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Review

# Epigenetics of cancer stem cells: Pathways and therapeutics

Samriddhi Shukla, Syed Musthapa Meeran *

*Laboratory of Cancer Epigenetics, Division of Endocrinology, CSIR–Central Drug Research Institute, Lucknow, India*

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ABSTRACT

*Background:* Epigenetic alterations including DNA methylation and histone modifications are the key factors in the differentiation of stem cells into different tissue subtypes. The generation of cancer stem cells (CSCs) in the process of carcinogenesis may also involve similar kind of epigenetic reprogramming where, in contrast, it leads to the loss of expression of genes specific to the differentiated state and regaining of stem cell-specific characteristics. The most important predicament with treatment of cancers includes the non-responsive quiescent CSC.
*Scope of review:* The distinctive capabilities of the CSCs make cancer treatment even more difficult as this population of cells tends to remain quiescent for longer intervals and then gets reactivated leading to tumor relapse. Therefore, the current review is aimed to focus on recent advances in understanding the relation of epigenetic reprogramming to the generation, self-renewal and proliferation of CSCs.
*Major conclusion:* CSC-targeted therapeutic approaches would improve the chances of patient survival by reducing the frequency of tumor relapse. Differentiation therapy is an emerging therapeutic approach in which the CSCs are induced to differentiate from their quiescent state to a mature differentiated form, through activation of differentiation-related signalling pathways, miRNA-mediated alteration and epigenetic differentiation therapy. Thus, understanding the origin of CSC and their epigenetic regulation is crucial to develop treatment strategy against not only for the heterogeneous population of cancer cells but also to CSCs.
*General significance:* Characterizing the epigenetic marks of CSCs and the associated signalling cascades might help in developing therapeutic strategies against chemo-resistant cancers.


## 1. Introduction

Mammalian development is a complex multi-step process starting with the totipotent embryo containing unlimited developmental potential and terminating into almost two hundred differentiated cell types specialized to perform physiologically different and specific functions. The cell fate and their developmental potentials are defined very well by C. H. Waddington's epigenetic landscape model [1], which shows a gradual decrease in the developmental potential of the cell per differentiation state. Initially, we followed the Waddington's epigenetic landscape model for cellular differentiation. According to this model, the cell loses its developmental plasticity irreversibly with each differentiation step. But, the nuclear transfer experiments with somatic cells proved higher degree of developmental plasticity than what was previously expected. Strikingly, these cells, which are completely different from each other in their differentiated forms, still retain the complete complement of the genes in their genome which they have inherited from their ancestral embryonic stem cell (ESC). Also, they still have the potential to become totipotent under specific circumstances. This process is accompanied by the erasure of different epigenetic marks such as CpG methylation and chromatin remodelling through different types of covalent histone modifications, termed as epigenetic reprogramming.

According to the presently available information, reprogramming of the epigenome is the major event initiated by the cellular signalling cascades, involved in animal development, functional differentiation and in the maintenance of stem cell properties. The information, we actually lack in, is the molecular interaction between the cell and its microenvironment which drives the epigenetic reprogramming of the cell nucleus. We still find it difficult to identify the forces working behind the conversion of an almost quiescent totipotent stem cell into a terminally differentiated, fully functional, somatic cell.

## 2. Cancer stem cells (CSCs)

### 2.1. Definition and characteristics

The biological function of normal adult somatic stem cells (SSCs) in their tissue counterparts is to provide a continuous supply of terminally differentiated, fully functional cells in the tissues with higher cellular turnover [2]. Similar to normal SSCs, CSCs also contain the unique biological characteristics of unlimited proliferation, self-renewal and

* Corresponding author at: Division of Endocrinology, CSIR–Central Drug Research Institute (CSIR–CDRI), Jankipuram Extn., Sector-10, Sitapur Road, Lucknow-226 031, India. Tel.: +91 522 2612411x4491; fax: +91 522 2623938.
*E-mail address:* s.musthapa@cdri.res.in (S.M. Meeran).

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differentiation into specialized cell types. The most recent definition of CSCs identifies these cells as "a small subset of cancerous population responsible for tumor initiation and growth, which also possess the characteristic properties of quiescence, indefinite self-renewal, intrinsic resistance to chemo- and radio-therapy and capability to give rise to differentiated progeny" [3]. CSCs display the distinctive characteristic of anchorage independence when grown in vitro. The most important growth advantage of CSCs is that these quiescent cells have indefinite proliferative potential leaving the two different types of progenies with asymmetrical distribution of growth potential. In addition to giving rise to the differentiated cancer cell populations with limited proliferative potential, they can also generate progenies having properties exactly similar to those of the parent CSC, this process is termed as self-renewal. Generally, the progenies with higher level of differentiation constitute the major fraction of the tumor mass. Another important and distinguishing feature of CSCs includes clonogenesis in vitro and tumorigenesis in vivo. In fact, the functional definition of CSCs depicts these cells as the cancer cell population capable of forming tumorous growth, following injection into the test animals.

### 2.2. Pathways

The SSCs and CSCs share common signalling pathways for the retention of their stem cell properties. The most important signalling pathways related with self-renewal characteristics include wingless and integration site growth factor (Wnt)/β-catenin signalling, notch signalling and sonic hedgehog (Shh) signalling. The Wnt family consists of inter-cellular signalling molecules which are involved in the regulation of embryonic development and are seen to be frequently altered during the process of epithelial to mesenchymal transition (EMT) in carcinogenesis [4,5]. Notch family is reportedly involved in the self-renewal and maintenance of stem cell characteristics by the activation of downstream effector molecules such as γ-secretase complex followed by the activation of *c-MYC*, an oncogene [6–8]. Shh is another important intercellular signalling molecule, functioning in the regulation of development and stem cell behavior. Researchers have identified the involvement of Shh in the regulation of the stem cell proliferation and cell-fate determination of neural stem cells and mesenchymal stem cells [9]. The regenerative proliferation activity of epithelial stem cells has been linked to the hedgehog signalling in many cancers including bladder cancer [9,10]. The major signalling pathways associated with stemness in CSCs are illustrated in Fig. 1.

### 2.3. Origin of CSCs

According to the somatic stem cell hypothesis on the origin of CSCs, the dormant stem cells present in the organs may give rise to CSCs due to mutation or inappropriate regulation of stem cell characteristics

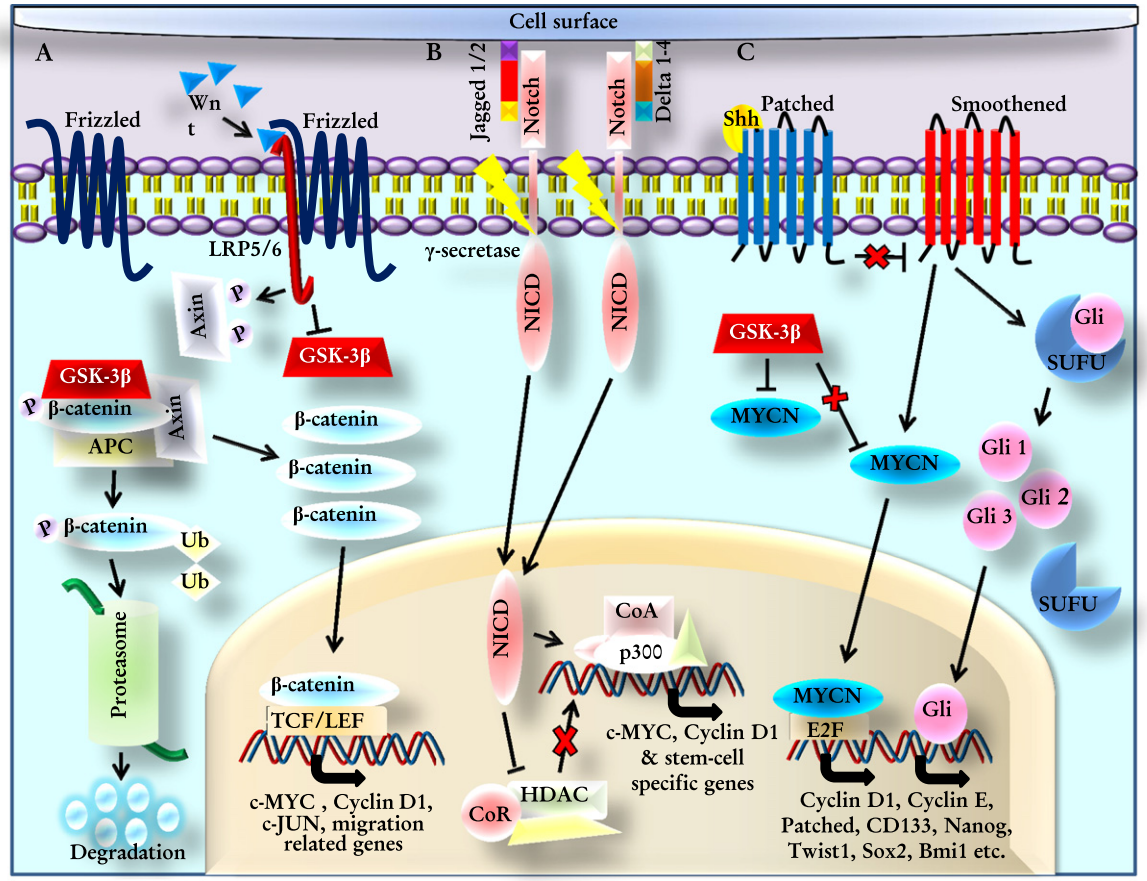


**Fig. 1.** The major signalling pathways associated with stemness in CSCs. A) In the absence of Wnt ligand, the cytoplasmic β-catenin remains bound in the destruction complex formed by Axin, APC, and GSK-3β proteins. GSK-3β phosphorylates this bound β-catenin leading to its ubiquitination followed by proteasomal degradation. In contrast, in the presence of Wnt ligands, the Frizzled receptors interact with LDL receptor-related proteins 5/6 (LRP5/6) and stabilize the cytoplasmic β-catenin by inhibiting GSK-3β and axin proteins. On cellular accumulation, β-catenin enters the nucleus and functions along with T-cell factor/Lymphoid enhancing factor (TCF/LEF) transcription factors in the activation of genes involved in cell growth, migration as well as stem cell specific genes such as *c-MYC*. B) The notch receptors after activation by Jagged1/2 or Delta 1–4 ligands activate the γ-secretase complex which, in turn, releases the intracellular domain of Notch receptor (NICD). The NICD then signals the activation of stem-cell specific genes by recruiting the co-activator complexes to the gene promoters. C) In the absence of sonic hedgehog ligand (Shh), the patched receptors inhibit the membrane incorporation of the smoothened receptor. It also allows the sequestering of the Gli proteins (Gli 1–3) by SuFu, finally leading towards their degradation. Shh binding inhibits Patched and Smoothened receptors that are incorporated in the cell membrane. Activated smoothened receptors relieve the Gli proteins, which then enter the nucleus and function as transcription factors leading to the expression of a variety of stem cell specific genes. This pathway also facilitates MYCN translocation into the nucleus leading to activation of multiple downstream target genes.

[11,12]. Recently, Wang et al. proposed the somatic stem cell misplacement theory of carcinogenesis which states that CSCs develop de novo from the misplaced somatic stem cells [13].

Progenitor cells are defined as the early descendents of stem cells which are distinct in characteristics such as the lowered capacity of self-renewal and limited replication [14,15]. Recently, there are multiple reports which have proven the presence of heterogeneous cell lineages among the tumor cell population which can be differentiated from a common progenitor cell [16,17].

Another view on origin of CSCs is that the somatic cells might function as the originating cells for tumor formation. First such evidence came from the study performed by Mintz et al. where they proved the teratogenic ability of early embryonic somatic cells through injections of these cells in the grafts of 6-day old mouse embryos [18]. Recent experimental evidences showing nuclear reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) have also supported this hypothesis [19–21].

Although, the hypotheses on the origin of CSCs are seemingly different, they are not mutually exclusive in principle. The tumor initiating cells (TICs) or CSCs, irrespective of their cells of origin, universally function in maintaining the self-renewal behavior, replication ability and the tumor heterogeneity. Abundance of CSCs varies from tumor to tumor, ranging from a small subpopulation to virtually all the cells of a tumor. Together, Fig. 2 summarizes the different hypotheses on the origin of CSCs.

## 3. Epigenetic reprogramming in origin of CSCs

During the process of somatic cell reprogramming, the over-expressions of transcription factors by retroviral, non-viral or chemical means followed by appropriate chromatin remodelling are required for the successful conversion of a somatic cell into stem cell state. Reprogramming occurs in step-wise fashion, starting with the binding of reprogramming factors which paves the way, next, for the binding of stem-cell specific transcription factors at their target DNA sites and silencing of differentiation-specific genes. Simultaneous chromatin remodelling changes regulate these transitional states from differentiated somatic cells with their specialized protein expressions to the undifferentiated stem cell states with the loss of most or all of the differentiation markers [22,23]. Similar nuclear reprogramming is also an indispensable requirement for the development of CSCs from their cell of origin, which might be a SSC, progenitor cell or a differentiated somatic cell. These TICs must undergo a similar series of methylation changes and chromatin remodelling to finally give rise to potential CSCs. Thus, during the process of carcinogenesis, the epigenetic modulators may function via two mechanisms; either, these factors may facilitate the binding of the over-expressed transcription factors by exposing the target oncogenic DNA sites, functioning as the passenger events of carcinogenesis; or, they might even initiate the transcription factor over-expression, thus playing the key functional role of driving events. Since, the epigenetic mechanisms are divided into three separate but inter-dependent parts; we will discuss their plausible role in the generation of CSCs. As most of our knowledge of CSCs relies on the information available in the field of stem cell biology, we will try to understand the role of epigenetics in the light of the available information.

### 3.1. Role of DNA methylation in epigenetic reprogramming of CSCs

DNA methylation refers to the addition of methyl groups at 5′-position of cytosine residues of the CpG dinucleotides present in the mammalian DNA mediated by three types of active DNA methyltransferases (DNMTs). DNMT1, a maintenance methyltransferase, functions in the maintenance of pre-existing methylation patterns by preferentially adding methyl groups to the hemi-methylated DNA during S-phase of

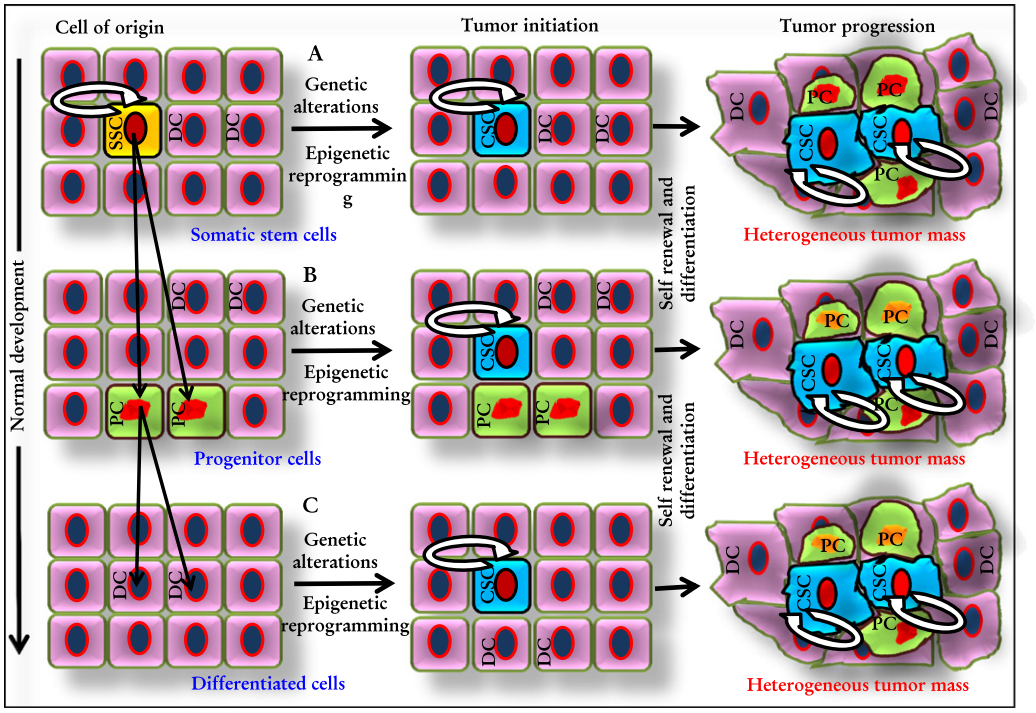


**Fig. 2.** Different theories on the origin of CSCs. During normal development, the somatic stem cells (SSCs), in addition to their self-renewal activity, give rise to a population of progenitor cells (PC), which further differentiate to form fully differentiated somatic cells (DC). A) Under the influence of certain genetic alterations and/or epigenetic reprogramming, these SSCs get converted into CSCs to finally form a heterogeneous tumor mass. B) Progenitor cells (PC) can also attain CSC-characteristics due to genetic or epigenetic reprogramming of their genomes, thus becoming capable of generating tumor mass with heterogeneous cell population. C) The fully differentiated somatic cells (DC) can also acquire CSC-characteristics through massive genetic and epigenetic reprogramming. The CSCs then give rise to and maintain the heterogeneous tumor mass.

cell cycle [24]. DNMT3a and DNMT3b, *de novo* methyltransferases, are important in development as these enzymes set the pattern of methylation of genes by targeting unmethylated CpG sites [25]. *De novo* methylation has been shown to play a very important role in the regulation of stem cell characteristics [26]. A recent glioblastoma iPSC reprogramming study showed that some of the tumors can be reprogrammed to lose their malignant behavior, by methylation-induced silencing of cancer-promoting pathways depending on their lineage identity [27]. In *DNMT3a*-null mice, each passage resulted in lowered differentiation capacity of hematopoietic stem cells (HSCs). DNMT3a was found to be indispensable for the proper differentiation of these cells [28]. Higher enrichment of DNMT3b at the CpG-islands (CGIs) of hypermethylated genes suggests that this enzyme functions more importantly in furnishing the aberrant methylation pattern observed in case of CSCs, which helps them to maintain undifferentiated state [29]. Active DNA demethylation is considered an ultimate requirement for cells to regain stem-cell state during induction of pluripotency. This active demethylation is probably achieved through the conversion of 5-methyl cytosine (5-mC) to thymine by activation-induced deaminase (AID) and by ten-eleven translocation (TET) proteins which convert 5-mC to 5-hydroxymethylcytosine (5-hmC) [30,31]. Abnormal DNA methylation events occur early during carcinogenesis resulting in premalignant states and field cancerization [32,33]. These altered methylation patterns include global hypomethylation changes and promoter-specific hypermethylation. Tumor suppressor gene $p16^{INK4A}$/*CDKN2A* is one such example of increasing frequency of promoter-specific gain of DNA hypermethylation during lung carcinogenesis starting from 17% of promoter methylation in lung airway basal cell hyperplasia to 24% methylation in squamous metaplasia and 50% methylation in carcinoma in situ [34].

The methylation patterns of CGIs of normal somatic cells differ largely from those of ESCs, iPSCs and CSCs. While most of the CGIs remain unmethylated in normal somatic cells, the iPSCs and CSCs display hypermethylation of CGIs in promoter regions of differentiation-specific and cancer-related genes [35]. ESCs maintain 'bivalent chromatin state' which is characterized by the presence of smaller regions of active chromatin mark, H3K4 trimethylation (H3K4me3) in the larger regions of inactive chromatin mark, H3K27 trimethylation (H3K27me3). These histone modification marks are important due to their role in the recruitment of DNMTs to the target genes [35]. The genes with bivalent chromatin often maintain a low state of transcription and later on, these might either switch towards transcriptionally active H3K4me3-enriched state or transcriptionally repressive H3K27me3-enriched state [36]. In cancer, most of the promoter hypermethylated genes fall in this category and are important in regulating the processes of self-renewal and differentiation [37]. Abnormal silencing of a single gene or multiple genes containing bivalent chromatin by enrichment of repressive mark H3K27me3 might help in the initiation and progression of carcinogenesis. The choice of genes to be silenced might be tissue-specific for different cancer types but this is a fundamental characteristic to the CSC population in every type of cancer. DNA hypermethylation in these genes with bivalent chromatin is generally accompanied by a lack of or decreased levels of both H3K4me3 and H3K27me3, leading to complete suppression of transcription [37,38].

During the process of somatic cell reprogramming, gradual alterations in the patterns of DNA methylation have been observed. The over-expression of pluripotency genes coincides with their promoter demethylation, while the cellular differentiation markers are progressively silenced by hypermethylation during the induction of pluripotency [39,40]. DNA methylation plays a very important role in endowing the cells with the loss of pluripotency and developmental plasticity as well as in imparting functional specificity to the cells [41]. The disruption of DNMT function has been shown to induce re-expression of many silenced tumor suppressor genes in different types of cancers as well as silencing of tumor promoter genes [42–44]. A comparison of DNA hypermethylation of an ESC and a transformed CSC cell line proved that the gene targets of polycomb group proteins (PcG), which are the regulators of lineage-specific gene transcription, were more prone to be hypermethylated in case of the CSCs. In acute myeloid leukemia (AML), a general association was found between DNA methylation and transcriptional silencing. The pattern of methylation of CGIs was also correlated with tumor prognosis [45]. Promoter methylation-mediated silencing of CSC-associated Wnt target genes, including *ASCL2* and *LGR5*, leads to poor prognosis in colorectal cancer and their re-expression leads to reduced tumor growth [46]. Recently, higher hypomethylation was reported in 68 differentially methylated regions (DMRs) of breast CSCs as compared to non-CSC populations and this hypomethylation was correlated to poor prognosis [47]. In the lung CSC-like populations, knockdown of DNMT1 reduced the stem cell properties, suggesting the possibility that DNMT1 inhibition might prove to be an important therapeutic strategy to eliminate the lung CSCs [48].

Deeper understanding of the role of different DNMTs in setting up the aberrant CGI methylation patterns will help the researchers to appropriately utilize the available DNMT inhibitors for reversing these hypermethylations. Prolonged exposure of stem/progenitor cells with higher doses of DNMT inhibitors has proven to induce differentiation in the progenitor cells [49]. Administration of DNMT inhibitor at low dose was reported to be capable of reducing the stem cell like properties of $ALDH^+$ ovarian CSCs, by reprogramming these CSCs to more differentiated state [50]. Similarly, DNMT inhibitors can be utilized to reset the CSCs towards a differentiated phenotype, thus rendering different types of cancers more susceptible to available therapeutic options.

### 3.2. Role of chromatin remodelling in epigenetic reprogramming of CSCs

The tight packaging of eukaryotic DNA into chromatin restricts the access of DNA-binding proteins to DNA; therefore, the opening of chromatin is pre-requisite for the gene expression. This process of chromatin opening, which is accomplished by multiple ATP-dependent, multi-enzyme complexes is known as chromatin remodelling and the complexes involved in these processes are known as chromatin remodelling complexes. Based on the structure and sequence of the ATPase subunits, these ATP-dependent chromatin remodelling complexes are subdivided into four families, which include — Switch/Sucrose nonfermentable (SWI/SNF), imitation SWI (ISWI), chromodomain helicase DNA-binding (CHD)/NuRD/Mi-2 and INO80 families [51].

The genes encoding the SWI/SNF remodelling complex family function in transcriptional regulation and DNA repair. Brahma-associated factor (BAF) complexes contain many different domains for protein–protein interactions, histone modification recognition domains and non-sequence specific DNA-binding domains. These complexes are capable of functioning both as transcriptional activators and repressors for the same set of genes [52]. *Brahma-related gene 1 (BRG1)*, which is involved in encoding the ATPase subunits of SWI/SNF complex is found to be indispensable for neuronic differentiation [53]. *BRG1* epigenetically regulates Wnt pathway, which is a major driving factor behind the intestinal tumorigenesis and loss of this gene prevents intestinal adenoma formation as well as reduces the TIC population [53]. Recently, *BRG* was found to be essential for leukemia maintenance, as AML cells lacking this gene tend to die faster than the *BRG*-expressing cells [54]. *BRG1* mutations are very common with a frequency of 20–40% in non-small cell lung cancer. This suggests that *BRG1* functions as a bona fide key tumor suppressor gene in lung tumorigenesis [55,56]. *BRG1* was found to repress pluripotency *OCT4* and *SOX2* gene targets, specifically those promoting pluripotency and differentiation [57,58]. Polycomb repressive complex (PRC)-1 contains ring finger protein 1A (RING1A) or ring finger protein 1B (RING1B), which catalyzes monoubiquitination reactions and PRC2 contains enhancer of zeste (EZH2), which catalyzes trimethylation of histone H3 at lysine 27. B lymphoma Mo-MLV insertion region 1 homolog (BMI-1) and EZH2 proteins of the PcG family have been correlated with poor prognosis in

many different cancers [59,60]. The loss of H3K27me3 in glioblastoma-CSCs causes altered activation of Wnt signalling pathway regulator, *ASCL1*, which is required for CSC maintenance and tumorigenicity [61]. PRC-2 is involved in the trimethylation of histone H3 at lysine 27 during embryonic development, ESC differentiation and cancer. Involvement of PRC-2 in ESC self-renewal was established by *Pcl3* knockdown-mediated induction of differentiation in ESCs. Recently, it was established that pharmacological inhibition of PRC2 by its inhibitor 3-Dezaneplanocin-A leads to lesser tumorigenicity and reduced tumor progression in prostate CSCs [62]. The PcG proteins function in gene repression and in the maintenance of stem-cell characteristics in CSCs, by often opposing the function of SWI/SNF complexes. The expression of these proteins is higher in CSCs in comparison to SSCs. Bivalency and regulation of gene expression in ESCs are dependent on the activity of Polycomb repressor complexes [63].

BMI-1 is required for the maintenance of adult stem cells and is involved in carcinogenesis of different cancer types. BMI-1 overexpression causes increased tumorigenicity and stem cell-like behavior of CSCs [64–66]. The SWI/SNF complex is the most frequently mutated chromatin remodelling complex in cancer, a finding more important due to the decisive role of these complexes in remodelling at gene promoters. Almost 20% of tumors from many different cancer types harbor mutations in genes encoding proteins of SWI/SNF family [67]. Experimental inhibition of the components of PRC-1 and -2 complexes has shown to have different effects on the reprogramming efficiency in human ESCs and fibroblasts undergoing reprogramming in vitro [68]. shRNA-mediated silencing of H3K27 methyltransferase, *EZH2* was reported to reduce reprogramming efficiency in the cultured ESCs and fibroblasts. On the other hand, the silencing of H3K9 methyltransferase, *SUV39H1*, H3K79 methyltransferase, *DOT1L* and transcription factor, *YY1*, was found to induce the nuclear reprogramming [68].

The ISWI remodelling complexes function in the regulation of cellular viability, fertility and proliferation. These complexes are subdivided into Nucleosome Remodelling Factor (NuRF) and CECR2-containing remodelling factor (CERF) complexes. These complexes primarily regulate the higher-order chromatin structure and organogenesis and their disruption results in the loss of survival or incomplete development [69–71]. The loss of the largest subunit of NuRF complex, *bromodomain PHD-finger transcription factor* (*BPTF*), leads to growth defects during early mouse embryogenesis [70]. The ATPase subunit of ISWI complex, SNF2H, was identified to be a component of large chromatin remodelling network highly expressed in ESCs [72]. The PHD-finger of BPTF present in the NuRF complex interacts with the H3K4me3 methylation marks in the chromatin leading to gene activation [73]. The precise role of the components of the ISWI complex in maintaining CSC-state is not yet known.

Many CHD remodelling complexes are involved in the regulation of tumor-associated genes. These complexes are divided into three subfamilies, CHD subfamily I comprising of CHD1 and 2; CHD subfamily II consisting of CHD3 and 4; and CHD subfamily III including CHD5–CHD9 [74]. A member of CHD subfamily I, CHD1, binds to the H3K4me2/3 and recruits transcription-initiator complex leading to gene activation. Downregulation of CHD1 leads to the loss of self-renewal and decreased expression of pluripotency gene, *OCT4* [75]. In addition, CHD1 is involved in the incorporation of histone variant H3.3 in chromatin of *Drosophila*, preventing the formation of heterochromatin foci thus maintaining the stem cell state [76]. Inhibitors of LSD1/KDM1, an H3K4me2/3 demethylase, suppress the stem cell properties of CSCs in vivo [77]. CHD subfamily II members form nucleosome-remodelling and histone deacetylase (NuRD) complexes and function in transcriptional repression. Metastasis-associated (MTA), methyl-CpG-binding domain (MBD) and retinoblastoma-associated binding protein (RbBP) are present in these complexes as accessory subunits. The loss of MBD3 facilitates the induction of pluripotency, suggesting its role in differentiation and lineage-specification [78,79]. CHD7, a well-studied member of CHD subfamily III, is proven to be involved in transcription of tissue-specific genes during development. This complex recruits the histone methyltransferases, ASH1 and TRX, to chromatin, which can reverse the gene repressing action of PcG proteins [80,81].

The INO80 remodelling complex family consists of INO80 and SWR1 complexes. These complexes in mammals are known as Snf2-related CBP activator protein (SRCAP) and TIP60–p400 complexes. The function of SRCAP involves the incorporation of histone variant H2A.Z in the chromatin which is required for proper lineage-commitment during development [82,83]. The TIP60–p400 complexes are involved in transcriptional regulation and DNA repair [84]. Downregulation of the proteins of these complexes induces premature differentiation and growth arrest of ESCs [85].

Overall, these observations prove the importance of chromatin remodelling in setting the highly complex patterns of spatial and temporal expressions of genes during development. During the induction of pluripotency, appropriate chromatin remodelling is required for efficient silencing of differentiation-specific genes and expression of stem cell-specific genes. Alterations in the expression patterns of the proteins forming these complexes are capable of tumor initiation. In addition, targeting these complexes alters the signalling cascade involved in the CSC and subsequent tumor development. Therefore, these complexes have the capability to continue the tumor progression initiated by some other factors or by induction of CSC. Inappropriate localization of these complexes can also lead to silencing of important tumor suppressor genes or activation of tumor promoter genes.

Fig. 3 depicts major DNA methylation and chromatin remodelling changes and different proteins and multi-protein complexes involved in the attainment of stemness in CSCs.

### 3.3. Role of microRNAs in epigenetic reprogramming of CSCs

The microRNAs (miRNAs) are small regulatory RNAs, which function in post-transcriptional regulation of gene expression by inhibiting the translation of mRNAs through inhibition of ribosome function, decapping and deadenylation of 5′ cap and poly (A) tails of mRNAs, finally leading to their degradation. These miRNAs, function in the retention of stem-cell properties in both normal SSCs and CSCs. Deregulation of these miRNA promotes tumorigenesis. The oncogenic miRNA, miR-21 functions in maintaining the stem cell-like properties as well as regulates the EMT transition of breast and ovarian cancer cells [86,87]. Another miRNA, miR-34a regulates the stem cell-specific Notch signalling in the colon CSCs [88]. MiR-34a inhibits CD44 expression in prostate CSCs leading to inhibition of stem cell proliferation and metastasis [89]. Re-expression of miR-34a in pancreatic CSCs and in human pancreatic cancer cell lines induced by the treatment with DNMT inhibitor, 5-aza-2′-deoxycytidine, and HDAC inhibitor, suberoylanilide hydroxamic acid, led to decreased cellular proliferation, cell cycle progression, EMT and self-renewal activities of these cells [90]. Suppression of miR-200c expression is required for normal stem cells to form mammary ducts and breast CSCs for tumor formation [91]. MiR-9 was found to be associated with EMT and breast CSC phenotype [92]. The loss of miR-203 is an important event followed by EMT in most of the breast cancer types in cells including breast CSCs. Re-expression of miR-203 in these cells induced differentiation and suppressed mesenchymal and stem cell properties [93]. Asymmetric cell division, a characteristic of CSCs required for self-renewal, is directed towards symmetry in the presence of tumor suppressor miR-146a in colorectal cancer [94]. Inhibition of oncogenic miR-126, which is involved in the maintenance of stem/progenitor function in AML, led to elimination of leukemic cells [95].

Recently, miRNAs have emerged as major regulators of stem cell behavior in both SSCs and CSCs and these miRNAs can prove to be better targets for cancer therapy. The similarity of biological functions of these molecules provides a strong proof of shared molecular mechanism in both SSCs and CSCs [51].

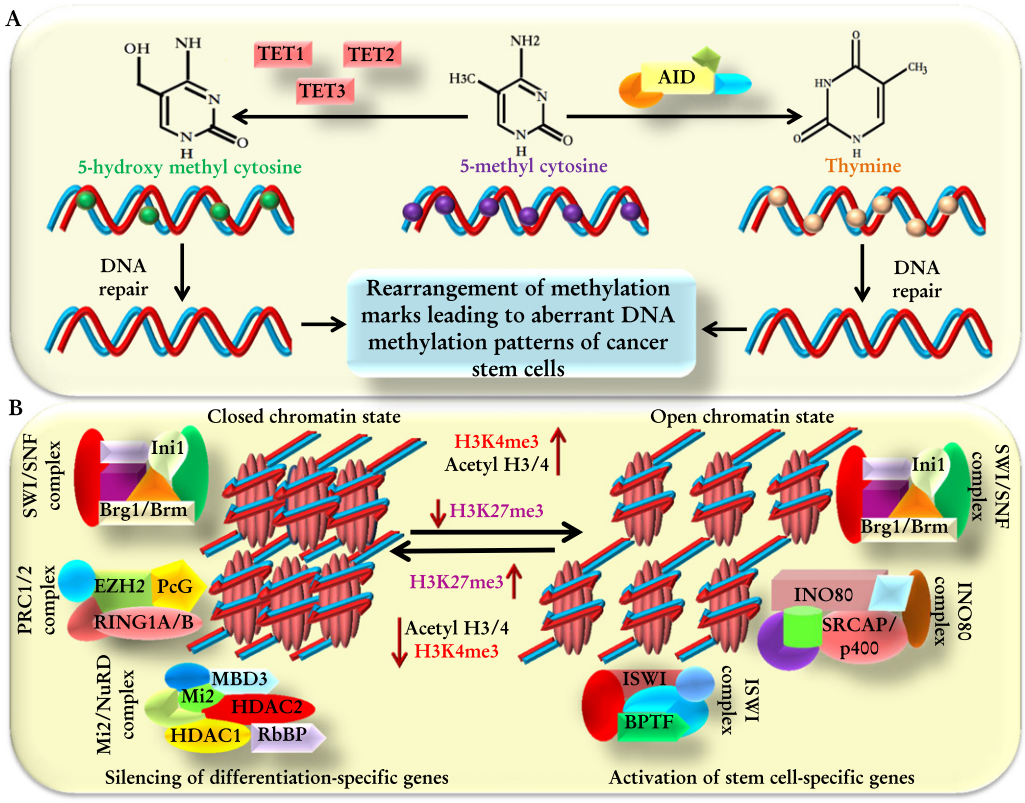


**Fig. 3.** Epigenetic reprogramming to achieve stemness. A) The 5-methyl cytosine residues are actively converted into 5-hydroxy methyl cytosine by ten eleven translocation (TET 1–3) proteins. The activation-induced deaminase (AID) also converts the 5-methyl cytosine into thymine. These residues are then removed in the process of DNA repair and a complete DNA demethylation signature is achieved. The rearrangement of these methylation marks leads to differential methylation observed in case of CSCs. B) The silencing of differentiation-specific genes is achieved by the compaction of chromatin with the help of SWI/SNF, Mi2/NuRD chromatin remodelling complexes and PRC1/2 repressor complexes. The compacted chromatin is enriched in the inactive mark H3K27me3, while the active marks, acetyl histone H3/4 and H3K4me3 are absent. The stem-cell specific genes are activated by the action of SWI/SNF, ISWI and INO80 chromatin remodelling complexes which increase the trimethylation of H3K4 (H3K4me3) and histone acetylation and decrease in H3K27me3 marks.

## 4. CSC markers and epigenetic biomarkers

Potential CSC biomarkers can be grouped into stem cell-like markers such as CD133, CD44, CD34 and CD24, pluripotency genes including *OCT3/4*, *Nanog*, *SOX2* and *MYC* and the markers of invasiveness such as vimentin, N-cadherin, snail, twist and Zeb1 as well as markers of drug resistance such as aldehyde dehydrogenase (ALDH) and ABC transporters [96]. Different combinations of these markers are characteristic for the identification of the different types of tumor tissues. For example, in prostate cancer, the expression of stem cell-like markers such as CD133, CD44, integrin-α2, nestin and CD49f has been associated with CSC phenotypes, whereas breast CSCs are generally identified as $CD44^{high}/CD24^{low}$ populations [96]. $CD24^{+}$ population present in nasopharyngeal carcinoma (NPC) possesses typical CSC characteristics of tumorigenesis and self-renewal. In addition, these cells exhibit the expression of pluripotency genes such as *SOX2*, *OCT4*, and *Nanog* and activation of stem-cell specific Wnt/β-catenin signalling. These results suggest that CD24 can be utilized as a CSC biomarker in NPC [97]. Pancreatic CSCs are identified by the presence of stem cell-like markers such as CD133, ALDH and the combination of $CD44^{+}CD24^{+}ESA^{+}$ cell surface antigens [98]. Overexpression of drug resistance-related proteins such as ABC transporters and stem cell-like marker CD34 is important in the identification of brain CSCs. ABC transporters together with other stem cell markers can be useful for the identification of brain cancer cells with abnormal progenitor properties [99]. Gastric CSCs show higher expression of drug-resistance genes such as *ALDH* and *multidrug resistance* (*MDR*) leading to increased resistance to chemotherapy. These cells also possess $CD44^{+}$ and $CD133^{+}$ phenotypes which are correlated with malignant transformation and increased invasiveness of this type of cancer. Thus, the presence of each or all of these markers might be indicative of gastric CSCs [100]. AML tumor populations expressing cell adhesion molecules N-cadherin and Tie2 along with stem cell-like markers $CD34^{+}CD38^{+}CD123^{+}$ are identified as leukemic CSCs. The interaction between these leukemic CSCs and tumor niche, mediated by the cell adhesion molecules, adds to the chemo-resistance [101]. Ovarian epithelial CSCs can be identified by the presence of stem cell markers Oct4, nestin, CD117 and CD44. The CSC populations displaying these stem cell markers show a state of dormancy [102]. Higher expression of four different CSC markers Nanog, OCT4, CD133 and nestin is the characteristic feature of prostate epithelial malignancy [103].

Interestingly, CD133 (AC133 or prominin 1), a universal CSC marker was found to be directly regulated by epigenetic modifications in ovarian cancer. $CD133^{+}$ population is distinguishable from $CD133^{-}$ population by retaining a promoter hypomethylated state [104]. $CD133^{+}$ brain tumor cells show the CSC-characteristics such as initiation of neurospheres exhibiting self-renewal, differentiation and proliferation. CD133 expression was found to be regulated through epigenetic mechanism in human gliomas, where hypomethylation of promoter regions of higher activity induced lower CD133 expression. [105]. Similar DNA hypomethylation was reported to regulate CD133 expression in colorectal and glioblastoma tumors. In these types of tumors, $CD133^{-}$ cells show higher methylation of promoter CGIs, while cells with higher CD133 expression lack such methylation [106]. Another study in hepatocellular carcinoma reported that TGF-β is involved in the epigenetic regulation of CD133 expression by inhibiting the expression of DNMT1 and DNMT3b, which causes demethylation of transcriptionally active region of *CD133* promoter [107]. CSCs isolated from breast and pancreatic cancer cell lines show higher expression of EZH2 as compared to the non-CSC populations. Recently, researchers have

shown that RNAi-mediated EZH2 knockdown leads to decreased frequency of CSCs in these cells, further confirming the role of EZH2 in the maintenance of CSC-phenotype. Therefore, EZH2 can be used as a functional CSC marker [59]. CUB-domain containing protein 1 (CDCP1) is another stem cell marker which is reported to be epigenetically regulated in breast cancer cell lines and clinical samples [108]. The loss of CDCP1 was reported to promote tumor invasiveness and poor prognosis in esophageal cancer [109]. CDCP1 was also found to induce EMT by repressing the epithelial phenotype in pancreatic cancer cells. In contrast, CDCP1 was found to be required for anchorage-independent growth, a major characteristic of CSCs, in lung cancer cells [110]. Intestinal and adenoma CSC marker, doublecortin-like kinase 1 (DCLK1) has been recently identified as epigenetic biomarker of colorectal cancer, which is regulated by promoter methylation. Promoter of *DCLK1* gene is highly methylated in colorectal cancer leading to the transcriptional silencing of this gene [111]. In spite of lack of detailed information on the epigenetically regulated biomarkers of CSCs, different types of CSC markers have been reviewed elaborately in previous reviews [51,96,112].

## 5. Epigenetics, stemness and chemoresistance

The CSCs have several growth advantages over the other cancer cell populations. An important advantage in this regard is the resistance to the chemotherapeutic drugs. Significantly higher expression of efflux transporters in both SSCs and CSCs has been observed. ABC transporters, typically membrane proteins, are involved in the ATP-dependent translocation of substrates against a concentration gradient. CSCs tend to express higher levels of these transporters to maintain constant outflux of the drug from the cell environment. It has been found that CSCs show expression of several ABC transporters, including ABCB1, ABCG2, and ABCC1 [113]. Hypermethylation of the promoter CGIs is an important regulatory mechanism for the expression of these ABCG2 transporters [114]. ABCB1 (P-glycoprotein), a product of *MDR* gene, is expressed in more than 50% of all of the drug-resistant tumors. This protein translocates a variety of hydrophobic compounds across the cell membrane and it is overexpressed either by gene amplification or by transcriptional upregulation, suggesting a role of epigenetic mechanisms. ABCC1, another important drug transporter involved in multidrug resistance, is regulated via the notch signalling and confers chemo-resistance to CSCs [115]. Other aspects of chemoresistance in CSCs include expression of ALDH activity and presence of alternative DNA damage response. Interestingly, DNMT and HDACs inhibitors as well as the combinations of these inhibitors are showing potential for chemo-sensitization of drug-resistant cells [115]. Recently, the regulatory role of miRNAs in the regulation of drug resistance in CSCs has also been established [116]. Therefore, epigenetic therapy alone or in combination with chemotherapy might be useful for the treatment of drug-resistant tumors. In addition, prolonged fasting has shown to promote hematopoietic-stem-cell-based regeneration and reverse immunosuppression by downregulation of IGF-PKA signalling. This study provides the evidence that fasting can protect cancer patients undergoing chemotherapy against the immunosuppression and mortality caused by chemotoxicity [117].

## 6. Conclusion and perspectives

Conventionally, the cancer therapeutic strategies often target the majority of the tumor population, but are incapable of targeting the CSCs. CSCs shield the tumors from these chemotherapeutic drugs with the help of properties of chemoresistance. The most important predicament with treatment of cancers includes the inability to precisely identify the stem cell-like population in the tumor. In spite of all the recent efforts from researchers, fast and accurate identification of CSCs in a heterogeneous population is yet to be achieved. The limitation arises from their similarities to the normal SSC population as well as the vast majority of differences among the CSCs of different tissue origins. These cells possess multiple biological mechanisms to evade the response to the chemotherapeutic drugs and thus render the disease almost untreatable. Studies to identify these quiescent populations and to reveal the molecular signalling pathways associated with the development of chemoresistance are the need of the hour. This information might help in both identification and targeting of the CSC population, thus empowering the currently available chemotherapeutic drugs and leading to the better identification of drug targets in drug-resistant tumors.

CSC-targeted therapeutic approaches might improve the chances of patient survival by reducing the frequency of tumor relapse. Differentiation therapy is an emerging therapeutic approach in which the CSCs are induced to differentiate from their quiescent state to a mature differentiated form, through activation of differentiation-related signalling pathways, miRNA-mediated alteration and epigenetic differentiation therapy. Epigenetic mechanisms play very important roles in providing the stem cell characteristics to the cancer cells as well as in the maintenance of these stem cell characters, which enhances the immortality of the tumors. Consistent with this observation, the inhibitors of the key epigenetic modulatory enzymes, such as DNMTs and HDACs, might help in targeting the CSCs in addition to targeting the bulk tumor population. The importance of tumor suppressor proteins involved in chromatin remodelling is well established and the restoration of the normal chromatin remodelling function by gene therapy might also be an important therapeutic strategy for the treatment of cancers with inappropriate chromatin remodelling. miRNA-mediated inhibition of stem-cell characters and loss of pluripotency as well as induction of tumor-suppressor genes are another potential options for targeting of CSC. Thus, for successful cancer treatment, eradication of all the different cancer cell populations is required and treatment strategies targeting both the bulk cancer cells as well as the CSCs are likely to emerge triumphant in the war against cancer.

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