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DNMT1 is a Required Genomic Regulator for Murine Liver Histogenesis and Regeneration

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Abstract

DNA methyltransferase 1 (DNMT1) is an essential regulator maintaining both epigenetic reprogramming during DNA replication and genome stability. We investigated the role of DNMT1 in the regulation of postnatal liver histogenesis under homeostasis and stress conditions. We generated *Dnmt1* conditional knockout mice (*Dnmt1^{alb}*) by crossing *Dnmt1^{fl/fl}* with Albumin-Cre (*Alb-Cre*) transgenic mice. Serum, liver tissues and primary hepatocytes were collected from 1–20 week old mice. The *Dnmt1^{alb}* phenotype was assessed by histology, confocal and electron microscopy, biochemistry as well as transcriptome and methylation profiling. Regenerative growth was induced by partial hepatectomy and exposure to CCl₄. The impact of *Dnmt1* knockdown was also analyzed in hepatic progenitor cell (HPC) lines; proliferation, apoptosis, DNA damage and sphere formation were assessed. *Dnmt1* loss in postnatal hepatocytes caused global hypomethylation, enhanced DNA damage response and initiated a senescence state causing a progressive inability to maintain tissue homeostasis and proliferate in response to injury. The liver regenerated via activation and repopulation from progenitors due to lineage-dependent differences in *Alb-Cre* expression, providing a basis for selection of less mature and therefore less damaged HPC progeny. Consistently, an efficient knockdown of *Dnmt1* in cultured HPCs caused severe DNA damage, cell cycle arrest, senescence and cell death. *Mx1-Cre*-driven deletion of *Dnmt1* in adult quiescent hepatocytes did not affect liver homeostasis.

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Conclusion—These results establish the indispensable role of DNMT1-mediated epigenetic regulation in postnatal liver growth and regeneration. The *Dnmt1*^{alb} mice provide a unique experimental model to study the role of senescence and contribution of progenitor cells to physiological and regenerative liver growth.

Keywords

DNA methylation; DNA damage; senescence; hepatic progenitor cells

Introduction

Reversible DNA methylation is a major epigenetic mechanism that regulates gene expression, cellular differentiation and development, and disruption of DNA methylation is observed during a range of human diseases, including cancer(1). In mammalian cells, methylation is catalyzed by members of DNA methyltransferases (DNMTs) that cooperatively establish tissue-specific methylation patterns. The most abundant is DNMT1 which is responsible for the maintenance of DNA methylation during replication and also contributes to de novo methylation activity(2, 3).

Mutational analyses in mice revealed that loss of *Dnmt1* caused cell-type-specific changes in gene expression that impact numerous pathways, including expression of imprinted genes, cell-cycle control, growth factor/receptor signal transduction and mobilization of retrotransposons(4,5). In support of the critical importance of DNMT1 functions, *Dnmt1*-null mice died soon after gastrulation with severely compromised liver development(6).

Recent tissue-specific gene deletion studies demonstrated that DNMT1 coordinates stem cell functions in a variety of somatic cell types thereby contributing to organ development. *Dnmt1* deletion caused p53-dependent apoptosis of mouse embryonic fibroblasts as well as pancreatic and intestinal progenitor cells, impaired self-renewal in hematopoietic and epidermal progenitor cells, and disrupted the timing and magnitude of astroglialogenesis(5,7–11).

In the liver, DNA methylation regulates tissue-specific gene transcription, hepatocyte differentiation and reprogramming of progenitor cells in collaboration with other epigenetic regulators controlling histone modifications(12). In mice, most of the epigenetic modifiers, including *Dnmt1*, are down-regulated by postnatal day 21 in parallel with cessation of hepatocyte proliferation, acquisition of the fully differentiated state and establishment of proper tissue architecture(13, 14). Accordingly, epigenetic profiling provided evidence that CpG methylation serves to stabilize gene-specific transcriptional states during postnatal liver ontogenesis(15). Here we employed liver-specific *Dnmt1* knockout mice to address the importance of DNMT1 for early postnatal lineage commitment and hepatic histogenesis. Our results demonstrate absolute DNMT1 requirement for maintaining genomic stability and functional maturity of hepatocytes during early postnatal liver growth and regeneration.

Materials and Methods

Animal Experiments

Liver specific deletion of *Dnmt1* was achieved by breeding *Dnmt1^{fl/fl}* mice(5) with homozygous *Alb-Cre* transgenic mice to generate *Dnmt1^{fl/fl};Alb-Cre⁺* (*Dnmt1^{alb}*). Genotyping was done by PCR analysis of tail genomic DNA (gDNA), and specificity of *Dnmt1* deletion was verified on gDNA from isolated hepatocytes. Male mice were used unless otherwise indicated. For acute liver injury, 4, 8 and 20 week-old mice received a single intraperitoneal injection of CCl₄ (10% in olive oil) (Sigma-Aldrich, St. Louis, MO) at 0.2 ml/kg. To induce CYP enzymes, 8 week-old mice received three daily doses of 100 mg/kg of PB (Sigma-Aldrich) and examined 24 hours after the last injection. To induce hepatocyte proliferation, 8 week-old mice were subjected to a standard partial hepatectomy. All procedures were performed according to protocols approved by the Animal Care and Use Committee of the National Institutes of Health.

Molecular and Gene Expression Analysis

Western blotting, isolation of genomic DNA and RNA, quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), were performed using standard methods. Sentrix Mouse Expression BeadChips version 2 (Illumina, San Diego, CA), EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, Irvine, CA), and nCounter® miRNA Expression Assay Kits (NanoString Technologies, Seattle, WA) were used for gene expression microarray, methylation specific microarray, and miRNA profiling.

Statistical Analysis

We applied Student's t test for all significance, unless otherwise denoted.

Additional Methods including Generation of *Dnmt1^{fl/fl};Mx1-Cre⁺* Mice; *Dnmt1* Knockdown by shRNA; Transmission Electron Microscopy; Gene Expression, Methylation and miRNA Profiling can be found online in the Supplementary Materials and Methods.

Results

Time Sequence of *Dnmt1* Knockout-Induced Events

To explore the epigenetic contribution to adult liver physiology, we generated liver-specific *Dnmt1* knockout mice by crossing *Dnmt1^{fl/fl}* with Albumin-Cre (*Alb-Cre*) transgenic mice (*Dnmt1^{alb}*) (Figure S1A). *Dnmt1* expression in mutant livers was reduced to less than 20% and 10% by 1 and 2 weeks of age, respectively (Figure S1B). Furthermore, we found a predicted upregulation of imprinted genes (*Igf2*, *H19*) and derepression of normally transcriptionally silent endogenous Intracisternal-A-particle (IAP) retroviral elements (Figure S1B)(4). *IAP* transcripts were elevated up to 100–1000-fold throughout the first 8 weeks after birth, and were reduced to about 10-fold by 20 weeks suggesting a less efficient *Dnmt1* gene deletion in older mice (see below). Electron microscopic observations confirmed the presence of mature IAP particles in *Dnmt1^{alb}* hepatocytes (Figure S1C). The *Dnmt1* deletion did not affect expression levels of the *de novo* DNMTs, *Dnmt3a/3b* (Figure

S1B) and decreased the genome-wide 5-methylcytosine content by 4–8 weeks with no significant differences at 12–20 weeks (Figure S1D).

The consequences of *Dnmt1* loss were evaluated by morphological, molecular, and functional studies during early postnatal hepatogenesis when mouse hepatocytes experience the greatest changes in *Dnmt1* expression, cease to proliferate and functionally mature(13). *Dnmt1^{alb}* pups were indistinguishable from *Dnmt1^{fl/fl}* littermates at birth. However, starting from an early age, *Dnmt1^{alb}* livers showed signs of progressive liver injury and developed striking structural abnormalities. By 8–12 weeks, *Dnmt1^{alb}* livers acquired a nodular organization, which persisted up to 60 weeks (end stage of observation) (Figure 1A–C). The nodules varied in size and were composed of more basophilic hepatocytes which were remarkably smaller than the surrounding giant hepatocytes with signs of degeneration (Figure 1B and S1C). The nodular growth was accompanied by periportal fibrosis and active infiltration of CD45⁺ inflammatory cells, resulting in modest liver enlargement by 8 weeks (Figure 1B and 1C). This coincided with a peak elevation in serum markers of liver injury (aspartate aminotransferase, alanine aminotransferase and bilirubin) (Figure 2A). The proportion of nodular parenchyma steadily increased concomitant with amelioration of inflammation and a partial resolution of fibrosis causing normalization of liver mass (Figure 1A–C). By 20 weeks, the nodular parenchyma constituted close to 100% of hepatic tissue with fibrosis limited to periportal septa around visible nodules.

To evaluate the genomic differences, we profiled the kinetic changes in gene expression in whole-liver samples from 4, 8 and 20 week-old *Dnmt1^{fl/fl}* and *Dnmt1^{alb}* mice. Consistent with a causal role for *Dnmt1* in gene silencing, *Dnmt1* loss was largely associated with gene derepression, including reactivation of imprinted genes and *IAP* (Figure 1D and Table S1). Gene Set Enrichment Analysis (GSEA) generated from a liver hypermethylation-specific gene signature confirmed a preferential activation of a large set of genes normally expected to be in a repressed state (Figure 1E)(16). In total, we identified 231 common differentially expressed genes (2-fold cutoff, $P < 0.001$, FDR < 0.05) taking into account age and genotype (Figure 1D). The gene expression changes were associated with remarkable cellular and architectural changes in *Dnmt1^{alb}* livers at 4 and 8 weeks of age. These genes were involved in biological processes associated with cell cycle regulation, immune response, DNA repair as well as cytoskeleton arrangement and cell adhesion (Figure 1F and Table S1). By 20 weeks, the transcript abundance of the common genes was greatly decreased in *Dnmt1^{alb}* livers, which clustered together with the age-matched controls, suggesting a near complete restoration of the transcriptomic landscape (Figure 1D).

Dnmt1 Deletion in Hepatocytes Triggers DNA Damage and Activates DNA Damage Response Pathways

Consistent with a crucial role for *Dnmt1* in genome stability, DNA damage was greatly elevated in mutant livers. Quantification of p-H2A.X fluorescence, a marker of DNA damage response (DDR) following double-strand breaks (DSBs), revealed on average 75–140-fold increase by 8 weeks of age which was declined by 12 weeks (Figure 2A and B, top)(17). The DNA damage was paralleled by a similar kinetics of p53 activation, a key component of DDR, upregulation of the proapoptotic markers (Figure 2C) and induction of

apoptosis (Figure 2A and 2B, bottom)(18). Functional annotation of key expression changes confirmed a significant enrichment of genes associated with a DDR and apoptosis gene signatures (Figure 2D). The magnitude of DNA damage and apoptosis was reduced in small nodular hepatocytes as compared to the peri-nodular parenchyma. Nonetheless, they were still more prone to DNA damage and died more often both from apoptosis (Figure 2A and 2E, left and middle) and necrosis (Figure S2B) than *Dnmt1^{fl/fl}* hepatocytes which at 8 weeks showed essentially no signs of cellular damage.

Loss of *Dnmt1*, an integral component of replication complexes, did not immediately halt DNA replication(2). During the first 4 weeks of age, *Dnmt1^{alb}* hepatocytes continued to proliferate even at a higher rate than the age-matched controls, most likely in an attempt to compensate for the enhanced cell death. However by 8 weeks, the mutant cells exhausted their proliferative capacities and were no longer able to sustain liver structural integrity (Figures 2A and 2E, right). By this time, the compensatory liver regeneration was shifted to a nodular growth characterized by a much higher proliferative activity as compared to the peri-nodular parenchyma.

DNA Damage Promotes Senescence State Followed by Clearance of Senescent Cells

There was a strong correlation between excessive genotoxic stress caused by *Dnmt1* deletion and induction of hepatocyte senescence. Among the canonical molecular triggers of senescence were p53, p21, p27, and most notably p16, encoding cyclin-dependent kinase inhibitor 2A (*Cdkn2a*) known to be activated by epigenetic derepression, while the expression of Skp2p45, a negative regulator of p27, was reduced (Figure 3A)(19). Morphologically, the *Dnmt1^{alb}* hepatocytes were stained with Sudan Black B (Figure 3B), reflecting a buildup of lysosomal mass, showed a progressive cell enlargement (Figure 2F, S2B and S2C) as well as increased autophagy as judged by electron microscopy (Figure 3C) and accumulation of autophagy marker LC3B (Figure 3A), all features of senescent cells(20–22). Furthermore, *Dnmt1^{alb}* livers showed a strong accumulation of inflammatory cells exclusively around the enlarged peri-nodular hepatocytes (Figure S2B and S2D), a robust induction of multiple cytokines and chemokines (Figure 3D and S2E) and enhanced macrophage recruitment (Figure 3E) consistent with acquisition of a senescence-associated secretory phenotype (SASP)(21). In addition, there was activation of TGF β signaling (Figure 3A and S2F), another SASP component known to trigger senescence via upregulating p21 and p27 and connective tissue growth factor (CTGF) (Figure 3A). Finally, when we carried out a standard partial hepatectomy experiment, only small nodular cells showed an adequate increase in proliferation whereas peri-nodular senescent hepatocytes were no longer responsive to growth stimulation (Figure 3F). These findings suggest a causal connection between DNA damage-induced senescence, increased secretion of pro-inflammatory signals, and effective elimination of *Dnmt1* mutant hepatocytes followed by a gradual reconstitution of damaged parenchyma via nodular regeneration.

Differentiation Defects in Postnatal Hepatocytes Caused by Dnmt1 Deletion

We next assessed the impact of *Dnmt1* deletion on functional maturity of hepatocytes. Since the genome-scale profiling indicated that many of the differentially expressed genes in *Dnmt1^{alb}* livers participated in xenobiotic metabolism or encoded transporter proteins of

the solute carrier (SLC) transporter family (Table S1), we compared the efficiency of xenobiotic detoxification in *Dnmt1^{fl/fl}* and *Dnmt1^{alb}* mice using a well-known carbon tetrachloride (CCl₄) model of acute liver injury(23).

Strikingly, the *Dnmt1^{alb}* mice were resistant to CCl₄-mediated hepatotoxicity and developed no or very little pericentral necrosis during the first 8 weeks of postnatal life (Figure S3A and S3B). The lack of necrosis was coincident with a strong repression of CYP2E1 expression, the major CYP enzyme responsible for CCl₄ bioactivation and a slight but consistent decrease in HNF4α, a master regulator of hepatocyte differentiation (Figure S3A and S3C)(23, 24).

To evaluate architectural maturation of hepatocytes, we then performed double immunofluorescence staining with E-cadherin and glutamine synthetase (GS), the markers of periportal (zone 1) and pericentral (zone 3) hepatocytes, respectively(25). Quantification of fluorescence signals revealed prominent changes in the zonal distribution of E-cadherin and GS staining in *Dnmt1^{alb}* livers (Figure S3D), reflecting disruption of proper liver zonation and gene expression. After a complete liver repopulation, the remodeled parenchyma gradually albeit partially regained functional competence (Figure S3A–D).

To strengthen these observations, we studied the induction of *Cyp* genes by phenobarbital (PB), a prototypical inducer of CYPs and other xenobiotic metabolizing enzymes(26). Since the response to PB is mediated at least in part by nuclear receptor constitutive androstane receptor (CAR) subjected to epigenetic regulation, we assessed the effect of PB on transcriptional activation of two CAR target genes, *Cyp2b10* and *Cyp3a11*(26, 27). Interestingly, *Dnmt1^{alb}* livers showed a two-fold decline in *Car* mRNA levels while the expression of pregnane X receptor (*Pxr*), another master regulator of xenobiotic metabolizing enzymes, was increased (Figure S3E). Loss of *Dnmt1* also resulted in a strong reduction of PB-mediated nuclear translocation of CAR protein (Figure S3F) and repressed the capacity to induce *Cyp2b10* and *Cyp3a11* genes (Figure S3E).

Recruitment of Hepatic Progenitor Cells and Origin of Nodular Cells

Widespread senescence and failure to regenerate *Dnmt1^{alb}* hepatocytes (Figure 3) caused a prominent activation of ductular reaction containing hepatic progenitor cells (HPCs), also referred to as oval cells, and a subsequent formation of foci of small basophilic hepatocytes(28). HPCs were identified by morphology, periportal location and staining with the known markers A6 and CK19(29, 30). HPCs were first detected at 4 weeks, became prominent by 8 weeks and diminished by 20 weeks when the parenchyma underwent full remodeling (Figure 4A and S4A). This coincided with kinetic changes in HPC proliferation (Figure 4B) and a significant enrichment of HPC gene signature (Figure S4B). The microarray data were validated by mRNA levels of the selected 'stemness' genes (Figure S4C)(30–32). The kinetic changes in HPCs accumulation were tightly associated with the activation of Kupffer and stellate cells (Figure 3D and S4D), the main cell components of HPC niche(33).

Electron microscopy supported lineage connection between the HPCs and newly-formed hepatocytes. Already at 4 weeks, we found a gradual transition to hepatocyte morphology

within the expanding progenitor/ductular cell population in close vicinity to the bile ducts (Figure S4E). In agreement with historical descriptions of oval cells in rodent liver, the first ductular hepatocytes (DHs) or hybrid hepatobiliary cells retained a close connection with the neighboring ductular cells via common lumen and shared basal membrane but have a distinctly different size and morphology (Figure S4E, b–f)(34, 35).

Of even greater significance, the DHs displayed a more prominent accumulation of IAP particles (Figure S4E, d–e). In comparison, the typical biliary epithelial cells (BECs) lining portal bile ducts (Figure S4E, a) and ductular oval cells (Figure S4E, c) had only few IAP particles in line with reported low levels of *Alb-Cre* promoter activity(36). The cells adopting hepatocyte morphology were less developmentally mature. They lost CK19 expression along with a ductular organization but continued to express a progenitor marker A6 (Figure 4A and 4C). The A6⁺ cells with hepatocyte morphology and immediate periportal location became positive for a classic hepatocyte marker HNF4α (Figure 4D). The frequency of A6⁺/HNF4α⁺ cells peaked at 8 weeks, when they formed repopulation nodules, and was inversely correlated with nodule size suggesting continuing lineage-specific maturation (Figure 4E). These results support the mobilization of HPCs as a predominant mechanism for restoration of the irreversibly damaged hepatocytes in *Dnmt1^{alb}* mice (Figure 4F).

HPC-Derived Hepatocytes Display Low Efficiency of *Dnmt1* Deletion

To address the mechanism for extended survival of the HPC-derived hepatocytes, we isolated hepatocytes and evaluated the *Dnmt1* deletion efficiency. The yield of viable *Dnmt1* mutant hepatocytes was very low at 4 and 8 weeks (Figure S5A) highlighting a survival disadvantage of cells with excised *Dnmt1* alleles. PCR-based genotyping of DNA showed the highest level of *Dnmt1* deletion at 4 and 8 weeks which was paralleled by a greatly reduced *Dnmt1* mRNA and protein levels (Figure S5B, S5C and S6A). The mRNA levels of *Dnmt3a/3b* did not change (Figure S5C) suggesting that these enzymes did not compensate for *Dnmt1* loss. However, by 12–20 weeks, when newly-formed hepatocytes reconstituted almost entire parenchyma, they contained mixed amounts of *Dnmt1* deleted and floxed alleles (Figure S5B, left). This was consistent with a partial recovery of *Dnmt1* mRNA and protein (Figure S5C and S6A) without detectable changes in expression levels of either *Cre* or *Alb* (Figure S5B and S5C). Nevertheless, the recombined *Dnmt1* allele persisted in older mice (Figure S5B), and the renewed parenchyma retained the *Dnmt1*-loss-mediated defects in hepatic architecture and differentiation as well as increased expression of the imprinted genes *Igf2* and *H19* (Figure S5C) indicating a “tug-of-war” between transcription and silencing of the *Alb-Cre* transgene in *Dnmt1^{alb}* mice.

To confirm the *Dnmt1* requirement for HPCs survival, we generated a stable *Dnmt1^{fl/fl}* HPC line expressing short hairpin RNAs (shRNAs) against *Dnmt1*, and clonally isolated cells which exhibited effective *Dnmt1* knockdown (>70–80%), as well as expected up-regulation of *Igf2* and *H19* and derepression of *IAP* (Figure 5A and 5B). Similar to *Dnmt1* mutant hepatocytes *in vivo*, the *Dnmt1* knockdown in cultured HPCs also triggered DDR signaling and caused inhibition of cell growth due to the activation of apoptotic and senescence

pathways (Figure 5C–E). Furthermore, these cells failed to initiate sphere growth under serum-free condition (Figure 5F).

Finally, we generated *Dnmt1^{fl/fl};Mx1-Cre* conditional knockout mice to achieve *Dnmt1* deletion in terminally differentiated hepatocytes (Figure S5D). Activation of a ubiquitous interferon-inducible *Mx1* promoter at 12 weeks of age reduced the *Dnmt1* mRNA levels and increased expression of *IAP* mRNA to the same degree as in *Dnmt1^{alb}* livers (Figure S5E). However, it did not produce any detectable changes in phenotype during the follow-up observation for 2 months (Figure S5F) consistent with a decreasing demand for *Dnmt1* expression with age(13). These results underscore a more stringent requirement for *Dnmt1* during hepatocyte-specific differentiation of HPCs as compared to the maintenance of mature hepatocyte phenotype and a strong competitive disadvantage of *Dnmt1* deleted cells at the time critical for physiological liver growth and regeneration.

Kinetics of Genome-wide Demethylation in *Dnmt1^{alb}* Hepatocytes

Given a mechanistic link between the DNMT1 and Polycomb group proteins in the epigenetic gene silencing, we also assessed the impact of *Dnmt1* genetic inactivation on Polycomb Repressive Complex 2 (PRC2) proteins involved in formation of a repressive chromatin state(37, 38). We found a significant reduction of EZH2 (Enhancer of Zeste homolog 2) and SUZ12 (Suppressor of Zeste 12 homolog) levels in hepatocytes isolated from 4 and 8 week-old *Dnmt1^{alb}* mice (Figure S6A). This was paralleled by a similar decline in the repressive histone marks H3K9me2 and H3K27me3 while the expression of active histone marks (H3K4me3 and H3K36me3) did not change (Figure S6B). Immunofluorescence staining confirmed that *Dnmt1* loss caused a strong down-regulation of EZH2 and H3K27me3 in the majority of mutant cells and was partially restored in the nodular hepatocytes (Figure S6C).

When we examined genome-wide CpG site methylation, 11,755 out of 485,000 CpG loci showed a significant change in methylation (Figure 6A). The unique CpG sites specific to each time point were represented by 4656 genes at 4 weeks, 1425 at 8 weeks and 1750 at 20 weeks, indicating a strong time-dependent impact of *Dnmt1* inactivation on the global CpG methylation levels. Loss of the methylation sites was predominant in 4 and 8 week-old hepatocytes, affecting 57% and 62% CpG loci, respectively (Figure 6B), as compared to 39% at 20 weeks.

CpG loci associated with CpG islands or regions in close proximity to islands, such as shores and shelves, comprised more than 60% of affected CpG sites (Figure 6C). However, the methylation state of CpGs outside this genomic region gradually increased at 8 and 20 weeks, whereas affected CpG islands decreased (Figure 6D).

Analysis of the differentially regulated networks specific to each age group showed that at 4 weeks the hypomethylated CpG loci corresponded to genes controlled by TGFβ, NF-κB, Cyclin D and POU5F1/OCT4 signaling involved in a variety of cellular activities, including cellular and tissue development, proliferation, cell death, and progenitor cell differentiation along with altered protein ubiquitination and inflammatory response (Figure S7A–C). While the global changes in methylation state were gradually decreased with time (Figure 6E and

6F), residual aberrations were associated with cellular damage. Even at 20 weeks, when a stabilized transcriptional program showed only few changes, an altered DNA methylation landscape prevailed, contributing to the continued proliferation, apoptosis and DNA repair (Figure S7A–E). Only few CpG loci were mutually regulated across all time points.

Furthermore, the hierarchical clustering revealed a preferential effect of *Dnmt1*^{alb} on the methylation profile at 4 weeks, including genes (*JARID2*, *ARID1B*, *GNAS* and *SMARCA6*) and miRNAs (*Lin28*, *Let7a3* and *Let7c*) involved in regulation of stem cells, transporters and chromatin-remodeling (Figure 6E).

A more direct miRNA expression profiling revealed 24 and 38 differentially expressed miRNAs in 4 and 8 week-old hepatocytes, 15 of which were commonly deregulated, and only one miRNA in 20-week group as compared to paired *Dnmt1*^{fl/fl} controls (Figure S8A). Of these miRNAs, five (miR-152, miR-29b, miR-322, miR-743a, miR-871) were under potential regulation of DNA methylation (Figure S8B, S8C and Table S2). Functional analysis of targets predicted by two different software (DIANA-microT v3.0 and miRDB) (Figure S8B–D) showed that many of the commonly activated miRNAs as well as those specific for 4-week and 8-week groups were involved in regulation of DDR and p53 signaling. Other deregulated miRNAs highly associated with the *Dnmt1*^{alb} phenotype participate in hepatic metabolism, fibrosis, inflammation, differentiation and activation of progenitor cells (Table S2). Overall, our genome-scale studies demonstrate that genetic inactivation of *Dnmt1* in hepatocytes induced global DNA methylation pattern changes affecting gene expression, histone modifications and miRNAs.

DISCUSSION

Here, we describe a mouse model in which *Alb-Cre*-driven inactivation of *Dnmt1* during early postnatal liver growth yields two different phenotypic outcomes depending on the degree of *Dnmt1* deletion. In hepatocytes, the loss of 80–90% of *Dnmt1* normal levels was incompatible with survival and ability to maintain tissue homeostasis. Most of *Dnmt1*^{alb} cells ceased to proliferate, entered the senescence state and eventually died due to massive genome impairment and activation of DNA damage checkpoints. Liver regenerated via repopulation from a progenitor compartment in which lineage-dependent differences in the differentiation state resulted in the variable *Alb-Cre* expression and thus in the extent of *Dnmt1* deletion, providing basis for selection of less mature and therefore less damaged HPC progeny (Figure 7). In support of this, a more efficient knockdown of *Dnmt1* in cultured HPCs caused severe DNA damage, cell cycle arrest, senescence and cell death. These data agree with previous studies reporting diverse phenotypic effects stemming from the variable *Dnmt1* expression in mammalian cells(39, 40). In striking contrast, *Mx1*-driven inactivation of *Dnmt1* in adult mice did not produce any phenotype consistent with ontogenic differences and differential requirement for *Dnmt1* in actively proliferating and quiescent tissues(8, 13).

Mechanistically, *Dnmt1* loss at the time of hepatocyte active proliferation and maturation caused genome-wide demethylation and deregulated genes controlling self-renewal and differentiation. There were temporal and quantitative correlations between genome-wide

demethylation and dysregulation of transcription with the kinetic changes in *Dnmt1^{alb}* phenotype. Many of the identified genes as well as upregulated miRNAs were involved in DNA damage and activation of p53 checkpoints. This is in line with the recent observations describing a link between genetic disruption of *Dnmt1* in perinatal intestinal progenitor cells, global hypomethylation and genomic instability(8). In addition, we found a strong up-regulation of IAP transposable elements associated with induction of DNA damage and apoptosis(5) suggesting that in our model *Dnmt1* deletion may activate both p53-dependent and independent apoptotic pathways.

Notably, *Dnmt1* loss also caused a strong time-dependent reduction in PRC2 proteins, establishing a direct connection between two key epigenetic repression systems(37). Several epigenetic studies demonstrated the requirement of PRC2 and repressive histone marks for DSB repair(17, 41). PRC2 proteins reportedly protect from the cell death by acting directly at sites of DNA damage in human osteosarcoma cells(17). PRC2 and H3K27me3 are also associated with the PTEN up-regulation following the repression of AKT/mTOR signaling in murine bone marrow cells(41). These functional observations suggest that *Dnmt1* deletion may magnify the extent of DNA damage via a concomitant down-regulation of PRC2 proteins and their downstream targets.

To minimize the rapid deterioration of tissue homeostasis due to massive DNA damage, *Dnmt1* mutant hepatocytes entered the state of replicative senescence characterized by hallmark features, such as H2AX phosphorylation, up-regulation of tumor suppressors (p53, TGF β) and cell cycle inhibitors (p16, p21, p27), prominent secretion of cytokines as well as cell enlargement and exhaustion of proliferative potential(21). *Dnmt1^{alb}* hepatocytes were eventually eliminated by a compensatory apoptosis and effective immune-mediated clearance. Importantly, conditional deletion of *Dnmt1* did not affect the non-parenchymal cells, including resident macrophages and recruited immune cells, the crucial cellular elements required for a resolution of tissue damage and transition to the regeneration phase(33).

Dnmt1^{alb} mice also present a dramatic example of liver regeneration via activation of the resident progenitor cells. This process occurred in a stepwise fashion involving a vigorous activation of HPCs and subsequent formation of foci of small hepatocytes gradually replacing the giant senescent hepatocytes. Currently, the relative contribution of hepatocytes and resident progenitor cells to liver regeneration is a subject of passionate debate. The lineage tracing experiments emphasized the remarkable plasticity of both hepatocytes and BECs(42). They also produced conflicting results regarding the contribution of HPC to the hepatocyte pool ranging from marginal to significant(31, 43–45). Our results support the activation of the existing HPCs as a predominant mechanism for restoration of the irreversibly damaged *Dnmt1^{alb}* livers. Despite the apparent low birthrate, the cumulative effect of progenitor-to-hepatocyte differentiation appears to be significant since the HPC progeny continued to proliferate as hepatocytes to replace the senescent dying old hepatocytes. Notably, the most recent study using a lineage tracing strategy demonstrated the efficiency of endogenous HPCs to regenerate the adult liver following a large scale parenchymal injury and senescence caused by the loss of *Mdm2* in hepatocytes(30). Hepatocyte-specific deficiency of PR-SET7, another key epigenetic modifier involved in

DNA repair, also caused a progenitor-mediated regeneration to compensate for extensive liver damage, reinforcing the essential role of maintaining genome integrity for liver homeostasis and regeneration(32).

Several lines of evidence support the role of HPCs in tissue repair in the *Dnmt1^{alb}* injury model. First, it seems unlikely that the *Dnmt1* mutant hepatocytes, which accumulate thousands of IAP particles, undergo a replicative senescence and are incapable of repairing themselves, can contribute to the restoration of liver mass. Second, consistent with generation of hepatocytes from HPCs, electron microscopy revealed the presence of DHs which were structurally connected with the neighboring ductular cells via a shared common lumen and a basal membrane. Third, these DHs were capable of upregulating IAP particles which argue against their origin from the *Dnmt1^{fl/fl}* hepatocytes escaping *Dnmt1* recombination. Fourth, a significant fraction of small nodular HNF4 α ⁺ hepatocytes retained expression of progenitor cell marker A6. Importantly, the frequency of A6⁺/HNF4 α ⁺ hepatocytes was inversely correlated with nodule size. Therefore, we favor the possibility that lineage-dependent differences in the *Alb-Cre* transgene activity may create diverse pattern of DNA methylation within the HPC progeny. Since the differences in DNA methylation dynamics and thus in the extent of DNA damage may define whether the cell survives and renews (e.g. HPC) or undergoes senescence and dies (e.g. mature hepatocyte), this epigenetic heterogeneity may provide a means for selecting the HPC offspring which are less mature and consequently expressing higher levels of *Dnmt1*. In support of this, the expanding nodular parenchyma exhibited a reduced range of mature hepatocyte properties, including repression of CAR and a number of CYP enzymes involved in xenobiotic metabolism. Furthermore, the mutant livers at the peak of injury at 4–8 weeks of age were unable to maintain structural gradients of liver functions along the portal-central vein axis demonstrating the essential role of *Dnmt1* for successful terminal differentiation.

In conclusion, we demonstrate the absolute requirement for DNA methyltransferase DNMT1 for maintaining genomic stability and functional maturity of hepatocytes during early postnatal liver growth. The *Dnmt1^{alb}* conditional knockout mice provide a unique experimental model to study the role of senescence and the contribution of progenitor cells to physiological and regenerative liver growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Alb albumin

ALT	alanine aminotransferase
AST	aspartate aminotransferase
BEC	biliary epithelial cell
CAR	constitutive androstane receptor
CCl₄	carbon tetrachloride
CK19	cytokeratin 19
CYP	cytochrome P450
DDR	DNA damage response
DH	ductular hepatocyte
DNMT	DNA methyltransferase phenotype
DSB	double-strand break
EpCAM	epithelial cell adhesion molecule
FACS	fluorescence-activated cell sorting
GS	glutamine synthetase
GSEA	gene set enrichment analysis
HNF4α	hepatocyte nuclear factor 4 alpha
HPC	hepatic progenitor cell
H2AX	H2A histone family member X
IAP	intracisternal-A-particle
Igf2	insulin-like growth factor 2
LC3B	microtubule-associated protein 1 light chain 3 beta
PARP	poly ADP ribose polymerase
PB	phenobarbital
PH	partial hepatectomy
PRC	polycomb repressive complex
SASP	senescence-associated secretory
SKP2	S-phase kinase-associated protein 2
TGFβ	transforming growth factor beta
TUNEL	TdT-mediated dUTP nick end labeling

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Authors names in bold designate shared co-first authorship.

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Figure 1. Ontogeny of liver injury and repair in *Dnmt1^{alb}* mice. (A) Changes in gross liver appearance. (B) H&E, Masson's Trichrome (MT) and CD45 staining. pv, portal vein; cv, central vein; N, nodule. Scale bars; 100μm. (C) Quantification of total nodular area, MT staining and CD45⁺ cells and age-dependent changes in liver/body mass. Nuclei counterstained with DAPI. Data are means ± SD (n=5). **P*<.01 versus age-matched *Dnmt1^{fl/fl}* mice. (D) Hierarchical clustering based on the significantly altered genes profiled in whole livers. (E) GSEA using hypermethylated 479-gene signature in liver cancer. The heatmap depicts the enrichment of the 84 most significant genes upregulated in *Dnmt1^{alb}*

compared to the expected repressed state in *Dnmt1^{fl/fl}* at week-8. (F) The highly significant functional networks identified by GeneGo analysis of the commonly altered 231-genes in whole liver. See also Figure S1 and Table S1.

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Figure 2.

Liver-specific *Dnmt1* deletion activates DNA damage response (DDR) pathways in hepatocytes. (A) H&E, p-H2A.X, TUNEL, and β-catenin/Ki67 staining at 8-weeks mice. pv, portal vein; N, nodule. Scale bars; 50μm. (B) Quantification of p-H2A.X⁺ (top) and TUNEL⁺ (bottom) hepatocytes. (C) Western blots of whole liver lysates for indicated proteins involved in DDR and apoptosis. Actin; loading control. FL, *Dnmt1^{fl/fl}*; KO, *Dnmt1^{Δalb}* (D) GSEA plots using DDR and apoptosis signatures (GO terms) in *Dnmt1^{Δalb}* livers as compared to *Dnmt1^{fl/fl}*. (E) Comparison of p-H2A.X (left) and TUNEL (middle) staining in nodular and perinodular parenchyma in *Dnmt1^{Δalb}* mice at 8-weeks and quantification of

Ki67⁺ hepatocytes (right). (F) Size distribution of hepatocyte nuclei in *Dnmt1^{fl/fl}* and *Dnmt1^{alb}* livers. Nuclei counterstained with DAPI. Data are means $\pm$ SD (n=5) (B, E). **P*<.01, ***P*<.05 versus age-matched *Dnmt1^{fl/fl}* mice. See also Figure S2.

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Figure 3.

Acquisition of senescent phenotype in *Dnmt1*^{alb} liver. (A) Western blots of whole liver lysates for indicated proteins involved in cell-cycle regulation, cellular senescence, TGFβ-signaling pathways and autophagy marker protein LC3B. Actin, loading control. FL, *Dnmt1*^{fl/fl}, KO, *Dnmt1*^{alb} (B) Sudan black B staining of liver sections in 8-weeks mice. Arrowheads, senescent cells. (C) Electron microscopy images of two hepatocytes undergoing autophagic cell death (a) and a Kupffer cell (b) containing autophagic vacuoles surrounded by double layer membranes in 8-weeks *Dnmt1*^{alb} liver. Boxes are selected regions for magnified new. Nu, nucleus, *, indicate collagen fibers. (D) Transcriptome analysis of kinetic changes in the expression of cytokine-related genes in *Dnmt1*^{alb} livers at

different age. (E) H&E staining (top) and double-immunofluorescence with F4/80 and CK19 (bottom) in *Dnmt1^{fl/fl}* and *Dnmt1^{alb}* livers. Graph shows quantification of F4/80 staining. (F) H&E (top) and immunofluorescence of Ki67 (bottom) before and 48 hours after partial hepatectomy (PH) in 8-weeks mice. Graph shows quantification of Ki67⁺ hepatocytes. Scale bars; 50µm (E, F). Nuclei counterstained with DAPI (E, F). pv, portal vein; cv, central vein; N, nodule. Data are means ± SD, (n=5) (E) and means ± SEM, (n=2) (F). **P*<.01 versus age-matched *Dnmt1^{fl/fl}* mice.

Figure 4.

Activation and differentiation of hepatic progenitor cells in *Dnmt1^{alb}* livers. (A) H&E staining and double-immunofluorescence with A6/CK19. Boxes are selected regions for magnified new (bottom images). (B) Double-immunofluorescence with CK19/PCNA in *Dnmt1^{fl/fl}* and *Dnmt1^{alb}* at 4-weeks. Inset shows higher magnification of boxed area. Graph shows quantification of CK19⁺/PCNA⁺ cells. (C) Kinetic changes in the frequency of A6⁺/CK19⁺ and A6⁺/CK19⁻ cells in *Dnmt1^{alb}* livers. (D) Double-immunofluorescence with A6/HNF4α. Graph shows quantification of A6⁺/HNF4α⁺ hepatocytes in the periportal areas in *Dnmt1^{alb}* livers. (E) Double-immunofluorescence with A6/HNF4α in small and large nodules. Graph shows the inverse correlation between the frequency of A6⁺/HNF4α⁺

hepatocytes and nodule size in *Dnmt1^{alb}* livers. (F) Scheme of kinetic changes in *Dnmt1^{alb}* phenotype defined by immunohistochemical staining, immunoblotting and transcriptomic analysis. Nuclei counterstained with DAPI. pv, portal vein; cv, central vein; N, nodule, white triangles, A6⁺/HNF4α⁺ hepatocytes. Data are means ± SD, (n=5) (B, C); (n=3) per 5 periportal areas (D) or 5 nodules each (E). **P*<.01 versus age-matched *Dnmt1^{fl/fl}* mice (B); &.#*P*<.01 versus the A6⁺/CK19⁺ and A6⁺/CK19⁻ cells (C); **P*<.01(D). See also Figure S3.

Figure 5.

Knockdown of *Dnmt1* results in DNA damage, apoptosis and senescence in hepatic progenitor cells (HPC) *in vitro*. (A) Experimental design for generation of HPC lines from normal *Dnmt1*^{fl/fl} livers. FACS sorting of isolated nonparenchymal cells to enrich for PE-EpCAM⁺/APC-Lineage⁻ HPC cells (top), sphere assay to select cells possessing self-renewal capacity (middle), single cell expansion in 2D culture (bottom). (B) The efficiency of *Dnmt1* knockdown by shRNA in *Dnmt1*^{fl/fl} HPC lines measured by quantitative RT-PCR, western blotting (WB) of whole cell lysates (left), and reactivation of intracisternal-A-particles (*Iap*) and imprinted genes (*Igf2* and *H19*) (right). (C) WB of whole cell lysates for indicated proteins involved in DNA damage response and apoptosis. (D) Immunofluorescence with p-H2A.X and cleaved PARP, and SA-β-gal staining. Insets show magnified views. Nuclei counterstained with DAPI. (E) Quantification of p-H2A.X and cleaved PARP positive cells, and SA-β-gal positive areas, as well as cell viability. (F) Bright field images of sphere-forming assay. Graphs show quantification of sphere numbers and sphere size. WT, untransduced HPCs; Scr, scrambled vector-transduced HPCs; sh1 and sh2,

two single-cell derived clonal HPC cell lines transduced with *Dnmt1* shRNA. Data are means $\pm$ SD, (n=3); * P <.01 versus WT. In C–E, the cells were cultured for 3 days. Scale bars; 50 μ m (D); 200 μ m (F). Actin, loading control (B, C).

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Figure 6. Aberrant DNA methylation in *Dnmt1*^{alb} hepatocytes. (A) Dendrogram for hierarchical clustering of the significantly altered CpG loci in *Dnmt1*^{alb} hepatocytes (n=3 per genotype/age) (top). Degree of unique genes represented by significantly altered CpG loci across the time points (bottom). (B) Fraction of hyper- versus hypomethylated CpG loci. (C) Location of significantly altered CpG loci in the promoter region. (D) Altered regulatory elements (top). The majority of changes were in CpG loci in enhancer regions. DHS, DNA hypersensitive sites; DMR, differential methylated regions. The percentage of altered loci adjusted to the total number of regulatory elements (grey). Location of CpG loci in gene

regions (bottom). TSS, transcription start site. (E) Hierarchical clustering based on a class comparison using random variance modeling. *P* values for significantly altered genes were computed using 10000 random permutations. A total of 614 genes were significant at $P < .001$ by Univariate F test. The heatmap was scaled to ± 0.1 (range of -0.6 to 0.7 in total change). (F) Schematic changes in transcriptome, methylation and miRNA expression underlining evolution of *Dnmt1^{alb}* phenotype. See also Figure S6–S7 and Table S2.

Figure 7. Graphic summary of major discoveries from the *Dnmt1*^{-/-}; *Alb-Cre*⁺ mouse. *Alb-Cre*-driven inactivation of *Dnmt1* during early postnatal liver growth yields two different phenotypic outcomes depending on the degree of *Dnmt1* deletion.