

REVIEW

Epigenetic regulation of cancer stem cell formation and maintenance

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Abstract

Cancerous tumours contain a rare subset of cells with stem-like properties that are termed cancer stem cells (CSCs). CSCs are defined by their ability to divide both symmetrically and asymmetrically, to initiate new tumour growth and to tolerate the foreign niches required for metastatic dissemination. Accumulating evidence suggests that tumours arise from cells with stem-like properties, the generation of CSCs is therefore likely to be an initiatory event in carcinogenesis. Furthermore, CSCs in established tumours exist in a dynamic and plastic state, with nonstem tumour cells thought to be capable of de-differentiation to CSCs. The regulation of the CSC state both during tumour initiation and within established tumours is a desirable therapeutic target and is mediated by epigenetic factors. In this review, we will explore the epigenetic parallels between induced pluripotency and the generation of CSCs, and discuss how the epigenetic regulation of CSCs opens up novel opportunities for therapeutic intervention.

KEYWORDS

cancer stem cells, cancer therapy, early detection of cancer, epigenetics, induced pluripotent stem cells, tumorigenesis

1 | INTRODUCTION

As with adult tissues, cancerous tumours also contain a rare subset of cells with stem-like properties that can function to regenerate the heterogeneous cell populations observed therein. These cancer stem cells

(CSCs) are defined by their ability to divide both symmetrically and asymmetrically, to initiate new tumour growth and to tolerate the foreign niches required for metastatic dissemination. As the tumour-initiating population, CSCs underpin the very nature of malignancy and studying their regulation is essential for understanding tumour formation, metastasis and relapse after therapy.

As it is not possible to isolate CSCs based on functional properties, CSC identification can be achieved by FACs sorting based on surrogate cell surface marker profiles and subsequent transplantation into immune-compromised mice to demonstrate enhanced tumorigenic potential. Using this strategy, CSCs have been identified in most cancers, first in acute myeloid leukaemia (AML) followed by breast cancer and other solid malignancies such as brain, colon and pancreatic cancer, and are purported to account for only a few per cent of the total cell population.¹⁻⁶ The existence of a rare population

Abbreviations: AML, acute myeloid leukaemia; ATP, adenosine triphosphate; BAF, BRG1- or BRM-associated factors; BER, base-excision repair; BET, bromodomain and extra terminal; BRD, bromodomain; CIMP, CpG island methylator phenotype; CSC, cancer stem cell; DNA, deoxyribose nucleic acid; DNMT, DNA methyltransferase; EMT, epithelial-mesenchymal transition; ESC, embryonic stem cell; FACs, fluorescence-activated cell sorting; GBM, glioblastoma multiforme; HDAC, histone deacetylase; IL6/17, interleukin 6/17; iPSC, induced pluripotent stem cell; lncRNA, long noncoding RNA; LSD1, lysine-specific demethylase 1; MET, mesenchymal-epithelial transition; miRNA/miR, microRNA; ncRNA, noncoding RNA; PDAC, pancreatic ductal adenocarcinoma; PRC, polycomb repressive complex; RISC, RNA-induced silencing complex; RNA, ribose nucleic acid; SAM, S-adenosyl methionine; SWI/SNF, SWI/SNF/sucrose nonfermentable; TAM, tumour-associated macrophage; TET, ten-eleven transcription factor; TGF, transforming growth factor; TME, tumour microenvironment; VEGF, vascular endothelial growth factor

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of CSCs supports the notion that a heterogeneous cancer arises from a single cell atop a cellular hierarchy. This also suggests that cancer arises from a cell with stem-like properties, as the cell-of-origin would require the asymmetric division to initiate and maintain tumour growth. Furthermore, the existing properties and long lifespan of a stem cell make it more likely than a differentiated cell to acquire a tumourigenic phenotype.⁷ Recent evidence supports the premise that cancer arises from the deregulation of existing stem cell populations. In an organ-wide study, Zhu et al induced oncogenic mutations specifically in CD133+ cells in the mouse. Tumours only arose in those organs where CD133 was proven to have generative capacity, that is, was an effective marker of a normal stem cell population (the liver, small intestine and stomach but not brain, kidney or pancreas). Furthermore, liver injury increased CD133+ cells and tumourigenic potential after transformation, thus indicating environmental factors can converge with genetic mutations to increase cancer incidence.⁸ Another study showed that deregulation of existing cell populations preceded tumour formation in an inflammatory model of bowel cancer. Chronic inflammation disrupted homeostasis in the large intestine so that the paneth cells de-differentiated to a stem-like population from which tumours could arise.⁹ These two studies are both examples of cancer arising from stem cells, be that an existing stem cell pool or one generated by extrinsic factors. CSC plasticity also persists during tumour growth as both stem and nonstem-like populations are capable of inter or intraconversion in response to extrinsic signals.¹⁰⁻¹² This de novo generation of the CSC phenotype has obvious

implications for therapeutic strategies, however, the molecular mechanisms involved are poorly understood. Creation of CSCs by definition requires a reversible but heritable process (asymmetric division), which strongly suggests a role for epigenetic regulation and there is mounting evidence in support of this, not least the importance of epigenetics in induced pluripotency.

Epigenetics refers to a number of mechanisms that control the reversible regulation of gene expression by changing the chromosome without altering the DNA sequence: DNA can be altered epigenetically by methylation and demethylation of CpG nucleotides. Epigenetic changes in the overall structure of chromatin occur through at least three interrelated mechanisms: posttranslational modifications of histones, ATP-dependent chromatin remodelling and the incorporation (or replacement) of specialised histone variants into chromatin. Finally, noncoding RNA can interact with transcriptional processes to alter gene expression. In addition to 2D processes, epigenetic regulation can also involve higher-order chromatin organisation including promoter-enhancer interactions, regulatory DNA loops and 3D chromatin localisation in the cell nucleus (Figure 1).

As these epigenetic mechanisms are important mediators of cellular identity, we will explore how the restructuring of such epigenetic barriers reinforces the stem-like state in both normal cells and cancer, and their relevance to tumour initiation (Figure 2). Furthermore, we will discuss how the epigenetic regulation of CSCs opens up novel opportunities for cancer detection and therapeutic intervention.

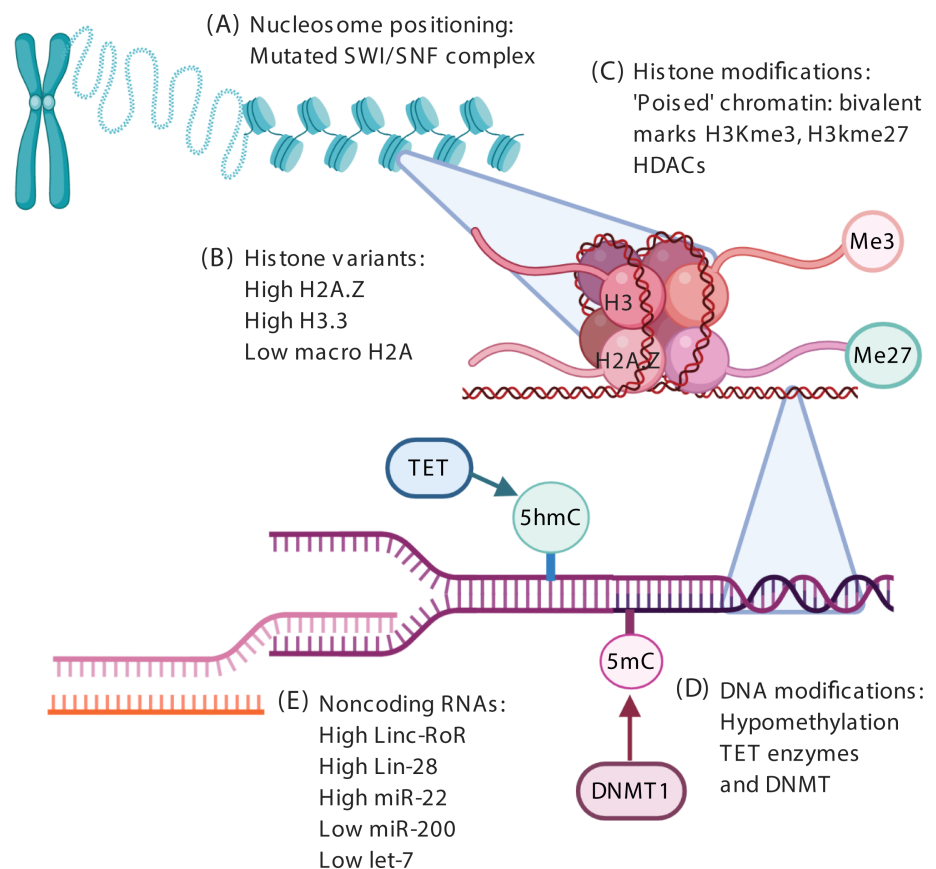


FIGURE 1 Epigenetic regulation of cancer stem cells. A variety of epigenetic mechanisms have been implicated in the generation of CSCs including changes in (A) nucleosome remodelling and associated complexes (eg, mutations in SWI/SNF). B, Histone variant deposition including higher H2A.Z and lower macroH2A. C, Histone modifications, in particular, bivalent histone marks D, Hypomethylation mediated by TET proteins E Altered expression of noncoding RNAs for example, high Lin28, low let-7 [Color figure can be viewed at wileyonlinelibrary.com]

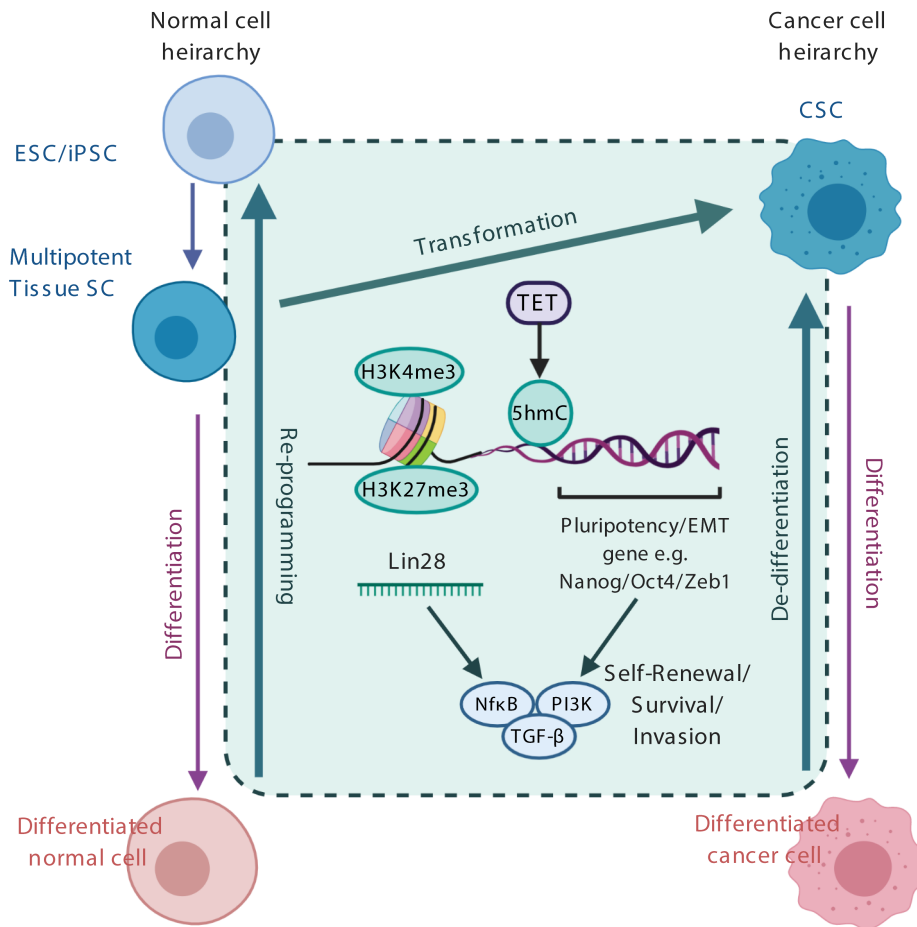


FIGURE 2 Epigenetic parallels between reprogramming, transformation and de-differentiation. Transition to stem-like states requires enhanced expression of pluripotency and EMT genes, which can be mediated at relevant loci by a combination of epigenetic processes including nucleosome loss at transcription start sites, histone variant deposition, histone modifications, in particular, bivalent histone marks to allow for rapid gene activation, demethylation by TET proteins and miRNA expression such as Lin-28. All converge on downstream signalling pathways such as TGFβ and NfκB to promote stem-like characteristics such as self-renewal, survival and invasion [Color figure can be viewed at wileyonlinelibrary.com]

2 | DNA METHYLATION AND DEMETHYLATION IN CSC PLASTICITY

DNA methylation is a form of gene silencing that occurs mainly to CpG dinucleotides which cluster in CpG islands; areas of high CpG density usually found at promoters. To methylate cytosines, DNA methyltransferases (DNMTs) catalyse the transfer of a methyl group from cofactor S-adenosylmethionine to the carbon of the cytosine ring to generate 5-methylcytosine (5mC). This functions to inhibit gene expression either by recruitment of methyl-CpG-binding domain proteins which in turn recruit histone-modifying and chromatin-remodelling complexes, or by preventing the recruitment of DNA-binding proteins, that is, transcription factors. To reactivate expression after silencing, 5-mCs can be oxidised to 5-hydroxymethylcytosine (5hmC) and back to the unmodified state by TET proteins and base excision repair (BER), to ultimately restore the unmethylated cytosine. TET protein-mediated demethylation can occur via either passive or active processes. Passive demethylation results from the failure to maintain 5mC marks across cell divisions (5hmC is not a substrate for DNMTs). Active demethylation is enzymatic, whereby TET proteins further oxidise 5hmC marks to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) then back to the unmodified state. 5fC bases are recognised by thymine-DNA-glycosylases (TDG) that excise mismatched pyrimidines that would then be replaced by an

unmodified cytosine by the BER pathway. Intermediate marks (5fC or 5caC) are much less abundant than 5hmC but may also have independent functions¹³ (Figure 3A).

Methylation patterns are altered in cancer; localised hypermethylation occurs in CpG islands of the promoters of tumour suppressor genes (the so-called “CpG island methylator phenotype”—CIMP), silencing their expression.¹⁴ However, global hypomethylation in intergenic regions causes oncogene activation and ultimately results in genomic instability.^{15,16} What happens in the CSC subset and what methylation changes are required to generate CSCs and initiate tumour growth?

In general, CpG methylation is required for differentiation whereas demethylation is essential for induction of the pluripotent state.¹⁷⁻¹⁹ However non-CpG methylation, although rarer, is associated with pluripotency and is lost upon development in all tissues except the brain.²⁰ There is evidence to suggest that loss of methylation is also required for the generation of CSCs and tumour initiation: data produced in mouse models demonstrate that global loss-of-imprinting (LOI) events alone promote the onset of cancer, including loss of growth inhibition by TGFβ.²¹ CSC formation from cancer cells was found to depend on loss of methylation of the Nanog promoter via DNMT1 inhibition.²² Loss-of-function mutations in DNMT3A also led to the expansion of preleukaemic SCs, indicating a role for dysregulated DNA methylation in inducing tumour-initiating cells.²³

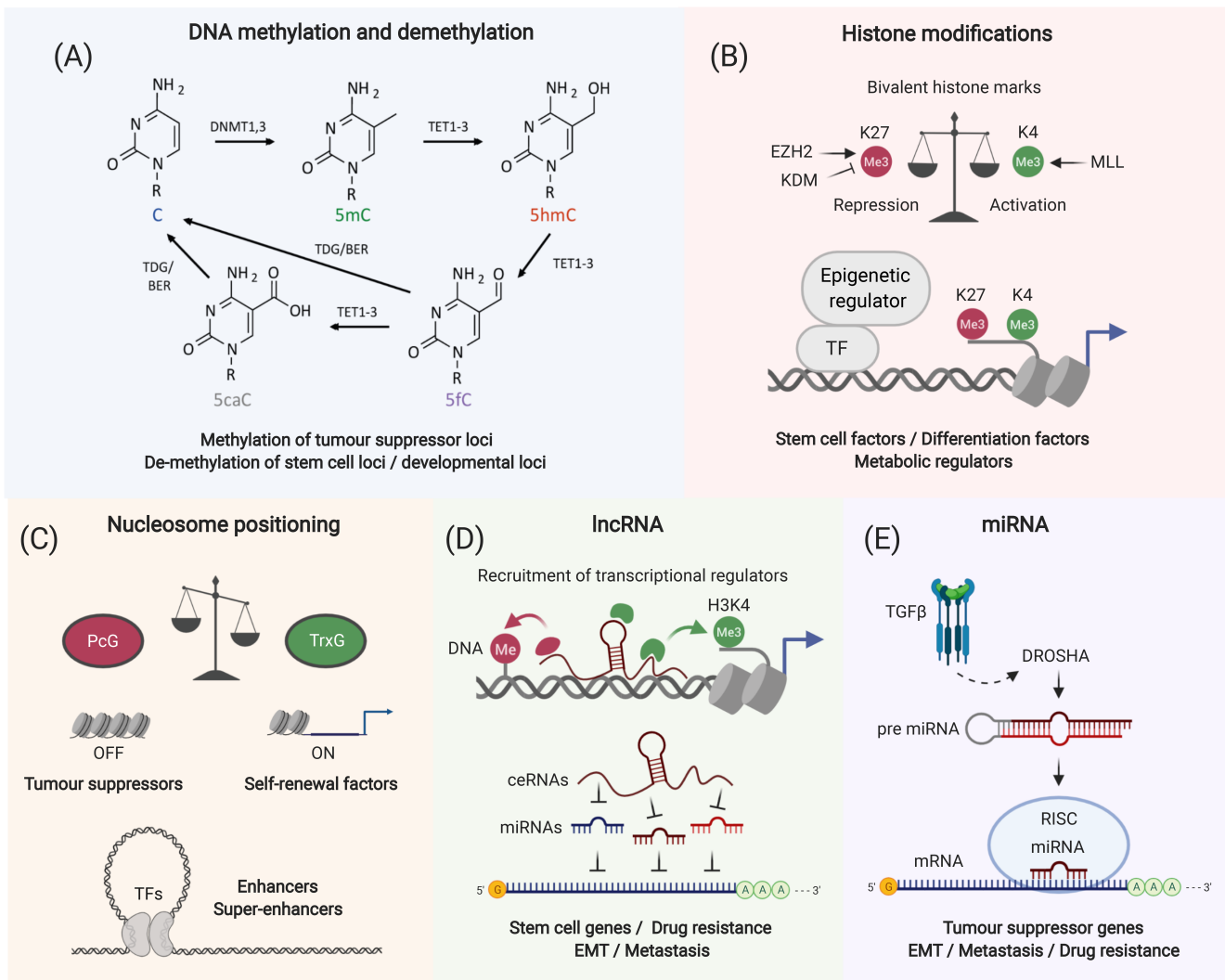


FIGURE 3 Epigenetic mechanisms in detail. A, Cycle of methylation and demethylation by DNMT and TET proteins, respectively. TET-mediated demethylation generates intermediate 5hmC, 5fC and 5caC marks that are converted to unmodified cytosine by the BER pathway. B, Bivalent histone marks; the presence of both activating H3K4me₃, and repressing H3K27me₃ marks at certain loci allow for rapid gene activation and are characteristic of stem cells. C, Nucleosome remodelling complexes can affect gene expression at relevant loci: TrxG complexes allow expression of self-renewal factors whereas PcG complexes prevent transcription of tumour suppressors. D, Long noncoding RNAs modify gene expression by affecting the recruitment of transcriptional regulators. E, MicroRNAs modify gene expression by directly affecting transcription [Color figure can be viewed at wileyonlinelibrary.com]

Some hypomethylation also continues to be associated with CSCs once the tumour has been established. For example, the CpG region of the CD133 gene promoter, a key gene involved in CSCs, was shown to be hypomethylated in several cancer types including breast, ovarian, colorectal and glioblastoma.^{24–26} Also, some important demethylated regions occur exclusively in CSCs compared to nonstem tumour cells. For example, in pancreatic ductal adenocarcinoma (PDAC), genes involved in CSC pathways, including GATA6, SOX9 and BMP4, were demethylated in the invasive (more stem-like) population.²⁷ Demethylation is highly important in pluripotency, both in ESCs and induced pluripotent stem cells (iPSCs). Reprogramming of somatic cells to a stem-like state can be achieved by expression of the Yamanaka factors (Oct3/4, Sox2, Klf4 and c-Myc), and is accompanied by epigenetic changes including demethylation. Reprogramming is

dependent on TET1, which affects Nanog levels and can even act as a substitute for Oct4. However, knockout of TET1 in ESCs did not perturb the pluripotent state.^{18,28–30} These studies show that TET1 is important in the establishment of pluripotency as opposed to the maintenance of this state, and suggests that TET proteins may also be involved in the generation of CSCs.

However, opposing roles for DNMT1 have been demonstrated as it can both promote or inhibit CSC formation. For example, DNMT1 was required for the initiation of colon cancer.³¹ DNMT was also important for CSC function in established leukaemia, breast, lung and PDAC tumours^{32–35} whereas inhibition of DNMT1 promoted CSCs and EMT in prostate cancer.³⁶ These discrepancies could be due to tumour-specific effects or local hypermethylation of tumour suppressor genes which may promote the generation of CSCs in some cases.³⁷

The importance of both hypomethylation and DNMT1 in CSCs suggests that specific localization of 5mC and 5hmC marks is likely to be a more important determinant of cellular identity than the overall global patterning. In particular, hypomethylation at pluripotency loci and hypermethylation at tumour suppressor genes or those involved in differentiation is important in the generation of CSCs.

3 | HISTONE MODIFICATIONS IN CSC PLASTICITY

The amino acid residues located on the N- and C-terminal tails of histones can be modified to influence gene expression, including by acetylation, methylation and ubiquitylation. When occurring on promoters or enhancer regions, these modifications confer chromatin states that affect gene expression by altering the ability of protein complexes to bind. Chromatin formation that permits protein binding and gene expression is known as euchromatin, and repressive is known as heterochromatin. These states are mediated by the Trithorax group (TrxG) proteins and Polycomb group (PcG) proteins, respectively. Modifications are named by the histone type (eg, H3) followed by the amino acid and modification, for example, K4 me3 (trimethylation on lysine 4). The most studied modifications are those that occur to histone 3 found primarily at active enhancers (H3K9ac, H3K27ac), promoters (H3K4me3) and within the bodies of actively transcribed genes (H3K36me3).³⁸ Repressive marks include H3K27me3 and H3K9me3, also found on enhancers (Figure 3B).

Alterations of the histone modification landscape are widely associated with SCs with approximately one-third of the genome differing in chromatin structure between differentiated cells and ESCs.³⁹ In general, the genome of pluripotent SCs is enriched for more transcriptionally permissive euchromatin and less heterochromatin relative to somatic cells. This means that PSCs contain more acetylated chromatin and smaller regions of the repressive marks H3K9me3 and H3K27me3 relative to differentiated cells.³⁹ Accordingly, certain protein modifying enzymes (eg, acetylases, demethylases) are important in PSCs and establishment of cell identity.¹³ In particular, the repressive complexes PRC1 and PRC2 are both required for induction of pluripotency.⁴⁰

Although there are many parallels between PSCs and CSCs, histone-modifying enzymes can have tumour-specific effects. The subunit of the Polycomb group complex 2 (PRC2) that catalyses H3K27me3 is the histone methyltransferase EZH2 (enhancer of zeste homologue 2). EZH2 inhibition has been shown to be sufficient for tumourigenesis and is associated with the generation of CSCs in some cancers.^{41,42} However, EZH2 has been found to be hyperactivated in breast cancer and PDAC.⁴³⁻⁴⁵ Loss-of-function mutations of the H3K27 demethylases UTX3 and JMJD3 were also found to have completely opposite effects on tumour initiation in T-ALL whereby loss of UTX3 acted as a tumour suppressor while the loss of JMJD3 promoted tumour initiation.⁴⁶

As well as methylation, other modifications have shown importance in CSCs. The BMI1 subunit of the polycomb repressive complex 1 (PRC1), which monoubiquitinates histone H2A on lysine 119 (H2AK119Ub1) was able to promote CSCs in leukaemia and

GBM.^{47,48} This was also the case for some HDACs: HDAC7 promoted tumourigenesis in the lung.⁴⁹ In addition to histone modifiers, dysregulation of some proteins that regulate chromatin structure, such as cohesins, are involved in promotion of stem cells and the generation of CSCs in leukaemias.⁵⁰ Furthermore, mutations that disrupt the function of chromatin-remodelling complexes, and are found at high frequency in cancers, can cause aberrant activation of stem cell-related pathways.^{51,52}

Nearly all the genes involved in ESC identity, including KLF4, Sox2, Oct4 and Nanog, are regulated by super-enhancers; large genomic regions with very high levels of transcription factors that are highly important in controlling pluripotency and differentiation.^{53,54} In turn, Sox2, Nanog and Oct4 co-bind to various enhancers and super-enhancers, thereby forming a self-maintaining gene circuitry that maintains pluripotency. Aberrant activity of histone modifiers can impair the function of enhancer regions and effect cell identity, with certain modifications promoting the acquisition of stem-like cells.^{55,56} One study highlights the importance of enhancer reprogramming in the generation of stem cell identity by an oncogenic event. Overexpression of the oncogenic MYC in luminal human mammary epithelial cells (HMECs) led to downregulation of transcription factors and consequent loss of activity at enhancer regions important for maintaining luminal identity. At the same time, new enhancer regions were activated inducing transcriptional reprogramming to the stem-like state. This enabled the HMECs to acquire stem-like features including self-renewal and multipotency.⁵⁷

Chromatin at promoters and enhancers can be classed as active, repressed or poised. Poised regions are bivalent in terms of histone modifications as they contain both H3K27me (repressive) and H3K4me (activating) histone marks. In ESCs, the majority of bivalency occurs in the promoters of transcription factors and half of bivalent domains have binding sites for pluripotency transcription factors.⁵⁸ In ESCs, bivalency tends to be lost upon differentiation with some enhancers becoming active (loss of H3K27me3, gain of H3K27ac) and some become repressed (H3K27me3 enrichment), this occurring in a cell type-specific manner.⁵⁹ As the fluid interconversion between cellular states (normal and stem) depends on the capacity of a cell to switch on and off cell-specific transcriptional programs, it is not surprising that poised chromatin is also important for determining cell identity and plasticity in CSCs. Indeed CSCs possess a more plastic and dynamic chromatin formation than differentiated cells.⁶⁰ In AML CSCs, genes involved in stem cell identity were bivalently marked with both H3K4me3 and H3K27me3, with H3K4me3 marks lost during differentiation.⁶¹ Bivalent marks at pluripotency loci have also been identified in solid cancers.^{62,63} In theory, poised chromatin can allow cellular identity to switch in any direction, that is, not only differentiation but also de-differentiation, and may therefore be important in the generation of CSCs. Bivalency also allows for the highly dynamic state so characteristic of pluripotency, and may be the single most important epigenetic process for conferring CSC plasticity. A direct link between CSC generation and bivalency was demonstrated in breast cancer where CSCs marked by high CD44 expression were generated from CD44-low cells by TGF β stimulation. This process was dependent on poised, bivalent chromatin at the promoter of the

ZEB1 gene, and conversion of bivalent to repressive marks rendered CD44-low cells insensitive to TGF β .⁶⁴

4 | NUCLEOSOME POSITIONING AND HISTONE VARIANTS IN CSC PLASTICITY

The basic unit of chromatin is the nucleosome, which is formed of 147 bp of DNA wrapped around eight histones, two each of histones H2A, H2B, H3 and H4. These appear like beads on a string of chromatin. Gene expression is regulated at the level of chromatin structure in an ATP-dependant process by chromatin modifiers which act to remove or slide assembled nucleosomes along with the DNA, and can also exchange histone H2A-H2B dimers with dimers of histone variants. The presence of nucleosomes naturally represses gene expression by preventing the access of transcription factors. The absence or loss of nucleosomes at a transcription start site (nucleosome-free region [NFR]) allows for assembly of the transcription machinery and rapid activation of gene expression.^{65,66} The position of nucleosomes must therefore be precisely regulated at promoters, enhancers and repressors. This is achieved by four known families of ATP-dependent chromatin remodelling complexes; switch/sucrose nonfermenting (SWI/ SNF), imitation switch (ISWI), inositol requiring 80 (INO80) and those with a NuRD/Mi-2/CHD helicase binding domain.^{52,67-69} Chromatin remodelling complexes use ATP hydrolysis to catalyse the assembly, sliding and ejecting of nucleosomes.⁶⁵ DNA-sequence specificity is achieved by interaction with transcription factors. How nucleosome reorganisation is achieved is not fully understood but it is thought that remodelling complexes cause DNA to loop or twist to disrupt connection with histones and thus forcing translocation across a nucleosome. Chromatin modifiers can also interact with methylated DNA and covalent histone modifications to affect global gene expression patterns and chromatin architecture^{70,71} (Figure 3C).

The chromatin architecture of SCs differs greatly from that of differentiated cells. While small-scale chromatin changes at DNA regulatory elements occur during transcriptional regulation in most cells, extensive remodelling of chromatin structure is required for cellular differentiation during embryonic development.⁷²⁻⁷⁴ In particular, the SWI/SNF remodelling complex is known to be important in ESCs and development: SWI/SNF complexes containing the ARID1A protein can remodel chromatin to inhibit expression of the pluripotency genes *Sox2* and *Oct4*, and promote differentiation to mesoderm, but not ectoderm.⁷⁵ Nucleosome positioning is also important in the reprogramming of somatic cells to pluripotency. Nucleosomal landscapes of induced PSCs become extensively altered during reprogramming, in particular, nucleosome occupancy is reduced at enhancers which colocalise with binding sites of key pluripotency transcription factors, including *Klf4*, *Oct4*, *Sox2* and *c-Myc*.⁷⁶

As nucleosome repositioning is required for the generation of the pluripotent state it may be important for the formation of CSCs. This may occur via aberrant functions of ATP-dependent chromatin remodelers, which has also been linked to cancer, especially in terms of the SWI/SNF complex, which is frequently found mutated.⁷⁷⁻⁷⁹ In leukaemia, the SWI/SNF complex appears to function as it does in

PSCs where the ATPase subunit BRG1 is required for nucleosome repositioning at the *Myc* enhancer to promote *Myc* expression, leading to enhanced self-renewal.^{80,81} However, in lung cancer, loss of either BRG1 or BRM ATPase subunits can promote cancer development.⁸² The SNF5 (BAF47) subunit of the SWI/SNF complex is considered a tumour suppressor and its inhibition is sufficient to drive malignant transformation by inactivating p16 and p21. In the case of the rare paediatric rhabdoid tumour, SNF5 mutation is an inherited lesion that is considered the sole cause of this highly aggressive cancer.⁸³ Rhabdoid tumours are poorly differentiated and highly metastatic suggesting a high proportion of CSC features. This strongly links aberrant SWI/SNF-dependent nucleosome remodelling with CSC generation. This process may also play a role in CSC generation in other cancers as mutations of *SNF5* have been identified in epithelioid sarcoma and renal medullary carcinoma.^{84,85}

ATP-dependent chromatin remodelling complexes can also regulate transcription via incorporation of histone variants into nucleosomes.⁸⁶ There are eight histone variants of H2A, and six of H3, which are deposited in specific locations along the genome. Histone variants can influence gene expression by directly altering the structure and stability of nucleosomes, or by recruiting readers of histone modifications to induce local chromatin changes. For example, nucleosomes that contain the histone variant H2A.Bbd, bind less DNA and are not as stable, resulting in less compact chromatin. Less stable nucleosomes including those that contain H2A.Bbd, H2A.Z or H3.3 are localised at active promoters, enhancers and insulators, and may serve to prevent the formation of stable nucleosomes around these regulatory regions and facilitate transcription.⁸⁷ In contrast, nucleosomes containing the histone variant macroH2A are relatively more stable and inhibit transcription.⁸⁸

High mobility and exchange of histone variants is a key feature of SCs and this dynamism is thought to contribute to SC plasticity.^{89,90} The expression of histone variants is tightly regulated in ES cells and during development, and variants have specialised roles in cell fate decisions and lineage-specification.^{73,91} These variants, in turn, have a corresponding influence on reprogramming: those expressed in early development (the less stable H3.3, TH2A and TH2B) facilitate transcription and promote reprogramming whereas those expressed in somatic cells (the more stable macroH2A) inhibit transcription and prevent the induction of pluripotency.⁹¹ As the histone variant macroH2A is considered an epigenetic barrier to reprogramming, a tumour suppressive function would be expected in cancer.⁹² Accordingly, overexpression of macroH2A reduced metastatic potential of melanoma and inhibition of macroH2A1 generated CSCs in hepatocellular carcinoma.^{93,94} Importantly, inhibition of macroH2A1 in bladder cancer increased stem-like cells.⁹⁵ In contrast, the less stable H2A.Z and H3.3 variants promote transcription and reprogramming, and both have been found to be overexpressed in cancer.⁹⁶ H2A.Z correlates with poor prognosis in melanoma, breast cancer and hepatocellular carcinoma where it has been linked with EMT.⁹⁷⁻⁹⁹ However, the role of H3.3 appears to differ in adult GBM where it is repressed in SCs and its overexpression impairs self-renewal. Furthermore, repression of H3.3 in nonstem cancer cells is sufficient to induce cell plasticity and generate a CSC state.¹⁰⁰

These findings suggest that the overexpression of labile histone variants such H2A.Z (and in some cases H3.3), coupled with the reduction of stable histone variants such as macroH2A could contribute to cancer cell plasticity and the generation of CSCs.

4.1 | Noncoding RNA and CSC plasticity

Noncoding RNAs (ncRNA) are transcribed from regions of the genome that do not encode for proteins. The resulting RNA transcripts function to regulate the expression of protein-coding genes and are therefore essential for control of cellular function and identity. ncRNAs can be divided into two major groups based on their size: small ncRNAs being 200 nucleotides or less, and long ncRNAs (lncRNAs) 200 nucleotides or more. Small ncRNAs can be further subcategorized based on length, function and subcellular localization and include microRNAs (miRNAs) and short interfering RNAs (siRNAs) amongst others.¹⁰¹ Long and short ncRNAs regulate gene expression by different modes of action.

4.2 | Long ncRNAs

lncRNAs can physically associate with DNA or proteins to either promote or repress gene expression. To promote transcription, lncRNAs can function as guides or scaffolds for the assembly of protein complexes at specific loci. To repress transcription lncRNAs can function as decoys, binding and preventing functions of RNA or protein targets¹⁰² (Figure 3D). Many lncRNAs are known to be involved in the regulation of pluripotency and cell fate transitions and are important in many types of adult stem cells. In particular, long intergenic ncRNAs (lincRNAs) which reside in gene deserts have tissue-specific expression patterns that make them key players in the establishment of cellular identity. Knockdown of those lincRNAs associated with pluripotency results in differentiation of PSCs.¹⁰³ Furthermore, the expression of many lncRNAs is altered during the early stages of reprogramming to iPSCs.¹⁰⁴ Some lncRNAs associated with pluripotency have also been found to be upregulated in CSCs, for example, H19, which is involved in both mesenchymal SCs and glioblastoma CSCs.^{105,106} Importantly, many lncRNAs have been implicated in the generation of CSCs via roles in cellular transformation and EMT. In particular, the lncRNA LINK-RoR (regulator of reprogramming) is elevated in iPSCs and modulates iPS-mediated reprogramming.¹⁰⁷ LINK-RoR is also more highly expressed in CSCs as compared to more differentiated cancer cells and is important in a wide range of cancer types.¹⁰⁸⁻¹¹³ Furthermore, LINK-RoR also induces EMT and may therefore be involved in the conversion of cancer cells to CSCs during tumorigenesis.^{109,114} Many other lncRNAs play an important role in EMT. In particular, the lncRNA HOTAIR is involved in the recruitment of PRC2 to Hox genes during development and is also upregulated in TGF-beta1-responsive cancer cells during EMT.^{115,116}

4.3 | Micro RNAs

MiRNAs function by recognising small sequences (6-8 nucleotides) at the 3'-untranslated region of multiple target mRNAs.¹¹⁷ To do this, miRNAs are assembled in the RNA-induced silencing complex (RISC), where complementarity between the miRNA and mRNA target sequence results in mRNA cleavage. If partial complementarity occurs, deadenylase complexes are recruited which remove or shorten the mRNA poly-A tail to impair translation¹⁰¹ (Figure 3E).

The importance of miRNAs in embryonic stem cells (ESCs) has been established by deletion of Dicer1 and DGCR8 (both critical for miRNA biogenesis) in mouse models. Dicer1 deletion is embryonic lethal whereas DGCR8-deficient mice have impaired differentiation due to failure to silence the stem markers, Oct4, Sox2 and Nanog.^{118,119} This phenotype can be rescued by expression of the let-7 family miRNAs, which are not expressed in ESCs and appear during differentiation.^{120,121} Let7 miRNA expression is controlled by a negative feedback loop with Lin-28, which is a marker of pluripotency and is also important in reprogramming.¹²² In PSCs and cancer cells, Lin-28 binds to let-7 precursors to block their maturation, whereas the let-7 family miRNAs target Lin-28 for degradation in differentiated cells.¹²¹

Let7 is also known as a negative regulator of CSCs especially in breast cancer where, as expected, let-7 was linked with differentiation. Inhibition of let-7 promoted CSC properties, mediated by increased RAS and HMGA2 expression.¹²³ In turn, Lin-28 has been shown to promote CSCs in breast and prostate cancer,¹²⁴⁻¹²⁷ and even induce transformation in multiple cancers.¹²⁸⁻¹³⁰ It was found that transformation of a normal breast cell line by the Src oncoprotein required an epigenetic switch whereby the resulting upregulation of NFkB by src directly activated transcription of Lin-28, which in turn reduced let-7 expression. Low let-7 expression allowed for elevated levels of IL6, which in turn reactivated NFkB. This epigenetic positive feedback loop was found to be essential for maintaining the transformed state of breast cancer cells.¹²⁸ These findings strongly suggest that the Lin-28/ let-7 miRNA axis may control the generation of CSCs in cancer as it does with pluripotency of noncancerous cells.

As well as directly affecting the transcription of pluripotency-associated genes, miRNAs can also affect CSC biology via cross-talk with other CSC pathways and epigenetic mechanisms. For example, the maturation of miR-21 via a DROSHA and RNA helicase p68 complex is induced by TGF- β signalling.¹³¹ The up-regulation of miR-21 promotes cancer invasion and metastasis by negatively regulating the expression of the tumour suppressor gene PDCD4.¹³² Furthermore, miR-22 promotes SCs in breast cancer through direct inhibition of TET activity, thus preventing demethylation of the mir-200 promoter and silencing mir-200 expression.¹³³ miRNA expression in SCs is also regulated by bivalent promoters. In particular, lineage-specific miRNAs appear to be controlled in this manner, suggesting that an important mechanism underlying cellular plasticity is mediated by miRNAs.¹³⁴

5 | THE ROLE OF EMT-MET PLASTICITY IN THE EPIGENETIC REGULATION OF CSCS

Many studies have associated EMT with the generation of CSCs.^{135,136} However two distinct but interconverting populations of CSCs have been identified in some cancers including breast and pancreatic; one being more epithelial-like and one more mesenchymal-like.^{6,12} The functional relevance of two CSC populations is thought to lie in the fact that metastasis requires phenotypic plasticity: mesenchymal-like properties are required for cellular dissemination whereas epithelial properties are required for attachment and proliferation within the foreign niche.¹³⁷ EMT-MET plasticity can be conferred and maintained by epigenetic mechanisms. For example, a TGF- β -induced EMT resulted in a downregulation of p53 and the miRNAs 200c and 183, and concomitant elevation of the stem cell-related genes Bmi1 and Klf4, which could be rescued by overexpression of miR-200c or p53.¹³⁸ Bivalent histone markings could also mediate EMT-MET plasticity as poised chromatin allows for flexible determination of gene expression.

CSC generation by EMT may also be influenced by the localised tissue environment via epigenetic modification. Cells present in the tumour CSC niche such as stromal and immune cells produce cytokines and growth factors which promote CSC characteristics. For example, tumour-associated macrophages (TAMs) secrete VEGF and IL6, and T cells secrete IL17 and TGF- β , all of which are able to promote CSCs via EMT.^{139,140} In addition, myeloid-derived stem cells in the TME secrete IL6 and nitric oxide that promote CSC characteristics via a Stat-3 and mir101-mediated EMT.^{141,142} Environmental cross-talk occurs both ways; epigenetic mechanisms in CSCs also alter their interaction with

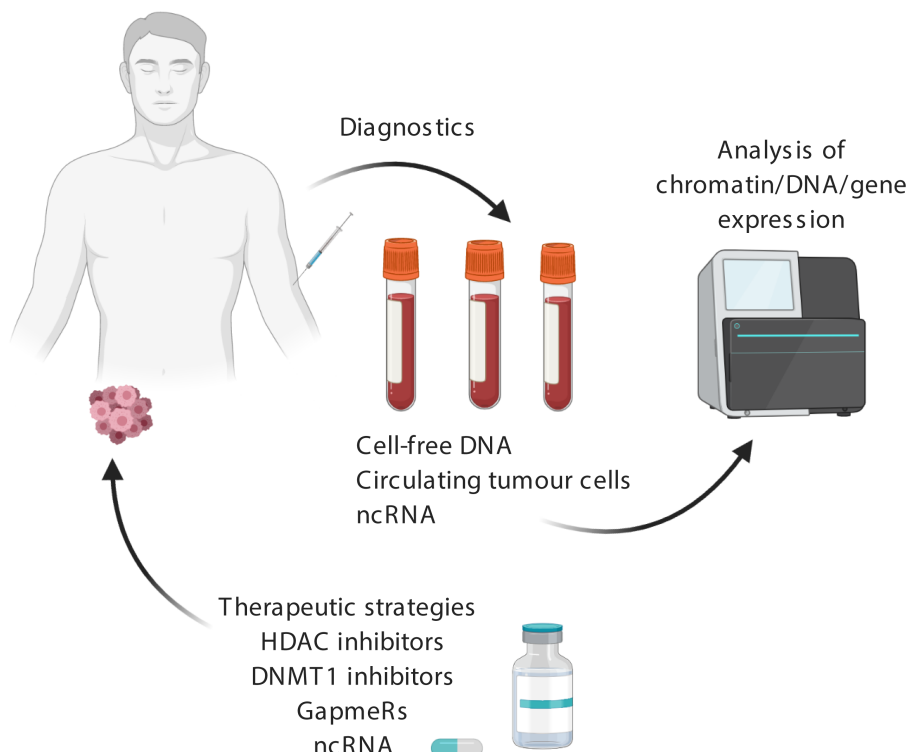
cells in the TME. For example, hypermethylation at the Tap promoter enhances the ability of CSCs to evade destruction by immune cells.¹⁴³

6 | TRANSLATIONAL RELEVANCE OF CSC EPIGENETICS

Many epigenetic aberrations identified as being involved in the generation of CSCs are likely early markers of cancer and therefore potentially useful as early detection methods. Early cancer detection could be achieved by analysis of patient blood samples, which is considered a relatively noninvasive procedure compared to a biopsy. Patient blood can contain cancer cells and cell-free DNA, which could be identified by characteristic genetic and epigenetic signatures as biomarkers of the cancerous state. Should such a process be found to be accurate, it may facilitate the detection of tumour signatures even when the tumour is not large enough to be picked up on a scan. This technique was originally designed for the identification of large genomic alterations, however, advances in the understanding of epigenetic aberrations in cancer have led to the pursuit of an epigenetic signature that may define the cancerous state. Although these may differ between cancers, epigenetic signatures associated with the generation of CSCs are likely candidates across cancer types due to the requirement for CSC generation in tumour initiation (Figure 4).

Most approaches have focussed on the methylation status of particular gene promoters but it is likely that the methylation status of several promoters would be required to serve as an accurate and reliable signature. For example, hypermethylation of the combination of four genes: *BCAT1*, *CDO1*, *TRIM58* and *ZNF177*, has been shown to

FIGURE 4 Translational relevance of epigenetic processes. Epigenetic mechanisms involved in CSC generation reveal factors with potential both as biomarkers for early diagnosis and as therapeutic targets. Early diagnosis could be achieved by analysis of cell-free DNA, circulating tumour cells or noncoding RNAs for the presence of characteristic epigenetic marks. Epigenetic marks could also be used as targets for therapeutic agents, such as DNMT1 or HDAC inhibitors. Alternatively, ncRNAs or gapmeRs could be used as therapeutics to modify gene expression [Color figure can be viewed at wileyonlinelibrary.com]



predict lung cancer.¹⁴⁴ Recently, methylation patterns were used to detect and localise over 50 different cancer types.²² Hypomethylation is a characteristic signature of cancer and CSCs, and considered an early event in tumorigenesis. Therefore, a global hypomethylation profile, defined by the presence of 5-hmC marks, may be a useful sign of tumour presence. In particular, hypomethylation of SC-associated genes such as Oct4, Sox2 and Nanog, together with or methylation of certain differentiation markers or tumour suppressor genes such as p53 may indicate the presence of CSCs and therefore early cancerous lesions.

An increase in histones and their associated covalent modifications have also been identified in the blood of cancer patients.¹⁴⁵ Bivalent histone signatures may be a promising avenue of exploration due to their specific association with CSCs. Unexpectedly, nucleosome positioning inferred from whole-genome sequencing of plasma DNA correlates with RNA levels and furthermore with RNA in tumour tissue. This suggests that nucleosome positioning could also accurately reflect gene expression and be used to predict tumour incidence.¹⁴⁶

A useful form of epigenetic cancer detection is ncRNA. Noncoding RNAs are readily secreted by the tumour into the microenvironment and subsequently reach the bloodstream and even distant tissue sites. LncRNAs and miRNAs have been detected in blood, urine and saliva, all of which facilitate diagnostic testing and many have been identified as potential biomarkers.^{147,148} Biomarkers have been identified through comparison of the pre and postoperative state, that is, a potentially accurate cancer biomarker should decrease upon removal of the tumour. Such candidates include mir-221 and mir-375 in breast and pancreatic cancer^{149,150} and mir-20, mir-21, mir-145 and mir-223 in early-stage lung cancer.¹⁵¹ Some miRNAs may even be able to distinguish certain subtypes of cancer, for example, lower levels of miR-16-5p, miR-21-5p and miR-199a-5p were found to be associated specifically with the presence of triple-negative breast cancer.¹⁵² Many of those miRNAs found upregulated in cancer are also associated with CSCs. For example, mir-221 was found to promote the generation of CSCs in breast cancer. Interestingly, this occurred via its role in the downregulation of DNMT3b, hence providing a link between a miRNA and reduced methylation, also associated with CSCs.¹⁵³ Mi-21 is also a promoter of CSCs in many cancers especially colorectal.^{154,155} These associations suggest that a role in CSCs may define what makes a useful and specific epigenetic biomarker for cancer.

Epigenetic targeting is an exciting area of translational cancer research offering many novel opportunities for therapeutic intervention. Many studies have explored the use of existing and novel compounds to target epigenetic marks to impair cancer cell growth and elicit an improved clinical response (Figure 4).

HDAC inhibitors are already widely considered to have anticancer effects and some are in clinical use. For example, SAHA (vorinostat) is approved for T-cell lymphoma and many others are in clinical trials for various malignancies either alone or in combination with other therapeutics.¹⁵⁶ Despite promising anticancer effects, results have been mixed and in some cases accompanied by substantial toxicity.^{157,158} However, preclinical studies have shown that a range of HDAC

inhibitors can preferentially target CSCs in cancer cell lines. In breast cancer, these effects were attributed specifically to inhibition of the HDACs 1 and 7.¹⁵⁹ However, the HDAC inhibitor valproic acid, was actually able to increase the number of CSCs in breast cancer via inducing de-differentiation to the stem-like state (ie, CSC generation).¹⁶⁰ These effects were mediated by the promotion of wnt-signaling. These conflicting results may reflect the specificities of different HDAC inhibitors, or the nonspecific effects of HDAC inhibitors in general, and may explain conflicting clinical data and toxicities. While there may be potential in pursuing HDAC inhibition as a CSC-targeting approach, much further work is required to elucidate underlying mechanisms of action and avoid adverse effects. An alternative approach to targeting histone acetylation has been sought through bromodomain inhibitors. Bromodomains (BRDs) are readers of histone acetylation that target chromatin-modifying enzymes to specific genomic sites to regulate transcription. The bromodomain and extra terminal (BET) family is made up of four different proteins that are important in cancer and also function as cell cycle regulators. The first developed inhibitor of the BET bromodomain family was JQ1.^{161,162} A combination of JQ1 and the HDAC inhibitor SAHA promoted apoptosis in a number of cancers including PDAC. JQ1 also promoted apoptosis and cell-cycle arrest of CSCs in glioma.^{163,164}

Targeting of histone modifications may also be achieved through LSD1 inhibitors. Lysine-specific demethylase 1A (LSD1) removes methyl groups from the histone 3 proteins (H3K4me2/1 and H3K9me2/1) resulting in transcriptional repression or activation, respectively.¹⁶⁵ LSD1 is overexpressed in cancer, where it functions to inhibit differentiation and enhance proliferation, invasiveness and cell motility.¹⁶⁶ Effective LSD1 inhibitors have been developed by mimicking the lysine groups of the LSD phenyl ring at two positions (NCL-1) or (NCL-2).¹⁶⁷ NCL-1 analogues have exhibited good anticancer activities across solid tumours including against CSCs.¹⁶⁸

Histone modification could also be targeted by inhibition of PRC components BMI-1 or EZH2. BMI-1 is a key regulatory component of (PRC1) and its function is strongly associated with the maintenance of CSCs.^{169,170} Two small molecule inhibitors of BMI-1, PTC-209 and QW24, both impaired the self-renewal of colorectal CSCs.¹⁷¹ EZH2 is an enzymatic catalytic subunit of polycomb repressive complex 2 (PRC2), which is also associated with CSC function.¹⁷² Several inhibitors of EZH2 methyltransferase activity have been developed with a 2-pyridone core designed to occupy the binding pocket for cosubstrate S-adenosyl-methionine (SAM) in EZH2. GSK343 is one such SAM-competitive inhibitor, which has been shown to inhibit stemness in patient-derived glioma lines.¹⁷³ However, targeting of EZH2 may be unpredictable as it has been shown to have opposing effects across cancer types.⁴¹⁻⁴⁶

Although an increasing number of ncRNAs are known to be involved in CSCs and cancer in general, and are therefore attractive drug targets, it is considered difficult to specifically target aberrant mRNAs or RNA-protein interactions with small molecules. Nevertheless, due to the high promise of such targets, a number of studies have attempted therapeutic modulation. The miRNA lin28 which is very important in CSC generation has been found

to be targeted by the compound “N-methyl-N-[3-(3-methyl[1,2,4]triazolo[4,3-b]pyridazin-6-yl)phenyl]acetamide”, which acts by blocking the interaction between lin-28 and let-7. This compound impaired Lin-28, rescued let-7 function and resulted in differentiation in ESCs, and reduced tumorsphere formation in cancer cell lines. It is likely that the reduction in tumorsphere formation was caused by differentiation of CSCs.¹⁷⁴ An alternative approach is the administration of synthetic miRNAs to restore functions lost in cancer, for example, the introduction of tumour suppressor miRNAs.¹⁷⁵

Another way of targeting DNA and RNA moieties is by using peptide nucleic acids (PNA). These are synthetic analogues of DNA that are highly stable and have a hybridization affinity toward natural DNA and RNA, and are therefore a potential approach for the modification of gene expression and epigenetic states.¹⁷⁶ This approach has been applied to the targeting of the lncRNA HOTAIR to block its interaction with EZH2, resulting in reduced invasiveness and increased sensitivity to chemotherapy.¹⁷⁷ These effects strongly suggest inhibition of the stem-cell compartment.

Despite promising results, therapeutic targeting of epigenetic marks should proceed with caution. A therapeutic strategy designed to target epigenetic readers and writers is by definition not a specific approach. Such an intervention would elicit gene expression changes on a global scale and is likely to incur off-target and adverse effects. Therefore much further investigation is required to identify a clinically viable approach. Epigenetic targeting that focuses on a specific gene or pathway changes (eg, miRNA and lncRNA) may be more easily targeted to CSC inhibition and therefore have much greater potential for efficacy.

7 | CONCLUSIONS AND FUTURE DIRECTIONS

The epigenetic processes involved in CSC generation have substantial implications for tumour initiation, metastasis and relapse. Many studies have focussed on the elucidation of mechanistic links between epigenetic processes and CSCs, and have identified attractive targets for cancer prevention, diagnosis and therapy. However, as always, these studies have highlighted many areas in which further investigation is required. In particular:

- To obtain a greater understanding of the epigenetic mechanisms which drive CSC formation from normal cells.
- To determine whether the mechanisms of CSC generation differ when deriving from normal or cancerous cells and their associated stem and nonstem subpopulations. Epigenetic mechanisms involved in the emergence of CSCs will likely differ depending on from which population they are derived, that is, from a normal cell, normal stem cell, pre-CSC or bulk tumour cell, as each of these identities has distinct epigenetic profiles.
- To ascertain the relative contribution of genetic and epigenetic effects to tumour initiation; can epigenetic events alone initiate the transformed state?

- To determine which are the most important environmental signals involved in CSC generation and how these can be modified or targeted for prevention and treatment.

CONFLICT OF INTEREST

The authors note no conflict of interest.

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