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Review:

DNA methylation in the tumor microenvironment*

Meng-wen ZHANG[1,2,3,4], Kenji FUJIWARA[3,4], Xu CHE[3,4], Shu ZHENG[†‡1,2], Lei ZHENG[†‡3,4]

([1]*The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China*)

([2]*The Key Laboratory of Cancer Prevention and Intervention, China National Ministry of Education, Hangzhou 310009, China*)

([3]*Department of Oncology, Johns Hopkins University School of Medicine, Baltimore 21231, USA*)

([4]*The Sidney Kimmel Cancer Center, Johns Hopkins University School of Medicine, Baltimore 21231, USA*)

[†]E-mail: zhengshu@zju.edu.cn; lzheng6@jhmi.edu

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Abstract: The tumor microenvironment (TME) plays an important role in supporting cancer progression. The TME is composed of tumor cells, the surrounding tumor-associated stromal cells, and the extracellular matrix (ECM). Crosstalk between the TME components contributes to tumorigenesis. Recently, one of our studies showed that pancreatic ductal adenocarcinoma (PDAC) cells can induce DNA methylation in cancer-associated fibroblasts (CAFs), thereby modifying tumor-stromal interactions in the TME, and subsequently creating a TME that supports tumor growth. Here we summarize recent studies about how DNA methylation affects tumorigenesis through regulating tumor-associated stromal components including fibroblasts and immune cells. We also discuss the potential for targeting DNA methylation for the treatment of cancers.

Key words: Tumor microenvironment (TME); DNA methylation; Cancer-associated fibroblasts; Cancer-associated immune cells; Epigenetic therapy

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1 Introduction

The tumor microenvironment (TME) is a complex aggregation of tumor cells, stromal cells, and the extracellular matrix (ECM) (Fig. 1). Stromal cells are a heterogeneous population of cells composed of fibroblasts, immune cells, and vascular endothelial cells (Feig *et al.*, 2012; Neesse *et al.*, 2015). Although they themselves do not have malignant biological capabilities such as unrestricted replicative potential and resistance to cell death, they seem to facilitate the development of cancer by providing a tumor-favoring microenvironment (Hanahan and Weinberg, 2011). The stromal compartment even occupies the majority of the tumor mass in some tumor types, including pancreatic ductal adenocarcinoma (PDAC) (Rucki and Zheng, 2014).

Tumor initiation and progression are driven by accumulated mutations and epigenetic alterations, such as histone modification, non-coding RNA (ncRNA) regulation, and DNA methylation (Fig. 2). Histone proteins undergo post-translational modifications, such as methylation, acetylation, phosphorylation, and ubiquitination (Cheung *et al.*, 2000; Sharma *et al.*, 2010), leading to activation or suppression of certain genes, heterochromatin formation, and DNA replication (Kouzarides, 2007). NcRNAs, which lack the capacity to encode proteins, have been shown to regulate gene expression by various epigenetic mechanisms (Garzon *et al.*, 2009; Gibb *et al.*, 2011). Most published studies have focused on microRNAs (miRNAs) and long ncRNAs (lncRNAs). More and more target genes that are regulated by human miRNAs have been identified (Iorio *et al.*, 2010). LncRNAs (more than 200 nt) are usually

‡ Corresponding authors

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ORCID: Meng-wen ZHANG, http://orcid.org/0000-0003-1589-9314

longer than miRNAs. They regulate gene expression in various ways including generating miRNAs (Cui *et al.*, 2002), inducing long-range chromatin looping (Ling *et al.*, 2013), and interacting with proteins (Xiang *et al.*, 2014).

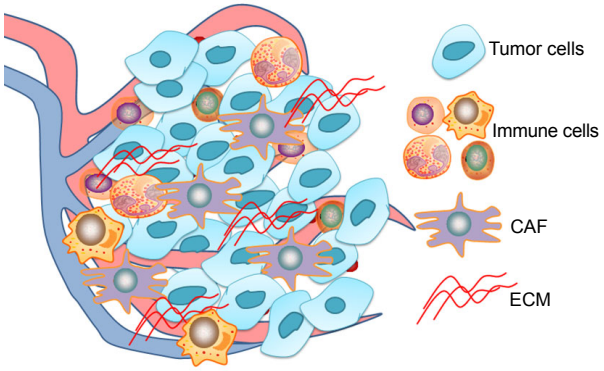

Fig. 1 Schematic representation of the TME
A typical tumor microenvironment (TME) is composed mainly of cancer cells, cancer-associated fibroblasts (CAFs), multiple types of immune cells (T lymphocytes, B cells, natural killer (NK) cells, macrophages, and antigen presenting cells), vessels, and extracellular matrix (ECM)

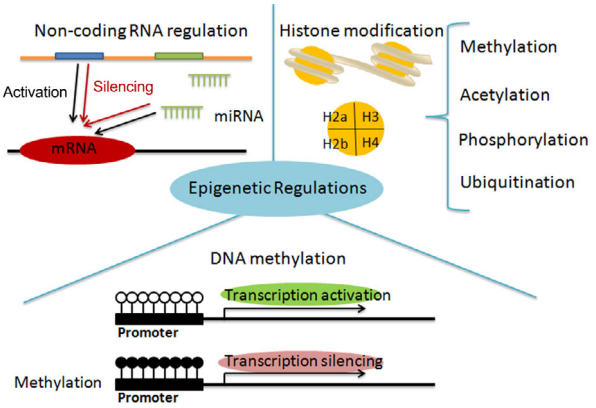

Fig. 2 Epigenetic regulation, such as histone modification, non-coding RNA regulation, and DNA methylation, leads to different gene expressions in the tumor microenvironment (TME)

DNA methylation is a stable and heritable change in gene structure without a change in DNA sequence (Bird, 2007; Easwaran *et al.*, 2014). Among all the epigenetic alterations, DNA methylation is the most frequently studied mechanism involved in regulating gene expression. DNA hypermethylation in the promoter region of tumor suppressor genes leads to the epigenetic silencing of those genes, and is a hallmark of cancer (Esteller, 2007; Gronbaek *et al.*, 2007; Vincent *et al.*, 2011).

There have been many studies of the malignant behavior of cancer cells and the epigenetic alterations in tumor cells or cancer stem cells (Liu *et al.*, 2015), but few have reported the role of DNA methylation in tumor-associated stromal cells. In this review we summarize recent studies demonstrating how DNA methylation regulates the phenotypes of tumor-associated stromal cells and discuss the potential of drugs targeting DNA-methylation for the treatment of specific cancers.

2 DNA methylation in cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) acquire their activated phenotype in the TME as a result of interactions with tumor cells or tumor cell-derived biomolecules. CAFs constitute the most abundant population in the tumor stroma, contribute to tumorigenesis by secreting various factors that regulate intercellular signaling in tumor cells, and mechanically remodel the cancer tissue (Karagiannis *et al.*, 2012; Xing *et al.*, 2015; Gascard and Tlsty, 2016; Ishii *et al.*, 2016).

Jiang *et al.* (2008) used methylation-sensitive single nucleotide polymorphism arrays (MSNPs) to analyze DNA methylation in stromal CAFs isolated from human gastric cancers and their accompanying reactive normal stromal cells. Their findings indicated that the global DNA hypomethylation of CAFs was similar to that of tumor cells. Only a few genes had hypermethylation in CAFs compared to normal fibroblasts. These hypermethylated genes (homeobox A9 (*HOXA9*) and *HOXB6*) were independently validated by bisulfite sequencing. Genome-wide human methylation microarray analysis was conducted on CAFs and paired control fibroblasts from non-small cell lung cancer patients by Vizoso *et al.* (2015). Compared with normal fibroblasts, they found global DNA hypomethylation in CAFs accompanied by some hypermethylated genes. These DNA methylation alterations had a selective impact on gene expression in the transforming growth factor-β (TGF-β) pathway. TGF-β signaling is well established in CAF activation (Zhi *et al.*, 2010), thus providing one

mechanism for the activation of CAFs under the control of methylation. Albrengues *et al.* (2015) found that normal fibroblasts can be reprogrammed to harbor a proinvasive phenotype by treatment with leukemia inducible factor (LIF), a proinflammatory cytokine secreted by tumor cells. LIF induced methylation in the promoter of the protein phosphatase regulator SHP1 (*SHP-1*) and the loss of *SHP-1*, resulting in the constitutive activation of Janus activated kinase 1/ signal transducer and activator of transcription 3 (JAK1/STAT3) signaling, which drives normal fibroblasts to become proinvasive CAFs. When CAFs were treated with a DNA methyltransferase (DNMT) inhibitor, 5-Aza-2'-deoxycytidine (decitabine, DAC), *SHP-1* expression was restored, JAK1/STAT3 signaling was inhibited, and the CAFs lost their protumoral capabilities. These studies suggest that DNA methylation plays a significant role in mediating the cancer promoting activities of fibroblasts.

The activation of CAFs is mechanistically similar to that of myofibroblasts. Both have a high expression of α-smooth muscle actin (α-SMA) (Hinz *et al.*, 2007). Myofibroblasts are the primary contributors to fibrosis, which drives some invasive cancers, especially liver cancer (Pinzani *et al.*, 2005; de Wever *et al.*, 2008). Many studies have explored methylation changes in fibrotic diseases such as hepatic, pulmonary, and renal fibrosis (Mann *et al.*, 2010; Rabinovich *et al.*, 2012; Tampe *et al.*, 2014). Gotze *et al.* (2015) found global DNA hypomethylation in the early stage of hepatic stellate cell (HSC) activation, similar to the findings in CAFs. The CAFs and HSCs used for methylation array analysis in these studies were passaged three to four times or cultured in serum-free medium for several days in vitro, which may itself have caused some changes. El Taghdouini *et al.* (2015) first reported comparative gene expression and promoter methylation in freshly isolated uncultured human liver cells and culture-activated HSCs, showing that activation of HSCs is partially associated with specific DNA methylation changes in vitro. Similar findings were obtained by Page *et al.* (2016), who showed that HSC transdifferentiation was accompanied by DNA methylation.

A recent study by Xiao *et al.* (2016) used merged analysis of DNA methylation arrays and gene expression arrays to identify genes that were both methylated and downregulated in PDAC-derived CAFs or human mesenchymal stem cells (MSCs) after co-culture with PDAC cells. They used the co-culture model to provide CAFs with direct contact with tumor cells and identified the genes whose methylation and expression patterns were reprogrammed by tumor cells. In particular, this study demonstrated that suppressor of cytokine signaling 1 (*SOCS1*) was silenced in CAFs by DNMT-dependent promoter methylation as a result of interacting with tumor cells of PDAC. The induction of *SOCS1* methylation in CAFs requires direct contact between tumor cells and CAFs. *SOCS1* methylation was reversed by DNMT1 knockdown in MSCs, pretreating MSCs with DAC or transfecting MSCs with miR29b, which was previously found to be able to induce *SOCS1* expression by promoter demethylation (Amodio *et al.*, 2013). The methylation and downregulated expression of *SOCS1* can activate STAT3 signaling, leading to high expression of insulin-like growth factor-1 (*IGF-1*) in CAFs. CAFs were shown to promote tumor progression and invasion through secreting the protumoral growth factor IGF-1 (Mueller and Fusenig, 2004; Ahmed and Farquharson, 2010).

Vizoso *et al.* (2015) identified hypermethylation-associated SMAD family member 3 (*SMAD3*) silencing in CAFs, which was associated with aberrant response to exogenous TGF-β1. Exogenous TGF-β1 can induce contractility and ECM expression in CAFs, but not in normal fibroblasts, suggesting that *SMAD3* silencing promotes hyperresponsiveness to TGF-β in CAFs. However, *SMAD3* depletion was shown to decrease contractility and ECM expression in other kinds of fibroblasts (Wang *et al.*, 2007). Yu *et al.* (2012) identified ADAM metallopeptidase domain 12 (*ADAM12*) as hypomethylated and overexpressed in CAFs derived from primary PDAC compared to normal fibroblasts. Consistently, overexpression of *ADAM12* in CAFs was reported to be protumoral (Iba *et al.*, 1999). All these studies demonstrate that both hyper- and hypomethylation of specific genes contribute to the activation of CAFs.

3 DNA methylation in immune cells

Tumor initiation and progression are often accompanied by the inhibition of anticancer response

and dysregulation of inflammatory activity (Berraondo *et al.*, 2016; Sukari *et al.*, 2016). Various kinds of solid tumors are characterized by the presence of immune cells, such as T lymphocytes, B cells, natural killer (NK) cells, macrophages, and antigen-presenting cells. These immune cells in the TME exhibit different behaviors and morphologies as a result of aberrant differentiation (Peric-Hupkes *et al.*, 2010). For example, macrophages are a heterogeneous population of immune cells with a myeloid lineage. They can differentiate into M1 or M2 phenotypes by epigenetically regulating lineage-specific enhancers and promoters. Accumulated epigenetic studies have demonstrated that DNA methylation plays an important role in lineage-specific changes in immune cells, influencing the expression of genes crucial for the identity of immune cells and promoting cellular responses to stimuli (Sorensen *et al.*, 2010; Smith and Meissner, 2013; Luperchio *et al.*, 2014).

Epigenetic modification plays an important role in regulating immune cell differentiation. Schuyler *et al.* (2016) analyzed 112 whole-genome bisulfite sequencing datasets from the BLUEPRINT Epigenome Project (http://www.blueprint-epigenome.eu) to identify the global DNA methylation trends among different lineages of immune cells and the cancers that arise from them, especially leukemia and lymphoma. They found distinct methylation patterns in the myeloid and lymphoid lineages in cancer tissues. The global methylation level increased during macrophage differentiation and activation, while it dropped in both T and B lymphocytes. Other studies also showed that different methylation patterns contribute to the activation of myeloid and lymphoid cells (Broske *et al.*, 2009; Bock *et al.*, 2012). Sido *et al.* (2015) found that Δ^9-tetrahydrocannabinol (THC), an exogenous cannabinoid, can induce hypermethylation of DNMT3a and DNMT3b in mouse cancer models. Furthermore, promoter regions of key functional genes (arginase 1 (*Arg1*) and *STAT3*) of myeloid-derived suppressor cells (MDSCs) were demethylated in THC-treated MDSCs as a result of the alteration in DNMT activity. The high expressions of *Arg1* and *STAT3* were associated with the highly immunosuppressive nature of activated MDSCs (Trikha and Carson, 2014). Thus, THC had mediated epigenetic changes to promote MDSC differentiation and function in the TME.

The methylation status of immune genes influences the tumor immune response in the TME, and correlates with the density of tumor-infiltrating lymphocytes. Janson *et al.* (2008) demonstrated the different methylation status of the interferon-γ (*IFN-γ*) gene promoter and upstream enhancer in naive $CD4^+$ T lymphocytes. However, the *IFN-γ* gene promoter and enhancer were demethylated in Th1 lymphocytes, where the expression of *IFN-γ* was induced. Th2-differentiated lymphocytes remained hypermethylated in the *IFN-γ* promotor and enhancer. These data suggested that Th1/Th2 differentiation may be mediated by methylation and demethylation of the *IFN-γ* gene. Further investigation of the colon cancer-derived tumor-infiltrating lymphocytes demonstrated that hypermethylation of the *IFN-γ* gene can prevent the maturation of Th1 lymphocytes. This may be the epigenetic mechanism for tumor-induced immunosuppression. By performing DNA methylation profiling in normal breast tissues and infiltrating ductal carcinomas, Dedeurwaerder *et al.* (2011) identified several hypomethylated genes correlated with high T lymphocyte infiltration, including lymphocyte transmembrane adaptor 1 (*LAX1*), signaling threshold regulating transmembrane adaptor 1 (*SIT1*), and ubiquitin associated and SH3 domain containing A (*UBASH3A*). Furthermore, the study revealed that hypermethylation of these genes accounted for an enhanced antitumor T-cell response, and had an independent prognostic value. Ke *et al.* (2016) established a co-culture system to study the effect of non-small-cell lung cancer cells on the phenotype and function of $CD4^+$ regulatory T cells (Tregs). The study showed that decreased activity of DNMTs in $CD4^+$ Tregs was accompanied by demethylation of the forkhead box P3 (*FOXP3*) gene promoter and downregulation of immune responses in the TME. Wieczorek *et al.* (2009) showed that DNA methylation of *FOXP3* was a more consistent and specific marker for the quantification of Tregs in lung and colorectal tumor tissues than gene or protein expression levels.

4 Epigenetic therapy targets stromal cells in the tumor microenvironment

A number of studies have confirmed the tight connection between DNA methylation and tumorigenesis.

Inactivation of tumor suppressor genes, mediated through DNA methylation, contributes to the tumorigenesis process. Drugs inhibiting DNA methylation have been developed in in vitro and animal experiments as a potential therapeutic approach to suppress tumor cells (van Kampen *et al.*, 2014; Chiappinelli *et al.*, 2016). Several of them have been approved by the Food and Drug Administration (FDA, USA) for the treatment of certain human cancers. 5-Azacytidine (5-Aza) and DAC can become incorporated into DNA during replication and inhibit the activity of DNMTs, leading to a widespread reduction in gene methylation (Batarseh, 2013). Both have shown clinical benefits in patients with myeloid malignancies (Pleyer and Greil, 2015). However, DNMT inhibitors showed less promising results in the treatment of solid tumors. Since DNMT inhibitors do not specifically target tumor cells, they would be expected to have effects on stromal cells in the TME.

Yu *et al.* (2012) used DAC to treat CAFs isolated from PDAC patients. They found that fewer genes were regulated by DNA hypermethylation status in CAFs than in tumor cells. Bian *et al.* (2012) demonstrated that DAC treatment can reduce phosphatase and tensin homolog (*PTEN*) promoter methylation and increase *PTEN* mRNA expression in HSCs following activation by TGF-β in vitro. *PTEN* silencing in stromal fibroblasts can accelerate the initiation and malignant transformation of mammary epithelial tumors in a mouse model (Trimboli *et al.*, 2009). Thus, it is possible that DAC can reverse *PTEN* silencing and halt the progression of breast cancer by increasing *PTEN* expression. Janson *et al.* (2008) found increased expression of *IFN-γ* in 5-Aza-treated tumor-infiltrating lymphocytes, suggesting that DNMT inhibitors may stimulate the immune response in the TME. Most studies have described the effect of DNMT inhibitors on stromal cells in vitro. Xiao *et al.* (2016) reported that DAC treatment can inhibit tumor growth in a subcutaneous tumor model. DAC can reverse the methylation of *SOCS1* in stromal fibroblasts induced by PDAC tumor cells. When MSCs were pretreated with DAC before co-inoculation with PDAC tumor cells into NOD/SCID mice subcutaneously, they lost their capacity to promote the growth of xenografts. Furthermore, xenografts grew slower in mice pretreated with DAC for 5 d before PDAC tumor inoculation.

5 Conclusions

The TME plays an important role in supporting tumor growth. A number of studies have investigated the effect of DNA methylation on the behavior of tumor-associated stromal cells. DNA methylation is involved in the activation of stromal cells, and the methylation of certain genes contributes to the pro-cancerous activity of stromal cells. Table 1 summarizes the genes regulated by DNA methylation in tumor-associated stromal cells as described above. DNMT inhibitors have significant effects on stromal

Table 1 DNA methylation in tumor-associated stromal cells

Reference	Cell type	Specific gene	Methylation status
Jiang *et al.*, 2008	Human gastric cancers CAFs	*HOXA9*, *HOXB6*	Hypermethylated
Vizoso *et al.*, 2015	Human lung cancer CAFs	*SMAD3*	Hypermethylated
Albrengues *et al.*, 2015	Human head and neck CAFs	*PTPN6*, *SHP-1*	Hypermethylated
Gotze *et al.*, 2015	Human activated HSCs	*CNR2*, *MMRN2*	Hypermethylated
El Taghdouini *et al.*, 2015	Human activated HSCs	*ACTG2*	Hypermethylated
El Taghdouini *et al.*, 2015	Human normal HSC	*APOB*	Hypermethylated
Xiao *et al.*, 2016	Human pancreatic cancer CAFs	*SOCS1*	Hypermethylated
Yu *et al.*, 2012	Human pancreatic cancer CAFs	*ADAM12*	Hypomethylated
Sido *et al.*, 2015	Mouse activated MDSCs	*Arg1*, *STAT3*	Hypomethylated
Janson *et al.*, 2008	Human colon cancer lymphocytes,	*IFNG*	Hypermethylated
Dedeurwaerder *et al.*, 2011	Human breast cancer lymphocytes,	*LAX1*, *SIT1*, *UBASH3A*	Hypomethylated
Ke *et al.*, 2016	Human lung cancer $CD4^+$ Tregs	*FOXP3*	Hypomethylated
Wieczorek *et al.*, 2009	Human lung and colorectal cancer Tregs	*FOXP3*	Hypomethylated
Lucas *et al.*, 2012	Human melanoma Tregs	*FOXP3*	Hypomethylated
Bian *et al.*, 2012	Human activated HSCs	*PTEN*	Hypermethylated

CAF: cancer-associated fibroblast; HSC: hepatic stellate cell; MDSC: myeloid-derived suppressor cell; Tregs: regulatory T cells

cells, but most studies have been conducted in vitro due to the lack of animal models for studying stromal cell epigenetics in the TME. More studies are needed to help understand the mechanisms that regulate the behavior of tumor-associated stromal cells through DNA methylation or demethylation. Stroma-targeted therapies may be a promising novel strategy for treating certain stroma-prominent malignant tumors.

Compliance with ethics guidelines

Meng-wen ZHANG, Kenji FUJIWARA, Xu CHE, Shu ZHENG, and Lei ZHENG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：肿瘤微环境中的甲基化调节机制

概　要：肿瘤微环境主要由肿瘤细胞、肿瘤相关间质细胞及细胞外基质组成。肿瘤细胞通过多种方式调控肿瘤微环境中的间质细胞，诱导间质细胞分化并发挥促肿瘤的作用，从而为肿瘤的生长及转移创造一个适宜的环境。DNA 甲基化异常是肿瘤的特点。目前关于肿瘤的甲基化调控机制已有大量报道，对于肿瘤细胞与微环境中间质细胞的相互作用机制也有了一些报道。然而，关于肿瘤细胞对微环境间质细胞的甲基化调控机制以及这种调控对肿瘤发生发展的影响并没有系统的论述。本综述总结了肿瘤细胞对微环境中间质细胞甲基化调控机制的最新研究进展，以及间质细胞发生的一些促肿瘤改变，从而全面阐释了肿瘤细胞和间质细胞间的相互作用，同时总结了肿瘤细胞对肿瘤微环境的表观遗传学调控，尤其是甲基化调控在肿瘤进展中发挥了重要的作用。干预肿瘤细胞对微环境中间质细胞的甲基化调节过程，可以发挥抗肿瘤的作用。

关键词：肿瘤微环境；DNA 甲基化；肿瘤相关成纤维细胞；肿瘤相关免疫细胞；表观遗传学治疗