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Teaser This paper is a demonstration of the potential of epigenetic approaches that will inevitably begin to move into more clinical trials for use in patients with liver diseases including hepatocellular carcinoma.

Molecular epigenetic targets for liver diseases: current challenges and future prospects

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Advanced chemotherapy fails to treat liver cancer but recent progress in understanding epigenetic modifications have witnessed promising clinical outcomes. Epigenetic alteration is the alteration of epigenomes (surrounding histone proteins) without changing the DNA sequence. Such epigenetic mechanisms include histone modifications such as methylation, acetylation, phosphorylation and sumoylation followed by changes in the genomic architecture. Current studies involving the understanding of small RNA molecules such as noncoding RNA and microRNA in modulating the chromatin architecture are explained in depth here, along with effects of some novel compounds from recent preclinical and clinical evidence. This review also discusses the current state-of-the-art strategies and the possible scope of investigation to improve the existing treatment methods for liver-related disorders.

Introduction

Epigenetic targets in the treatment of liver diseases currently represent one of the most attractive approaches in the search for new therapeutics [1]. Unlike the present gene therapy approaches, epigenetic therapy has the potential to switch off gene expression of the aggressive diseases without changing the primary DNA sequence [1,2]. Presently, there is one epigenetic drug on the market for treatment of liver cancer [1,3], whereas multiple drugs targeting the epigenetic modifications are available for cancer therapy overall [4,5]. Several compounds are in clinical development and hundreds of compounds are in laboratory evaluation and preclinical stages [6]. It is a great hope that those compounds that are either in clinical stages or the preclinical testing stage will be part of the therapeutic armamentarium in the near future. These drug candidates are eagerly awaited and could prove highly beneficial for the growing number of liver disease

Robert Zeidler currently works as a PhD student in the Department of Cell Techniques and Applied Stem Cell Biology at the Center for Biotechnology and Biomedicine, University of Leipzig. His research fields are epigentic targets in liver diseases, especially relating to the role of histone and DNA modifications and miRNA in the development of hepatic cancer. Additionally, he studies medicine at Leipzig University where he also received his MSc in chemistry in 2013.

Augustinus Bader is a German physician and biomedical scientist, and one of the leading experts in the field of stem cell research. His clinically most relevant inventions include a biological process that imitates bionic principles for stem cell activation and tissue regeneration. Most of his patents have a global coverage, with 27 currently active patent families with over 200 international filings. In 2010, Augustinus received the Cicatrix Prize, the largest European scientific prize organized by a patient organization, for the development of a therapeutic method to prevent scar formation following severe thermal injuries.

Shibashish Giri is a stem cell scientist in research and therapy. Currently, he works as a Deputy head of Applied Stem Cell Biology and Cell Technology, Center for Biotechnology and Biomedicine, Medical Faculty, University of Leipzig, Germany. He has 12 years of research experience on location and isolation of endogenous stem cells in humans and animal models, activation of endogenous stem cells for cell or organ regeneration, long noncoding RNA (lncRNA), microRNA (miRNA), epigenetic models, scarfree skin regeneration, treatments for hair loss and establishment of in vitro fatty liver models for fatty liver diseases. He has published 32 peer-reviewed articles on stem cell research and therapy.

This article is dedicated to the memory of Prof. Harald A. Lange.
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patients, particularly for those who do not qualify for liver transplantation. Accumulating clinical results show the great benefits for cancer patients [4,5]. It is anticipated that elucidating and understanding the role of epigenetic modifications in liver diseases will provide new therapeutic targets. Some of the main targets of epigenetic modifications are: (i) chromatin structure; (ii) noncoding RNA. The chromatin structure involves histone modifications including DNA methylation. DNA methylation is a process of addition of methyl groups at the cytosine residues predominantly in the promoter region of the target genes. The histone modifications are: acetylation, methylation, phosphorylation, unbiquitination, ribosylation and sumoylation. Histone modifications occur by addition of chemical groups involving or driven by five key enzymes. The five key enzymes are: histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), DNA methyltransferases (DNMTs) and histone demethylases (HDMs). These enzymes have been identified and well characterized for mediating histone modifications. New therapeutic strategies to modulate epigenetic regulation are now being extensively investigated for liver diseases, particularly for hepatocellular carcinoma patients [1,3]. Although the knowledge remains in stages of infancy, the increasing understanding of epigenetic mechanisms and liver-disease-associated epigenetic modulation mechanisms could provide future direction for better drug development [6]. The basic target is DNA methylation – adding a methyl group while histone modification occurs by adding chemical groups. These five epigenetic enzymes are the most significant pharmaceutical targets. Epigenetic modulation follows the common principle of readers, writers and erasers. The readers are acetyl lysine recognition and methyl lysine recognition. The writers are DNMTs, Ten-Eleven Translocation (TET) oxygenases (formation of hydroxymethyl-C), HATs, HMTs, among others; and erasers are HDACs and HDMs.

Crosstalk between DNA methylation and histone modification is also an important bonafide therapeutic target for prevention and treatment of liver diseases [1]. In fact, in DNA methylation, methylated protein and histone-modifying enzymes are involved in crosstalk which is crucial to understand their interaction for moving forward to develop drugs that will target the specific pathways [7,8]. Histone modification controls the transcriptional landscape inside a cell [7,8]. Noncoding RNA such as microRNA (miRNA), short interfering RNA (siRNA) and Piwi-interacting RNA (piRNA) are known to be involved in epigenetics processes [9]. Short- and long-chain coding have important roles in histone modification and DNA methylation, as well as targeting and silencing [7,8]. Epigenetics is a natural process and is associated with cancer. DNA hypomethylation activates the oncogenes and DNA hypermethylation initiates the silencing of genes. New and ongoing research on the role of short noncoding RNA, piRNA, siRNA and miRNA for epigenetic modulation to shutdown aggressive disease gene expression is highlighted and discussed in this review. Currently, several companies (e.g., GlaxoSmithKline, Epizyme, Eisai, Celgene, Cellzome, Chroma Therapeutics, Abbott) are actively participating in a number of deals and partnerships for development of epigenetic drugs by investing millions of US$ [10].

Because the prevalence of liver disease is increasing and expected to increase in the coming years, treatment options for liver disease are in many cases insufficient. To improve such treatment methods an increased understanding of epigenetics and its modulation is necessary. In this review, recent advances in molecular targets on epigenomes of normal and cancer phenotypes are discussed. In addition, investigations of the effects of current small molecules for epigenetic research are now emerging. Indeed, epigenetic modulation has already been identified as one of the key factors in liver diseases and related complications, which are further discussed. The promising *in vitro* and *in vivo* studies on epigenetics modulations of academic and industrial laboratories are discussed relating to future trends. Gailhouste *et al.* [11] identified that miR-148a is highly expressed in adult liver as hepatospecific miRNA but is downregulated in biopsies of hepatocellular carcinoma (HCC) patients, mouse and human HCC cell lines. In a mouse fetal hepatoblast model, the authors demonstrated the dual role of miR-148a. In the first scenario, miR-148a targets DNMT for hepatic differentiation, and in a second scenario through RNA interference it recognizes adult liver phenotype by silencing DNMT1. The authors also demonstrated that overexpression of miR-148a significantly enhances albumin production and inhibition of aggressive HCC cells. Similar findings are also reported by Long *et al.* [12] regarding the overexpression of miR-148a and a drastic inhibition of HCC cell proliferation and cell cycle progression.

miRNAs and their implications in liver cancer

miRNAs are small nucleic acid molecules that are 18–21 nucleotides long and are known to negatively regulate gene expression either by translational inhibition or mRNA degradation. However, a recent surge in understanding miRNA-mediated gene regulation has also enabled significant advances in understanding HCC and its related complications. Liu *et al.* [13] collected human HCC tissues and para-cancerous tissues from 63 paired patients who were undergoing hepatobiliary surgery and analyzed these tissues for the expression of miR-30a. They observed lower expression of miR-30a in patients with metastasis compared with the patients without metastasis. Further, the authors also compared the miR-30a expression in HCC cells with LO2 normal human liver cells and the role of miR-30a in cell migration, invasion and changes in epithelial–mesenchymal transition (EMT). EMT is an *in vitro* process for initiation of metastasis for cancer progression. They observed that the miR-30a expression is downregulated in HCC cells compared with its expression in LO2 normal human liver cells. Interestingly, reduction of miR-30a led to enhanced cell migration, invasion and EMT changes. They identified SNAI1, is the direct target of miR-30a which could offer promising therapeutic applications for reducing invasion and metastasis of liver cancer. Profiling miRNA expression in HCC in human liver cancer patients is represented in Fig. 1.

Yan *et al.* [14] collected primary tumor cells from livers of 32 Chinese HCC patients who underwent curative liver resection for primary tumor cells. The authors used a high-throughput miRNA microarray analysis for different expression of miRNA. Their results showed that, among the 41 miRNAs that were found, miR-148a is inversely proportional to the degree of metastasis in HCC patients. They attributed these findings to the involvement of the Wnt signal pathway for the miR-148a-mediated inhibition of EMT and cancer-stem-cell-like properties of HCC cells. They reported the potential inhibition of metastasis of

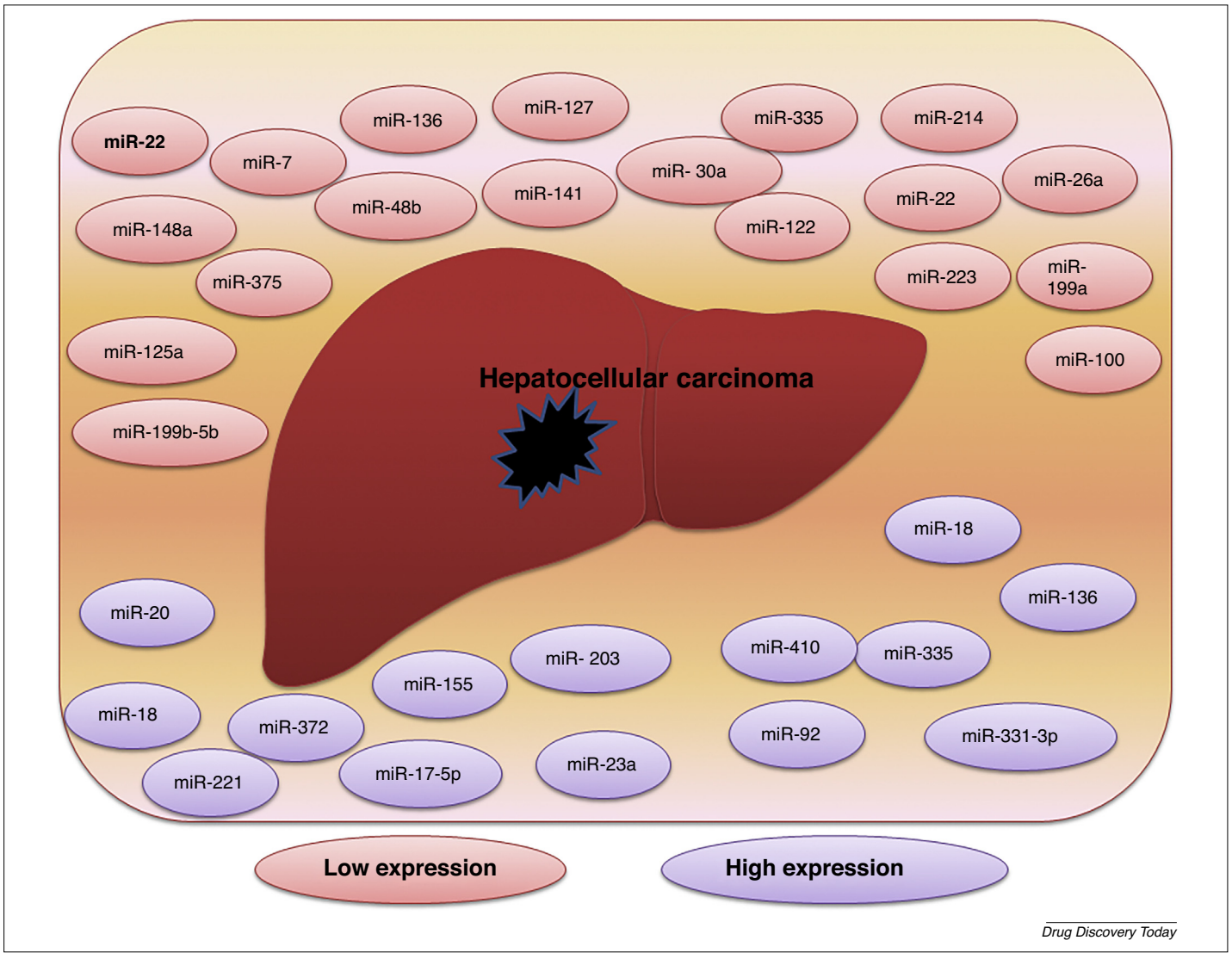

FIGURE 1

Profiling microRNA expression in hepatocellular carcinoma in human liver cancer patients.

HCC cells by blocking EMT, migratory capacity and cancer stem cell properties by overexpression of miR-148a. The results confirmed that Wnt1 is a target gene of miR-148a in HCC cells.

miR-199a-3p, miR-199b-5b, miR-125a, miR-122, miR-26a and let-7 members are consistently downregulated in the majority of HCC patients, and thus probably support tumor suppression. The clinical use of miRNA-based therapeutics could be developed for patients with liver cancer. miR-125a, miR-92, miR-20, miR-18, miR-372, miR-221, miR-17-5p, miR-155, miR-203 and miR-18 are probably involved in tumor initiation and are highly upregulated in patients with HCC. These upregulated miRNAs are targeted for inhibition to block cancer initiation, progression and migration. In 2005, Krutzfeldt and colleagues [15] silenced miR-122 in mouse liver using intravenous administration of specific antagomirs. In 2008, Elmen *et al.* [16] inhibited miR-122 expression by administration of anti-miRNA oligonucleotides in the liver of adult non-human primates without any evidence of toxicity. Park *et al.* [17] conducted a preclinical investigation in an orthotropic mouse model of HCC for therapeutic efficacy of anti-miR-221 oligonucleotides against the oncogenic microRNA miR-221. They found that a cholesterol-modified isoform of anti-miR-221 (chol-anti-miR-221) inhibited miR-221 following reduced proliferation of tumor cells, increased cell-cycle arrest and increased mouse survival. Callegari *et al.* [18] investigated the role of *in vivo* delivery of anti-miR-221 oligonucleotides to downregulate miR-221 levels. A significant reduction of the number and size of tumor nodules was observed. Kota *et al.* [19] selected miR-26a to evaluate its antitumorigenic properties for liver cancer *in vivo* using a mouse model of HCC. After systematic administration of miR-26a, inhibition of cancer cell proliferation and increased tumor-specific apoptosis without toxicity was seen. The potential therapeutic utility of cholesterol-conjugated 2′-*O*-methyl-modified miR-375 mimics (chol-miR-375) was evaluated in a mouse model and observed to significantly reduce of the growth of hepatoma xenografts in nude mice [20]. Xiong *et al.* [21] purchased RNA oligoribonucleotides and designed miRNA duplexes corresponding to mature miR-29 as described by Lim *et al.* [22]. They evaluated the effect of enhanced miR-29 expression on the ability of HCC cells to

form tumors *in vivo*. They identified two direct targets (Bcl-2 and Mcl-1) and the mitochondrial pathway of miR-29 for increased apoptosis of HCC cells. The replication of hepatitis C virus (HCV) relies on a liver-specific miRNA: miR-122. Landford *et al.* [23] investigated the feasibility and safety of prolonged administration of a locked nucleic acid (LNA) oligonucleotide drug for silencing of miR-122 in chronically infected chimpanzees. Further studies demonstrating the therapeutic utility of miRNA have been reported using *in vitro* preparations. For example, restoration of miR-31 reduced the formation and colonization efficiency of liver metastases [24].

Therefore, miR-331-3p could be a sensitive biomarker and a valuable therapeutic target because miR-331-3p is overexpressed in HCC [25]. The authors used a clinically tested polyethylenimine (PEI) -derived *in vivo* jetPEI® transfection reagent (Polyplus-Transfection®, Illkirch, France) for delivering anti-miR-331-3p in a HCC xenograft mouse model to evaluate the therapeutic potential of anti-miR-331-3p. PEI-based delivery of miR-331-3p through systemic administration could have important therapeutic potential for HCC treatment. Identification of tumor-specific miRNAs during colorectal cancer (CRC) progression and metastasis is an important step for designing effective therapeutic targets. Chen *et al.* [26] identified that miR-214 is an important regulator of CRC liver metastasis and showed the downregulation of miR-214 which is associated with CRC proliferation and metastasis. Overexpression of fibroblast growth factor receptor 1 (FGFR1) is observed in CRC tissues compared with adjacent normal tissues as well as in liver metastases. The overexpression of FGFR1 is inversely proportional to the expression levels of miR-214 and FGFR1 in CRC patients. It showed that downregulation of miR-100 could lead to tumor progression in HCC [27]. Chen *et al.* [28] investigated the role of miR-129-2 in hepatitis B virus (HBV)-infection-related HCC and methylation-mediated repression of miR-129-2 which stimulates oncogenic SOX4 expression in HCC. Reduced expression of miR-335 was found in HCC by aberrant DNA methylation [29]. The authors identified miRNA genes that are silenced by DNA hypermethylation in HCC. The expression levels of miR-335 were significantly lower in primary HCC tumors in comparison with their non-tumor tissue [29]. Downregulation of miR-223 was observed in HCC [30]. It was demonstrated that miR-223 effectively inhibited the HCC metastasis in an orthotopically implanted model of metastasis. Sulfatide epigenetically regulates miR-223 for migration of human HCC cells [30]. Targeting the miRNA biogenesis pathway is a new strategy and offers new targets for cancer treatments. miR-26a promotes miRNA biogenesis for suppression of tumor growth and metastasis [31]. The authors discovered that miR-26a directly targets Zcchc11 and Lin28B, and enhances miRNA biogenesis, and therefore inhibits tumorigenesis and metastasis of liver cancer as well as other types of cancer [31]. Systemic delivery of miR-124 could be a clinically viable anticancer therapeutic approach for treatment of liver cancer. miRNA delivery is more efficient in liver in comparison with other tissues. The authors evaluated the therapeutic and preventive effects of miR-124 for tumor-suppressive effects in human liver cancers. Systemic administration of miR-124 suppresses hepatocellular carcinogenesis [32]. Reduced expression of oncogenic miRNAs in HCC cell lines was seen by treatment with the pan-deacetylase inhibitor panobinostat [33]. Deacetylase inhibitors (DACi) are a new class of epigenetic drugs for cancer treatment. Panobinostat, a novel potent DACi, demonstrates its anticancer effect by suppressing these miRNAs [33]. Circulating miRNAs are the most sensitive biomarkers in cancer patients. The authors established global circulating miRNA profiles – miR-1228 could be a favorable stable endogenous control for monitoring the circulating miRNA in cancer patients. Steady expression of miR-1228 was observed in the blood of cancer patients [34], hence miR-1228 is a stable and sensitive marker for cancer detection. Systematic identification of long noncoding RNA (lncRNA) is a new concept for designing therapeutics for HCC. Hence, lncRNAs have important roles in carcinogenesis because they influence HCC initiation, progression and treatment [35]. A number of artificial miRNAs (amiRNA) have been used for HCC gene therapy for significant inhibition of invasion and induction of apoptosis of HCC cells. Generation of HCC-targeting amiRNA is also possible using natural miRNA precursors [36]. amiRNA could be a promising alternative to current therapeutics. The link of inflammation to cancer is a complex network rather than a simple linear pathway. miR-21 and miR-181b-1 epigenetically switch from inflammation to cancer [37]. c-Met receptor tyrosine kinase is a promising therapeutic target for HCC. Controlling the c-Met expression in cancer by miRNA was reported in HCC [38]. The authors investigated the potential contribution of miR-181a-5p to control c-Met overexpression in HCC. Downregulation of miR-181a-5p was shown in HCC [38]. Epigenetic alterations of miR-22 and miR-29b were observed in early preneoplastic livers in a rat model [39]. This study suggested the downregulation of the Mat1a and Mthfr genes which could be the main drivers of events that promote liver carcinogenesis followed by epigenetic abnormalities. Liver-tumor-initiating cells (T-ICs) are precarious factors for hepatocarcinogenesis. Epigenetic modification of miR-429 has been shown to boost T-ICs [40]. HCC involves genetic and epigenetic changes. An increasing amount of RNA is associated with A-to-I modifications, and RNA editing could be a causal basis of various cancers including HCC. Transcriptome diversity regulation by RNA editing in HCC was reviewed elsewhere [41]. miR-100 has a significant role in pancreatic cancer development [42]. Higher expression of histone demethylase retinoblastoma binding protein 2 is seen in HCC which is negatively regulated by hsa-miR-212 [43]. miR-29b was found to inhibit metastasis in hepatocellular carcinogenesis [44]. Downregulation of miR-30a is directly related with tumor cell migration and invasion [45]. The authors also identified a SNAI1 transcription factor that is a direct target of miR-30a. miR-224 enhances the proliferation and metastasis of HCC cells [46]. Inhibition of HCC cell proliferation is possible by miR-520c-3p [47]. miR-122 is considered an antitumor agent against HCC [48]. Restoration of miR-122 completely inhibits HCC tumors in an in vivo mouse model [49]. Modulation of miR-29 expression is associated with the HCC epigenome [50]. Upregulation of miR-21 represses HCC [51] and miR-125b reduced the tumorigenic potential of HCC cells [52]. Ha-ras and β-catenin oncoproteins have major roles in mouse liver tumors [53]. miRNA can control hepatocarcinogenesis by regulating hepatocyte nuclear factor 4α-inflammatory signals [54]. The miR-23a is involved in regulation of the anti-HCC [55]. High expression of miR-410 is seen in liver and colorectal tumors that enhance tumor cell growth [56]. Sorafenib, the tyrosine protein kinase inhibitor, has been approved by the

FDA for the treatment of advanced HCC [57]. In addition, various miRNA isoforms have been investigated in cirrhotic liver and hepatocellular carcinoma [58]. miR-141 has the potential to suppress HCC progression [59]. Xiao *et al.* [60] investigated the anticancer activity of miR-34a modulator in cell culture and animal models and finally found a compound named Rubone for activation of miR-34a expression in HCC cells. The miR-148a-mediated inhibition of metastasis of HCC is observed [61]. Yan *et al.* [62] presented the tumor-suppressive role of miR-375 in cancer progression. miR-26a suppresses tumor growth and metastasis of HCC [63] and miR-433 inhibits liver cancer cell migration [64]. miR-122 is considered as a tumor suppressor in hepatocarcinogenesis [65]. Zha *et al.* [66] showed that miR-134 significantly inhibits invasion of HCC cells and metastasis *in vitro* and *in vivo*. Propofol reduces the invasiveness in HCC cells by downregulation of matrix metalloproteinase (MMP)-9 expression by miR-199a [67]. miR-7 arrests the cell cycle in G1 phase of HCC cells, a possible target for liver cancer [68]. miR-148b expression was reduced in HCC which is directly linked to tumor invasion and progression [69]. Demonstration of miR-375 and miR-136 promotes cell migration in HBV-associated HCC [70]. Downregulation of miR-127 was observed in 69.7% of HCC tissues in comparison with adjacent normal tissues [71]. Zhou *et al.* [72] demonstrated the downregulation of miR-22 in HCC [72]. Taken altogether, miRNA have novel roles from diagnostics to therapeutics in liver disease [73]. Hence, miRNA exhibit dual roles acting as therapeutic targets as well as therapeutic agents [74].

Silencing of the tumor suppressor gene retinoblastoma protein (RB1) in different types of human cancer, including HCC, is common. However, mutations of the RB1 gene in HCC were reported in human HCC [75]. The authors reported a systematic screen for the identification of imprinted genes deregulated and revealed that RB1 shows abnormalities and a high proportion [75] (40%) of the HCC specimens (16/40) showed hyper- or hypomethylation of the RB1 gene [75]. Dermatopontin (DPT) is generally found in several human cancers including HCC. The authors examined DPT expression in 202 HCC samples by immunohistochemical staining and found that DPT expression was significantly downregulated and therefore is a potential biomarker of tumor metastasis. They demonstrated DPT-suppressed HCC cell proliferation and growth and metastasis *in vivo* [76]. Furthermore, they proved the inhibitory effects of DPT on HCC motility. Silencing of key genes by DNA hypermethylation is an important part of carcinogenesis. The authors identified hypermethylated genes in HCC using 45 pairs of HCC and adjacent nontumorous tissues and six normal liver tissues and found EYA4 functions as a prognostic molecular marker in HCC [77]. Secreted Frizzled-related proteins (SFRPs) are antagonists of the Wnt signaling pathway epigenetically downregulated in hepatocarcinogenesis. However, dysregulation of SFRPs induced by HBV X protein (HBx) was studied in HBV-HCC [78]. They showed that SFRP1 and SFRP5 expression were intensely decreased by HBx in hepatoma cells and identified that SFRP1 and SFRP5 promoters were hypermethylated in HBx-expressing hepatoma cells and HBV-HCC tissues [78]. Transcriptional intermediary factor 1 gamma (TIF1γ) has a dual role as either a potential tumor suppressor or tumor promoter in cancer. The crucial role of TIF1γ in the progression of HCC was reported in advanced HCC tissues, compared with adjacent noncancerous tissues [79]. The expression of TIF1γ is low in HCC in advanced HCC tissues as opposed to adjacent noncancerous tissues [78]. The shorter overall survival times were observed in HCC patients with low TIF1γ expression. They showed that the downregulation of TIF1γ in HCC was caused by hypermethylation of CpG islands in the TIF1γ promoter [79].

DNA- and histone-modifications

DNA methylation is a crucial epigenetic modification that is often altered in cancer. Liu *et al.* [80] analyzed the conversion of 5-methylcytosine (5 mC) to 5-hydroxymethylcytosine (5 hmC) in HCC tissues and non-tumor tissues. They reported that the level of 5 hmC was decreased in HCC tissues relative to non-tumor tissues and that the 5 hmC level is associated with tumor size, Alpha-fetoprotein (AFP) level and poor overall survival. A decreased 5 hmC level during cancer development was determined in contrast to the 5 mC level in a rat model of diethylnitrosamine (DEN)-induced liver cancer. Furthermore, they showed that only TET1 expression is upregulated in HCC. This indicates that 5 hmC can be a prognostic marker for HCC and that decreased expression of TET1 might be a mechanism underlying 5 hmC loss in HCC.

Sun *et al.* [81] investigated the influence of DNA methylation and histone acetylation on the gene expression and signaling pathways in HepG2 cells. The main goal of their study was to identify the potential role of epigenetic modification in the development of HCC and its treatment. They identified different types of expressed genes associated with DNA methylation and histone deacetylation blockage. They reported that inhibition of DNA methylation and histone deacetylation could be an effective treatment for hepatic cancer.

Kondo *et al.* [82] examined the epigenetic alterations during hepatocarcinogenesis in cancerous tissues and in corresponding noncancerous liver tissues from HCC patients and found high expression of G9a and EZH2 in cancerous tissues. They studied the DNA methylation levels in the promotors of P16, RASSF1a, progesterone receptor (PGR) and estrogen receptor α (ERα). All genes showed aberrant methylation profiles, and the patients had substantially higher methylation levels overall in liver tissues. Therefore, the methylation of P16 was cancer-specific. Furthermore, they treated HepG2, Huh7 and Hep3B cells with the DNMT inhibitor DAC and the HDAC inhibitor TSA to investigate silencing by either histone methylation or DNA acetylation. DAC efficiently reactivated P16 and RASSF1, which are the typical targets of DNA methylation and H3-K9 diMe, whereas TSA effectively increased PGR and ERα gene expression as targets of H3-K27 triMe. They demonstrated that promotor silencing of the tumor-suppressing genes P16 and RASSF1a depended on DNA methylation and histone H3-K9 methylation. The silencing of the PGR and ERα genes was more closely related to H3-K27 methylation.

Side population (SP) cells are also a special subpopulation of HCC cells with high numbers of cancer stem cells. Zhai *et al.* [83] examined the genome-wide DNA methylation profile of SP cells to determine the role of epigenetic regulation in sustaining HCC SP cells toward tumorigenesis. They isolated SP cells from Huh7 and PLC/PRF/5 cell lines, assessed the tumorigenicity in NOD/SCID mice and analyzed the genome methylation status by DNA methylation microarray analysis. Subcutaneous inoculation of SP cells yielded tumors in 60% of the NOD/SCID mice, whereas no tumor

developed following inoculation of a 1000-fold higher amount of non-SP (NSP) cells. Genome-wide DNA methylation microarray analysis showed that 72 and 181 genes were hypermethylated and hypomethylated, respectively, in Huh7 and PLC/PRF/5 SP cells compared to NSP cells. Different methylation levels for a wide range of gene promotors in SP and NSP cells were demonstrated. Furthermore, the authors investigated the genes B-cell-translocation gene 2 (BTG2), four-and-a-half LIM domains 1 (FHL1) and growth arrest and DNA-damage-inducible gamma (GADD45G) with differential methylation between SP and NSP cells and analyzed their function in signaling pathways. They confirmed a differential DNA methylation status of SP cells compared with NSP cells. The differentially methylated genes in SP cells were involved in 12 signaling pathways.

Maemura *et al.* [84] demonstrated an important function of delta-like 3 (DLL3) in hepatocarcinogenesis by examining the silencing of DLL3 by methylation and investigating its roles in HCC; they found that DLL3 expression is associated with cell growth suppression in HCC. The authors also investigated the methylation status of the apoptosis-inducing gene DLL3 in HCC cell lines. The mRNA expression of DLL3 of ten HCC cell lines was determined by PCR in two cell lines (Huh1 and HuH2) without DLL3 mRNA expression. The methylation status of the DLL3 CpG islands was analyzed by methylation-specific PCR detecting apparent methylation in four cell lines (HuH2, Hep3B, Kim1 and FLC4). Treatment with DAC reactivated the expression in five cell lines (HuH1, HuH2, HuH4, Alex and Kim1), and addition of TSA showed an increased effect for some cell lines. Colony formation and TUNEL-tests demonstrated suppressed cell growth by induction of apoptosis. The restored expression of DLL3 by demethylation led to apoptosis in HuH2 cells via a Notch1-independent pathway.

Xiao *et al.* [85] attempted to show the effects of MS-275 on the release and function of exosome-related immune molecules in HepG2 cells. The authors concluded that enhancement of the nonspecific immune response of exosomes derived from HepG2 cells by the histone deacetylase inhibitor (HDACi) drug MS-275 is a novel tumor vaccine approach against liver cancer. They investigated the influence of MS-275 on the release of exosome-related immune molecules for tumor-specific antigen chaperones.

Zhang *et al.* [86] examined the expression of the retinoblastoma-interacting zinc finger gene (RIZ1) which is inactivated in many cancers in 48 HCC tissues, corresponding noncancerous tissues and six HCC cell lines (HepG2, Hep3B, Huh7, SK-HEP-1, SNU182 and SNU449). Their results suggested that promoter methylation and H3K9 modifications contribute to silencing of the RIZ1 gene in HCC. They also showed the restoration of RIZ1 by 5-Aza-dC. Methylation-specific PCR revealed RIZ1 promotor methylation in 32 HCC tissues with complete loss of RIZ1 immunoreactivity compared with three noncancerous tissues and four HCC cell lines: HepG2, Huh7, SNU182 and SNU449. Treatment of HepG2 cells with TSA or DAC showed no demethylating effects to the RIZ1 promotor but restored the RIZ1 mRNA by HDAC1 downregulation; the combination of both showed a partial reversal of promotor methylation. Furthermore, a ChIP assay revealed an increase in H3K9 acetylation owing to a decrease in H3K9 trimethylation.

Chapell *et al.* [87] explained the importance of epigenetic events, rather than mutations in cancer-related genes, in contributing to the high incidence of liver tumors in a mouse model of fibrosis-associated liver cancer. They analyzed the methylation levels of the five tumor suppressor genes: cyclin-dependent kinase inhibitor 2A (Cdkn2a), *O*6-methylguanine-DNA methyltransferase (Mgmt), suppressor of cytokine signaling 1 (Socs1), cadherin 1 (Cdh1) and PR domain containing 2 with ZNF domain (RIZ1), in a mouse model. They treated mice with *N*-nitrosodiethylamine (DEN), CCl4 or both to induce hepatic cancer. Hypermethylation analysis by methylation-specific PCR revealed all genes were heavily methylated in liver tumors in $DEN^{+}CCl4$-treated mice, but only expression of RIZ1 and Mgmt was decreased by promotor hypermethylation. Additionally, they investigated the activity of histone-methylating enzymes, identifying a decrease in H3K9 trimethylation after treatment with $DEN^{+}CCl4$, whereas H3K27 and H4K20 were only slightly affected. They showed DNMT1 and DNMT3a to be upregulated after treatment with $DEN^{+}CCl4$, whereas histone lysine (K)-specific demethylase (Kdm4a and Kdm4b) genes were downregulated. Their results demonstrate that epigenetic changes play an important part in HCC development.

Stem-cell-like transcriptional gene networks are associated with cancer development. Wang *et al.* [88] believed that reactivation of pluripotency circuits (particularly NANOG) leads to cancer progression followed by abnormal epigenetic alterations. They examined 15 HCC samples and several cancer cell lines to investigate the reactivation processes of pluripotency regulatory circuits during cancer progression. The pluripotency-associated genes NANOG, OCT4, c-MYC, KLF4 and SOX2 were analyzed for CpG methylation by bisulfite sequencing analysis, which revealed NANOG hypomethylation and gene upregulation in HCC. *In vitro* tests using a NANOG-overexpressing orthotropic tumor mouse model confirmed this pro-metastatic role. Demethylation of NANOG promotor was observed in $CD133^{+}$high cells. Additionally, cross-regulation via reprogramming of promoter methylation between OCT4 and NANOG was demonstrated and revealed the pluripotency circuits in cancer cells as a regulatory mechanism for cancer progression.

Downregulation of Gls2 in human liver and colon cancer cells is observed in primary HCC tissues [89] and is correlated to its promoter hypermethylation. The authors proved that ectopic expression of Gls2 reduced cancer cell growth via cell cycle arrest. They analyzed 20 HCC and five CRC tissues for mRNA expression of glutaminase 2 (Gls2). The Gls2 promotor methylation was analyzed via methylation-specific PCR and bisulfite genome sequencing which showed low expression of Gls2 as a result of hypermethylation. By treating several HCC and CRC cell lines with DAC for demethylation of the Gls2 promotor, mRNA expression was dramatically restored. After transfection of SMMC-7721 and HCT116 with Gls2-expressing vectors, a cell growth assay by colony formation test was performed to identify the biological function of upregulated Gls2. It was demonstrated that an upregulation of Gls2 significantly reduced the number of cell colonies and decreased the cell growth rate as a result of G2/M arrest.

Ye *et al.* [90] examined the regulation of BCL2-antagonist/killer 1 (Bak) through Zinc-binding protein-89 (ZBP-89) via epigenetic regulation mechanisms. A western blot expression analysis was performed on 103 liver cancer tissues and revealed high expression

levels of DNMT1 and HDAC3, whereas Bak expression was reduced and lower levels of ZBP-89 were detected via immunohistochemical staining in poorly differentiated cancer. Treating HepG2 cells with VPA and Zebu led to increased Bak expression via poly(ADP-ribose) polymerase (PARP) cleavage-mediated apoptosis. Furthermore, ZBP-89 overexpression clearly induced expression of Bak and inhibited HDAC and DNMT activity significantly. The influence of HDAC and DNMT on Bak was shown using an siRNA knockdown. Enhanced Bak expression by downregulation of HDAC3 and DNMT1 was demonstrated. The expression of Bak was enhanced by ZBP-89, VPA and Zebu and tumor growth was inhibited in a xenograft mouse model. The authors demonstrated that ZBP-89 stimulated Bak expression through an epigenetic mechanism in HCC.

Magerl *et al.* [91] investigated the methylation of histones in different cancer types as prognostic values. They analyzed carcinomas of the hepatic and gastrointestinal tract using immunohistochemical staining for the dimethylation of histone H3 at lysine 4 (H3K4diMe), H3K4 methylating (Ash2 complex) and demethylating (LSD1) enzymes. They showed that HCC underlies completely different active enzyme complexes compared with gastrointestinal cancers. They observed high levels of H3K4diMe in most cancers except HCC. Comparing H3K4diMe modification and LSD1 expression, Ash2 complex was highly expressed in most HCCs. This showed that there is a complex epigenetic regulation system between H3K4diMe, Ash2 and LSD1.

He *et al.* [92] investigated the coherence between expression levels of H3K4me3 in HCC and the clinicopathologic variables and outcome. HCC samples from 168 patients were analyzed for expression of H3K4me3, HMT and MYND domain-containing protein 3 (SMYD3) by western blot and immunohistochemical staining. The authors showed high expression levels of H3K4me3 and SMYD3 in HCC cell lines. This was correlated with poor patient survival, particularly in early stages of HCC. Furthermore, the authors showed coherence between SMYD3 expression and H3K4me3 upregulation. By validating these findings through an independent group of 147 HCC samples, they demonstrated H3K4me3 upregulation as a reliable marker for prognosis of patient survival.

Zopf *et al.* [93] investigated the influence of the histone deacetylase inhibitor panobinostat on the expression of DNMT1, DNMT3a and DNMT3b in HepG2 and Hep3B cell lines. After treatment of the cell lines with 0.1 μM panobinostat, they found a significant downregulation of DNMT1 and DNMT3a in both cell lines. They confirmed a low methylation status of the RASSF1A and APC genes, which are generally highly methylated owing to DNMT activity, as well as a xenograft mouse model confirming a lowered DNMT1 and DNMT3a activity. These findings demonstrate the effect of DNMT genes on transcriptional control by HDAC-dependent mechanisms.

Anwar *et al.* [94] identified the DLK1-MEG3 locus frequently deregulated in HCC. They analyzed the expression of MEG3 and DLK1 in 40 HCC samples and observed coherence between increased DNA methylation and reduced MEG3 expression as well as a decrease in DNA methylation and DLK1 expression. A siRNA-mediated knockdown of DNMT1 showed lowered methylation of MEG3-DMR and an increase in MEG3 RNA expression. By allelic-specific expression analysis, allelic switching was accompanied by gain or loss of DNA methylation primarily at IG-DMR1. This revealed the DLK1-MEG3 locus for monitoring the response to epigenetic therapy.

Hsu *et al.* [95] analyzed the promotor methylation and expression of PTPRO and its function in human HCC. Using MassARRAY® analysis of 24 HCC samples and bisulfite sequencing of 17 HCCs, they examined the PTPRO promotor and found significantly higher methylation which resulted in a lower expression of PTPRO. They identified the valosin-containing protein (VCP) as a main substrate for PTPRO using the MS-coupled *in vitro* substrate-trapping assay. This work clarified that the tumor suppressor function of protein tyrosine phosphatase receptor type-O (PTPRO) in HCC is mediated by reduced phosphorylation of VCP.

Acun *et al.* [96] analyzed the expression of the Smad-interacting protein-1 (SIP1), a transcription factor that is involved in transforming growth factor β/bone morphogenetic protein signaling. Downregulation of SIP1 was demonstrated in five of 14 HCC cell lines, Hep3B and HepG2 cells and 17 of 23 HCC tumors [96]. For exclusion of mutations in the HCC cell lines, mutation screening was performed showing no allelic deletions or somatic mutations for SIP1, which confirmed that epigenetic changes are responsible for downregulated SIP1 expression. HepG2, Hep3B and PLC cells were treated with DAC and TSA to evaluate promotor methylation; this treatment restored the SIP1 expression in these cell lines. Additionally, an *in silico* analysis provided three possible SIP1 promotor regions. Bisulfite restriction analysis was conducted in 39 HCC samples to assess methylation levels and tumor-specific hypermethylation of the SIP1 promotor region was found. The authors showed SIP1 to be epigenetically silenced in HCC and demonstrated SIP1 to be a potential suppressor of HCC. Epigenetic targets for liver disease are outlined in Table 1a (DNMT1), Table 1b (HDAC), Table 1c (HDAC DNMT) and Table 1d (others).

Epigenetic profiles in hepatocarcinoma

The relationship between DNA methylation and histone modification has been described in several physiological systems and disease states. Recent investigations have also included miRNA as new players adding to another layer of complexity [97], thereby reinforcing the effects of epigenetic changes. It is well understood that miRNAs play a crucial part in various diseases such as infection and cancer. There have been studies reported where differential expression of miRNA has been shown toward tissue-specific disease outcomes. The majority of studies focus on high-throughput analysis of miRNA expression levels directly from diseased models that lack their correlation with specific cellular processes. Liver cancer or HCC is a complex disorder with multiple underlying pathogenic mechanisms caused by an array of risk factors. The lack of robust molecular markers for HCC diagnosis and treatment assessment has posed a major challenge. As discussed, the expression of a large number of genes, proteins and other molecules attributing to diverse cellular processes and pathways are disturbed in HCC. Such conditions pose a challenge for healthcare personnel to establish a set of tests or a method to provide accurate assessment under clinical settings.

One of the main objectives of array-based studies for liver cancer is to determine the factors that contribute toward progression of cancer from normal tissue to metastasis. This would require a thorough understanding of the genome–phenome relationships based on multiple factors such as environment, host genetic

TABLE 1

Epigentic targets for liver diseases: (a) DNMT1, (b) HDAC, (c) HDAC DNMT and (d) others

Type of liver disease	Molecular target of disease/ defect gene	Epigenetic targets/ epigenetic modulations	*In vitro/in vivo* model	Epigenetic agents	Methods	Major preclinical outcome (advantages)	Refs
(a) DNMT1							
HCC, HCA, FNH	RB1	DNMT1	*In vivo*	siRNA, DAC	Methylation analysis of 40 HCCs, 10 HCAs and 5 FNHs, and the adjacent liver tissues from 34 HCCs, 8 HCAs, 2 FNHs and 5 healthy samples	40% of the HCC specimens (16/40) showed hyper- or hypo-methylation at the CpG island in intron 2 of the RB1 gene	[75]
HCC	ERG, HOXA11, EYA4	DNMT	*In vivo*		145 HCC compared to 6 healthy liver tissues analyzed for hypermethylation of various genes by immunohistochemical staining and bisulfite sequencing	EYA4 gene highly methylated in HCC; methylation level corresponded to tumor size and overall survival; might prove to be a good diagnostic/prognostic marker. HOXA11 gene expressions correlated to a short disease-free time whereas ERG showed no correlation	[77]
HCC	TIF1γ	DNMT	*In vitro* and *in vivo*	–	Expression analysis of TIF1γ in HCC samples compared to noncancerous tissues; methylation analysis of CpG islands in TIF1γ promotor region	Downregulation of TIF1γ in HCC by hypermethylation of CpG islands in the TIF1γ promoter leading to shorter overall survival times and higher recurrence. TIF1γ could be a powerful prognostic biomarker in HCC	[79]
HCC	Cdkn2a (cyclin-dependent kinase inhibitor 2A), Mgmt (*O*6-methylguanine-DNA methyltransferase), Socs1 (suppressor of cytokine signaling 1), Cdh1 (cadherin1), RIZ1 (PR domain containing 2, with ZNF domain)	H3K9me3 H3K29 H4K20 DNMT1 DNMT3a	*In vivo*	–	Mice treated with *N*-nitrosodiethylamine (DEN), CCl_4 or both to induce hepatic cancer. The methylation status of CpG islands of Cdkn2a, Mgmt, Socs1, Cdh1 and RIZ1 determined by methylation-specific PCR; histone modification analysis for H3K9, H3K29 and H4K20 trimethylation; qRT-PCR to identify the active genes for DNA and histone methylation	All genes heavily methylated in liver tumors in DEN^+CCl4-treated mice, but only expression of RIZ1 and Mgmt was decreased by promotor hypermethylation; decrease in H3K9 trimethylation after treatment with DEN^+CCl4, whereas H3K27 and H4K20 were only slightly affected; DNMT1 and DNMT3a upregulated after treatment with DEN^+CCl4, whereas histone lysine (K)-specific demethylases (Kdm4a and Kdm4b) genes were downregulated	[87]
HCC	Pluripotency-associated genes NANOG (Nanog homeobox), OCT4 (octamer-binding transcription factor 4), c-MYC (myelocytomatosis oncogene), KLF4 (Kruppel-like factor 4) and SOX2 [sex-determining region Y (SRY)-box 2]	DNMT	*In vitro* and *in vivo*	Cross-regulation by interaction analysis between OCT4 and NANOG	15 HCC samples and several cancer cell lines analyzed for CpG methylation by bisulfite sequencing analysis; NANOG-overexpressing orthotropic tumor mouse model; methylation analysis of NANOG promotor in $CD133^+$ high cells; cross-regulation analysis between OCT4 and NANOG	NANOG hypomethylated in HCC resulting in gene upregulation; orthotropic tumor mouse model confirmed its pro-metastatic role; NANOG overexpressed in $CD113^+$ high cells; OCT4 and NANOG are cross-regulated which revealed the pluripotency circuits in cancer cells as one possible cancer stem cell development process	[88]

TABLE 1 (*Continued*)

Type of liver disease	Molecular target of disease/ defect gene	Epigenetic targets/ epigenetic modulations	*In vitro/in vivo* model	Epigenetic agents	Methods	Major preclinical outcome (advantages)	Refs
HCC, CRC (colorectal cancer)	Gls2 (Glutaminase 2)	DNMT	*In vitro*	DAC	20 HCC and 5 CRC tissues analyzed for mRNA expression of Gls2; Gls2 promotor methylation via methylation specific PCR and bisulfite genome sequencing; DAC treatment of HCC and CRC cell lines for demethylation of Gls2 promotor; cell growth assay by colony formation test after Gls2 expressing vector transfection	Low expression of Gls2 observed as result of hypermethylated promotors; DAC treatment restored the mRNA expression of Gls2 in HCC and CRC cell lines; transfection of HCC (SMMC-7721) and CRC cells lines (HCT116) with Gls2 expressing vector transfection reduced the number of cell colonies and significantly decreased cell growth rate resulted by G2/M arrest	[89]
HCC	DLK1-MEG3 locus	DNMT1	*In vitro and in vivo*	siRNA	Expression analysis of MEG3 and DLK1; siRNA-mediated knockdown of DNMT1; allelic switching analysis for IG-DMR1	Increased DNA methylation reduced MEG3 expression, decreased methylation decreased DLK1 expression; siRNA knockdown of DNMT1 lowered MEG3-DMR methylation and increased MEG3 RNA expression	[94]
(b) HDAC							
HCC	Release of exosome-related immune molecules	HDAC	*In vitro*	Histone deacetylase inhibitor MS-275	HepG2 cells treated with MS-275, mRNA analysis by PCR and western blot for HSP70, MICA, GAPDH and MICB; cytotoxicity assay against natural killer cells	MS-275 treated HepG2 cells showed upregulated mRNA expression of HSP70 and MICB	[85]
HCC	RIZ1 (retinoblastoma-interacting zinc finger gene)	H3K9 (histone H3 lysine 9), HDAC1, HDAC3	*In vitro* and *in vivo*	DAC, TSA	48 HCC samples and six cell lines (HepG2, Hep3B, Huh7, SK-HEP-1, SNU182 and SNU449) analyzed for RIZ1 promotor methylation level by methylation-specific PCR; HepG2 cells treated with DAC and TSA to possibly restore RIZ1 expression	32 HCC tissues showed a complete loss of RIZ1 immunoreactivity TSA/DAC treatment showed no demethylating effects on RIZ1 promotor but mRNA restoration; both showed a partial reversal of promotor methylation	[86]
HCC	DNMT1, DNMT3a, DNMT3b, RASSF1A,APC	HDAC	*in vitro* and *in vivo*	Pabinostat (DAC inhibitor)	Treatment of HepG2 and HepB3 cells with panobinostat; expression analysis of DNMT1, DNMT3a, DNMT3b; methylation-specific PCR for RASSF1A and APC; xenograft mouse model with HepG2 cells for DNMT activity	Panobinostat resulted in fast downregulation of DNMT1 and DNMT3a in both cell lines; low expression levels of DNMT1, DNMT3a (mRNA and protein) in xenograft mouse model; decreased methylation levels of RASSF1A and APC	[93]
(c) HDAC DNMT							
HCC	DPT	DNMT, HDAC	*In vitro* and *in vivo*	DAC, TSA	Expression analysis of demethylated HCC cell lines SMMC-7721, Huh7, MHCC-97H and THLE-2; proliferation analysis (*in vitro*) and metastasis analysis (*in vivo*) during DPT overexpression	DPT silenced by promotor methylation in CpG-island; suppressed HCC cell proliferation (*in vitro*) and tumor growth (*in vivo*); reduced cell migration, invasion and metastasis by $\alpha 3\beta 1$ integrin-Rho GTPase signaling	[76]

HBV-related HCC	SFRP1, SFRP5 (secreted Frizzled-related proteins, SFRP)	DNMT, HDAC	*In vitro* and *in vivo*	DAC, TSA	SFRP-expression analysis of HBV-HCC tissues (and paired adjacent non-tumorous tissues) and of HBx-expressing HCC cells upon exposure to DAC and TSA; methylation-specific PCR (MSP) analysis of CpG islands in SFRP promotor regions of HBV-HCC cells	SFRP1 and SFRP5 were silenced by HBx-related upregulation of DNMT1, DNMT3a and HDAC, whereas other SFRPs were not affected. Downregulation of DMNTs partially restored SFRP	[78]
HCC	Gene expression profile of HepG2 cells	DNMTHDAC	*In vitro*	DAC, TSA	HepG2 cells treated with DAC, TSA or of both followed by an expression analysis to identify differentially expressed genes, changes in signal pathways and potential target sites for regulatory transcription factors	Inhibition of DNA methylation has a more significant contribution than histone acetylation to gene expression. TSA treatment showed changes in TGF-β signaling pathway; AZA treatment affected. Transduction-related integrin-mediated cell adhesion, the AMPK signaling pathway and the $\alpha 6\beta 4$ signaling pathway	[81]
HCC	P16, RASSF1a, PGR (progesterone receptor) and ERα (estrogen receptor α)	DNMTHDAC	*In vitro* and *in vivo*	DAC, TSA	Methylation analysis of P16, RASSF1a, PGR and ERα promotors in 23 paired HCC tissues and adjacent noncancerous liver tissue. HepG2, Huh7 and Hep3B cell lines were treated with the DAC and HDAC to investigate the silencing mechanism by either histone methylation or DNA acetylation	All genes showed aberrant methylation profiles, revealing methylation of P16 revealed as cancer-specific. DAC efficiently reactivated P16 and RASSF1, targets of DNA methylation and H3-K9 diMe, whereas TSA effectively increased PGR and ERα gene expression as targets of H3-K27 triMe	[82]
HCC	DLL3 (delta-like 3)	DNMT, HDAC	*In vitro*	DAC, TSA	Methylation status-analysis of apoptosis-inducing gene DLL3 in HCC cell lines (HuH1, HuH2, HuH4, HuH7, Li7, Hep3B, HT17, FLC4, Alex and Kim1) by methylation-specific PCR; restoration of DLL3 gene activity by DAC/TSA treatment (HuH2 and Kim1) and cell growth/apoptosis analysis for HuH2 cell lines by colony formation and TUNEL test	Apparent methylation of DLL3 in four cell lines (HuH2, Hep3B, Kim1 and FLC4); DAC/TSA treatment reactivated DLL3 expression in HuH1, HuH2, HuH4, Alex and Kim1; suppressed cell growth by induction of apoptosis through Noch1-independent pathway	[84]
HCC	Pluripotency-associated genes NANOG (Nanog homeobox), OCT4 (octamer-binding transcription factor 4), c-MYC (myelocytomatosis oncogene), KLF4 (Kruppel-like factor 4) and SOX2 [sex-determining region Y (SRY)-box 2]	DNMT	*In vitro* and *in vivo*	Cross-regulation by interaction analysis between OCT4 and NANOG	15 HCC samples and several cancer cell lines analyzed for CpG methylation by bisulfite sequencing analysis; NANOG-overexpressing orthotropic tumor mouse model; methylation analysis of NANOG promotor in $CD133^+$ high cells; cross-regulation analysis between OCT4 and NANOG	NANOG hypomethylated in HCC resulting in gene upregulation; orthotropic tumor mouse model confirmed its pro-metastatic role;NANOG overexpressed in $CD113^+$ high cells; OCT4 and NANOG are cross-regulated which revealed the pluripotency circuits in cancer cells as one possible cancer stem cell development process	[88]

TABLE 1 (*Continued*)

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HCC	ZBP-89 (zinc-binding protein-89)	HDAC3 and DNMT1	*In vitro* and *in vivo*	DAC, VPA (valpranoic acid), Zebu (zebularine)	Expression analysis of 103 HCC samples for Bak, ZBP-89, DNMT1 and HDAC3 protein levels; VPA and Zebu treatment of HepG2 cells for Bak function analysis; siRNA knockdown of DNMT1 and HDAC3 to analyze their effect on Bak expression; xenograft mouse model to analyze the Bak expression under influence of ZBP-89, VPA or Zebu	Bak expression reduced and a lower level of ZBP-89 detected in HCC tissues; increased Bak expression via PARP cleavage-mediated apoptosis observed after VPA and Zebu treatment of HepG2 cells; enhanced Bak expression by siRNA knockdown of HDAC3 and DNMT1; in xenograft mouse model, expression of Bak was enhanced by ZBP-89, VPA and Zebu	[90]
HCC	SIP1	DNMT,HDAC	*In vitro and in vivo*	DAC, TSA	Expression analysis by RT-PCR and immunohistochemistry; mutations analysis of SIP1 in 14 HCC cell lines and 23 HCC tumors; mutation analysis of SIP1 in 14 HCC cell lines; promotor methylation analysis in HepG2, Hep3 B and PLC cell lines and 39 HCCs	Downregulation of SIP1 in 5 HCC cell lines and 17 HCCs; no mutation of SIP1 in all HCC cell lines; high promotor methylation levels in the three cell lines and the HCC tumors	[96]
(d) Others							
HCC	TET1, TET2, TET3 (ten-eleven translocation enzymes)	Conversion of 5			methylcytosine (5 mC) to 5-hydroxymethylcytosine (5 hmC) leading to demethylation	*In vivo*	
Immunofluorescent staining for 5 hmC levels in 146 HCC samples and non-tumorous counterparts; 5 hmC analysis in a rat model for diethylnitrosamine (DEN)-induced liver cancer	Level of 5 hmC decreased in HCC tissues compared with non-tumor tissues; 5 hmC associated with tumor size, AFP level and poor survival. In diethylnitrosamine (DEN)-induced liver cancer, 5 hmC level was decreased during cancer development relative to 5 mC	[80]					

HCC	Genome-wide screening for methylation status; e.g., BTG2 (B-cell translocation gene2), FHL1 (four and a half LIM domains 1), GADD45G (growth arrest and DNA-damage-inducible gamma)	–	*In vitro* and *in vivo*	–	Genome-wide DNA methylation profile of side population (SP) cells of HCC by DNA methylation microarray analysis. Isolation of SP cells from Huh7 and PLC/PRF/5 cell lines, assessing tumorigenicity in NOD/SCID mice	Subcutaneous inoculation of SP cells yielded tumors in 60% of NOD/SCID mice, NSP cells did not lead to tumors. 72/181 genes hypermethylated/hypomethylated, respectively, in both cell lines as compared with their corresponding NSP cells. For example BTG2, FHL1 and GADD45G showed different methylation between SP and NSP cells; differentially methylated genes in SP cells were involved in 12 signaling pathways	[83]
HCC, CCC, PDAC, gastric carcinoma, neuroendocrine carcinoma	H3K4diMe, Ash2 (absent, small or homeotic discs2), LSD1 (lysine-specific histone demethylase 1)	Expression levels of H3K4diMe, Ash2 and LSD1 in different cancer tissues	*In vivo*		Analysis of carcinomas of the hepatic and gastrointestinal tract by immunohistochemical staining for H3K4diMe, Ash2 and LSD1	High levels of H3K4diMe were observed in most cancers except HCC; correlating to H3K4diMe modification and LSD1 expression the Ash2 complex was highly expressed most of all HCC	[91]
HCC	H3K4me3, SMYD3 (MYND-domain-containing protein 3)	Expression levels of H3K4diMe and SMYD3	*In vivo*	–	Expression analysis of H3K4me3 and SMYD3 by western blot and immunohistochemical staining in 168 HCC samples; correlation of expression to patient overall survival time; validation of the findings to another 147 HCC samples	High expression levels of H3K4me3 and SMYD3 in HCC cell lines correlated with poor patient survival, particularly in early-stage HCC coherence between SMYD3 expression and H3K4me3 upregulation	[92]
HCC	PTPRO (protein tyrosine phosphatase receptor type-O)	–	*In vitro and in vivo*	–	Expression analysis of PTPRO by MassARRAY® and bisulfite sequencing; MS-coupled *in vitro* substrate-trapping assay to identify substrates of PTPRO	PTPRO promotor is more methylated resulting in a lower expression of PTPRO; valosin-containing protein (VCP) is a main substrate for PTPRO and thus functions as a tumor suppressor in HCC	[95]

Abbreviations: HCC, hepatocellular cancer; HCA, human adenocarcinoma; FNH, focal nodular hyperplasia; CRC, colorectal cancer; CCC, intrahepatic cholangiocarcinomas and extrahepatic adenocarcinomas of the biliary tract; PDAC, pancreatic ductal adenocarcinoma; DAC, 5-aza-2-deoxycytidine; TSA, trichostatin-A; DPT, dermatopontin; TIF1γ, transcriptional intermediary factor 1 gamma.

features and lifestyle. Molecular profiling of genes, proteins and other molecules is aimed at deciphering the genotype–phenotype relationship with the goal of developing new therapies for human disease. The thorough understanding of epigenetic modifications and their downstream effects will enable us significantly in contributing toward the avenues of developing personalized medicine. Omics technologies are expanding rapidly, and these new analytical strategies – combined with more-efficient tissue procurement, protein isolation and separation methods, as well as development of data analysis tools – are expected to increase our ability to detect novel biomarkers for the diagnosis, prognosis and treatment of HCC.

miRNAs as therapeutic targets for HCC would be highly novel and of great importance. Systemic administration of antisense LNA oligonucleotides specific to miR-122 was recently shown to modulate miRNA and target gene expression in the liver and result in the significant loss of HCV with minimal toxicities in a non-human primate [98]. This study was encouraging enough to demonstrate the practicality of this approach for targeting aberrantly overexpressed miRNA in the liver under diseased conditions. Under normal conditions miR-26a is known to target cyclins D2 and E2 and induce G1 arrest when expressed in human liver cancer cells. It is well established that miR-26a expression level is frequently lost or downregulated in HCC conditions [99]. An elegant strategy involving replacing of the reduced levels of miR-26a by using a self-complimentary adenoviral overexpression system dramatically nullified the disease progression. These results support the idea of targeting tumor-suppressing miRNA or aberrantly expressed miRNA as a powerful and highly specific anticancer therapeutic technique for HCC.

A better understanding of epigenetic regulatory mechanism of miRNA expression will help to elucidate the complex network of epigenetic modifications and design innovative strategies for cancer treatment. Although DNA methylation is the best-studied epigenetic mechanism for miRNA deregulation, it is still largely unknown which miRNAs are altered owning to histone modification. This is partially because of the lack of effective detection methods and relatively strict requirements for obtaining and examining clinical samples. Notably, the expanding role of additional epigenetic factors such as SWI/SNF, MLL1, among others, in regulating genotypic changes has recently received further attention. One of the other approaches for overcoming the challenges of organ transplant and molecular therapeutic approaches could be tissue regeneration. Chronic human liver disease is often represented with an increase in fibrotic scar deposition and the outpacing of liver healing by regeneration. Currently, our knowledge on the epigenetic control of liver regeneration is limited. However, a recent study showed that loss of Arid1a, a component of the chromatin remodeling complex SWI–SNF, resulted in improved liver regeneration after partial hepatectomy in mice [100]. Alterations in the SWI–SNF complex, an ATP-dependent chromatin-remodeling complex, are also associated with cancer development [101]. Abrogation of SWI–SNF function through alterations in its various subunits can result in malignant transformation.

The rationale of epigenetic therapy is to reverse the causal epigenetic aberrations that occur in cancer, leading to the restoration of a normal epigenome. A plethora of epigenetic drugs have been designed and discovered in the past decade that can reverse key epigenetic changes in DNA methylation and histone modification aberrations that result in cancer [102]. Epigenetic drugs that inhibit tumor growth by several mechanisms including restoration of the expression of silenced tumor-suppressor genes as a result of epigenetic modifications and miRNA could prove promising options for cancer treatment [103]. Applications involving DNA methylation and histone deacetylation inhibitors can be administered or tested synergistically to suppress growth of cancer cell lines *in vitro* and *in vivo*. Many epigenetic drugs have shown promising results in clinical trials for cancer [104,105]. In fact, a HDACi was used in a clinical trial for juvenile idiopathic arthritis [106]. Another intriguing therapeutic avenue would enforce expression of multiple miRNAs that can act synergistically for a specific disease [107]. Upregulation of G9a histone methylation was observed in human HCC, contributing to epigenetic silencing of the tumor-suppressor gene RARRES3 in liver cancer [108]. Such targets like G9a could be a novel approach for HCC treatment [108]. Taken together, alterations in epigenetic markers and miRNA are crucial to the molecular mechanisms underlying carcinogenesis and autoimmunity, and further elucidating the complex layers of regulation might lead to novel treatments for these diseases.

DNA methylation inhibitors were among the first epigenetic drugs proposed for use as cancer therapeutics. The ability of these drugs to be incorporated into DNA also raises concerns regarding their potential toxic effect on normal cells. Therefore, an alternative approach involving the development of non-nucleoside compounds, which can effectively inhibit DNA methylation without being incorporated into DNA, is also being actively pursued. Development of several small-molecule inhibitors such as SGI-1027, RG108 and MG98 is a step in that direction [109,110]. These molecules can achieve their inhibitory effects by either blocking catalytic or cofactor-binding sites of DNMTs or by targeting their regulatory messenger RNA sequences; however, the weak inhibitory potential of these drugs indicates a need for the development of more-potent inhibitory compounds in the future. Such shortcomings and existing challenges open up avenues for a better understanding and characterization of miRNA function in regulating the epigenome landscape and possible implications as therapeutics.

Future prospects

The past two decades have witnessed a revolutionary development in the field of epigenetic targets for the treatment of various human diseases, including cancer. Several epigenetic therapies have already been approved by the FDA for untreatable human diseases and several compounds are undergoing preclinical investigation and clinical trial testing [111,112]. For example, first-generation cancer epigenetic agents have been approved for the treatment of cancer patients based on their role in DNA methylation (Dacogen® and Vidaza®) and broad-spectrum HDAC inhibition (vorinostat and romidepsin) [113]. It is important to understand the nuclear organization, DNA methylation, histone modification and gene expression patterns of cancer cells compared with normal cells. This will not only help to develop treatments but also aid in identification of disease markers. In particular, the liver has extensive potential for spontaneous regulation. A healthy liver can be cut in half and regenerate to its

original size within weeks. The mechanism of this regeneration remains unclear, and thus understanding of the epigenetic mechanisms that regulate liver regeneration is needed. Epigenetic information is naturally contained in cells and these mechanisms are natural processes and are often used for organ regeneration. The epigenetic signature normally follows multiple processes such as DNA methylation, histone modification (methylation, acetylation, phosphorylation, etc.), nucleosome positioning and miRNA expression, among others. Furthermore, lifestyle (diet, smoking, alcohol consumption) and surrounding environment cause epigenetic changes and this information is stored in the epigenome. Previous studies have shown that prolonged folate deficiency can induce methylation in the liver leading to hepatic carcinoma. Epigenetic drug discovery in existing and future clinical trials with epigenetic modifiers might be able to cure different types of diseases and disorders. It is important to discover a disease-associated protein target, controlled by DNA methylation, histone modification, chromatin remodeling, transcriptional control and noncoding RNA. Regulation of gene expression by epigenetics sites as a reversible process without changing the DNA sequence is represented in Fig. 2. The reversible nature of epigenetic changes provides an advantage when developing therapeutic drugs. Understanding the causes of epigenetic variation in normal and diseased cells as well as in human polymorphisms is essential. Surrounding environments, food habits and lifestyles have also influenced the epigenome over time. The prevention of mutations by DNA repair pathways for cancer prevention is reviewed elsewhere [114]. Some ingestion studies have also reported that it is possible to silence the genes by food habit. Diet acts as an important factor which can sustainably affect the activity state of genes. Epigenetic modification is associated with obesity and predicts a fatty liver [115], as well as impaired glucose metabolism and morbid obesity. In a recent mouse model experiment, it was reported that the epigenetic modification of the Igfbp2gene in early life can cause a fatty liver later in life. The epigenetic memory box is shown in Fig. 3.

Epigenetic targets are welcome news not only for research scientists and clinicians but also for the general public. A healthy lifestyle is important for better health, and one might be able to silence aggressive disease genes by practicing healthy lifestyle

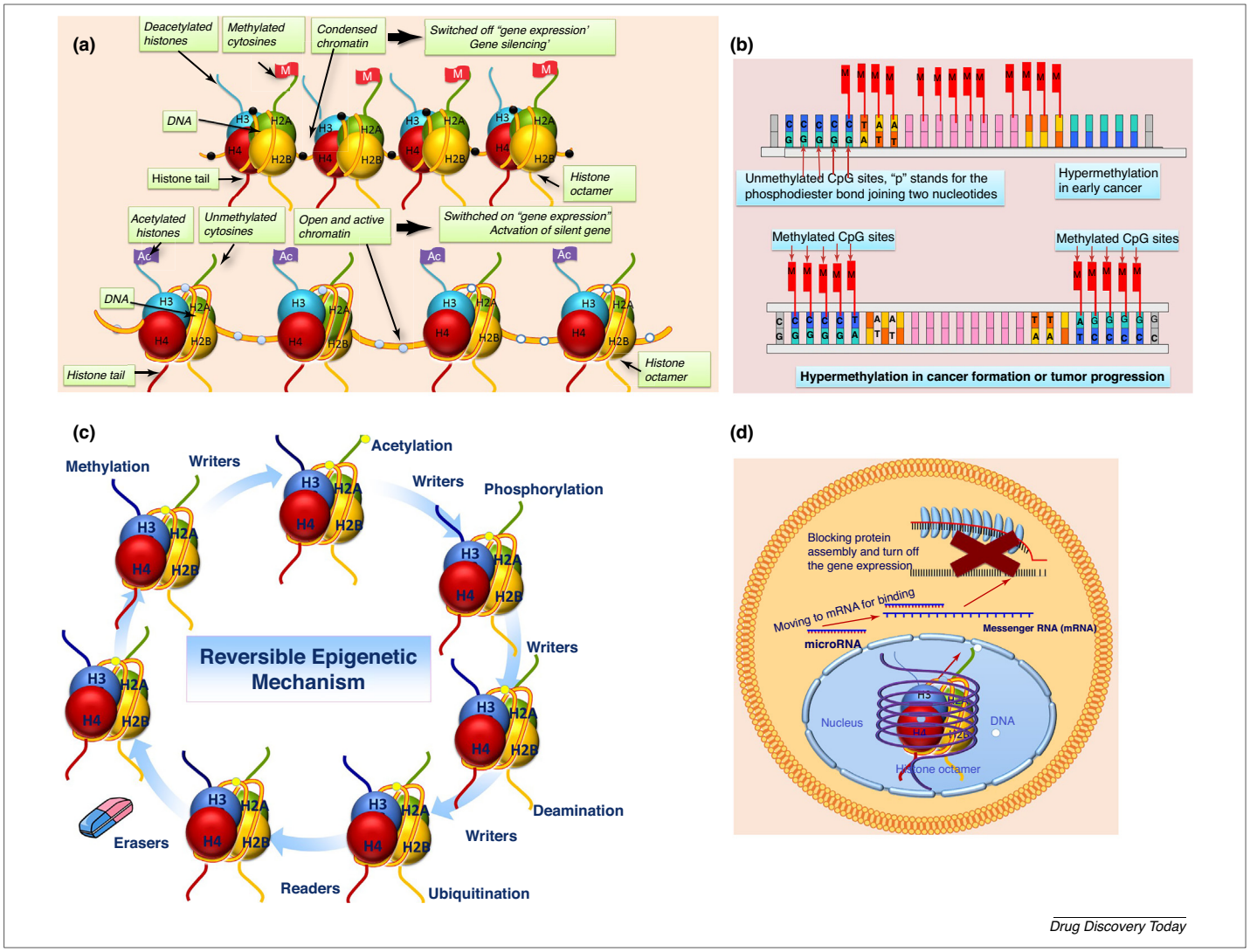

FIGURE 2

Regulation of gene expression by epigenetics sites in reversible processes without changing the DNA sequence.

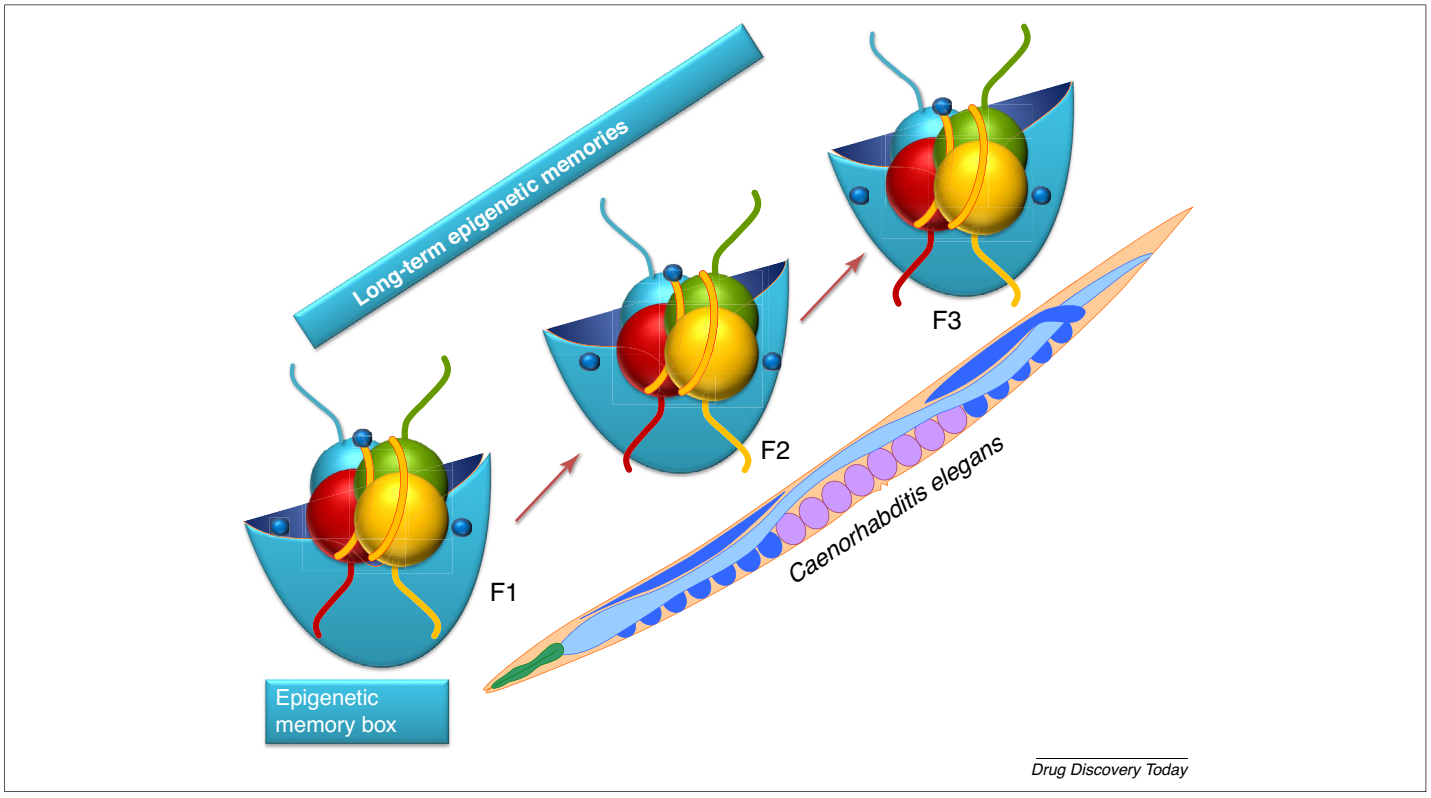

FIGURE 3

Epigenetic memory box: inheritable changes in gene expression not directly coded in our DNA but recorded in the epigenome, which could pass down to our children and our children's children. Such epigenome recording memory could help to protect for cancer prevention or initiation of cancer depending upon the type of records that are stored in the epigenome. F1 is for first generation, F2 is for second generation, F3 is for third generation.

changes. Therefore, it is of the utmost importance to communicate and educate the role of epigenetics in health and disease to the global population. It is important to delineate and understand the epigenetic complexities of genomic cohorts from diverse geography. Although the scope of epigenetic therapeutic approaches remains promising, some of the inherent challenges endure. The challenges pertaining to such an approach include cytotoxicity mediated by the drugs and poor drug delivery methods.

Concluding remarks

Epigenetic modifications are one of the fastest growing targets for liver cancer drug development. Advances in discovering molecular therapeutics will significantly help current patients living with the disease and will also add to the patients who undergo graft rejection (organ rejection – a synonym for rejection of transplantation). Some epigenetic drugs are approved for clinical use but several compounds are currently in advanced stages of clinical drug development pipelines and hundreds of potential drug candidates are currently under rigorous investigation in the lab-based setting. There has been a significant increase in research into epigenetic modulation. The effect of small molecules in DNA methylation and histone modifications has rapidly translated into clinical indentations, and thus epigenetic targets are important sites to target for drug development. This paper highlights the discovery of epigenetic modulations that are most potent and selective. By improving the understanding of epigenetic modulations among the global population, there are opportunities to improve patient outcomes for liver diseases on a global scale. This could represent the new generation of small molecules for the treatments of liver patients. It is the right time to jump from conventional hepatocyte research to epigenetic hepatocyte research to develop epigenetic drugs for the treatment of currently untreatable liver diseases. Considering the challenges mentioned above, we strongly believe in improving the current therapeutic approach involving small-molecule-based therapies targeting the transcription level and engineering an efficient means of organ- or tissue-specific drug delivery methods. Therefore, our review opens up an expanding field of opportunities to understand and investigate the much criticized epigenome landscape and strategies to check its regulation which will be crucial in developing better strategies toward treatment and prevention of liver-related disorders.

Conflicts of interest

The authors have nothing to declare.

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