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REVIEW

Epigenetic therapy of cancer stem and progenitor cells by targeting DNA methylation machineries

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Abstract

Recent advances in stem cell biology have shed light on how normal stem and progenitor cells can evolve to acquire malignant characteristics during tumorigenesis. The cancer counterparts of normal stem and progenitor cells might be occurred through alterations of stem cell fates including an increase in self-renewal capability and a decrease in differentiation and/or apoptosis. This oncogenic evolution of cancer stem and progenitor cells, which often associates with aggressive phenotypes of the tumorigenic cells, is controlled in part by dysregulated epigenetic mechanisms including aberrant DNA methylation leading to abnormal epigenetic memory. Epigenetic therapy by targeting DNA methyltransferases (DNMT) 1, DNMT3A and DNMT3B *via* 5-Azacytidine (Aza) and 5-Aza-2′-deoxycytidine (Aza-dC) has proved to be successful toward treatment of hematologic neoplasms especially for patients with myelodysplastic syndrome. In this review, I summarize the current knowledge of mechanisms underlying the inhibition of DNA methylation by Aza and Aza-dC, and of their apoptotic- and differentiation-inducing effects on cancer stem and progenitor cells in leukemia, medulloblastoma, glioblastoma, neuroblastoma, prostate cancer, pancreatic cancer and testicular germ cell tumors. Since cancer stem and progenitor cells are implicated in cancer aggressiveness such as tumor formation, progression, metastasis and recurrence, I propose that effective therapeutic strategies might be achieved through eradication of cancer stem and progenitor cells by targeting the DNA methylation machineries to interfere their "malignant memory".

Key words: Cancer stem and progenitor cells; DNA methylation; Epigenetic therapy; Aza-cytidine; Aza-deoxycytidine

Core tip: Several types of cancers can be developed from genetic and/or epigenetic instabilities of stem and progenitor cells. Epigenetic abnormality involving dysregulation of DNA methylation has been reported to implicate in cancer aggressiveness. Inhibition of DNA methyltransferase activity by DNA methylation inhibitors has shown promising results toward treatment of myelodysplastic syndrome, a disease associated with leukemic stem cells. This review summarizes evidences which are pertinent to the antitumorigenic potential of DNA methylation inhibitors 5-Azacytidine and 5-Aza-2′-deoxycytidine on cancer stem and progenitor cells including those of leukemia and other solid tumors.

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INTRODUCTION

The study of stem cell biology, especially that of hematopoiesis, has provided an insight into the biology of cancers[1,2]. In particular, normal stem cells and cancer cells share one of their characteristics, which is an ability to proliferate without differentiation, the property known as self-renewal. Three concepts of cancer biology are proposed based on the study of hematopoietic stem cells and their tumorigenic counterparts as the paradigm: (1) normal stem cells and cancer cells might possess similar mechanisms to control their proliferation or self-renewal; (2) cancer cells can be derived by differentiation of normal stem cells *via* mutation of transit-amplifying cells or progenitors; and (3) there might be cancer stem cells inside tumors where the tumorigenesis occurred with normal stem cells[3]. Leukemia, which is the cancers of hematopoietic system, has provided the best evidence that normal stem cells are the target of oncogenic evolution, and that leukemogenesis is driven by cancer stem cells. For example, the tumor initiating cells of myelodysplastic syndrome (MDS), which can develop into acute myeloid leukemia (AML), are cancer stem cells, at least those with genetic deletion of chromosome 5q[4]. Cancer stem and progenitor cells have been suggested to involve with relapse in many types of cancers[5-13]. This tumorigenic recurrence of cancer stem and progenitor cells, which often associates with more aggressive phenotypes compared with differentiated cancer cells, can occur *via* accumulation of genetic mutations and/or epigenetic defects leading to selective advantage and hence the oncogenic evolution of cancer stem and progenitor cells.

DEFECTS OF DNA METHYLATION IN CANCERS

Gene expression must be tightly regulated by both genetic and epigenetic mechanisms in non-cancerous normal cells, thereby avoiding malignant transformation. Both genetic- and epigenetic-dependent mechanisms ensure that expression of genes, whose function is involved in cell proliferation, apoptosis, self-renewal and differentiation, is normally controlled in temporal- and spatial-dependent manners. Deregulated epigenetic mechanisms have been shown to implicate in tumorigenesis. One epigenetic mechanism, which has been prominently focused in cancer research, is DNA methylation which also represents one of the most active fields of epigenetic research.

DNA methylation is one of the most common defects in epigenetic regulation observed in tumorigenesis. Earlier studies have suggested that cancer cells possess global DNA hypomethylation, which results in chromosomal instability[14]. However, in the past decade most cancer epigenetics studies have paid attention to regulatory region-associated DNA hypermethylation[15]. DNA hypermethylation of CpG dinucleotides has been well-documented in many types of cancer such as leukemia and colon cancer, and has been proposed as a hallmark of tumorigenesis[16]. Specifically, DNA hypermethylation at promoters of tumor suppressor- and differentiation-associated genes has been frequently reported. The DNA hypermethylation of those genes then leads to a reduced gene expression, which in turn provides a selective advantage to cancer cells. Thus, down-regulation of the genes by DNA hypermethylation might result in the emergence of cancer stem and progenitor cells[17,18].

DNA methylation has been widely accepted to mediate malignant memory of cancer cells due to its stable inheritance during cell division. Gene regulatory network specific to cancer stem and progenitor cells might play a direct role in dysregulation of DNA methylation. For example, the pluripotency-associated transcription factors OCT4 and NANOG have been shown to directly up-regulate expression of the maintenance DNA methyltransferase (DNMT) DNMT1 in mesenchymal stem cells[19]. In fact, maintenance of DNA methylation has been reported to be critical for cancer stem cell properties in leukemic, colon and lung cancer stem cells[20-22], which might be required to suppress expression of differentiation- and apoptosis-related genes. For instance, *p16* and *MLH1* are frequently hypermethylated in certain types of cancer. Of interesting is *MLH1*, which is a member of DNA mismatch repair pathway. Thus the epigenetic instability of *MLH1* might be a cause of genetic instability of cancer stem and progenitor cells. The epigenetic defects of other genes involved in different pathways related to cell migration, hormone receptors, and cell cycle control, have also been described in tumorigenesis[18]. Different patterns of DNA hypermethylation have also been found in different types of cancers, which might be explained by cellular function of the cells in respective tissues[16]. In addition, the pattern of DNA hypermethylation might be influenced by an interaction between DNMTs and polycomb repressive complex 2 (PRC2) protein subunits[23], since different tumors also have different patterns of PRC2 binding[17,24,25]. Epigenetic therapy based on DNA methylation inhibitors including 5-Azacytidine (Aza) and 5-Aza-2'-deoxycytidine (Aza-dC) has promised a therapeutic regimen to patients with DNA hypermethylation-induced cancer diseases[15]. Therefore, understanding how DNMTs transfer a methyl group to genomic DNA, how they function in cancer cells, and how the drugs inhibit DNMT activity is crucial for development of superior anti-cancer therapy.

MOLECULAR MECHANISM UNDERLYING DNA METHYLTRANSFERASE ACTIVITY

DNA methylation is responsible for multiple chromatin-related processes including regulation gene expression by adding a methyl group from S-adenosyl methionine

(SAM) to the C5 position of a cytosine base mostly within CpG dinucleotide context at gene regulatory regions such as promoters and CpG islands. The CpG methylation is catalyzed by DNMTs. Five DNMT family proteins, *i.e.*, DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L, have been identified in mammalian genomes[26]. Among these, DNMT2 does not function as a methyltransferase for DNA but as a RNA methyltransferase[27,28]. In addition, DNMT3L does not possess the methyltransferase catalytic domain[29], leaving only DNMT1, DNMT3A and DNMT3B that are functional DNA methyltransferases[30]. Two mechanisms underlying DNA methylation have been reported. The first mechanism is maintenance DNA methylation pattern, of which all three DNMTs have the ability to copy DNA methylation pattern from DNA template to daughter DNA strand[31-37]. In one study, Sharma *et al*[34] have proposed a homeostatic inheritance system for maintenance of DNA methylation, whereby high levels of 5-methylcytosine stabilize DNMT3A and DNMT3B and prevent them from proteolysis[34]. The second mechanism is *de novo* DNA methylation which can only be performed by DNMT3A and DNMT3B. Apart from repressing gene expression *via* methylation at gene promoters, DNA methylation also plays important roles in many epigenetic phenomena involved in development for example genomic imprinting, X chromosome inactivation and higher-order chromatin organization[38,39]. Hence, apart from cancers, dysregulation of DNA methylation are also implicated in pathogenesis of many diseases.

Several DNMT domains play important role in the methylation catalysis. Region Ⅳ harbors a prolyl-cysteinyl dipeptide that functions as the active site (Figure 1). Regions Ⅰ and Ⅹ share amino acid residues, which are critical for binding of SAM. Region Ⅴ contains glutamyl/asparaginyl residue, which functions to protonate nitrogen 3 (N3) of targeted cytosine during the methyltransferase reaction. The enzymatic mechanism of DNA methylation begins with flipping cytosine out of the DNA helix upon binding with a DNMT. The conformational change of the cytosine nitrogenous base ensures that carbon 5 (C5) of the base aligns at the catalytic pocket with a bound SAM, and that C6 is at close proximity to a cysteine residue at the active site of the enzymes. The base flipping leads to formation of a covalent bond between C6 of the cytosine in DNA and the cysteine residue mediated by nucleophilic attack of sulfhydryl group of the cysteine to the C6 resulting in a change from C5 = C6 double bond into C5-C6 single bond (Figure 2A). The chemical bonding between C6 and cysteine then leads to an increase in electron flow toward C5 position of cytosine. At the same time, a methyl group from SAM is transferred to C5 generating C5-CH_3 of the cytosine and S-adenosyl-homocysteine as the products. The proton at C5 position is then abstracted due to its weak bonding with C5 resulting in β-elimination at the C5-C6 single bond and in the reformation of the C5 = C6 double bond, which return a single electron to the cysteine of DNMT and discharge to the enzyme[30]. The C5-cytosine is now become methylated giving rise to methylcytosine.

INHIBITION OF DNMTS BY AZA-DC

The ability of Aza and Aza-dC to induce not only cell death but also differentiation had encouraged the exploitation of the compounds for treatment of cancers associated with stem/progenitor cells such as leukemia[40,41]. Unlike Aza which can be incorporated into both DNA and RNA, Aza-dC is only incorporated into DNA and is therefore a specific inhibitor for DNA but not RNA methyltransferases[42,43]. Consequently, the difference between the two epigenetic drugs results in differential cellular responses and causes distinct changes in the gene expression patterns of cells treated with Aza-dC and Aza[44,45].

Intracellular uptake of Aza-dC is mediated by the human concentrative nucleoside transporter 1 HCNT1, also known as SLC28A1[46]. Once inside cells, Aza-dC is then phosphorylated by deoxycytidine kinase yielding Aza-dCMP. Aza-dCMP is further phosphorylated to Aza-dCDP and subsequent Aza-dCTP, the active substrate form for DNA replication, by dCMP kinase and diphosphokinase, respectively. Hence the irreversible inhibition of Aza-dC on DNA methylation takes place only if Aza-dCTP is used as a substrate by DNA polymerases during DNA replication at S phase of the cell cycle, but not from free Aza-dC itself. Within DNA and with the presence of DNMTs, which bind to genomic DNA, the Aza-dC base can make a covalent bond with a DNMT enzyme (Figure 2B), disrupting the function of the enzyme. Mechanistically, because the nitrogen atom at position 5 (N5) of Aza-dC has a higher electronegativity than C5 of cytosine, the incorporated Aza-dC can covalently trap the DNMT enzyme. A single bond between N5-C6 is formed upon the nucleophilic attack of sulfhydryl group to the C6. However, unlike C5 in cytosine, the N5 can abstract a proton, and hence stably form a single bond to the proton with a strong affinity due to its high electronegativity. The DNMT is therefore trapped with the incorporated Aza-dC through a covalent bond between C6 and the cysteine residue leading to a DNA-DNMT adduct. Thus only in the presence of DNMTs can Aza-dC function to inhibit DNA methylation activity[47]. At chromatin level, gene reactivation activity of Aza-dC has been mechanistically demonstrated by Jones *et al*[31]. The authors devised a novel technique called Acce*SS*ible[48]. They find that approximately 2% of chromatin regions, which are demethylated after Aza-dC treatment in a colon cancer cell line, govern a feature of open chromatin[48] possibly associated with deposition of the histone variant H2A.Z[49]. Importantly, they show that of all genes reactivated by Aza-dC treatment, 90% of them possess DNA demethylation and chromatin accessibility supporting the role of the DNA methylation inhibitor as a gene reactivator. Interestingly, many reactivated genes are also tumor suppressor genes[48].

Several reports have demonstrated that depletion of DNMT1, DNMT3A or DNMT3B can counteract the cytotoxic and apoptotic effect of Aza-dC on treated cancer cells, confirming the essential role of DNMTs in mediating the Aza-dC response[47,50-52]. In contrast to

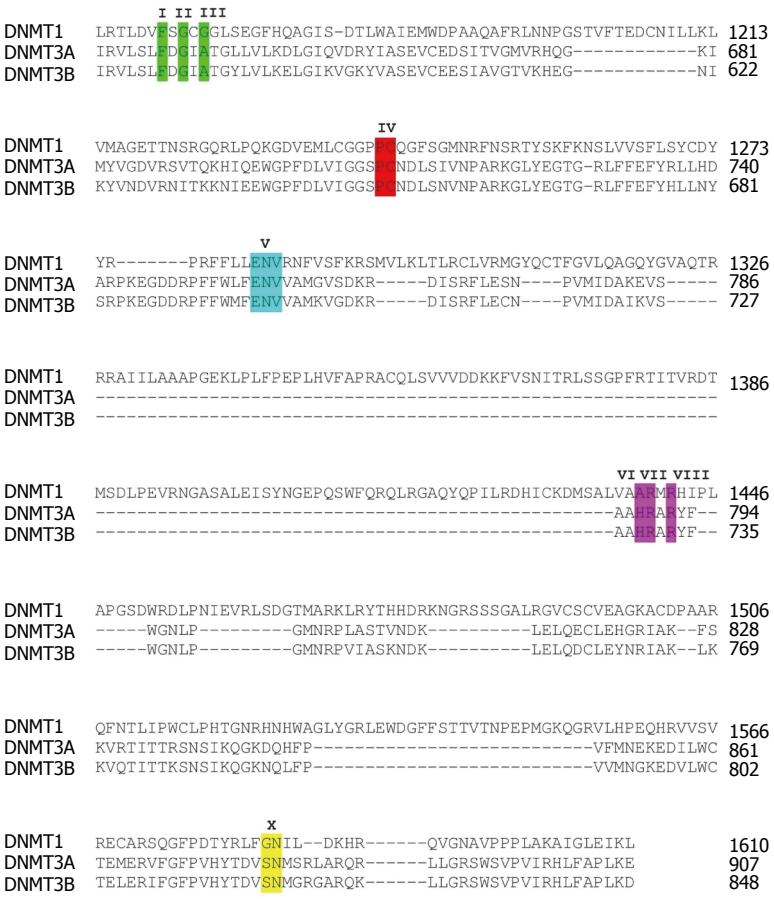

Figure 1 High similarity of critical amino acid residues at the catalytic domain of DNA methyltransferases. Multiple alignment of amino acids within the catalytic domain of three catalytically active human DNA methyltransferases DNMT1, DNMT3A and DNMT3B is shown. Highlighted amino acids with roman letters shown above indicate critical residues of the catalytic domain. DNMT: DNA methyltransferases.

the apoptotic response mediated by DNMTs, whether DNMTs also mediate stem cell differentiation induced by Aza-dC is not well understood. Epigenetic therapy by induced differentiation offers an option for treating malignancies, in which cancer tissues contain both cancer stem/progenitor cells and differentiated cancer cells, such as chronic leukemia, teratocarcinoma, and other solid tumors. Thus elucidating how cell differentiation can be triggered by Aza-dC or Aza could have an enormous impact on epigenetic therapy targeting cancer stem and progenitor cells.

INHIBITION OF EPIGENETIC PATHWAYS IN CANCER STEM AND PROGENITOR CELLS

Leukemia

Leukemia is considered to be the prototypical model for development of epigenetic therapy. MDS together with AML have been identified for being diseases of cancer stem and progenitor cells[4,53], which might contribute to relapse and resistance of chemotherapies. In fact, the DNMT inhibitor Aza is the first epigenetic drug which is approved by the Food and Drug Administration for treatment of MDS. Aza and Aza-dC have initially been shown to induce differentiation of Friend erythroleukemia cells[54]. In this study, the authors show that Aza-dC has a greater effect on reduction of cell proliferation compared with Aza when used at the same concentration, which might be associated with an increase in iron uptake and heme biosynthesis[55]. Later on, Pinto *et al*[56] have reported the first evidence of Aza-dC as a differentiation inducing agent of primary cells derived from patients with AML[56]. The differentiation inducing effect of Aza and Aza-dC has proven them to be promising therapeutic approaches aiming at induction of leukemic stem and progenitor cells toward non-malignant differentiated cells[57,58].

The induction ability of myeloid differentiation by Aza-dC in myeloid leukemic cells correlates with an up-regulation of CD15, myeloperoxidase, lysozyme and the tumor suppressor p15[59]. Several mechanisms have been proposed to underline Aza- and Aza-dC-induced differentiation of myeloid leukemia. For example, Aza-dC facilitates tumor necrosis factor alpha-induced monocytic differentiation of two AML cell lines NB4 and U937 by demethylation of *DIF2* (also known as *IER3*) promoter and thus up-regulation of the gene[60]. Similarly, an upstream regulatory element within PU.1 promoter has been shown to be demethylated by Aza leading to an up-regulation of PU.1 and its target genes[61]. Co-treatment of $CD34^+$ cells derived from MDS patients by Aza and granulocyte colony-stimulating factor led to myeloid differentiation[61]. Another study has shown that Aza-dC induces expression of olfactomedin 4 (*OLFM4*), of which an over-expression led to apoptosis and differentiation HL60 cell line[62]. Treatment of myeloid leukemia cells with Aza-dC also led to an induction of

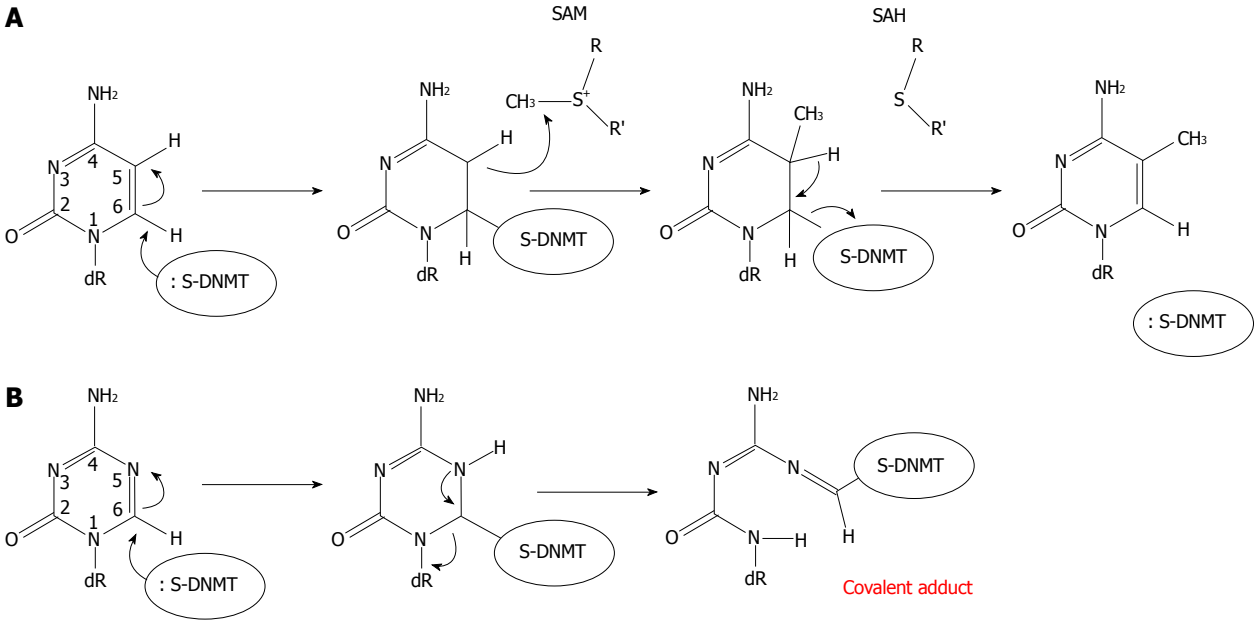

Figure 2 Mechanism of DNA methylation and its inhibition by 5-Aza-2'-deoxycytidine *via* covalent trapping. A: Cytosine methylation by a DNMT is initiated by the formation of a covalent bond between the cytosine base and a cysteine residue at the active site of the methyltransferase. SAM then transfers a methyl group to the cytosine generating a methylcytosine. B: A DNMT is covalently trapped by the DNA-incorporated 5-Aza-2'-deoxycytidine (Aza-dC) adduct. The sulfhydryl group of the cysteine residue at the active site of DNMT forms a covalent bond with C6 of the Aza-dC in DNA. However, the active methyl group from SAM cannot be transferred to Aza-dC by DNMT. This covalent trapping mechanism then leads to a gradual loss of free DNMTs, because they are covalently linked with Aza-dC within DNA. Therefore, the loss of free DNMTs then precedes passive DNA demethylation. Thus cells expressing high levels of DNMTs will respond to Aza-dC at a higher extent compared with cells expressing low levels of DNMTs (see Figure 3). R: Methionine/cysteine; R': Adenosine; Dr: Deoxyribose-5-phosphate; DNMT: DNA methyltransferases.

differentiation-associated genes including the erythroid-lineage transcription factor GATA1, which inhibits proliferation of the cancer cells[20]. A link between DNA hypermethylation and AML1/ETO-mediated repression of microRNA expression has been suggested as a means to inhibit apoptosis and differentiation of leukemic stem and progenitor cells[63,64]. In AML and chronic myeloid leukemia cell lines, Aza treatment reactivated expression of microRNA-193a, which suppresses translation of oncogenes including c-kit. Furthermore, an ectopic over-expression of microRNA-193a in primary cells of AML blasts led to apoptosis and differentiation of the cells[63]. Remarkably, Tsai *et al*[65] have recently shown that application of Aza or Aza-dC at low and non-acute toxic doses has successfully reduced stem cell characteristics of leukemia and also other cancer stem and progenitor cell lines and of primary tumors, without adverse effects on normal human bone marrow cells[65]. The low concentrations of the epigenetic drugs can demethylate both CpG island- and non-CpG island-containing promoters, which up-regulate expression of tumor suppressor- and differentiation-associated genes.

Recently, a failure of Aza to eradicate cancer stem and progenitor cells in patients with MDS and AML has been reported[66]. However, it has yet to be determined the mechanisms underlying this failure in order to improve new strategies of Aza treatment, and to design new drugs and clinical regimes that can completely diminish the cancer stem and progenitor cells of MDS and AML, either through an induction of cell death or differentiation. Interestingly, tranylcypromine, an inhibitor targeting histone H3 lysine 4 demethylase LSD1 (also known as KDM1A) which is pivotal for maintaining the stem cell state of AML cells[67], shows a promising result when combined with retinoic acid to induce myeloid differentiation of AML cells[68]. Therefore, combination therapies between DNA methylation inhibitors, preferentially with low doses[65], and other molecules which induce apoptosis and/or differentiation of cancer stem and progenitor cells of MDS and AML cells might offer an alternative direction to target the malignant cells in MDS and AML patients.

Although the effect of DNMT inhibitors on cytotoxicity and differentiation of lymphoblastic leukemia is less studied, a recent finding has suggested the role of Aza-dC in induction of apoptosis and differentiation of B-cell acute lymphoblastic leukemia (B-ALL) cell line NALM-6 and T-cell acute lymphoblastic leukemia (T-ALL) cell line CCRF-CEM[69]. The apoptotic induction by Aza-dC in B-ALL cells might be due to reactivation of *HES5* gene, which has been shown to be hypermethylated in primary B-ALL cells[70]. Further investigations are needed to ascertain whether Aza and Aza-dC induces differentiation of primary B-ALL and T-ALL cells, and to elucidate mechanisms responsible for the eradication of cancer stem and progenitor cells of B-ALL and T-ALL by DNMT inhibitors.

Medulloblastoma

Medulloblastoma is the most common malignant

brain tumor found in children, and is a disease of cancer stem cells which possess activity of cell signaling pathways resemble to that of embryonic stages of neural progenitors[71]. Recent genome-wide DNA methylation analyses have suggested that DNA methylation plays a major role in the pathogenesis of medulloblastoma by repression of genes involved in developmental regulation thus avoiding cellular differentiation[72,73]. In addition, DNA methylation might also be important for medulloblastoma to avoid cell death, since an inhibition of DNA methylation sensitized medulloblastoma cell lines to undergo apoptosis induced by interferon gamma and TNF-related apoptosis-inducingligand (TRAIL)[74]. Treatment of medulloblastoma cell lines by Aza-dC has been shown to induce expression of *SPINT2*, which is an inhibitor of hepatocyte growth factor signaling and is hypermethylated in a number of patients with medulloblastoma[75]. Interestingly, an ectopic expression of *SPINT2* led to a decrease in proliferation, colony survival and cell migration of medulloblastoma cell lines. Moreover, mice transplanted with *SPINT2* over-expressing medulloblastoma cells show a prolonged survival compared with controls suggesting that *SPINT2* functions as a tumor suppressor *in vivo*[75]. Similar to *SPINT2*, expression of *CRABP2*, which encodes a retinoic acid binding protein, is silenced by DNA methylation in medulloblastoma. Treatment of the cancer cells by Aza-dC restored expression of *CRABP2*, which in turn allowed neuronal differentiation of medulloblastoma cells induced by retinoic acid[76]. Co-treatment of Aza-dC and the histone deacetylase (HDAC) inhibitor valproic acid also resulted in a reduction of medulloblastoma formation in *Patched* mutant mouse models, and extended survival of the mice[77]. Thus further preclinical studies on combined therapies using DNA methylation inhibitors and HDAC inhibitors might be worthwhile for treatment of patients with medulloblastoma.

Glioblastoma

While medulloblastoma is the most prevalent brain tumor in children, glioblastoma is the most common and aggressive brain tumor found in adults. The role of DNA methylation in repression of genes involved in differentiation seems to be similar between glioblastoma and medulloblastoma. For example, DNA hypermethylation has been reported at CpG island upstream of *CRABP2*. Inhibition of DNA methylation by Aza-dC treatment in primary glioblastoma resulted in activation of *CRABP2* expression and differentiation of the cells[76,78]. However, in contrast to medulloblastoma[74], treatment of glioblastoma stem-like cells by Aza-dC failed to sensitize the cells toward TRAIL-induced apoptosis, although expression of caspase-8 was induced by Aza-dC[79]. A connection of DNA methylation with p53 pathway has been described, in which Aza-dC treatment led to demethylation at p53 promoter. As the result, p53 then directly binds to the promoter region of the G protein-coupled formylpeptide receptor, and represses its expression leading to differentiation of glioblastoma cells[80]. Aza-dC has also been reported to induce expression of microRNA-137, which is involved in anti-proliferation and differentiation of glioblastoma stem cells[81].

Neuroblastoma

Neuroblastoma has been proposed to develop from undifferentiated neural crest progenitor cells, which possess the potency to differentiate into peripheral neural and mesenchymal lineages[82]. It is a pediatric cancer, which might be explained by the embryonic origin of neural crest progenitor cells. DNA methylation has been reported to epigenetically silence expression of caspase-8, which is a mediator of apoptosis in neuroblastoma[83]. Co-treatment of Aza-dC and either chemotherapeutic drugs cisplatin or etoposide induced cell death possibly through caspase-8 reactivation[84]. In addition, as mentioned above for medulloblastoma[74], the same study also shows that a combined treatment of Aza-dC and interferon gamma led to apoptotic induction *via* TRAIL pathway in neuroblastoma cell lines[74]. It would be of interest to further investigate whether Aza-dC and interferon gamma might synergistically and extensively induce differentiation of the cancer progenitor cells as neuroblastoma have been shown to differentiate by Aza-dC treatment[85,86].

Prostate cancer

Androgen receptor (AR) is an important survival factor in prostate cancer. However, in some cases of prostate cancer, AR can function as a tumor suppressor gene[87]. The inhibitory role of AR in aggressiveness of prostate cancer is supported by loss of AR expression *via* promoter DNA methylation[88-91]. Interestingly, expression of AR is also reduced in prostate cancer stem cells particularly with high CD133 expression level[92]. Treatment of prostate cancer stem cells with Aza-dC led to an up-regulation of AR and a decrease in proliferation and tumor formation. Moreover, Aza-dC treatment also induced differentiation of the cancer stem cells, which is correlated with a reduced expression of stem cell-associated genes *OCT4* and *NANOG*[92]. Thus, this study suggests that the inhibition of DNA methylation might be clinically translated for patients having prostate cancers with androgen-independent feature.

Pancreatic cancer

Pancreatic cancer stem cells have been shown to be sensitive to Aza-dC, which reduces tumor sphere formation and induces apoptosis of the cancer stem cells through activation of caspase-3 pathway[93]. The tumor suppressor activity of Aza-dC on pancreatic cancer stem cells is due to its ability to induce expression of microRNA-34, which in turn suppresses expression of the growth factor VEGF-B[93]. In addition to the apoptotic induction effect, Aza-dC has also been demonstrated to induce differentiation of the pancreatic cancer progenitor cell lines MIA PaCa-2[94] and PANC1[95]. Further investigations on how Aza-dC induces differentiation of pancreatic cancer stem and progenitor cells at molecular level are important toward its clinical translation.

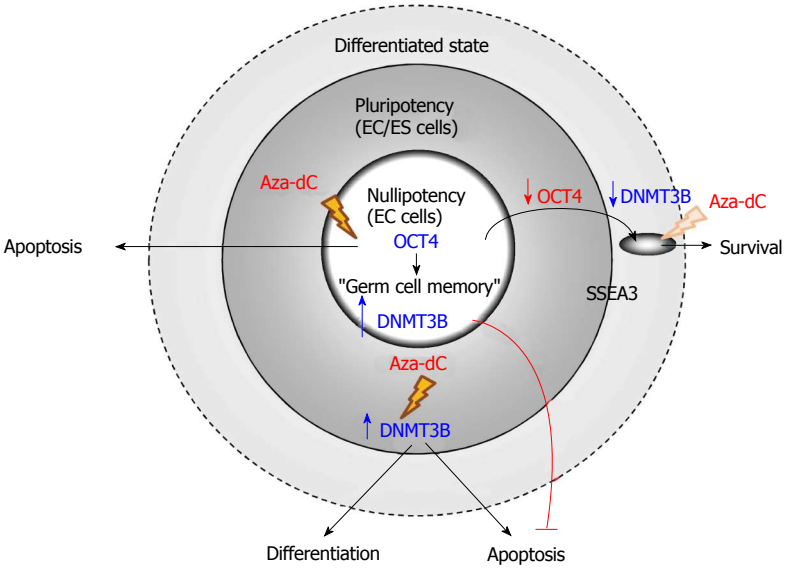

Figure 3 5-Aza-2'-deoxycytidine induces apoptosis of human teratocarcinoma stem cells but not their differentiated counterparts. Human nullipotent embryonal carcinoma (EC) cells, the stem cells of teratocarcinoma, are highly sensitive to 5-Aza-2'-deoxycytidine (Aza-dC), which induces apoptosis of the cancer stem cells. On the other hand, OCT4-knockdown differentiated nullipotent EC cells are resistant toward Aza-dC treatment. In human pluripotent EC cells NTERA2, DNMT3B mediates apoptosis induced by Aza-dC, whereas the enzyme mediates differentiation of human ES cells induced by the inhibitor. DNMT: DNA methyltransferases; ES: Embryonicstem; OCT4: Octamer-binding transcription factor 4.

Testicular germ cell tumors

Germ cell tumors (GCTs) with stem cell characteristics comprise of seminoma and non-seminoma, which belong to type Ⅱ testicular GCTs. Seminoma and non-seminoma have been proposed to develop from carcinoma *in situ*, also known as intratubular germ-cell neoplasia unclassified lesion or testicular intratubular neoplasia, which is the malignant counterpart of gonocytes[96,97]. Although the human seminoma-like cell line TCam-2 is resistant to Aza-dC even applied at 10 μmol/L[98], human embryonal carcinoma (EC) cells, the stem cells of teratocarcinoma, are extremely sensitive to the epigenetic drug of which only 10 nmol/L is sufficient to reduce viability of EC cells. This cytoxicity of Aza-dC on human EC cells is mediated by DNMT3B, which is a marker of human pluripotent stem cells[99] and is highly expressed in human EC cells[51]. Subsequently, DNMT3B has been reported to play a significant role in activation of DNA damage response and expression of p53 target genes induced by Aza-dC in human pluripotent EC cell line NTERA2[100].

Albeit having the cytotoxic effect on the cancer stem cells, Aza-dC fails to induce apoptosis of differentiated cells derived from human nullipotent EC cells, which can be isolated from teratocarcinoma more frequently than their pluripotent counterparts[52]. Although human nullipotent EC cells undergo apoptosis induced by Aza-dC, they do not differentiate. On the other hand, Aza-dC induces both differentiation and apoptosis of human pluripotent EC cells and also human embryonic stem (ES) cells. The ability to induce apoptosis of Aza-dC depends on DNMT3B in human pluripotent EC cells but not in their nullipotent counterparts (Figure 3). Similarly, the ability to induce differentiation of Aza-dC in human ES cells also depends on DNMT3B, supporting the role of DNMT3B in facilitating differentiation of human ES cells[101]. The failure of Aza-dC to induce differentiation of human nullipotent EC cells suggests that mechanism(s) other than DNA methylation is responsible for maintaining the stem cell state of nullipotent EC cells[102], which might still possess a remnant of germ cell memory[103]. Therefore, to effectively eradicate teratocarcinoma comprising of EC cells and their associated teratoma tissues, combined therapies between Aza-dC and agents that target the differentiated cancer cells should be considered.

CONCLUSION

Progresses in an understanding of normal stem cell biology have revolutionized the concept of cancer stem and progenitor cells, which might be the key toward success in treatment of a number of cancer diseases by targeting at cancer stem and progenitor cells. Aza and Aza-dC, although being the drugs currently used for treatment of hematologic malignancy, have held a great promise for treatment of solid tumors[65]. Future studies on mechanisms underlying differentiation and/or apoptosis induced by Aza and Aza-dC on cancer stem and progenitor cells are required for effective treatment of the epigenetic drugs. Several questions emerge including; (1) Do gene regulatory networks, which are specifically associated with cancer counterparts of stem and progenitor cells, up-regulate expression of DNMTs? (2) Which signaling pathways and their downstream target genes are functionally perturbed by Aza and Aza-dC treatment? (3) Are cell death pathways other than apoptosis involved in the anti-proliferation effect? (4) Are Aza and Aza-dC effective to eradicate cancer stem and progenitor cells in xenograft tumors? (5) In case of relapse, do recurrent cancer stem and progenitor cells evolve a distinct "malignant memory" at epigenomic levels compared with the cancer cells isolated before admission? and, if so, (6) Does the oncogenic evolution lead to more aggressive phenotypes such as a reduced differentiation potential of cancer stem and progenitor cells ? Treatments with Aza and Aza-dC can lead to certain toxicity and side effects to hematopoietic, nervous as well as metabolic systems[104-107]. Nevertheless, they have been recognized as having lower toxicity compared

to traditional chemotherapy. In fact, low-dose treatment of Aza-dC in patients with MDS[108] and solid tumors[109] resulted in complete response of the patients. Thus using low-dose DNA methylation inhibitors may provide a safe therapeutic choice[65] especially for elderly patients[107,110]. Whether long-termed treatments of low-dose Aza and Aza-dC would perturb fates of stem and progenitor cells without side effects and toxicity in patients have yet to be elucidated. Addressing these questions will not only provide an understanding of cancer stem cell biology but also shed light on development of novel clinical regimes.

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