates gene transcription through proteins that specifically recognise and bind these modifications (Martin and Zhang, 2005). Histone methylation is a dynamic, reversible process which is maintained by the balance of histone methyltransferases (HMTs) and histone demethylases (HDMs) (Kouzarides, 2007). Furthermore, some of these enzymes can directly bind specific DNA sequences and some can modify particular lysine residues (Kouzarides, 2007). The majority of HMTs contain a SET domain that catalyses the addition of methyl groups to lysine residues (Rea et al., 2000). HMTs recruit S-adenosyl-L-methionine (SAM) as a cofactor, which donates the methyl group for the methylation event (Rea et al., 2000).

The methylation of H3K9 (H3K9me) was the first mechanism of gene repression to be linked to HKM (Rea et al., 2000). Studies in *Drosophila* showed that the gene *Su(var)39*, later shown to encode a H3K9 HMT, had an important role in the regulation of position-effect variegation (Tschiersch et al., 1994; Rea et al., 2000) and similar enzymes were subsequently discovered in humans (SUV39H1/H2, G9a and Riz1 among others) (Shilatifard, 2008). H3K9 methylation is important for chromatin condensation and heterochromatin formation. H3K9me is recognised and bound by heterochromatin protein 1, which recruits SUV39H, reinforcing the silencing process (Shilatifard, 2008). H3K9me1 and H3K9me2 are associated with euchromatic gene repression, whereas H3K9me3 is associated with stably silenced heterochromatin (Martin and Zhang, 2005). H3K9me2 marks contribute to the maintenance of gene repression in differentiated tissues in large genomic regions known as 'large organised chromatin K9–modifications (LOCKs)', which require the activity of the methyltransferase G9a (Wen et al., 2009). H3K9me marks are removed by the lysine-specific demethylase-1 (LSD1) and members of the Jumonji C domain (JMJD) family of KDMs (Hublitz et al., 2009).

Another important repressive mark is H3K27 methylation which plays an essential role in embryogenesis, cell differentiation and organogenesis (Hublitz et al., 2009). H3K27me3 is associated with constitutive heterochromatin and maintenance of gene repression during early development (Bernstein et al., 2006). In ESCs, H3K27 methylation usually overrides the effect of H3K4me3 in

bivalent regions, maintaining them in a repressed state. Upon differentiation, these regions become exclusively marked by either of these modifications, leading to gene activation or repression (Bernstein et al., 2006). H3K27me2/me3 marks are usually present at silent promoters and absent from active promoters and gene regions (Barski et al., 2007). H3K27me1 marks, however, are higher at active promoters, especially downstream of the transcription start site (TSS) (Barski et al., 2007). H3K27 methylation is catalysed by the Polycomb repressive complex 2 (PRC2) (Sawarkar and Paro, 2010). PRC2 is composed mainly of suppressor of zeste 12 (SUZ12), embryonic ectoderm development and enhancer of Zeste homologue 2(EZH2), which is the catalytic component with HMT activity. Core components of the Polycomb complexes do not bind DNA, but are thought to be directed and anchored by DNA-binding proteins, such as Zeste or Jarid2 (Jumonji, AT rich interactive domain 2), and non-coding RNAs such as HOTAIR (Gupta et al., 2010; Pasini et al., 2010; Sawarkar and Paro, 2010). Two H3K27 demethylases, UTX and JMJD3, were described and are found in complexes with the mixed lineage leukaemia (MLL) proteins, which have H3K4 HMT activity (Hublitz et al., 2009).

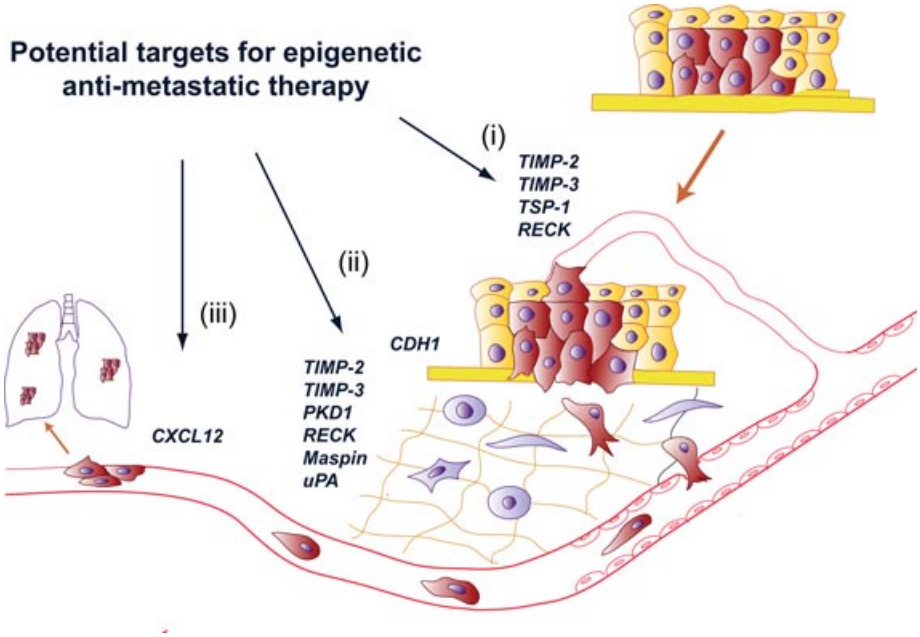
H3K4 methylation (H3K4me) is present in euchromatic regions and is usually associated with transcriptional activation (Pokholok et al., 2005). H3K4me3 occurs principally at the 5′ end of actively transcribed genes, near the TSS. H3K4me2 is more spread throughout genes, peaking towards the middle of the coding region of transcribed genes, and H3K4me1 is more abundant at the 3′ ends (Pokholok et al., 2005). H3K4me2 marks can be present at both active and inactive euchromatic genes, whereas H3K4me3 is present exclusively at active genes (Santos-Rosa et al., 2002). H3K4me favours transcriptional activation by facilitating H3 acetylation and recruitment of RNA polymerase II, but it also antagonises gene repression by preventing the binding of the nucleosome remodelling and deacetylase co-repressor complexes (such as NuRD), and interfering with substrate recognition by the SUV39H methyltransferases (Lachner and Jenuwein, 2002). The balance between HMTs and HDMs is important for the dynamics of H3K4me and the regulation of gene transcription. More than ten H3K4 HMTs have been identified (Hublitz et al., 2009), including the MLL1–4 proteins, Set 1a and Set 1b, Ash1L, Set7/9, and SMYD family members (SMYD1 and SMYD3). The MLL proteins (Trithorax homologues in *Drosophila*) are important for the regulation of developmental genes such as the Hox cluster, and deficiency of MLL1 or MLL2 causes embryonic lethality. The Set1 family is responsible for the majority of H3K4me in mammalian cells and Set1a regulates several housekeeping genes (Hublitz et al., 2009). The role of the other H3K4 HMT in different physiological or pathological processes is starting to be revealed. LSD1 was the first HDM to be discovered (Shi et al., 2004). Initially, proposed as a H3K4-specific demethylase, it was later found to cause H3K9 demethylation when associated to the androgen receptor, participating in the activation of androgen receptor targets (Metzger et al., 2005). Other H3K4 demethylases (JHDM1A–B and Jarid1A–1B–1C–1D) were recently identified, some of which play important roles during the development and have tissue-specific expression (Lan et al., 2008).

Epigenetic contributions to cancer progression and metastasis

Metastasis formation is a multi-step process (Foulds, 1958; Valastyan and Weinberg, 2011), with progressive accumulation of genetic and epigenetic alterations in cancer cells that provide a selective advantage to metastasise (Fearon and Vogelstein, 1990). A benign tumour becomes malignant when it acquires the capacity to invade and form distant metastases (Weinberg, 2007). This process involves distinct crucial steps (Fidler, 2003; Weinberg, 2007). Initially, progressive tumour growth, supported by increased angiogenesis, leads to localised invasion after infiltrating the basement membrane. Cells then migrate and traverse the extracellular matrix (ECM) invading nearby structures. In order to enter the circulation, these cells intravasate into lymphatic or blood vessels. Cancer cells that manage to survive the hostile circulatory environment and subsequently disseminate to distant anatomical sites can extravasate and form micrometastases. These foci can proliferate and colonise the new organ-forming macrometastases and interfering with normal organ function (Fidler, 2003; Weinberg, 2007) (Figure 1).

Several groups have identified metastasis genes or gene expression signatures that can predict

Figure 1 | Genes regulated by DNA methylation during cancer progression and metastasis
Progressive steps from initial tumour formation to establishment of metastasis include (i) tumour growth, angiogenesis and localised invasion; (ii) intravasation and survival; and (iii) extravasation and formation of distant tumours. Several genes that promote these processes are regulated by DNA methylation and constitute potential targets for epigenetic anti-metastatic therapy.



prognosis and metastasis in cancer patients and are being used in clinical practice (McDermott et al., 2011). van 't Veer et al. (2002), for example, identified a 'poor prognosis' signature using DNA microarray analysis, predictive of a short interval to metastasis in sporadic breast cancer patients with negative lymph nodes at diagnosis. Nguyen and co-workers classified the cancer genes into three main groups according to their roles in the metastatic process (Yang et al., 2004; Nguyen and Massagué, 2007; Yang and Weinberg, 2008; Nguyen et al., 2009): (i) metastasis initiation genes promote changes that allow cancer cells to leave the primary tumour and enter the circulation by regulating the epithelial–mesenchymal transition (EMT), migration, invasion, angiogenesis and intravasation (Nguyen et al., 2009); (ii) metastasis progression genes are important for survival in the circulation and for extravasation, and can contribute to the initial steps in the metastatic process; and (iii) metastasis virulence genes allow cancer cells to successfully colonise distant organs by enabling them to survive in a particular environment (Nguyen et al., 2009).

The progression genes are often specific to the cancer cell type and may have different functions in the primary and distal tumours, as is the case with *MMP-1* which promotes vascular remodelling in primary breast carcinomas but also contributes to lung extravasation (Minn et al., 2005). Downregulation of 'metastasis-suppressor genes' could contribute as well to metastasis progression (Steeg, 2003). These genes inhibit metastasis formation without affecting primary tumour growth. Few of these genes have been identified, such as *Kiss-1* or *NM23* in breast cancer cells and melanoma (Steeg, 2003). The virulence genes likely give cancer cells a selective advantage only in the metastatic sites, but not in the primary tumours; for example, parathyroid-hormone-related protein and interleukin-11 promote osteolytic metastatic lesions of breast cancer cells, but do not provide an advantage in the primary site (Yin et al., 1999; Kang et al., 2003). This set of genes would not be detectable in the search for metastasis predictive signatures in the primary tumour because they are thought to be induced when cells metastasise

Table 1 | Abnormal DNA methylation patterns in cancer cells

DNA hypomethylation	Consequence
Global hypomethylation	Reactivation of endoparasitic and repetitive genomic sequences Chromosomal and genomic instability
Hypomethylation of gene bodies	Activation of incorrect sites of transcription initiation
Loss of promoter methylation	Activation of metastasis and tumour promoting genes
DNA hypermethylation	**Consequence**
Promoter CpG island (CpGI) methylation	Tumour-suppressor gene (TSG) silencing Inhibition of transcription factors regulating TSGs Inactivation of metastasis suppressors
CpGI shore methylation	Abnormal transcriptional inactivation
Loss of imprinting	Deregulation of imprinted genes

to a specific organ (Nguyen et al., 2009). Hence, the identification of genetic biomarkers predicting metastasis and 'poor prognosis' gene expression signatures in specific types of cancers could be useful to guide personalised cancer therapy in the near future. More studies are needed to integrate this conceptual framework with molecular characterisation of the epigenome of metastatic cells.

DNA methylation in cancer initiation and metastasis

Altered DNA methylation is a well-established molecular hallmark of cancer cells (Portela and Esteller, 2010) (Table 1). The first epigenetic alteration identified in cancer cells was global loss of DNA methylation (Feinberg and Vogelstein, 1983). The hypomethylated state induces chromosomal instability and abnormal gene expression by the reactivation of repetitive genomic sequences and endoparasitic sequences (Gaudet et al., 2003; Esteller, 2007). Loss of gene-body methylation probably alters gene expression by allowing transcription initiation at erroneous sites (Portela and Esteller, 2010). Promoter hypomethylation of tumour-promoting genes appears to contribute to cancer progression. However, a major abnormality in cancer cells is the hypermethylation of promoter CpGIs of tumour-suppressor genes (TSGs) (Greger et al., 1989; Herman et al., 1994). This mechanism contributes to both sporadic and hereditary tumours and can be responsible for the 'second hit' of inactivation of the two-hit Knudson model (Esteller et al., 2001). Many genes regulating different cellular processes (e.g. proliferation, DNA repair, cell adhesion and apoptosis) are silenced by promoter hypermethylation in various human cancers (Esteller, 2007). DNA hypermethylation is thought to occur early in tumourigenesis, contributing to the genetic instability that predisposes cancer cells to acquire new mutations (Ballestar and Esteller, 2008). Loss of imprinting due to hypermethylation of imprinting centres also increases the risk of tumourigenesis (Sakatani et al., 2005). For example, gain of methylation of the imprinting centre in the maternal allele of the *H19–Igf2* locus causes abnormal activation of the silent *Igf2* copy and increases the risk of developing colorectal cancer by altering differentiation of the intestinal epithelium (Sakatani et al., 2005). DNMT overexpression is frequently observed in several tumour types, but mutations are uncommon (Miremadi et al., 2007). Inactivating mutations in the *DNMT3A* gene were recently found in AML patients and were associated with poor outcome (Ley et al., 2010). Also, Tet2 mutations appear to alter myeloid differentiation and favour the development of myeloid malignancies (Ko et al., 2010). One important remaining question is how the DNA methylation machinery is disrupted in cancer cells and how specific genes or genomic regions are targeted for methylation.

The advent of whole-genome techniques to study cancer cell methylomes has revealed that DNA methylation is not only important for oncogenesis, but also alters gene expression in later cancer stages (Rodenhiser, 2009). Our current knowledge of the contribution of DNA methylation to metastasis concerns mainly global and gene-specific DNA methylation changes (Rodenhiser, 2009). The recent discovery of Tet enzymes and active DNA demethylation raises the questions whether they play a clear role in regulating metastasis genes and whether they could be useful predictors of metastasis or progression. In the context of individual cancers, DNA methylation appears to regulate some metastasis initiation or metastasis progression genes that promote events such as angiogenesis, EMT, migration, invasion and extravasation (Figure 1). Yet, few metastasis virulence genes, regulating distant colonisation, have been found to be regulated by this mechanism.

Methylation of metastasis initiation genes

Regulation of ECM and angiogenesis

In the early stages of cancer progression, tumour cells induce degradation of the ECM by matrix metalloproteinases (MMPs) for angiogenesis. Downregulation of the genes encoding tissue inhibitor of metalloproteinases contributes to this process by loss of MMP regulation and release of angiogenic factors such as fibroblast growth factor-2 (FGF–2) and vascular endothelial growth factor (VEGF) (Seo et al., 2003). *TIMP-2* is suppressed in some solid tumours and lymphoid malignancies by promoter CpGI hypermethylation (Galm et al., 2005; Pulukuri et al., 2007). *TIMP-2* suppression by DNA methylation was observed in several metastatic prostate cancer lines, compared with normal prostate cells or non-invasive prostate cancer cells. Treatment with the demethylating agent 5-aza-CdR or the histone deacetylase inhibitor trichostatin A induced *TIMP2* mRNA re-expression and a significant decrease in their *in vitro* invasive capacity (Pulukuri et al., 2007). *TIMP-3* was also found to be suppressed by DNA methylation during initial, local progression of gastric and oesophageal cancers, correlating with poor patient survival (Gu et al., 2008). Decreased *TIMP-3* expression by promoter hypermethylation was also detected in kidney, brain, colon, breast and lung cancer (Sahin et al., 2010). The ECM component thrombospondin-1 (TSP-1) is a potent angiogenesis inhibitor; it regulates endothelial cell migration and survival, and limits the release of matrix-bound VEGF (Lawler, 2002). *TSP-1* inactivation by promoter hypermethylation was found in gastric, colon and pancreatic carcinoma and glioblastoma (Sahin et al., 2010). In gastric cancer patients, the level of methylation of *TSP-1* in tumour samples was significantly associated with vascular invasion, distant metastasis and a worse prognosis (Miyamoto et al., 2007). Treatment of gastric cancer cells with 5-aza-dC led to demethylation and re-expression of *TSP-1*.

Regulation of cell adhesion and invasion

Cancer cell dissemination relies on cellular invasion through tissue barriers (e.g. basement membrane, interstitial stroma and endothelial barrier) and requires changes in cell adhesion, cell morphology and cell migration (Friedl and Wolf, 2003). EMT is a key process in early tumour dissemination and metastasis formation in carcinomas, by allowing cells of epithelial origin to infiltrate adjacent tissues and disseminate to distant sites (Christiansen and Rajasekaran, 2006). This process involves changes in cell morphology and epithelial differentiation markers (e.g. E-cadherin, β-catenin) are typically downregulated (Yang et al., 2004; Yang and Weinberg, 2008). Loss of E-cadherin (which maintains cell-to-cell adhesion) is considered a hallmark of EMT (Yang and Weinberg, 2008). Although somatic and germline mutations are found in some cancers, the main mechanism of E-cadherin inactivation is by DNA methylation (Yoshiura et al., 1995; Berx et al., 1998; Lujambio and Esteller, 2009). Treatment of breast and prostate cancer cell lines with 5-Aza-CdR partially restores *CDH1* and *E-cadherin* expression in cells with CDH1 inactivation by DNA methylation (Graff et al., 1995). Reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) is a metastasis-suppressor gene which inhibits angiogenesis and distant metastasis and has prognostic impact in colon, lung, breast and pancreatic cancer (Shoushtari et al., 2011). Downregulation of *RECK* by promoter methylation was observed in colon and lung cancer cells (Chang et al., 2006; Cho et al., 2007). 5-Aza-CR treatment restored RECK expression and suppressed cellular invasion. Interestingly, oncogenic *RAS* activation in mouse fibroblasts could induce DNMT3B binding and methylation of the RECK promoter that was reversed with 5-Aza-CR (Chang et al., 2006).

Serine protease function is important for tumour cell invasiveness. Maspin, a member of the serine protease inhibitor (serpin) superfamily, displays altered methylation patterns that contribute to cancer progression (Sheng et al., 1996). *Maspin* is frequently downregulated during breast and oral cancer progression (Xia et al., 2000; Maass et al., 2001) and 5-aza-dC treatment restored *Maspin* expression in breast cancer cells (Domann et al., 2000). In patients with oral squamous cell carcinoma, higher *Maspin* expression was associated with better overall survival rates (Xia et al., 2000), but this was not the case in gastric and ovarian cancers (Sood et al., 2002; Akiyama et al., 2003; Terashima et al., 2005). The serine protease urokinase plasminogen activator (uPA) catalyses the conversion of plasminogen to active plasmin and was implicated in invasion and metastasis in prostate and breast cancers (Rabbani and Mazar, 2001; Guo et al., 2002; Pakneshan et al., 2003). Elevated uPA expression due to promoter

hypomethylation in breast and prostate cancer cells contributes to a more aggressive phenotype (Guo et al., 2002; Pakneshan et al., 2003). This could be mimicked experimentally by blocking methylation with 5-Aza-CR (Pakneshan et al., 2003). The methylation status of the uPA promoter was proposed as a prognosis indicator in breast cancer patients because a low level of methylation and high uPA expression correlated with more aggressive histological stages in tumour biopsies (Pakneshan et al., 2004). uPA was also shown to be necessary for tumour cell intravasation, a later step in the metastatic cascade (Kim et al., 1998).

Several molecules that mediate organ-specific colonisation have been identified (e.g. cytokines, adhesion molecules and proteases) (Kang et al., 2003; Minn et al., 2005; Kaplan et al., 2006). Human breast cancer cells, for example, express high levels of the chemokine receptor CXCR4 and are attracted by organs expressing the CXCL12 ligand (e.g. bone marrow, lymph nodes, liver and lungs). CXCL2, which is normally expressed in mammary epithelial cells was found to be silenced by DNA methylation in several breast cancer cell lines (Wendt et al., 2008). Paradoxically, it appeared that primary breast cancer cell lines that lack CXCL2 expression have an increased response to endocrine signals driving metastasis (Wendt et al., 2008). Re-expression of CXCL2 in these cells led to inhibition of lung metastasis when injected in severe combined immunodeficiency(SCID) mice.

Further identification of genes that are essential for the metastatic process and are regulated by DNA methylation will probably be useful for the development of future anti-metastatic therapies. We are aware that concomitant hypermethylation and hypomethylation changes of different genes contribute to metastasis; therefore, it is necessary to develop therapies that modify gene-specific patterns and not global DNA methylation, which can have undesired effects.

Histone lysine methylation and metastasis

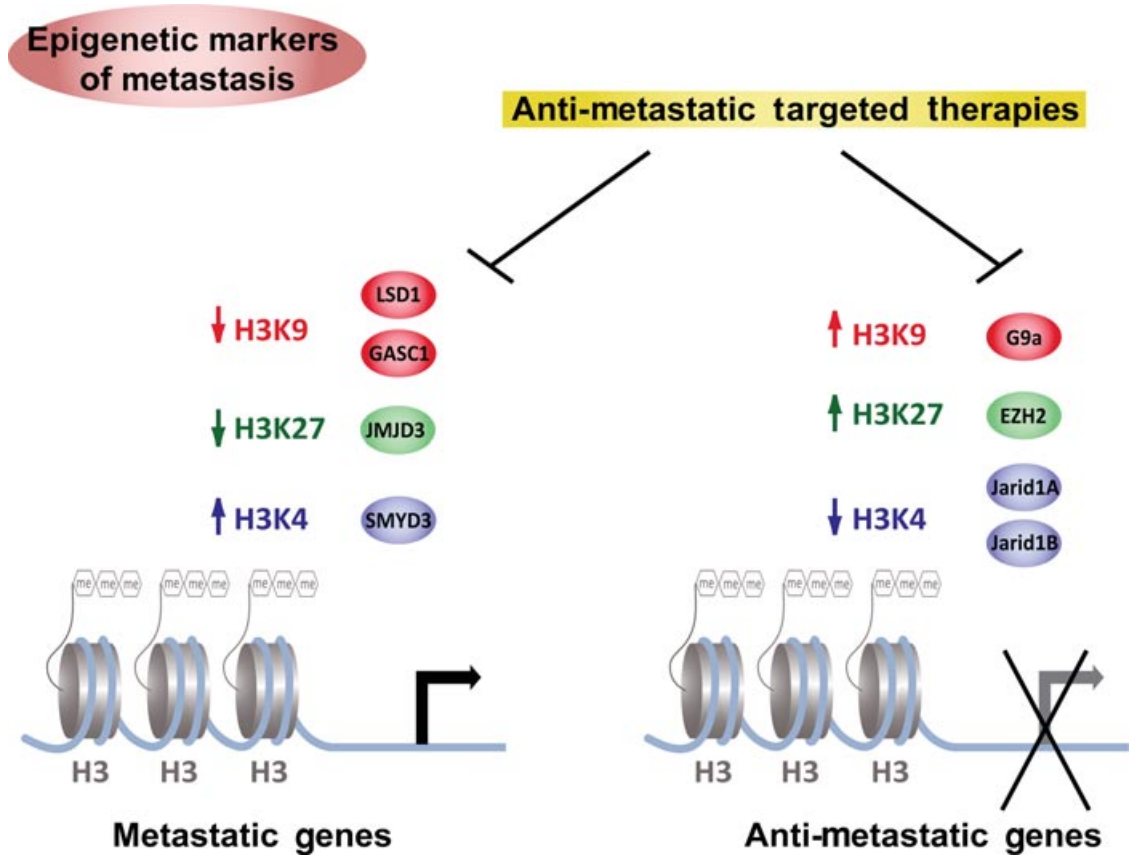
Global changes in HKM patterns, as well as modifications at specific gene promoters, are commonly observed in cancer cells. The landscape of histone modifications and the molecules that shape this landscape are attracting increasing attention in cancer research. Intense investigation of HMTs and HDMs has revealed their contribution to oncogenesis in many cancer types. Some of these enzymes are master regulators of tumour-associated genes, but only a few of these enzymes have been directly linked to the metastatic process (Figure 2). Their differential expression in primary and metastatic tumours suggests that they may play roles in metastasis formation.

Repressive histone modifications

Increases in repressive histone marks constitute another layer of abnormal silencing of TSGs which may function in conjunction with DNA hypermethylation (Nguyen et al., 2002; Kondo et al., 2003; Wozniak et al., 2006). Histone H3K9 methylation is thought to precede DNA methylation, which then 'locks in' a silenced state (Bachman et al., 2003). Possible crosstalk mechanisms are supported by the observation that treatment of bladder, colorectal or breast cancer cell lines with the DNA demethylating agents, 5-Aza-CdR or 5Aza-dC, led to a decrease in H3K9me marks and re-expression of silenced TSGs (Nguyen et al., 2002; Kondo et al., 2003; Wozniak et al., 2006). Both global and gene-specific H3K9 methylation modifications can influence the metastatic phenotype of cancer cells. Elevated H3K9me3 correlated positively with tumour stage, cancer recurrence and poor survival rate in gastric adenocarcinoma (Park et al., 2008). Also, H3K9 methylation was linked to the silencing of two anti-metastatic genes, desmocollin 3 (*DSC3*) and *Maspin* in breast cancer cells (Wozniak et al., 2006). HMT enzymes responsible for H3K9 methylation are overexpressed in diverse cancers and linked to metastasis. For example, G9a expression correlated with poor prognosis in lung cancer patients (Chen et al., 2010). G9a promotes invasion and metastasis in lung cancer cells by silencing the cell adhesion molecule *EP-CAM* (Chen et al., 2010). H3K9me2 marks on the promoter led to binding of other transcriptional repressors (DNMT1, HP1 and HDAC1). Inhibition of G9a in lung cancer cells reduced their invasive and metastatic properties, whereas G9a ectopic expression in poorly invasive lung cancer cells increased their metastatic capacity. SETDB1, another H3K9 HMT, is amplified in melanoma, breast, lung, liver and ovarian cancers (Ceol et al., 2011) and its overexpression accelerated melanoma onset and tumour aggressiveness in a zebrafish model (Ceol et al., 2011).

Figure 2 | Histone lysine-modifying enzymes involved in metastasis

Upregulation of modifiers of lysine (K) methylation (me) of histone H3K9, H3K27 and H3K4, which activate metastatic genes or repress anti-metastatic genes, could serve as biomarkers of metastatic potential in cancer cells. They constitute potential targets for targeted anti-metastatic therapy.



H3K27 methylation also cooperates with DNA methylation to induce gene silencing in cancer cells (Schlesinger et al., 2006; Martinez-Garcia and Licht, 2010). H3K27me3 established by Polycomb during development may pre-mark certain genes for *de novo* DNA methylation in colon cancer cells (Schlesinger et al., 2006). Epigenetic programs controlled by PRC2 are associated with aggressive cancer phenotypes and contribute to metastasis in patients (Glinsky, 2005; Glinsky et al., 2005; Sparmann and van Lohuizen, 2006). Multi-gene Polycomb silencing pathway signatures in primary tumours were linked to metastatic dissemination and poor clinical outcome in prostate and breast cancer patients (Glinsky, 2005; Glinsky et al., 2005; Yu et al., 2007). The catalytic PRC2 component EZH2 was one of the first histone-modifying enzymes associated with metastasis (Varambally et al., 2002). EZH2 is frequently overexpressed in prostate, breast, colon, skin and lung cancer, and is involved in silencing TSGs (Bachmann et al., 2006; Chi et al., 2010). High EZH2 expression was associated with larger tumour size and was an independent predictor of metastasis in familial breast cancer (Alford et al., 2011). EZH2 levels in prostate tissues increased with cancer stage progression, being higher in metastatic cancer and were associated with inferior clinical outcome (Varambally et al., 2002). Mutations in EZH2 were identified in follicular lymphoma and diffuse large B-cell lymphoma (Morin et al., 2010). Another PRC2 component SUZ12 was also found to be upregulated in colon and breast tumours (Kirmizis et al., 2003; Berdasco and Esteller, 2010). PRC2 function has been linked to silencing of the angiogenesis inhibitor, vasohibin-1, in

tumour-associated endothelial cells (Lu et al., 2010). Interestingly, EZH2 formed a co-repressor complex with deacetylases HDAC1/HDAC2 and SNAIL transcription factor that silenced the expression of *E-cadherin* (Tong et al., 2011). EZH2 also mediates epigenetic silencing of the gene encoding a Ras GTPase-activating protein *DAB2IP* in prostate cancer cells, driving EMT and the metastatic phenotype (Min et al., 2010).

Altered histone demethylation may also play a key role in tumourigenesis. The HDM, LSD1, has both tumour-promoting and tumour-suppressive effects because of its dual action of removing repressive H3K9 marks or activating H3K4 marks. LSD1 is overexpressed in several types of cancer (e.g. prostate, bladder, lung and colorectal carcinoma) (Metzger et al., 2005; Hayami et al., 2011) and can promote proliferation by erasing H3K9me marks in prostate cancer (Metzger et al., 2005; Lim et al., 2010) or inhibit the metastatic phenotype of breast cancer cells by repressing genes involved in cell migration, invasion and EMT (Wang et al., 2009). The differences may be explained by differences in LSD1-chromatin-associated complexes (Wang et al., 2009). Alterations in the genes encoding H3K27 HDMs, UTX and JMJD3, were also reported in multiple cancers and were associated with transcriptional changes and an effects on cell proliferation and metastasis (Xiang et al., 2007a; van Haaften et al., 2009).

Activating histone modifications

The SET domain HMT SMYD3 is upregulated in various cancers (colorectal, hepatocellular, breast and cervical carcinoma) and highly metastatic pancreatic cells (Hamamoto et al., 2004; Liu et al., 2007; Nakamura et al., 2007; Wang et al., 2008; Ren et al., 2010). SMYD3 is unusual in that it has a catalytic domain which methylates H3K4 as well as a DNA-binding domain that might target specific genes promoters. SMYD3 appears to transcriptionally activate genes that regulate cancer phenotypes such as proliferation, immortalisation and migration. SMYD3 activates *c-Met*, a hepatocyte growth factor receptor involved in metastasis (Birchmeier et al., 2003; Zou et al., 2009). Silencing of SMYD3 by short hairpin RNA (shRNA) in hepatocellular and breast cancer cell lines inhibited *c-Met* expression, cell migration and invasion (Zou et al., 2009). We recently found that SMYD3 directly regulates the expression of the metastasis-associated gene *MMP-9* in human fibrosarcoma cells, and is essential for the invasive property of these cells *in vitro* and *in vivo* (Cock-Rada et al., 2012; Medjkane et al., 2012).

Aberrant expression of H3K4 demethylases can also contribute to oncogenesis (Blair et al., 2011). For example, JARID1B (PLU-1/KDM5B) is overexpressed in breast, prostate, lung and bladder cancer (Xiang et al., 2007b; Yamane et al., 2007; Blair et al., 2011) and silences TSGs, such as *BRCA1*, inducing breast cancer cell proliferation (Yamane et al., 2007). Evidence suggests that JARID1B marks a small subpopulation of slow-cycling melanoma cells with tumour-initiating capacity (Roesch et al., 2005) and knock-down experiments showed that JARID1B was required for continuous *in vivo* tumour growth and metastasis formation in serial transplantation assays. Other members of the JARID family have been reported to be overexpressed in gastric cancer or non-small cell lung cancer (Sharma et al., 2010b; Zeng et al., 2010; Blair et al., 2011) or even mutated in clear cell renal carcinoma (Dalgliesh et al., 2010). But it remains uncertain whether these events affect global chromatin structure or have targeted effects on specific gene loci related to the transformed phenotypes.

MicroRNAs as epigenetic regulators

In addition to DNA and histone methylation events, other epigenetic mechanisms likely contribute to the metastatic process. An active area of research is the study of microRNAs (miRNAs), short, non-coding RNAs that control gene expression post-transcriptionally by inhibiting protein translation or degrading target mRNA transcripts. An extensive discussion of the role of miRNAs in cancer and metastasis is beyond the scope of this review and we refer to the reader to several excellent recent reviews (Lujambio and Esteller, 2009; Zhang et al., 2010; Kasinski and Slack, 2011). miRNAs can function as oncogenes or tumour suppressors depending on the pathways they target and could one day be used themselves as anti-cancer therapeutics (Kasinski and Slack, 2011). Furthermore, genetic screens have highlighted the role of specific miRNAs in EMT and metastatic disseminated (e.g. miR-200 loss has been associated with aggressive tumours, and miR-31 has an anti-metastatic effect on breast cancer dissemination) (Kasinski and Slack, 2011). Interestingly, there

are also several well described examples of miRNAs targeting epigenetic regulators of metastasis. For example, loss of miR-101 is associated with upregulation of EZH2 PcG protein in prostate cancers (Varambally et al., 2008). And the DNA methylating enzymes themselves may be regulated by miRNAs in cancer (Sandhu et al., 2012). As the genes encoding these regulatory non-coding RNAs may themselves be regulated by DNA methylation, miRNA networks likely play an important and complex role in regulating cancer cell phenotyes and tumour progression.

Epigenetic drug treatment for cancer

The fact that epigenetic alterations are potentially reversible offers an attractive strategy for the development of epigenetic drugs that restore the non-cancerous methylation landscape. Clearly, an important consideration is the extent to which the changes in epigenetic-modifying enzymes produce global or targeted effects on gene expression (Medjkane et al., 2012). Epigenetic-modifying drugs are mainly used in combination treatment in cancer patients (Kelly et al., 2010). One of their current drawbacks is the lack of specificity, as the drugs in clinical trials modulate global changes in gene expression but not gene-specific alterations. However, they offer the possibility of restoring TSG expression or loss-of-function phenotypes, while other drugs mainly target overexpression or gain-of-function phenotypes. Combining epigenetic drugs with conventional chemotherapy appears to increase their therapeutic efficacy. For example, 5-Aza-CdR reactivates pro-apoptotic genes inducing chemosensitivity to cytotoxic agents (Soengas et al., 2001). Also, HDAC inhibition sensitises DNA to exogenous genotoxic damage, induces generation of reactive oxygen species and alters chromosome segregation (Eot-Houllier et al., 2009). A major challenge in the epigenetics field will be to develop drugs that modulate gene-specific epigenetic events. An alternative approach is by targeting chromatin-modifying enzymes that regulate a subset of genes involved in the control of different cancer phenotypes (Figure 2). Some of these enzymes are overexpressed almost exclusively in cancer cells, making them ideal candidates for epigenetic-targeted therapies. In this context, our recent finding that SMYD3 specifically regulates expression of the metastatic *MMP-9* gene without affecting global H3K4me3 levels suggests that targeting specific enzymes may provide an effective means to tackle metastatic disease (Cock-Rada et al., 2012; Medjkane et al., 2012). The analysis of the crystal structure and functional studies of these novel identified enzymes provides an important tool for the development of specific inhibitors using sophisticated *in silico* drug design strategies (Sirinupong et al., 2010). However, the search for therapeutic compounds that selectively inhibit HMTs or demethylases is still at the beginning. Synthetic or natural compounds such as the SAM analogues, *S*-adenosylhomocysteine (SAH) or the bacterial product sinefungin have been found to inhibit global HMT activity but they can also block other reactions that use SAM such as DNA methylation (Spannhoff et al., 2009). Although these compounds have been used extensively as research tools, their lack of specificity hinders their application in the clinic. Several more specific compounds have been recently discovered using high throughput screening analysis. Chaetocin, a fungal metabolite, was found to inhibit SUV3-9H and to a less extent G9a (Greiner et al., 2005). A selective inhibitor of G9a, BIX-01294, was shown to reduce global H3K9me2 and decrease these marks at G9a target genes in mouse ESCs and fibroblasts (Kubicek et al., 2007). A more potent and selective G9a/GLP inhibitor, UNC0638, showed stronger effects with less toxicity in different cancer cell lines (Vedadi et al., 2011). It caused a marked reduction of global H3K9me2 levels, similar to levels observed with shRNA knockdown of G9a/GLP in breast cancer cell lines. It reduced the clonogenicity of MCF7 cells, and decreased H3K9me2 marks at known G9a-regulated promoters. In mouse ESCs, UNC0638 treatment also reactivated G9a-silenced genes (Vedadi et al., 2011).

Several groups have investigated LSD1 inhibitors *in vitro* and *in vivo*. LSD1 is an amino-oxidase that shares homology with the neural monoamine oxidase (MAO)-A and MAO-B (Hoffmann et al., 2012). Therefore, inhibitors of MAOs used as psychiatric drugs, such as tranylcypromine, pargyline or phenelzine, were initially tested as LSD1 inhibitors, but some failed in further studies or displayed significant off-target effects (Hoffmann et al., 2012). Biguanide and bisguanidine polyamine analogues are also potent inhibitors of LSD1. When used in colon cancer cells, they led to re-expression of

aberrantly silenced genes, inducing H3K4me and reducing H3K9 methylation marks at promoters of re-expressed genes (Huang et al., 2007). Treatment with a polyamine analogue, PG11150, combined with DNA methylation inhibitors, led to a synergistic effect on gene re-expression (Huang et al., 2009). Interestingly, when administered *in vivo* to athymic nude mice, xenografted with human colorectal cancer cells, both PG11150 and 5-Aza-CR showed a marked reduction in tumour growth. However, the greatest anti-tumour effect was observed with the combination of both treatments, with no significant toxicity, suggesting a potential powerful combination of these epigenetic-modifying approaches to treat cancer (Huang et al., 2009).

Several pharmaceutical companies are trying to develop direct inhibitors of EZH2 because of its pathogenic role in several types of cancer and the association of high EZH2 expression with poor clinical outcome (Wagner and Jung, 2012). An indirect EZH2 inhibitor, DZNeP, which is a SAH-hydrolase inhibitor, showed promising effects both *in vitro* and *in vivo*, decreasing cancer cell invasion and angiogenesis in brain and prostate cancer (Crea et al., 2012). However, a more specific EZH2 inhibitor is needed because this compound has global HMT inhibition properties (Miranda et al., 2009). Two recent studies offer promise for more selective EZH2 inhibitors which could be effective to treat EZH2 mutant lymphomas; Knutson et al. (2012) reported the discovery of EPZ005687, a potent inhibitor of EZH2 {*K*(i) of 24 nM} which may have effects against lymphomas with specific EZH2 mutations; and researchers from GlaxoSmithKline have developed GSK126, a potent, highly selective, *S*-adenosyl-methionine-competitive, small-molecule inhibitor of EZH2 methyltransferase activity which inhibits the proliferation of EZH2 mutant DLBCL cell lines (McCabe et al., 2012). Despite ongoing efforts to find specific and potent inhibitors of HMTs or HDMs in a cancer context, most studies have not passed the *in vitro* phase because of a lack of potency, specificity or dual actions of these compounds.

Mouse xenograft models have been useful to test anti-tumour and anti-metastatic effects of epigenetic-modifying drugs approved for clinical use, by treating immunosuppressed mice with these compounds after subcutaneous or intravenous injection of human cancer cells, and evaluating tumour size or metastasis formation (Ganesan et al., 2009; McCabe et al., 2012). The efficacy of epigenetic-modifying drugs combined with chemotherapeutic agents is also evaluated in mouse models, as well as their toxicity or the drug concentration needed to induce the desired effect in pre-clinical phases (Sausville and Burger, 2006; Festuccia et al., 2009). However, caution should be taken in translating these results into the clinic because tumour biology (growth, progression and metastasis) and treatment response in mouse models differ substantially from tumour biology in cancer patients (Ellis and Fidler, 2010).

Conclusion and perspectives

The last few decades, since the groundbreaking discoveries of oncogenes and TSGs, have led to unprecedented insight into the role of genetic lesions in cancer and tumour progression. Although much of this work has focused on tumour-initiating mutations, the molecular events that drive metastasis are much less clearly understood. Genetic screening has become a useful tool for clinicians to identify family members at risk and in some cases choose appropriate clinical protocols. But mutations are irreversibly locked into the cancer genome and rarely offer clear prognosis for disease progression. In contrast, epigenetic events may serve as effective biomarkers to follow disease evolution and eventually provide 'drugable' targets for reversing the epimutational effects and the associated phenotypes. We are convinced that the molecular machinery that regulates chromatin states and the definition of the genome-wide methylation landscape of tumour progression will begin to offer new hope for cancer patients.

Selective HMT or HDM compounds, used alone or in combination with other anti-cancer therapies, are promising approaches to treat cancer. The newly discovered function of Tet enzymes could offer additional potential drug targets with broad therapeutic applications.

Cancer is such a heterogeneous disease that an integrated approach is urgently needed to fight this devastating disease. With the advent of high-throughput platforms to study the genetic and epigenetic landscape of cancer cells, coupled with targeted molecular approaches, more effective patient-tailored treatments will probably be available in the near future, providing a better chance of killing cancer cells with less toxicity for the patients.

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Conflict of interest statement

The authors have declared no conflict of interest.

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