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Review

Role of epigenetics in liver-specific gene transcription, hepatocyte differentiation and stem cell reprogrammation☆

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Controlling both growth and differentiation of stem cells and their differentiated somatic progeny is a challenge in numerous fields, from preclinical drug development to clinical therapy. Recently, new insights into the underlying molecular mechanisms have unveiled key regulatory roles of epigenetic marks driving cellular pluripotency, differentiation and self-renewal/proliferation. Indeed, the transcription of genes, governing cell-fate decisions during development and maintenance of a cell's differentiated status in adult life, critically depends on the chromatin accessibility of transcription factors to genomic regulatory and coding regions. In this review, we discuss the epigenetic control of (liver-specific) gene-transcription and the intricate interplay between chromatin modulation, including histone (de)acetylation and DNA (de)methylation, and liver-enriched transcription factors. Special attention is paid to their role in directing hepatic differentiation of primary hepatocytes and stem cells *in vitro*.

***Keywords*: Epigenetics; DNA methyltransferase inhibitor; Histone deacetylase inhibitor; Liver-enriched transcription factors; Stem cells; Primary hepatocytes; Differentiation**

1. Introduction

Hepatocytes contain a rich source of xenobiotic biotransformation enzymes and consequently, the liver represents a primary target for xenobiotic-induced acute and systemic toxicity. Hence, hepatocytes are the ultimate source for toxicological screening/profiling of potential drug candidates. The drawback, however, is

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Abbreviations: ADSC, adipose tissue-derived stem cells; ALB, albumin; AFP, alpha-fetoprotein; AhR, aryl hydrocarbon receptor; 5-AzaC, 5-Azacytidine or azacytidine; 5-Aza-dC, 5-Aza-2′-deoxycytidine or decitabine; C/EBP, CCAAT/Enhancer Binding Protein; CYP, cytochrome P450; CBP, CREB-binding protein; CpGs, cytosine-guanine dinucleotides; DHAC, 5-6-Dihydro-5-azacytidine; 4-Me2N-BAVAH, 5-(4-dimethylaminobenzoyl)-aminovaleric acid hydroxamide; DNMTs, DNA methyltransferases; DNMTi, DNMT inhibitors; ES, embryonic stem cells; EGF, epidermal growth factor; EGCG, (-)-epigallocatechin-3-gallate; HCC, hepatocellular carcinoma; HNF, hepatocyte nuclear factor; HATs, histone acetyl transferases; HDACs, histone deacetylases; HDACi, hydroxamate-based HDAC inhibitors; iPS, induced pluripotent cells; LETFs, liver-specific transcription factors; MSC, mesenchymal stem/progenitor cells; MBD, methylated DNA-binding protein; P/CAF, p300/CBP-associated factor; PGC-1α, PPAR-gamma coactivator 1alpha; zebularine, 2-Pyrimidone-1-β-D-riboside; TSA, Trichostatin A; VPA, valproic acid.

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that upon isolation and subsequent culture of hepatocytes, a rapid and substantial decline of hepatic functionality occurs. In particular, the xenobiotic biotransformation capacity undergoes phenotypic changes [1]. This progressive loss of a differentiated hepatic phenotype *in vitro* plausibly results from a proliferative response, elicited during hepatocyte isolation from the liver. In fact, the cell cycle entrance triggers the activation of several signal transduction pathways, ultimately leading to profound alterations in gene expression [2]. The acquisition and stabilisation of a differentiated hepatic geno- and consequently phenotype, i.e. liver-specific gene/protein expression, very often rely on the concerted binding of liver-enriched transcription factors (LETFs) and other *trans*-acting DNA-binding proteins to well-defined regulatory and coding regions of target genes [3–5]. As DNA is tightly condensed into chromatin fibers by histones and other proteins, modulation of chromatin compaction is a prerequisite to facilitate binding of transcription factors and consequential transcriptional activation [6,7]. Epigenetic events, including covalent histone modifications and DNA methylation, are therefore broadly acknowledged to play a fundamental role in the organisation of chromatin architecture and hence in the strict control of gene transcription [8,9]. For example, in proliferating hepatocellular carcinoma (HCC) and HCC-derived hepatoma cell lines, inhibition of histone deacetylation and DNA methylation is found to drastically down- and up-regulate genes involved in cellular proliferation and xenobiotic metabolism, respectively [10,11]. Consequently, it was thought that epigenetic events may display a predominant role in the acquisition and maintenance of the hepatocyte's differentiated phenotype of dedifferentiating primary hepatocytes *in vitro*. Alternatively, stem cells have been proposed to produce functional hepatocytes as well. New insights into the molecular mechanisms governing the balance between self-renewal/proliferation and lineage-directed differentiation of embryonic stem cells have unveiled the presence of epigenetic marks as being key regulatory players [9,12]. In fact, progression from unsoiled stem cells towards their differentiated progeny is characterized by alterations in the epigenetic landscapes of gene regulatory and coding regions. [9,12–15]. More specifically, locus-specific modifications on histones and DNA, progressively silence the transcription of pluripotent genes (euchromatic → heterochromatic state), whilst typical differentiated, lineage-specific genes become activated (heterochromatic → euchromatic state) [9,12–15]. Anticipation with nuclear chromatin might thus involve a key strategy for cell fate re-programmation.

In this review, we will discuss the key regulatory role of epigenetic modification in gene transcription, with particular focus on the maintenance and the acquisition of a differentiated geno/phenotype of primary hepatocytes and stem cells, i.e. pluripotent embryonic stem cells (ESCs) and multipotent mesenchymal stem/progenitor cells (MSC), respectively.

2. Epigenetic control of gene transcription

2.1. Structural chromatin modifications by histone acetylation/deacetylation

The chromatin higher order structure can be subjected to a number of reversible posttranslational modifications [16]. Although the functional relevance of the individual reactions is often unclear, it is generally believed that the global repertoire of histone tail modifications constitutes a (epigenetic) code, which affects chromatin structure and/or gene expression [17]. To date, histone acetylation, methylation, phosphorylation/ubiquination/sumoylation, ADP-ribosylation and glycosylation of histones were reported [6,16]. Histone acetylation, the best-understood posttranslational histone modification, is discussed in the following paragraphs.

2.1.1. Role of histone deacetylases in the regulation of gene expression

Two opposing enzyme activities, i.e. histone acetyl transferases (HATs) (recently also referred to as lysine (K)-acetyltransfereases or briefly KATs [18]) and histone deacetylases (HDACs), determine the acetylation status of the lysine residues at the N-terminal histone tails extending out of the nucleosome [6]. Upon acetylation (Fig. 1), the positive charges on the side chains of these lysine residues are partially neutralised, thereby weakening the interaction with the negatively charged phosphate groups in the DNA backbone and affecting the nucleosome stability. The degree of acetylation of core histones can thus modulate DNA accessibility and chromatin activity in transcription, replication, recombination and repair [19]. Whereas actively transcribed genes are characterized by highly acetylated core histones, hypoacetylated histones are preferentially found in transcriptionally silenced chromatin regions [20]. Consequently, the long-standing paradigm existed that HDAC inhibition, leading to histone hyperacetylation, was exclusively associated with transcriptional activation. Yet, evidence is accumulating of HDACs functioning as both transcriptional activators or repressors. Indeed, by removal of acetyl groups from histone tails, HDACs do not only modulate the physical interaction between histones and DNA in the nucleosomal units, but also the message encrypted in the histones' posttranscriptional modifications, and thus the epigenetic/histone code [21]. Consequently, specific effector proteins, e.g. transcription factors, are recruited resulting in further transcriptional stimulation or silencing,

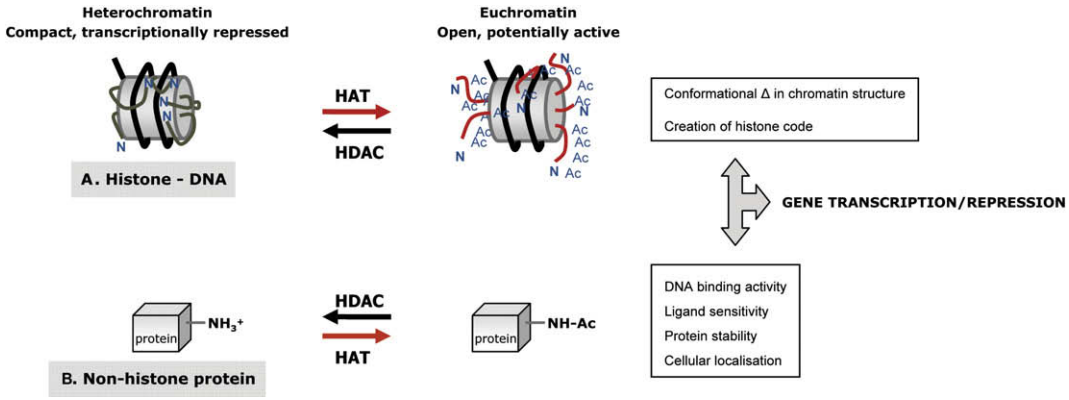

Fig. 1. HAT/HDAC-mediated transcriptional control. (A), Histone-related pathway: HATs/HDACs acetylate/deacetylate histones resulting in reduced/augmented chromatin compaction and alternations of the histone code, respectively. (B) Non-histone related pathway: HDACs directly interfere with non-histone protein targets, including transcription factors, nuclear hormone receptors, nuclear import factors, structural proteins and adhesion proteins. Deacetylation of latter non-histone proteins might affect diverse aspects of their protein physiology, resulting in either decreased or increased activity of the target protein. Both pathways interconnect with each other. The transcriptional outcome thus relies on the sum of all – transcription-stimulating/inhibiting – actions.

depending on the message comprised. Additionally, HDACs have targets other than histones, including the transcription factors p53, c-myc, NF-κB, YY-1, E2F and GATA family [22]. HDAC-mediated deacetylation of these non-histone proteins may affect their stability, localization, DNA-binding activity or ability to interact with other proteins [23]. As a result, the activity of the target protein might be augmented/reduced (Fig. 1).

In light of these data, it is clear that HDACs operate at more than one level in the transcriptional regulation scheme. We refer to Smith for a scrupulous review regarding the transcriptional machinery underlying HDACs-mediated transcriptional (in)activation [24].

2.1.2. Classification of histone deacetylases and their role in differentiation programs

Today, 18 HDACs have been characterized. Based on sequence similarity, they can be classified into four distinct classes. Class I (HDAC-1,-2,-3,-8) and class II HDACs (HDAC-4,-5,-6,-7,-9,-10) closely resemble the yeast RPD3 and HDA1 proteins, respectively. Class III HDACs or 'sirtuins' are structurally unrelated to the other HDACs and apparently insensitive to hydroxamate-based HDAC inhibitors (HDACi) such as Trichostatin A (TSA) [22]. HDAC-11 is the single representative of mammalian class IV HDACs. It displays conserved residues in the catalytic core that share some, yet insufficient, homology to both class I and II enzymes [25–27]. Most HDACs lack intrinsic DNA-binding activities and are therefore capable of homo- and hetero-dimerisation. The HDAC catalytic domain is formed by a stretch of ca. 390 amino acids consisting of a set of conserved amino acids, which differ between class I and class II HDACs [6,28]. The active site consists of a gently curved tubular pocket, a zinc-binding site and two Asp-His charge relay systems [29].

Class I HDACs (HDACs-1,-2,-3,-8) are generally small nuclear enzymes [27] that participate in cell cycle progression [25] and the regulation of housekeeping genes [28]. Class II HDACs (HDACs-4,-5,-6,-7,-9,-10) share some domain similarity with yeast HDA1 [25,27]. They can be subdivided into class IIa HDACs (HDACs-4,-5,-7,-9) and class IIb HDACs (HDAC-6 and -10) [30]. With the exception of HDAC-10, class II HDACs show a restricted tissue-specific expression pattern with the highest expression found in heart, brain and skeletal muscle [28]. They are larger than class I HDACs and shuttle between cytoplasm and nucleus. In the nucleus, they mediate cellular proliferation and transcriptional repression of differentiation-related genes, leading to loss of the differentiated phenotype [25]. This was particularly shown for class IIa HDACs-4,-5,-7,-9 isoforms in muscle cell differentiation [31]. Class IIa HDACs, and principally HDAC-4, are also implemented in stress signaling processes, such as cardiac/chondrocyte hypertrophy and neuronal cell death. [32–34]. Most of these functions are mediated by interactions with the MEF2 transcription factor family [32]. In contrast to other HDACs, Class IIb HDAC-6 can accomplish cytoplasm-located functions as well. More specifically, HDAC-6 functions at the crossroads between two cellular signaling systems, i.e. protein lysine acetylation and ubiquitination [35]. This unique feature expounds its protective role against the accumulation of cytotoxic misfolded protein aggregates within cells [35]. In addition, HDAC-6 and class I HDAC-3 regulate osteoblast differentiation and bone formation via interaction with transcriptional regulators such as Runx2 [35,36]. Likewise, class I HDAC-1, in complex with myoD, mediates repression of muscle-specific gene expression in undifferentiated myoblasts [37]. In general, class I HDACs overexpression

coincides with increased cell proliferation and a concomitant shift towards dedifferentiation, while levels drop during differentiation processes. For example, in normal small intestine, the HDAC-3 expression is maximal in proliferating cells at the crypt base and is markedly decreased at the villus tip, harbouring more differentiated cells [38]. In comparison, 90% of cells residing in adenomas of small intestine are HDAC-3^{+}. Basically, overexpression of distinct HDACs appears in various tumor specimens, e.g. HDAC-1 in prostate, gastric and colon tumors, HDAC-2 in colorectal, cervical and gastric cancer [26], and HDAC-3 in colon cancer [38]. Specific inhibition of HDAC-3 by RNA interference could inhibit proliferation of colon cancer cell lines and increase both expression and activity of the differentiation marker alkaline phosphatase [38]. As for liver-specific functions of HDACs, recently a crucial role was credited to HDAC-3 in liver homeostasis and development. In this respect, HDAC-3 absence in zebrafish leads to abnormalities in liver development, [39] whilst conditioned deletion of HDAC-3 in mice induces severe disruption of carbohydrate and lipid metabolism, resulting in organ hypertrophy and hepatocellular damage [40]. HDAC-1 overexpression in transgenic mice, on the other hand, results in a high incidence of hepatic steatosis and nuclear pleomorphism concomitant with altered expression of genes involved in cell cycle, apoptosis, and lipid metabolism such as p53, PPARγ, Bak and p21 [41,42]. Also, a number of studies provide evidence for the involvement of HDACs and HATs in the transcriptional regulation of liver-specific genes by LETFs. This issue will be thoroughly discussed in a later section.

Briefly, these data indicate that modulating the expression of specific HDACs might involve a strategy to (re)activate differentiation programs.

2.1.3. HDAC inhibitors: types and effects

Currently, several structurally diverse compounds both natural and synthetic, are known as HDACi. These include short-chain fatty acids, (non)-cyclic hydroxamates, (non)-epoxyketone-containing cyclic tetrapeptides, benzamides and miscellaneous structures [43]. Hydroxamate-based inhibitors of classes I and II are promising since they were repeatedly shown to selectively inhibit tumor growth in animals at low (micromolar) and apparently non-toxic doses [32]. Basically, in recent years, HDACi have emerged as promising therapeutics for the treatment of several malignancies, including leukaemia, solid tumors and non-solid cancers such as multiple myeloma [27]. In that respect, Vorinostat® has recently been approved by the FDA for the treatment of advanced primary cutaneous T-cell lymphoma, whilst several other hydroxamate-containing HDACi are being tested in phases I and II clinical trials for their therapeutic potential [44,45]. Having seen the growth-inhibiting and differentiation-promoting features of hydroxamate-based HDACi in tumor cells, including hepatoma cells [10,11,46–48], our group successfully applied these compounds to stabilize the differentiated phenotype of normal primary hepatocytes *in vitro* [1,2,49–52]. This will be discussed later in this review.

2.2. DNA methylation

2.2.1. Role of DNA methyltransferases in the regulation of gene expression

Reversible DNA methylation occurs at the cytosine–guanine dinucleotides (CpGs) in the DNA and includes addition of a methyl group to the carbon-5 position of cytosine [53]. DNA methylation patterns are established by DNA methyltransferases (DNMTs), catalyzing the addition of a methyl group derived from the methyl donor *S*-adenosyl methionine [17,54]. The catalytic activity of these enzymes is accomplished by a highly conserved C-terminal domain, present in all DNMTs.

In the mammalian genome, CpGs are not uniformly distributed. CpG islands, comprising >1 CpG per 80 base pairs [55], are particulary present in/near gene promotor regions. They are usually unmethylated, thereby allowing gene expression [53–55]. The distribution of (un)methylated CpGs differs, however, within distinct cell types due to the interplay between DNA methylation/demethylation, giving a cell-type specific DNA methylation pattern [17]. Passive DNA demethylation occurs during DNA replication by chemically blocking DNMTs [17]. The exact mechanism of active DNA replication-independent demethylation still remains elusive. Recent data suggest the involvement of identical enzymes in both the establishment of DNA methylation and demethylation patterns [56]. For example, the methylated DNA-binding protein MBD2 also displays demethylase activity [57]. Additional information is needed to unravel this tangled web.

2.2.2. Classification of DNA methyltransferases and their role in differentiation programs

Based on structural differences in their regulatory N-terminal domain, three distinct families of DNMTs, i.e. DNMT1, DNMT2 and DNMT3, have currently been identified. All are expressed in human liver tissue [58].

DNMT1 is the most abundant DNMT in mammals and mainly methylates hemimethylated GpGs. This 'maintenance' DNMT is particulary involved in maintaining DNA methylation patterns during DNA replication [54,58,59]. It also shows activity towards unmethylated DNA and plays a role in *de novo* DNA methylation [60]. DNMT2 is the least distinguished DNMT and lacks the regulatory N-terminal domain present in other DNMT enzyme families [59]. Its associ-

ated intrinsic DNMT activity and potential RNA methyltransferase activity suggest a possible role in epigenetic regulation [61,62]. The DNMT3 family contains three different DNMTs, i.e. DNMT3a, DNMT3b and DNMT3L. DNMT3a and DNMT3b, characterized as *de novo* DNMTs, mainly methylate unmethylated CpGs and establish new DNA methylation patterns during early embryonal development [54]. They plausibly cooperate with DNMT1 to maintain the DNA methylation pattern [63]. DNMT3L is a methyltransferase-like protein without intrinsic DNMT activity [59,64]. It interacts with DNMT3a/DNMT3b and directly modulates their catalytic activity [65].

DNMTs play a crucial role in the onset of chromatin remodelling and gene expression regulation. They are responsible for maintaining telomere integrity [66] and methylation pattern acquisition during gametogenesis, embryogenesis and somatic tissue development [67]. Several studies performed in tumor cell lines, including HepG2 cells [68,69], also indicate the occurrence of cell cycle arrest, apoptosis and cellular differentiation upon DNMT inhibition. Accordingly, it has been suggested that modulation of the expression of specific DNMTs might involve a strategy to target the differentiation status in developing and proliferating and consequently dedifferentiating cells.

2.2.3. DNMT inhibitors: types and effects

Today, a number of synthetic and natural DNMT inhibitors (DNMTi) exist. (i) The group of nucleoside analogue DNMTi contains several structural analogues of deoxycytidine, including 5-azacytidine (azacytidine, 5-AzaC), 5-Aza-2′-deoxycytidine (decitabine, 5-Aza-dC), arabinosyl-5-azacytidine (fazarabine), 5-6-dihydro-5-azacytidine (DHAC) and 2-pyrimidone-1-β-D-riboside (zebularine) [70] (Fig. 2). These analogues, with the exception of zebularine, are modified at the carbon-5 position of the pyrimidine base cytosine [71]. After phosphorylation and incorporation in DNA/RNA, they form covalent bounds with DNA methyltransferases, resulting in passive demethylation upon replication [72]. The use of nucleoside analogue DNMTi in tumor cells results, like HDACi, in cell cycle arrest, induction of apoptosis and differentiation [10,69]. In addition, latter DNMTi were applied by our group to maintain differentiation in normal primary hepatocytes [73]. This issue will be discussed in the next section.

(ii) The non-nucleoside analogue DNMTi represent a heterogeneous group of DNMTi enclosing molecules such as derivatives of 4-aminobenzoic acid (procaine and procainamide), the main compound in green tea, (-)-epigallocatechin-3-gallate (EGCG) [74,75] and psammaplins from the sponge *Pseudoceratina purpurea* [76]. Procain and procainamide directly bind to CpG-rich DNA, interrupting the interaction between DNMTs and their target DNA sequences. EGCG and psammaplins are both involved in several cellular processes and also affect enzymes other than DNMT [75]. Until now, the DNMTs inhibition mechanisms of EGCG and psammaplins remain unclear.

2.3. Interplay between DNA methylation and histone acetylation

A tight correlation exists between [gene expression and DNA methylation] and [chromatin structure and DNA methylation]. Suppression of gene expression is frequently associated with methylated DNA and a dense chromatin structure, whereas active transcription is associated with unmethylated DNA and hyperacetylated open chromatin [17] (Fig. 3). Initially, DNA methylation was thought to unidirectionally affect chromatin structure. However, recent data in cancer cells now suggest a mutual interplay between both epigenetic modifications [17]. For example, in several cancer cell lines, combinations of DNMTi and HDACi have synergistic effects on the cellular homeostasis [69,77,78]. Accordingly, so far, several 'cocktails' are in clinical trials as chemotherapeutics [79]. Also, *in vitro*, our group discovered a synergistic effect of DNMTi and HDACi with respect to the differentiated phenotype of normal primary cells [73]. This will be further explained in the next section.

In summary, our findings show that transcription of genes, governing maintenance of a cell's differentiated

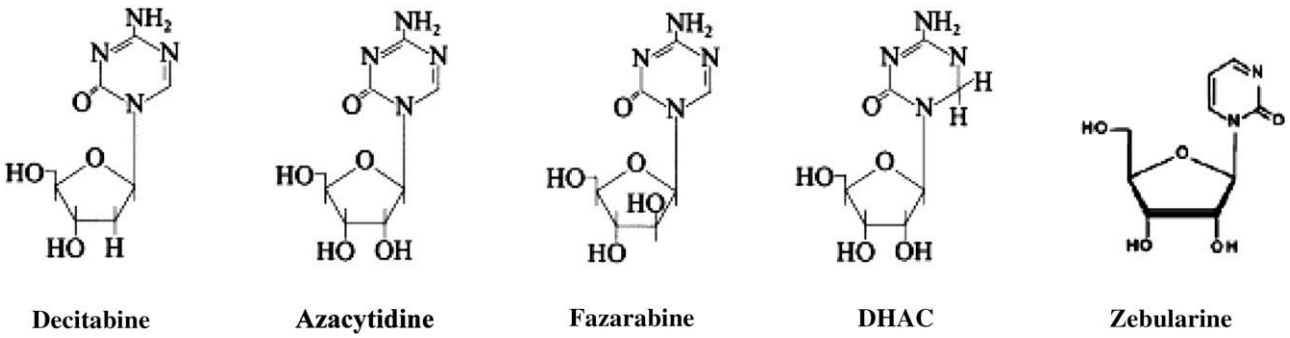

Fig. 2. Nucleoside analogue DNMTi.

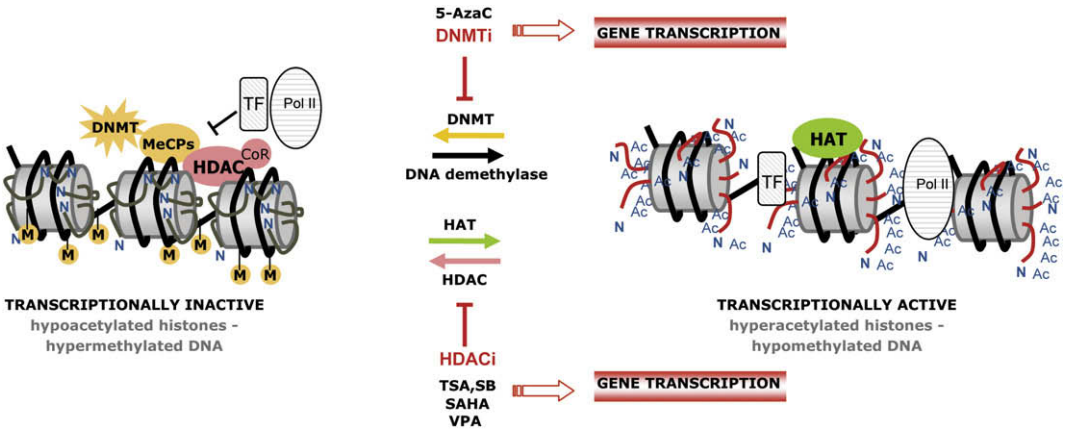

Fig. 3. Epigenetic control of gene transcription. Inhibition of gene transcription typically corresponds to hypermethylated CpG islands in gene promoter regions and deacetylated histone tails at local chromatin domains. The indirect mechanism of gene silencing may involve binding of methyl-binding proteins (MeCp) to methylated cytosine and subsequent recruitment of HDAC-corepressor (CoR) complexes, resulting in a non-permissive heterochromatin status that blocks binding of transcription factors (TF) and polymerase II RNA complexes (PolII) to target promoter sequences. The direct mechanism may involve the direct interference of TF with HDAC or methylated CpG sites within the promoter. HDAC inhibitors (HDACi) and DNMT inhibitors (DNMTi) modulate the chromatin structure. They create an open, transcriptionally active euchromatin configuration at gene coding and regulatory regions, accessible for TF, thereby facilitating gene transcription. *Abbreviations*: 5-AzaC, decitabine; M, 5-methyl cytosine at CpGs; SB, sodium butyrate; TSA, trichostatin A; VPA, valproic acid.

status in adult life and development, can be accomplished via targeting the expression of DNMTs and/or HDACs with increased chromatin accessibility of transcription factors to their target DNA as a result.

3. Epigenetic modifiers as potent differentiation-promoting compounds in vitro

3.1. Acquisition and stabilisation of a differentiated hepatic phenotype in vitro: an interplay between chromatin remodelling and liver-enriched transcription factors

Of major interest, at least from a pharmaco-toxicological point of view, is the ability of hepatocytes to protect the organism from toxic chemical insults. Hepatocytes dispose of an ingenious multi-step enzymatic clearance system, i.e. xenobiotic biotransformation, and therefore constitute the main cell type of interest for *in vitro* hepatotoxicity and drug metabolism studies to date [80].

Ex vivo, though, cell–cell and cell–extracellular matrix disruptions, resulting from collagenase perfusion and subsequent oxidative stress response, trigger the activation of several 'proliferative' signaling cascades [81]. Unlike hepatocytes *in vivo*, primary hepatocytes in culture are unable to completely redifferentiate upon proliferation, resulting in a loss of the differentiated phenotype and concomitant deterioration of cytochrome P450 (CYP)-mediated xenobiotic biotransformation capacity [2,81]. Another essential factor is the substantial decline in LETFs, controlling the transcription of numerous liver-specific genes [3–5,53,81–84]. Indeed, hepatocyte proliferation and differentiation are predominantly regulated at the transcriptional level [85]. Basically, eukaryotic gene transcription relies on the combinatorial binding of multiple specific *trans*-acting DNA-binding proteins, i.e. transcription factors, to particular DNA-sequence motifs in regulatory elements of a specific gene. Efficient gene expression is further often determined by interplays between different transcription factors, either adjacently or distantly located on the promoter, and by protein–protein interactions between transcription factors and coactivators/corepressors [7,13,82]. Additionally, efficient binding of transcription factors and associated proteins to their cognate DNA-sequences requires a permissive chromatin configuration in order to drive gene expression The dynamic modulation of the chromatin architecture by e.g. DNA methylation and/or covalent histone modifications represents thus a basic machinery for transcriptional activation, repression and derepression [7–9,13–15] (Fig. 3).

In hepatocytes, the liver-enriched transcription factors play an elemental role in hepatocyte-specific gene expression [3–5,82,85], and are as such key regulators of liver development, architecture and physiology. These *trans*-acting DNA-binding proteins are predominantly, but not exclusively, expressed in liver. It is hypothesized that the coordinated and timely expression of LETFs, in concert with ubiquitously expressed transcription factors such as NF1, Oct-1, Hex and other LETFs, is prerequisite for hepatocyte differentiation and constitutive liver-specific gene expression, including CYP-mediated xenobiotic biotransformation [4,82,84,85]. Evidence is accumulating that recruitment of coactivators/corepres-

sors, able to modulate the local chromatin configuration through post-translational histone modifications, mainly determine their transactivation potential. In this context, the transcriptional activation of LETFs critically depends on the recruitment of co-activator proteins with intrinsic HAT activity, such as CREB-binding protein (CBP), p300/CBP-associated factor (P/CAF) and SRC1, whereas co-repressor complexes containing HDAC negatively regulate liver-specific gene expression [86–107]. In detail, HNF-4α, directly interacts with SRC1, CBP and p300, resulting in its increased transcriptional activity [86–92]. The level of upregulation is isoform-dependent [92,93]. In human hepatoma cells, transactivation of CYP1A1, CYP1A2 and CYP2C9 by HNF-4α relies on the presence of the PPAR-gamma coactivator 1alpha (PGC-1α) [94]. PGC-1α, a key regulator of hepatic gluconeogenesis, lacks HAT-activity, but enables transcription through the assembly of a complex, containing SRC1 and CBP/p300 [95]. Likewise, in differentiating Caco2-cells, Rb strengthens HNF-4-dependent activation of the α-antitrypsin gene through reinforcement of the SRC-coactivator function [96]. Conversely, interaction with SMRT or p53, recruiting HDACs to transcription factors, represses HNF-4α activity [92,97,98]. HNF-1α, on the other hand, physically interacts with the HATs CBP/p300, P/CAF, SRC-1, and RAC3 [99,100]. CBP and PCAF, on one hand, and CBP and p300, on the other hand, synergistically upregulate HNF-1-mediated transactivation [100,101], whilst association of HNF-1α with HDAC1 – through NCoR– impairs its transcriptional activity. Treatment with the HDACi TSA disrupts latter corepressor complex, enhancing in turn HNF-1α-mediated transcription [102]. A good example of LETFs acting in a cooperative, synergistic regulatory network is the interaction between HNF-6 and HNF-3. In this respect, HNF-6 potentiates HNF-3β transcriptional activity by recruiting p300/CBP HAT proteins, [103] whilst HNF-6-dependent transcription is stimulated by complex formation between HNF-6 and C/EBPα, also recruiting coactivator CBP [104]. The transactivation potential of C/EBPα, in turn, is promoted by direct interaction with either CBP/p300 or Rb [105–107]. Apparently, this binding to C/EBPα robustly stimulates nucleosomal HAT activity of CBP [108]. C/EBPβ-dependent transactivation is further mediated by direct acetylation through association with the HATs p300 and PCAF [109–112]. Conversely, interaction between SMRT or subcomponents of the Sin3 complex, e.g. Sin3a, and HDAC1 represses its transcriptional activity [112]. Of particular interest is HNF3 as, in contrast to other LETFs, it directly affects chromatin conformations of numerous hepatic genes such as albumin (ALB) and α-foetoprotein (AFP), likely without interference with intermediary coactivators bearing HAT-activity or ATP-dependent enzymes. More specifically, the C-terminal domain of the protein binds to histones H3/H4 within highly compacted chromatin, creating a local, open nucleosomal domain, which facilitates further interactions between transcription factors, such as GATA4 and other LETFs, and DNA [113–115]. This HNF3-mediated transcriptional competence is designated as a prerequisite for the onset of liver ontogeny, and more specifically for the developmental activation of genes required for hepatocyte differentiation and function.

3.2. Effect of HDAC and DNMT inhibition on liver-specific gene expression

An overview of the most important hepatic genes affected by HDACi and DNMTi in various *in vitro* models is presented in Table 1. Out of these data, it appears that both the cellular origin and the type, concentration and exposure time of epigenetic modifier used, critically determine the final outcome. In transformed cells, including colon cells [116–118], glioma cells [119,120], pancreatic cells [121], breast cells [122] and hepatoma cells [11], HDACi-mediated growth arrest is frequently, at least *in vitro*, associated with induction of differentiation. As such, HDAC inhibition upregulates C/EBPα, HNF-1α, HNF-3α, HNF-3β and HNF-4α levels in various hepatoma cells, resulting in increased CYP expression [11]. Yet, in spite of this beneficial outcome, the contribution of HDACi in long-term *in vitro* models is hampered by co-occurrence of cell death. Indeed, HDACi, including TSA, butyrate, valproic acid, SAHA, OSU-HDAC42 and ITF2357, induce both *in vitro* and *in vivo* apoptosis in hepatoma cells [11,46–48]. For example, SAHA reduces, dose- and time-dependently, the viability of HepG2 and Huh6 hepatoma cells as a result of concomitant activation of both extrinsic and intrinsic apoptosis signaling cascades [123]. In contrast, primary hepatocytes are relatively well-resistant against HDACi-induced apoptosis [2,49,50,124,125]. In this context, exposure of normal primary rat hepatocytes to TSA and 5-(4-dimethylaminobenzoyl)-aminovaleric acid hydroxamide (4-Me2N-BAVAH) upregulates C/EBPα and HNF-4α expression [49], which in turn increases CYP protein and activity levels (Figs. 4 and 5), gap junctional communication and ALB secretion, without any evidence of cell death [2,49,51,52,126,127]. Moreover, in epidermal growth factor (EGF)-stimulated primary rat hepatocytes, we could demonstrate that TSA and 4-Me_2N-BAVAH even delay the onset of spontaneous apoptosis as evidenced by reduced pro-caspase-3 processing, decreased pro-apoptotic Bid and Bax levels and increased anti-apoptotic Bcl_{xL} expression [2,50].

Further indication for the involvement of histone acetylation in the transcriptional activation of hepatic genes is supported by enhanced activity of several LETFs, including HNF-3γ, HNF-4α and C/EBPα, on the

Table 1
Effects of epigenetic modifiers on the expression of important liver genes in various *in vitro* models.

	Epigenetic modifier	Species	Model	Regulation[a]	Reference
HDAC inhibition					
Phase I biotransformation					
CYP1A1	TSA	Rat	Primary hepatocytes	↑	[49]
		Human	Mammary carcinoma MCF-7 cells	↑	[186]
		Human	HeLa cells	↑	[186]
	SAHA	Human	Mammary carcinoma MCF-7 cells	↑	[187]
CYP1A2	TSA	Human	Mammary carcinoma MCF-7 cells	↑	[186]
		Human	HeLa cells	↑	[186]
		Mouse	Primary hepatocytes	↑	[188]
	Butyrate	Mouse	Primary hepatocytes	↑	[188]
CYP1B1	TSA	Human	Mammary carcinoma MCF-7 cells	↑	[186]
		Human	HeLa cells	↑	[186]
		Human	HepG2 hepatoma cells	≈	[191]
CYP2B1/2	TSA	Rat	Primary hepatocytes	↑	[49]
	Valproate	Rat	Primary hepatocytes	↑	[190]
CYP3A4	TSA	Human	HepG2 hepatoma cells	≈	[129]
		Human	HepG2 hepatoma cells	↑	[131]
CYP3A2	TSA	Rat	Primary hepatocytes	↑	[49]
ADH1A	TSA	Human	HepG2 hepatoma cells	≈	[191]
			HeLa cells	≈	
ADH1B	TSA	Human	HepG2 hepatoma cells	≈	[191]
			HeLa cells	≈	
ADH1C	TSA	Human	HepG2 hepatoma cells	↑	[191]
			HeLa cells	≈	
FMO3	TSA	Human	HepG2 hepatoma cells	≈	[192]
Phase II biotransformation					
GSTP1	TSA	Human	Hep3B hepatoma cells	≈	[193]
	Butyrate	Human	Colon carcinoma HT29 cells	↑	[194]
	Butyrate	Human	Primary colon cells	≈	[195]
GSTA1/2	Butyrate	Human	Colon carcinoma HT29 cells	↑	[194]
		Human	Primary colon cells	↑	[195]
GSTA4	TSA, MS-275, VPA	Mouse	MC3T3-E1 preosteoblasts	↑	[196]
GSTM2	Butyrate	Human	Colon carcinoma HT29 cells	↑	[194]
GSTT1/2	Butyrate	Human	Colon carcinoma HT29 cells	≈	[194]
		Human	Primary colon cells	↑	[195]
UGT2B7	VPA	Human	Prostate carcinoma LNCaP cells	↑	[197]
UGT2B11	VPA	Human	Prostate carcinoma LNCaP cells	↑	[197]
SULT2B1	TSA	Human	HaCaT keratinocytes	↑	[198]
Ammonia removal	TSA	Human	HepG2 and Huh-7 hepatoma cells	↑	[11]
Albumin synthesis/secretion	TSA	Human	HepG2 and Huh-7 hepatoma cells	↑	[11]
		Rat	Primary hepatocytes	↑	[2,49]
Gap junctional intercellular communication					
Cx32	TSA	Human	Huh-7 hepatoma cells	≈	[199]
		Human	Neural progenitor cells	↑	[200]
		Human	kB nasopharyngeal tumor cells	≈	[201]
		Human	Prostate carcinoma cells	↑	[202]
		Human	Normal prostate epithelial cells	↑	[202]
		Rat	Primary hepatocytes	↑	[52]
	4-Me_2N-BAVAH	Rat	Primary hepatocytes	↑	[51]
Cx26	TSA	Human	Huh-7 hepatoma cells	≈	[199]
		Rat	Primary hepatocytes	↓	[52]
	4-Me_2N-BAVAH	Rat	Primary hepatocytes	↓	[51]
Cx43	TSA	Human	Huh-7 hepatoma cells	↓	[199]
		Rat	Primary hepatocytes	↑	[52]
	SAHA	Rat	*Ras* transformed WB-F344 liver epithelial cells	↑	[203]
		Human	Peritoneal mesothelial cells	↑	[203]
		Rat	C6 glioma cells	↑	[204]
	Phenylbutyrate	Human	Glioblastoma cells	↑	[120]
		Human	Glioblastoma cells	↑	[205]
		Human	Neural progenitor cells	↑	[200]
		Human	kB nasopharyngeal tumor cells	↑	[201]

Table 1 (*continued*)

	Epigenetic modifier	Species	Model	Regulation[a]	Reference
		Rat	Glioma cells	≈	[205]
	Sodium butyrate	Human	kB nasopharyngeal tumor cells	↑	[201]
		Human	Glioblastoma cells	≈	[205]
	4-Me_2N-BAVAH	Rat	Primary hepatocytes	↓	[51]
Liver-enriched transcription factors					
C/EBPα	TSA	Human	HepG2 hepatoma cells	↑	[10]
		Rat	Primary hepatocytes	↑	[49]
	SAHA	Human	Pancreatic carcinoma PANC-1 cells	↑	[121]
C/EBP*β*	Butyrate, TSA	Rat	Intestinal epithelial cell line IEC-6	≈	[206]
C/EBP*δ*	Butyrate, TSA	Rat	Intestinal epithelial cell line IEC-6	≈	[206]
HNF1α	TSA	Rat	Primary hepatocytes	≈	[49]
HNF1*β*	Depsipeptide	Human	Papillary thyroid cancer cells	↓	[207]
HNF4α	TSA	Rat	Primary hepatocytes	↑	[49]
Other					
Apolipoprotein CIII	TSA	Human	HepG2 and Huh-7 hepatoma cells	↑	[11]
HCFX	TSA	Human	HepG2 and Huh-7 hepatoma cells	↑	[11]
Glutamine synthetase	TSA	Human	HepG2 and Huh-7 hepatoma cells	↑	[11]
DNMT inhibition					
Phase I biotransformation					
CYP1A1	5-Aza-dC	Human	Mammary carcinoma MCF-7 cells	↑	[186]
		Human	HeLa cells	↑	[186]
CYP1A2	5-Aza-dC	Human	Mammary carcinoma MCF-7 cells	↑	[186]
		Human	HeLa cells	↑	[186]
		Mouse	Primary hepatocytes	≈	[208]
		Mouse	Hepa1c1c7 hepatoma cells	≈	[208]
CYP1B1	5-Aza-dC	Human	Mammary carcinoma MCF-7 cells	↑	[186]
		Human	HeLa cells	↑	[186]
	5-AzaC	Human	HepG2 hepatoma cells	≈	[189]
CYP3A4	5-Aza-dC	Human	HepG2 hepatoma cells	↑	[10]
CYP3A5	5-Aza-dC	Human	HepG2 hepatoma cells	↑	[10]
CYP3A7	5-Aza-dC	Human	HepG2 hepatoma cells	↑	[10]
FMO3	5-Aza-dC	Human	HepG2 hepatoma cells	↑	[192]
Phase II biotransformation					
GSTP1	5-Aza-dC	Human	Hep3B hepatoma cells	↑	[193]
	Procainamide	Human	LNCaP prostate cancer cells	↑	[74]
UGT1A6	5-Aza-dC	Human	HepG2 hepatoma cells	↑	[10]
UGT2B15	5-Aza-dC	Human	HepG2 hepatoma cells	↑	[10]
UGT2B28	5-Aza-dC	Human	HepG2 hepatoma cells	↓	[10]
Gap junctional intercellular communication					
Cx32	5-Aza-dC	Human	Caki-2 renal cell carcinoma cells	↑	[209]
		Human	Caki-2 renal cell carcinoma cells	↑	[210]
		Human	HK-2 renal tubular cells	≈	[209]
Cx26	5-Aza-dC	Human	Mammary carcinoma cells	↑	[211]
		Human	Mammary carcinoma cells	≈	[212]
		Human	Lung cancer cells	↑	[213]
		Human	Esophageal cells	≈	[214]
Cx43	5-Aza-dC	Human	Esophageal cancer cells	≈	[214]
		Human	Cervical adenocarcinoma cells	↑	[215]
		Human	CNE-1 nasopharyngeal cancer cells	↑	[216]
Liver-enriched transcription factors					
C/EBPα	5-Aza-dC	Human	HepG2 hepatoma cells	≈	[10]
C/EBP*β*	5-Aza-dC	Human	HepG2 hepatoma cells	↓	[10]
C/EBP*γ*	5-Aza-dC	Human	HepG2 hepatoma cells	↓	[10]
Other					
GLUT2	5-Aza-dC	Mouse	Primary hepatocytes	↑	[217]
		Mouse	Hepa1c1c7 hepatoma cells	↑	[217]
ADH1A	5-Aza-dC	Human	HepG2 hepatoma cells	≈	[191]
			HeLa cells	≈	

(*continued on next page*)

Table 1 (*continued*)

	Epigenetic modifier	Species	Model	Regulation[a]	Reference
ADH1B	5-Aza-dC	Human	HepG2 hepatoma cells	↑	[191]
			HeLa cells	≈	
ADH1C	5-Aza-dC	Human	HepG2 hepatoma cells	↑	[191]
			HeLa cells	≈	
HDAC + DNMT inhibition					
Liver-enriched transcription factors					
C/EBPα	TSA + 5-Aza-dC	Human	HepG2 hepatoma cells	↑	[10]
Phase I biotransformation					
ADH1A	TSA + 5-Aza-dC	Human	HepG2 hepatoma cells	≈	[191]
			HeLa cells	≈	
ADH1B	TSA + 5-Aza-dC	Human	HepG2 hepatoma cells	↑	[191]
			HeLa cells	≈	
ADH1C	TSA + 5-Aza-dC	Human	HepG2 hepatoma cells	↑	[191]
			HeLa cells	≈	

[a]≈, unchanged; ↑, upregulation; ↓, downregulation.
Abbreviations: ADH, alcohol dehydrogenase; 5-AzaC, 5-Azacytidine; 5-Aza-dC, 5-Aza-2′deoxycytidine, decitabine; C/EBP, CCAAT/enhancer binding protein; Cx, connexin; CYP, cytochrome P450; FMO, flavine monooxygenase; GLUT, glucose transporter; GST, glutathione *S*-transferase; HNF, hepatocyte nuclear factor; 4-Me_2N-BAVAH, 5-(4-dimethylamniobenzoyl)-aminovaleric acid hydroxamate; SAHA, suberoylanilide hydroxamic acid; SULT, sulfotransferase; TSA, Trichostatin A; UGT, uridine guanyl transferase.

promoter of CYP2C8, CYP2C19, CYP3A4, CYP3A5, CYP3A7, CYP7A1 and glucose-6-phosphatase upon exposure to TSA or short chain fatty acids, such as butyrate [57,128,129]. In addition, HDAC inhibition is implemented in the aryl hydrocarbon receptor (AhR)-mediated induction of CYP1A1 and CYP1A2 [130], and CYP3A4 induction by rifampicin [131]. Next to genes coding for xenobiotic biotransformation enzymes, HDACi also stimulate the transcription of other liver-specific genes, including phosphoenol-pyruvate carboxy-kinase [57], HCFX [11], apolipoprotein CIII [11], glucose-6-phosphatase [57] and glutamine synthetase [11]. They also promote ALB synthesis and secretion rate, ammonia removal and gap junctional intercellular communication [2,11,50–52] (Table 1).

DNA methylation marks, on the other hand, are crucial for developmental and tissue-specific transcription of numerous liver-specific genes, including ALB [132], AFP [133], Cx43 [134], Cx32 [134], human CYP2E1 [135], human CYP1A2 [136], rat CYP2D3 and CYP2D5 [137], mouse CYP2D9 [138], mouse CYP2A4 [139], and human SULT1A1 [140]. In addition, in HepG2 cells, CYP3A4, CYP3A5 and CYP3A7 levels were raised upon DNMT inhibition by 5-Aza-dC, whilst C/EBPβ and C/EBPγ were decreased. Combined exposure to TSA and 5-Aza-dC, but not to 5-Aza-dC alone upregu-

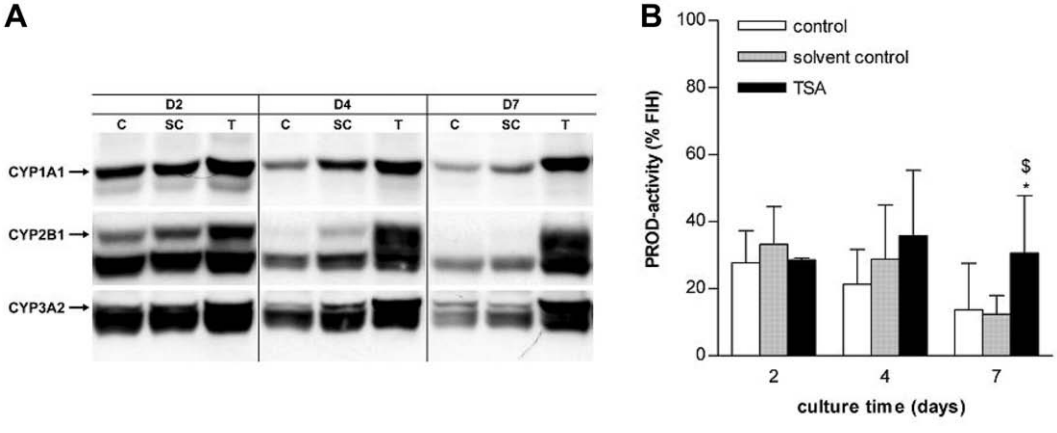

Fig. 4. Effects of TSA on phase I CYP-dependent biotransformation activity. Hepatocytes were cultured and remained either unexposed [C] or were exposed to 0.083% (v/v) ethanol as solvent control [SC] or 25 μM TSA [T] for 7 days. (A) After 2 [D2], 4 [D4] and 7 days [D7], CYP1A1, CYP2B1 and CYP3A2 protein expression were analysed by means of immunoblotting. In order to control for equal loading of proteins, expression levels of HNF-1α were determined, as latter LETF, in contrast to C/EBPα and HNF-4, is not affected by culture time or exposure to TSA. Representative images for three independent experiments are shown. (B) After 2, 4 and 7 days of culture PROD (CYP2B1)-dependent activities were measured. Data are expressed as percentage of the values found for freshly isolated primary rat hepatocytes, i.e. 7.7 ± 3.1 pmol/min/mg microsomal protein. Values represent mean ± SD.

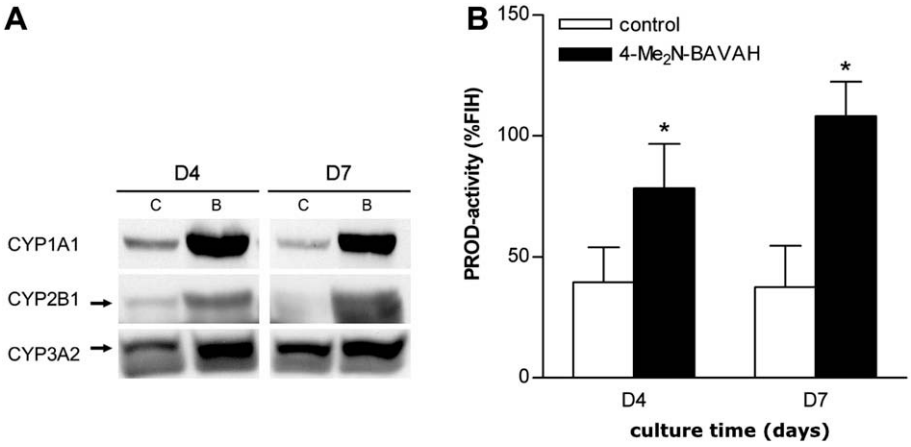

Fig. 5. Effects of 4-Me_2 *N*-BAVAH on phase I CYP-dependent biotransformation activity. Cultured hepatocytes were either exposed to 0.05% (v/v) ethanol as a solvent control [C] or to 50 μM 4-Me_2N-BAVAH [B] for 7 days. (A) After 4 [D4] and 7 days [D7], CYP1A1, CYP2B1 and CYP3A2 protein expression were analysed by means of immunoblotting. Representative images for three independent experiments are shown. (B) After 4 and 7 days of culture PROD (CYP2B1)-dependent activities were measured. Data are expressed as percentage of the values found for freshly isolated primary rat hepatocytes, i.e. 7.7 ± 3.1 pmol/min/mg microsomal protein. Values represent mean ± SD ($n \geqslant 3$). ($^{*}p < 0.05$ compared to control values, paired Student's *t*-test).

lated C/EBPα expression in HepG2 cells [10]. On the other hand, in EGF-stimulated primary rat hepatocytes, both combined and single exposure to 5-Aza-dC and 4-Me2N-BAVAH resulted in dose-dependent inhibition of the DNA replication and improvement of the hepatic functionality/phenotype, as evidenced by enhanced ALB secretion and elevated CYP1A1 protein expression [73]. Yet, upon combined application, lower concentrations of the respective epigenetic modifiers are needed when compared to single treatment in order to observe the same –or even a more pronounced– effect, pointing to a synergetic or even synergistic behaviour of DNMTi and HDACi with respect to important liver-specific processes [73]. Moreover, the interplay between HDACi/DNMTi and the expression of hepatic genes emphasizes a plausible involvement of chromatin remodelling agents in the acquisition/maintenance of a differentiated hepatic phenotype in healthy hepatocytes.

3.3. Epigenetic modifiers: a key factor to (re)program stem cells in vitro?

3.3.1. Stem cell signaling cascades

In vivo, stem cells inhabit restricted niches within an organ or tissue, directing their self-renewal, differentiation and cell fate [141–143]. In particular, adult tissue is renewed through asymmetric division of stem/progenitor cells, thereby forming one cell that remains a stem cell and another cell that differentiates into a mature cell type with specialised functions [141–143]. Batteries of developmental regulatory signaling molecules and transcription factors, including Wnts, fibroblast growth factors, Notch, sonic hedhodge, etc. may play a role [85,142,144]. More specifically, the coordinated signaling between stem cells, non-stem niche cells, the scaffold, and integration of stem cell-autonomous characteristics-including a dynamic interplay between transcription, epigenetic control and posttranscriptional regulators represent an interactive system, organized to facilitate cell-fate decisions in a spatio-temporal manner [85,142,144]. Identification of these *in vivo* signaling patterns is crucial for eliciting distinct responses from cultured stem cells and directing lineage-specific cell growth and differentiation *in vitro*. Lately, evidence is growing that particularly chromatin remodelling or alteration of epigenetic marks, including histone acetylation/methylation and DNA methylation are part of the core machinery required for nuclear reprogramming and cell-fate conversion [9,12–15,145,146].

3.3.2. The epigenetic control of stem cell differentiation

Pluripotent ES and more lineage-restricted adult stem/progenitor cells differ in their global gene expression status. Stemness genes, active in pluripotent embryonic stem cells are gradually silenced, whilst lineage-specific genes are switched on upon progression of development [12,14,15,146]. This discrepancy in gene expression profiling might be ascribed to alterations in the nuclear and chromatin architecture, resulting in selective accessibility of transcription factors towards specific DNA-binding sites. Each stage of lineage-directed development is thus featured by a dynamic interplay between unique repertoires of (lineage-specific) transcription factors and epigenetic regulators. This epigenetic code forms the base of the stem cell identity and determines its responsiveness to extrinsic signals at successive developmental stages [9,82,85,114]. Alternatively, extracellular growth factors might directly affect the chromatin status as well and as such facilitate or impede the differentiation competence of stem/progenitor

cells [147]. Hence, the hypothesis was concocted that cell fate might be reprogrammed by altering the epigenetic code/marks.

In this regard, a recent breakthrough was achieved by Takahashi and Yamanaka, who successfully reprogrammed mouse embryonic/adult fibroblasts to ES-like stem cells, referred to as induced pluripotent cells (iPS), via viral mediated transduction of Oct4, Sox2, c-Myc and Klf4 [148]. These four transcription factors act as core regulators of the transcriptional circuitry to maintain pluripotency in ES. The theory exists that their ectopic expression in lineage-committed somatic cells induces alterations in the histone code and DNA methylation status of stemness genes such as Oct4, Sox2 and Nanog [146,148]. Although the underlying mechanism remains elusive, latter realization emphasizes the tight epigenetic control of transcriptional machinery regulating pluripotency and lineage-specific differentiation. More specifically, on/off switch of cell fate reprogrammation and transcription of lineage-specific genes may be poised by dynamic open/closed configuration of histone and nuclear architecture at specific binding sites for transcription factors.

Since HDACi and/or DNMTi upregulate the transactivation potential of liver-enriched transcription factors in a plethora of hepatoma cells and primary hepatocytes [cf. Table 1], being key regulators of liver embryogenesis and liver-specific gene expression in particular, addition of HDACi and/or DNMTi to stem/progenitor cells, preferentially co-conditioned with hepatogenic growth factors and cytokines, is thought to comprise a potential strategy for driving differentiation programs and more specifically for directing hepatic differentiation of stem/progenitor cells. An overview of currently applied epigenetics-based strategies for *in vitro* hepatic differentiation of ES and MSC is given in Table 2.

3.3.3. Pluripotent embryonic stem cells and multipotent mesenchymal stem/progenitor cells as source of hepatocytes

3.3.3.1. Pluripotent embryonic stem cells. ES harbour a unique pluripotent versatility compared with fetal and adult multi/bipotent stem/progenitor cell populations. They posses the unrestricted capacity to form cell types of the three germ layers, including neuroectodermal cells, cardiomyocytes and hepatocytes. Yet, spontaneous ES differentiation is encountered by lack of organization and inherent heterogeneity [149–151]. Introduction of chromatin remodelling agents, biologically-derived signals such as purified growth factors, or other lineage-selective agents, though, could enrich for specific cell populations [150]. For example, exposure to 5 mM sodium butyrate enriches ES cultures for 10–15% to pure hepatic cells [152]. Priming with alternating concentrations of sodium butyrate (0.5–1 mM) in the presence of Activin A even results in 10–70% enrichment [153]. Basically, combined application of epigenetic modification and stepwise exposure to cytokine stimuli considerably contribute to homogeneity of the end-population and acquisition of hepatic functionality [153].

3.3.3.2. Multipotent mesenchymal stem cells. In recent years, evidence has been provided that MSC(-like) cells from various sources (bone marrow/adipose tissue/placenta/umbilical cord) could occasionally overcome lineage borders and differentiate into endodermal (hepatocytes) and ectodermal (neural cells) cell types upon coordinated *in vitro* stimulation [154–160]. New insights into the underlying mechanisms indicate that next to lineage-specific cytokines/growth factors (their concentrations, mode of presentation, and order of application) [144], alterations of the epigenetic traits and chromatin code of specific gene regulatory regions are essential for bypassing cell fate determinism and reprogramming cell fate [9,13,15,145,146]. In this context, we found as first that addition of 1 μM TSA to cultured human bone marrow MSC, pre-treated for 6 days with hepatogenic-stimulating agents, triggers their 'trans'differentiation into cells with similar phenotypic and functional characteristics as primary hepatocytes [154]. In line with our results, Seo et al. showed enhanced hepatic differentiation upon addition of 0.1% dimethylsulfoxide to human adipose tissue-derived stem cells (ADSC), prestimulated for 10 days with a mixture of hepatogenic cytokines [161]. Likewise, neuroectodermal and cardiomyocyte direction could be accomplished via co-exposure to HDACi along with neural stimulating agents [162] and shear stress [163,164], respectively. Recently also DNMTi, either alone or in combination with HDACi, were introduced to alter cell fate [155,165–167]. Basically, DNMTi function as preconditioning agents prior to hepatic differentiation [155,167,168], whereas HDACi act as stimulants during or post-differentiation [154,156,161,169]. In general, chromatin remodelling seems thus a potential innovative strategy to overcome cell fate determinism, cross lineage borders and favour lineage-specific differentiation. We expect that this field will emerge in the upcoming years.

Next to successful differentiations, also failures have been reported. For example, 1 mM valproic acid (VPA), 100 nM TSA and 1 μM sodium butyrate failed to promote oligodendrocyte or astrocyte differentiation in rat neural progenitors under respective stimulating conditions [162], whilst they could trigger differentiation into neural cells in a neural stimulating microenvironment [162]. In addition, Jori and group reported that 2 mM VPA, but not 50 nM TSA, could stimulate neural transition of MSC [170]. On the other hand, we found that functional hepatic differentiation of bone marrow MSC was especially successful upon exposure of 1 μM TSA to 6 days preconditioned cells.

Table 2
Detailed epigenetics-based strategies for *in vitro* differentiation of ES and MSC-like cells into hepatic cells.

Origin	Hepatic differentiation conditions					Hepatic features		Refs.
	Cell density	Cell–matrix/ cell–cell interaction	Serum	Growth factors-cytokines nonepigenetic additives	Differentiation-inducing agents	RNA + protein level	Functionality level	
EMBRYONIC STEM CELLS								
				Hepatic progenitor cells				
	NS	Gelatin	15% FBS	(3)*D11–17/23:* 10 ng/ml HGF until confluent	(1)*D0–4*: 0.8% DMSO (2)*D4–10*: 2.5 mM SB	AFP, α1AT, CK18/19 GGT, HNF3 β, DPPIV		[182]
mES(D3)				**Hepatocytes**				
	NS	Collagen type I	10% FBS	4)*D17/23–23/29:* insulin, dex nicotinamide 20 ng/ml EGF 10 ng/ml HGF (5)from D23/29: 10 ng/ml OSM, dex		ALB, G6P↑, TAT↑	Glycogen storage ALB secretion	
hES	NS	PAU-coating, nonwoven PTFE	NS	*D0–3*:100 ng/ml bFGF *D3–11*:100 ng/ml HGF *D11–14*:dex	*D3–11: 1*% DMSO	ALB	Lidocaine metabolism ALB and urea production	[173]
hEBs		Matrigel	20%FBS		5 mM SB	−: AFP +: ALB, α1AT, CK8/18	Glycogen storage Inducible CYP450 activity	[152]
mES (D3)	1×10^4 cells/cm^2	*D0–10*:Gelatin collagen type I polystyrene	*D0–10*: 20% FBS		*D0–4*: 1% DMSO *D4–10*: 2.5 mM SB	+: ALB, K18, DPPIV ADH, CYP3A13, CYP27A1	Glycolysis Glycogen storage Urea production CYP activity	[183]
	1×10^4 cells/cm^2 D11: subculture at 15×10^3 cells/cm^2	*D0–10:* gelatin from D11: 5% polyacrylamide	*D0–10:*20% FBS from D11:/		*D0–4:* 1% DMSO *D4-10:* 2.5 mM SB From D11:/		Glycogen storage ↑urea production ↑ALB secretion	[184]
mES (D3)	Phase I Pre-differentiation = no confluence	Gelatin	15%FBS	(3) from D10 until confluence: 10 ng/ml HGF	(1)*D0–4*: 0.8% DMSO (2)*D4–10:* 2.5 mM SB	+: CK19, ALB, α1AT HNF3*β* -: SSEA1, AFP		[182]
	Phase II Differentiation upon confluence and subculture	Collagen type I	10%FBS	(4) Upon subculture: *D0–6*: insulin, dex nicotinamide, 20 ng/ml EGF 10 ng/ml HGF (5) From *D6–12/18*: (5) From *D6–12/18*: 10 ng/ml OSM, dex		+: AFP, ALB, CK18, α1AT HNF3*β*, HNF4, TAT -: CK19	Glycogen storage ALB secretion	
hES	Differentiation onset pon 50–70% confluence D3/5:1/2 split	Matrigel	*D0–3/5:*/ *D3/5–D10/12*:SR from *D10/12*: 8.3% FBS	*D0–3/5:* 100 ng/ml activin A From D10/12:insulin, hydrocortisone, 10 ng/ml HGF, 20 ng/ml OSM	*D0-D1/2*:1 mM SB *D1/2–3/5*: 0.5 mM SB *D3/5–D10/12*: 1% DMSO	*D0–3/5:* CXCR4, HNF3*β*, Sox17 D3/5–D10/12: HNF4α↑, HNF1↑, TTR↑ AFP From *D10/12:*ALB, ApoF, CAR, TO, TAT↑, CYP3A4/7, CYP2C9/19	Glycogen storage ALB/fibrinogen/ fibronectin/A2M secretion inducible CYP activity	[153]
mES Monkey ES	mES:10^9 cells/ cm^3 lumen V monkey ES: 4×10^3 cells/cm^3 lumen V	Organoid culture in hollow fibers	20% FBS		*D9*: 1 mM SB	+: CPS↑	ALB secretion ammonia removal	[174]

(*continued on next page*)

Table 2 (*continued*)

Origin	Hepatic differentiation conditions					Hepatic features		Refs.
	Cell density	Cell–matrix/ cell–cell interaction	Serum	Growth factors-cytokines nonepigenetic additives	Differentiation-inducing agents	RNA + protein level	Functionality level	
MESENCHYMAL(-LIKE) STEM/PROGENITOR CELLS								
BM (tibias + femora, C57/BL6 mice)	1×10^4MSC/cm^2 $+5.2 \times 10^4$ liver cells/cm^2	Coculture with nonparenchymal liver cells on collagen	>coculture: 20% FBS Upon coculture: 5% liver failure patients sera	*12 h before coculture:* dex Upon coculture: nicotinamide, insulin, dex, 50 ng/ml HGF, 20 ng/ml OSM	*12 h before coculture::* 5 mM 5-AzaC *Upon coculture:* 1% DMSO	ALB, CK18/8/19, TAT		[156]
ADSC (abdominoplasty, 19–55 years human adults; P3–5)	$2.5–3 \times 10^{-4}$ cells/cm^2	FN	/	10 ng/ml HGF 10 ng/ml OSM, ITS 10 ng/ml EGF, dex	*From D10 on:* supplement of 0.1% DMSO	AFP, ALB	LDL uptake urea production	[161]
BM (iliac crest, human adults)	100–200cells/ cm^2	NS	2% FCS	D1:40 ng/ml HGF, 20 ng/ml EGF	*D0*:preincubation with 20 km 5-AzaC	Cx32, HepPar1, CYP3A4, CPS CK18, ALB, PEPCK	Glycogen storage Urea secretion	[185]
ADSC (subcutaneous/ peritoneal, female donor) BM (iliac crest and femora, human)	Differentiation onset upon 100% confluence					Cx32, CYP3A4, CPS CK18, ALB, PEPCK, DPPIV	Glycogen storage Urea secretion	[168]
BM (healthy human; P4–5)	22×10^3MSC/cm^2 differentiation onset upon 100% confluence	Collagen type I	*Predifferentiation:* 2% FBS	*D0-2*: 10 ng/ml FGF4 *D3–5*: 20 ng/ml HGF from D6 on:ITS, dex 20 ng/ml HGF	From D6 on: supplement of 1 μM TSA	ALB↑, CK18↑, HNF1α↑, MRP2↑ C/EBPα↑	ALB secretion↑, inducible CYP activity Urea secretion	[154]
ADSC (peritonial, Fischer 344 rats)	200–300 cells/cm^2 Differention onset: upon 95% confluence:	FN		(2)*D1:* hepatocyte growth medium	D0: preincubation with 20 μM 5-AzaC	AFP, ALB, CK18/19, CYP1A1, HepPar1, Cx32, DPPIV, PCK1	Glycogen storage Urea production	[155]
UCB (hTERT retroviral infected)	2.1×10^3 cells/cm^2	NS	*D1–22:* 10% FBS	*D1–22:* 10 ng/ml FGF2 20 ng/ml HGF 20 ng/ml OSM	D0: preincubation with 1 μM5-AzaC	ALB, C/EBPα/β↑, CYP1A1/2, PEPCK↑, wnt↓	Glycogen storage Urea secretion	[167]
BM (tibias + femora, SD rats)	22×10^3MSC/cm^2 differentiation onset upon 100% confluence	Collagen type I	*Predifferentiation:* 2% FBS	*D0–2:* 10 ng/ml FGF4 *D3–5:* 20 ng/ml HGF from *D6* on:ITS, dex 20 ng/ml HGF	*From D6 on:* supplement of 1 μM TSA	AFP↑, ALB↑, CK18↑, HNF1α↑, HNF3β	ALB secretion↑	[169]

Abbreviations: α1AT, alpha1-antitrypsin; ADH, alcohol dehydrogenase; ADSC, adipose tissue-derived stem cells; AFP, alpha-fetoprotein; ALB, albumin; A2M, α2macroglobulin; ApoF, apolipoprotein factor; 5-AzaC, 5-azacitidine; bFGF, basic fibroblast growth factor; BM, bone marrow; CAR, constitutive androstane receptor; C/EBP, CCAAT enhancer binding protein; CK, cytokeratin; CPS CPS-1, carbamyl phosphate synthetase; Cx, connexin; CYP, cytochrome P450-dependent monooxygenases; dex, dexamethasone; DMSO, dimethylsulfoxide; DPPIV, dipeptidyl-peptidase IV; EGF, epidermal growth factor; ES, embryonic stem cells; FBS, fetal bovine serum; FCS, fetal calf serum; FGF, fibroblast growth factor; FN, fibronectin; GGT, γ-glutamyltransferase; G6P, glucose-6-phosphatase; h, human; HepPar1, hepatocyte paraffin 1; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; ITS, insulin-transferrin-selenious acid; LDL, low density lipoprotein; m, mouse/murine; MRP, multidrug resistance protein; MSC, mesenchymal stem cells; NS, not specified; OSM, oncostatin M; P, passage; PAU, poly-amino-urethane; PEPCK, phosphoenol-pyruvate carboxykinase; PTFE, polytetrafluoroethylene; SB, sodium byturate; SD, Sprague–Dawley; Sox17, Sry-related HMG box transcription factor; SR, serum replacement; SSEA, stage-specific embryonic antigen; TAT, tyrosine amino-transferase; TERT, telomerase reverse transcriptase; TO, tryptophan-2,3-dioxygenase; TSA, trichostatin A; TTR, transthyretin; UCB, umbilical cord blood; 1.,2.,3; indicate order of serial steps.
↓, downregulation; ↑, upregulation; −, negative; +, positive.

Successful cell fate manipulation thus highly relies on (i) the microenvironment (cell–cell contact, cell densities), (ii) the appropriate type of epigenetic modifier and (iii) optimal fine-tuning of its dose and timing – onset and duration – of exposure [154,161,169,171]. The suitability of HDACi and/or DNMTi to promote hepatic (trans)differentiation requires a delicate balance between (i) proliferation and differentiation, (ii) biological activity, pharmacokinetic properties and toxicological characteristics, and finally (iii) apoptosis and cell survival. At least in some cases, failure of lineage-specific differentiation could be ascribed to inaccurate timing of exposure and dosage of chromatin modulating agents. Basically, although not generally [153], pre-stimulation of the cells towards the intended selected direction prior to introduction of HDACi, may comprise a key determinant to cross lineage boundaries and achieve promoted transdifferentiation into a specific lineage by means of HDAC inhibition [154,161,164,169,172–174]. An up-to-date overview of both successful and failed epigenetics-induced cellular (re)programmations of progenitor cells is given in Table 3.

3.3.4. Stem-cell derived hepatocytes or hepatocyte-like cells?

The differentiation of embryonic/fetal hepatoblasts into adult hepatocytes *in vivo* basically implies consecutive expression of early (HNF-3β, AFP, transthyretin), midlate (HNF-1α, HNF-4α, ALB, cytokeratin 18) and late (tryptophan-2,3-dioxygenase, tyrosine amino-trans-

ferase, C/EBPα, CYPs) markers [3,5,82,85,113,114,142,144,175,176]. Most metabolic and detoxifying enzymes only become functional during the terminal step of liver organogenesis, i.e. peri/postnatal. Therefore, functional assays for enzymes, related to specific functions of the adult liver [80,177], must be carried out in order to state the mature status of resultant stem cell-derived hepatocyte-like cells. At present, functional analysis is particularly focused on ALB-secretion, urea metabolism and glycogen uptake. Little attention has been paid to other metabolic functions, including CYP450-dependent enzymatic activity and responsiveness to prototype inducers such as phenobarbital (human: CYP2B6, CYP3A4, rat CYP2B1/2), rifampicin (CYP3A4) and 3-methylcholantrene (human and rat CYP1A1/2). Bearing in mind that inducible CYP-dependent activity is considered to be a key determinant of the functional hepatic phenotype [80,178], characterization should preferably comprise the above mentioned metabolic functionality assays. Alternatively, metabolic profiling using well-known molecules (e.g. paracetamol) might shed light on their potency as *in vitro* models for preclinical toxicological screening of drug candidates. Recently, Hay and colleagues, in fact succeeded to produce a nearly (≈71%) pure population of functional, metabolism competent hepatocytes out of human ES via fine-tuned preconditioning with HDACi and growth factor stimuli [153]. The resultant cells display active and inducible CYP isozymes, capable of converting various substrates, e.g. midazolam, bufuralol, phenacetin, tolbutamide and rifampicine, to their respective metabolites, thereby supporting their potential use as preclinical *in vitro* systems for toxicity screening of drugs [153].

The ultimate demonstration of hepatic functionality is no doubt *in vivo* transplantation of *ex vivo* generated hepatic cells in (immunodeficient) animal models suffering from liver injury. Sgodda and coworkers recently confirmed the functional integration of hepatic cells derived from MSC, that were primed with 5-AzaC prior to combined exposure to hepatocyte growth factor and EGF [155]. Despite seemingly irrefutable evidence that stem/progenitor cells could contribute to liver reconstitution, caution should be taken with production of false positives due to application of inaccurate labelling techniques [179]. Also, one should keep in mind that, apart from generating fully functional stem-cell derived hepatocytes, other mechanisms including the bystander effect, fusion, partial transdifferentiation and horizontal gene transfer [180,181] might be responsible.

4. Conclusion

During preclinical drug development, early screening of promising drug candidates with respect to their metabolism, pharmacokinetics and potential (toxic) interactions is encouraged to diminish the number of failures at later stages. *In vitro* techniques, including hepatocyte-based *in vitro* models, are currently being applied. Hepatocytes in culture, however, enter the cell cycle and irreversibly dedifferentiate. Their limited viability and major loss of xenobiotic biotransformation capacity strongly limit their applicability. Understanding how to control differentiation and proliferation of these primary cells is a key-challenge. Next to targeting the differentiated status of adult primary hepatocytes, stem/progenitor cell technology has been proposed as an alternative to produce functional hepatocytes. Until recently the mechanisms governing lineage-directed and terminal differentiation in these stem/progenitor cells and adult primay cells, respectively, largely have remained unknown. In this review, we provide a better understanding of the intracellular regulation of directed and established liver-specific gene transcription.

In vivo, a constellation of intra- and extra-cellular signaling pathways is known to govern the balance of growth and differentiation in all cells. Stem cells, their differentiated progeny-including adult hepatocytes, and elements of their microenvironment make up a structural, (epi)genetic controlled machinery that coordinates normal homeostatic functioning of these cells [3,5,85,113,114,142–144]. By extensively reviewing the literature, we realised that next to the reconstruction of extracellular communication pathways *in vivo* (lineage-specific factors, including growth factors, cytokines, hormones, glucocorticoids, cell–cell and cell–matrix interactions), interference at the intracellular level via chromatin remodelling agents might involve a strategy to control lineage-specific gene expression, and consequently (i) the multilineage differentiation potency of stem cells and (ii) the acquisition of a differentiated genotype in adult cells [9,12–15,82,85,114,49,52,128,129,135,137,145,146]. Gene expression is in fact largely regulated by epigenetic modifications of DNA and chromatin on genomic regulatory and coding regions. In general, acetylation of core histones is associated with transcriptional activation, whereas DNA methylation is associated with gene silencing [17,20]. However, as reviewed in Tables 1–3, differential effects are observed depending on the factors studied e.g. the origin of the cells, the property evaluated, the type of epigenetic modifier used and the exposure time. Nevertheless, from the presented data it appears that in several stem cells-derived and primary hepatic-based models both HDACi and DNMTi are potent modulators of liver-specific functions and cellular contacts, and as such could significantly contribute to the acquisition and maintenance of the hepatocyte-specific geno/phenotype in culture.

Up to now, HDACi and DNMTi are mostly applied separately, though, their combined exposure is advanta-

Table 3
Accomplished and failed HDAC and DNMT inhibitor-induced *in vitro* and *in vivo* differentiation of stem cells into various cell types.

Epigenetic modifier	Culture conditions	Stem cell type	Intended cell type	Observed features	Reference
(A) Successful ('trans')differentiation					
HDAC inhibitor					
TSA*	1 μM TSA Exposure from day 6 of differentiation onwards Hepatic stimulating medium	Human bone marrow MSC	Hepatocyte-like cells	Increased Alb, CK18, HNF1α, MRP2, C/EBPα expression and Alb secretion	[154]
TSA*	1 μM TSA Exposure from day 6 of differentiation onwards Hepatic stimulating medium	Rat MPC	Hepatocyte-like cells	Increased AFP, HNF, Alb, CK18, C/EBPα, CYPs expression and Alb secretion	[169]
DMSO*	0.1% DMSO Exposure from D10 of differentiation Hepatic stimulating medium	Human ADSC	Hepatocyte-like cells	Alb and AFP expression, urea production	[161]
DMSO*	(1) 1% DMSO Exposure from D3–11 of differentiation Hepatic stimulating medium	Human ES	Hepatocyte-like cells	Alb expression, lidocaine metabolism, Alb and urea production	[173]
DMSO + sodium butyrate*	(1) 1% DMSO Exposure from D0–4 of differentiation (2) 2.5 mM sodium butyrate Exposure from D4–10 of differentiation	Murine ES (D3)	Hepatocyte-like cells	Alb, CK18, DPPIV, ADH, CYP expression,	[183,184]
DMSO + sodium butyrate*	(1) 0.8% DMSO Exposure from D0–4 of differentiation (2) 2.5 mM sodium butyrate Exposure from D4–10 of differentiation From D11: hepatic stimulating medium	Murine ES (D3)	Hepatocyte-like cells	AFP, ALB, α1AT, CK18, CK19, GGT, HNF3b, G6P, TAT and DPPIV expression, glycogen storage, ALB secretion	[182]
Sodium butyrate*	1 mM Sodium butyrate Exposure from D9 of culture Unspecified differentiation medium	urine and monkey ES	Hepatocyte-like cells	Alb expression, urea and Alb secretion	[174]
Sodium butyrate + DMSO*	(1) 1 mM sodium butyrate Exposure upon 50–70% confluence for 24–48 h (2) 0.5 mM sodium butyrate Exposure from D1-2 of differentiation for 48-72 h (3) 1% DMSO Exposure from D3 to D5 of differentiation upon subculture for 7days Hepatic stimulating medium	Human ES	Hepatocyte-like cells	Expression of CYPs, HNF3b, HNF4a, HNF1a,b, TTR, AFP, Alb, ApoF, CAR, TO, TAT, glycogen storage, Alb/fibrinogen/ fibronectin/A2M secretion, CYP activity	[153]
TSA* + shear stress	10 ng/ml TSA Shear stress: concentration not specified 24 h exposure after 7 days of differentiation Unspecified differentiation medium	ES (129/Ola derived)	Cardiomyocytes	Increased acetylated GATA-4, β-MHC, Nkx2.5 and ANF expression	[164]

Table 3 (*continued*)

Epigenetic modifier	Culture conditions	Stem cell type	Intended cell type	Observed features	Reference
TSA[*] + shear stress	32 nM TSA Exposure from onset of differentiation Shear stress: concentration not specified 1–24 h exposure to TSA pre-incubated cells Unspecified differentiation medium	Murine ES (D3)	Cardiovascular cells	Increased VEGFR2, PECAM, SMA, SM-2 C, α-SA, etc. expression	[163]
TSA[*]	80 nM TSA 24 h exposure after 7 days of differentiation Non-stimulating medium	Embryos cloned from cow donor cells	Blastocysts	Change in morphology	[172]
TSA[*] VPA[*] Sodium butyrate[*]	100 nM TSA 0.3–1 mM VPA 1 μM Sodium butyrate 4 days exposure upon onset of differentiation Neural stimulating medium	Rat neural progenitors	Neural cells	Increased Tuj1 or MAP2ab expression	[162]
VPA + DMSO[*]	2 mM VPA, 2% DMSO Exposure from differentiation onwards Neural stimulating medium	Rat bone marrow MSC	Neurons and astrocytes	Increased expression of NSE and NF, AchE activity	[170]
VPA[*]	0.5–3 mM VPA Exposure during first 3 days of differentiation and first 4 days prior to differentiation	Human bone marrow MSC	Osteoblasts	Increased osterix, osteopontin BMP-2 and Runx2 expression	[218]
DNMT inhibitor					
5-AzaC[*,$]	20 μM 5-AzaC Exposure for 24 h upon 95%confluence prior to onset of differentiation Upon 24 h: Hepatic stimulating medium	Rat ADSC	Hepatocyte-like cells	Expression of DPPIV, Alb, CYP1A1, HepPar1, AFP, CK19, PCK1, CK18	[155]
5-AzaC[*,$]	20 μM 5-AzaC Exposure for 24 h prior to hepatic stimulation onset of differentiation at 100% confluency Hepatic stimulating medium	Human ADSC Human bone marrow MSC	Hepatocyte-like cells	Expression of Alb, CYP3A4, HepPar1, CK18, Cx32, PEPCK, CPS and Cx32, urea production, glycogen storage	[168,185]
5-AzaC[*,$]	1 μM 5-AzaC Exposure for 24 h prior to hepatic stimulation Hepatic stimulating medium	Human umbilical cord blood MSC	Hepatocyte-like cells	Expression of Alb, C/EBPa, CYP1A1/2, glycogen storage	[167]
HDAC + DNMT inhibitor					
TSA + 5-Aza-dC[$]	150 nM TSA + 500 nM 5-Aza-dC 2days exposure prior to transplantation Neural stem cell stimulating medium	Murine neurosperes	Haematopoietic cells	Expression of CD45, CD3, CD19, MAC1, CD4, CD8	[166]
TSA + 5-Aza-dC[$]	5 ng/ml TSA + 500 nM 5-Aza-dC 5-Aza-dC: exposure from 16 to 48 h cultivation in medium promoting exit of G0/G1 phase TSA: exposure from 48 h cultivation in medium promoting terminal differentiation	Human $CD34^+$ HSC	$CD34^+/CD90^+$ HPC	Marrow repopulating capability, self-renewing potential	[165]

(*continued on next page*)

Table 3 (*continued*)

Epigenetic modifier	Culture conditions	Stem cell type	Intended cell type	Observed features	Reference
DMSO + AzaC*	1% DMSO + 5 mM 5-AzaC AzaC: exposure for 12 h before coculture DMSO: exposure throughout culture time Hepatic stimulating medium Coculture with nonparenchymal liver cells	Murine bone marrow MSC	Hepatocyte-like cells	Expression of Alb, TAT, CK18/8, CK19	[156]
(B) Failure of ('trans')differentiation					
HDAC inhibitor					
TSA + VPA + DMSO*	50 nM TSA, 2 mM VPA, 2% DMSO Exposure upon D1 of differentiation onwards Neural stimulating medium	Rat bone marrow MSC	Neurons and astrocytes	Decreased expression of NSE and NF, Decreased AchE activity	[170]
Butyrate*	2 mM Butyrate	Mononuclear cells from murine/human peripheral blood/ bone marrow/spleen	Endothelial cells	Failure of HoxA9, VEGFR2 expression	[219]
TSA* MS-275*	1–2.5 μM TSA 3–10 μM MS-275 Exposure upon onset of differentiation Endothelial stimulating medium				
TSA*	5 or 10 nM TSA Exposure upon onset of differentiation Unspecified differentiation medium	Murine ES	Embryoid bodies	Steady alkaline phosphatase activity	[220]
VPA*	0.3–1 mM VPA	Rat neural progenitors	Astrocytes, oligodendrocytes	Failure of Rip and GFAP expression	[162]
TSA* Sodium butyrate*	100 nM TSA 1 μM Sodium butyrate Exposure for 4 days upon onset of differentiation Oligodendrocyte and astrocyte stimulating medium				
VPA*	0.5–2 mM VPA	Mouse 3T3-L1 preadipocytes	Adipocytes	Reduced PPARγ, SREBP1a, C/EBPα expression	[221]
TSA*	3 nM TSA Exposure after 2 days of confluence Differentiation inducing medium	Primary human preadipocytes			
(C) Time-specified related failurel accomplishment of ('trans')differentiation					
HDAC inhibitor					
VPA§	300 mg/kg VPA Injection: every 12 h, for 4 times - In postnatal weeks 1–2 - From postnatal week 3 onwards	Pups neural progenitor cells	Oligodendrocytes	 - Suppression of myelination - No suppression of myelination	[171]
TSA*	5–400 nM TSA	Mouse myoblasts	Myotubes		[222]

Table 3 (*continued*)

Epigenetic modifier	Culture conditions	Stem cell type	Intended cell type	Observed features	Reference
Sodium butyrate*	0.1–10 mM Sodium butyrate				
VPA*	0.1–10 mM VPA				
	Differentiation inducing medium				
	- Exposure from onset of differentiation			- Prevention of muscle differentiation	
	- Exposure after onset of differentiation			- Progression of terminal muscle differentiation and prevention of reorganisation of constitutive heterochromatin	
TSA*	0.1, 1 and 10 ng/ml TSA	Rat oligo-dendrocyte progenitors	oligodendrocytes		[223]
	Pre-treated neuroectodermal stimulating medium				
	- Exposure up to to 12–24 h upon mitogen removal			- Steady state expression of A2B5, no oligodendrocyte maturation	
	- Exposure after 24 h upon mitogen removal			- Failure of GAIC and PLP expression, oligodendrocyte maturation (20)	

Abbreviations: α1AT, alpha1-antitrypsin; AChE, acetylcholinesterase; ADH, alcohol dehydrogenase; ADSC, adipose tissue-derived stromal cells; AFP, alpha-fetoprotein; ALB, albumin; A2M, alpha-2 macroglobulin; ANF, atrial natriuretic factor; ApoF, apolipoprotein; α-SA, alpha-sarcomeric actin; AzaC, 5-azacytidine; 5-Aza-dC, decitabine 5-Aza-2′deoxycytidine; β-MHC, beta-myosin heavy chain; BMP-2, bone morphogenic protein; CAR, constitutive androstane receptor; CD, cluster of differentiation; C/EBP, CCAAT enhancer binding protein; CK, cytokeratin; CPS, carbamyl phosphate synthetase; Cx, connexin; CYP, cytochrome P450; DMSO, dimethylsulfoxide; DPPIV, dipeptidyl peptidase IV; DNMT inhibitor, DNA methyltransferase inhibitor; ES, embryonic stem cells; GAlC, galactocerebroside; GFAP, glial fibrillary acidic protein; HDAC inhibitor, histone deacetylase inhibitor; HNF, hepatocyte-nuclear factor; HSC, haematopoietic stem cells; Hox, homeobox; LDL, low density lipoprotein; MAC1, macrophage antigen-1; MAP, microtubule-associated protein; MPC, mesenchymal progenitor cells; MRP, multidrug resistance protein; MSC, mesenchymal stem cells; NF, neurofilament; Nkx2.5, NK2 transcription factor related locus 5; NSE, neuron specific enolase; PCK1, phospho-enolpyruvate carboxykinase; PeB, peripheral blood; PECAM, platelet and endothelial cell adhesion molecule; PEPCK, phosphoenol-pyruvate carboxykinase; PLP, proteolipid protein; PPARγ, peroxisome proliferator-activated receptor gamma; Rip, oligodendrocyte marker; Runx2, transcription factor vertebrate homologue of the Drosophila runt gene 2; SMA, smooth muscle actin; SREBP, sterol regulatory element binding protein; SS, shear stress; TAT, tyrosine aminotransferase; TO, tryptophan dioxygenase; TSA, trichostatin A; TTR, transthyretin; VEGFR2, vascular endothelial growth factor receptor type 2; VPA, valproic acid.

$ in vivo.

* in vitro.

geous [73] and is expected to gain in importance in the following years. Still, more in depth studies are needed to unravel the tangled web of epigenetic marks and lineage-specific transcription, governing the multipotent status of stem cells and the terminal differentiated geno/phenotype of adult cell types.

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