Original Research
Brief Communication

Expression of histone variant, H2A.1 is associated with the undifferentiated state of hepatocyte

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Abstract

Recent studies suggest the incorporation of histone variants into the chromatin regulate cellular proliferation, differentiation, and de-differentiation. We have earlier reported the increase of H2A.1 variant during sequential de-differentiation of hepatocyte to hepatocellular carcinoma. Here, we decipher the alterations in expression of H2A.1 and H2A.2 variants during rat liver embryogenesis and regeneration. The expression of H2A.1 and H2A.2, at protein and mRNA level, does not alter in normal cellular proliferation associated with regeneration of liver post PH. In contrast, gradual decrease of H2A.1 with increase of H2A.2 is observed during differentiation of embryonic to adult liver. Furthermore, the accumulation of H2A.1 is higher in embryonic stem cells compared to normal adult liver. Collectively, these data support a strong correlation of H2A.1 expression with undifferentiated cells and overall epigenetic reprogramming in dedifferentiation and maturation of undifferentiated cells, rather than with normal cellular proliferation.

Keywords: H2A.1, H2A.2, differentiation, de-differentiation, normal cellular proliferation

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Introduction

Chromatin, a dynamic structure, is composed of nucleosome having octameric histone core of H2A, H2B, H3, and H4 wrapped by 146 bp of DNA. Histones have variants along with the canonical forms and can be categorized on the basis of sequence similarity and replication behaviour.[1–3] Studies on histone variants, H2AZ, and macroH2A are emerging in embryonic development and cancer, suggesting their role in epigenetic reprogramming. Furthermore, macroH2A association with the repression of gene expression correlates with the occupancy of H3K27me3 during maintenance of pluripotency.[4] H2A.Z has been shown to over-express in cancers and has also associated with development and differentiation.[5–7] However, macroH2A has been reported to suppress tumor progression, and its loss positively correlates with malignant phenotype of cancer.[8] Furthermore, the redundancy of macroH2A has been shown by developmental defects in zebra fish embryos[9] and cell-fate decisions in human male pluripotent cells.[10] Previous report from laboratory has also shown over-expression of H2A.1 with decrease in expression of H2A.2 during de-differentiation of mature hepatocytes to HCC.[11] Recently, replication-dependent histone coded by human HIST1H2AC gene locus, sequence similar to rat H2A.1, has been associated with cell proliferation and tumorigenicty of bladder cancer cells.[12]

In order to define alteration in H2A.1 and H2A.2 variants in the same organ system during development, de-differentiation, and normal cellular proliferation, their comparative profiling at RNA and protein level was studied during liver regeneration post PH and differentiating embryonic liver. Liver has remarkable capacity to regenerate and, therefore, serves as an excellent model for normal cellular proliferation, whereas HCC development occurs due to uncontrolled proliferation of oval cell or de-differentiation of hepatocytes and, during embryonic liver development from hepatoblasts to hepatocytes, can be used to delineate the process of differentiation.

Our studies have shown no alteration in H2A.1/H2A.2 ratio, at protein and transcript level, during normal cellular proliferation. However, studies on differentiating embryonic liver from e15 to post-natal liver show decreased expression of H2A.1 with increased level of H2A.2 variant in adult liver. Collectively, the profile of H2A.1 and H2A.2 suggests increased level of H2A.1 with undifferentiated hepatocytes to probably impart epigenetic reprogramming for performing overlapping chromatin function for differentiation in different physio-pathological states.

Materials and methods

All the experiments were carried out on male Sprauge–Dawley rats (*Rattus norvegicus*). The proposal to use the

animals was duly approved by Institute Animal Ethics Committee, ACTREC, and the Committee, which is endorsed by the committee for the Purpose of Control and Supervision on Animals, Government of India. All excised tissues were washed with normal saline, snap frozen in liquid nitrogen, and stored at −80°C for further experiments unless otherwise mentioned. Tissues were fixed in formalin for hematoxylin and eosin staining for histopathological analysis (Supplementary Figure 1).

Animal experiments

The normal and *N*-nitrosodiethylamine-treated animals (n = 5, for each set of experiments) were euthanized after 4 months of treatment.[11,13] The animals (22–24 weeks) for liver regeneration studies were subjected to two-third PH. The surgeries were performed under sterile conditions as per protocol.[14] A horizontal incision was made, approximately 2–3 cm long, just below the Xiphoid cartilage. Left lateral and median lobes of liver (50–70% liver) were resected, and muscle and skin layers were closed. Animals were provided with an external source of heat during recovery and were maintained post operatively under optimal conditions. Animals were sacrificed, and regenerating liver tissues were collected at different time points (n = 5, for different time points and each set of experiment) (16, 24, 36, 48 and 72 h) post PH in synchrony with DNA synthesis and were processed as mentioned earlier. Furthermore, for embryonic liver development studies, pregnant rats (n = 6, for each set of experiments) confirmed by the vaginal plug were obtained. The developing liver (n = 6, for each time point) was excised on e15, e18, D0 (day of birth), 1 week, and 3 weeks and were processed as mentioned earlier.

Resolution of histones on AUT PAGE

Histones were extracted by acid-extraction method from nuclei purified by sucrose density-gradient centrifugation.[15] Histone (25 μg) and their variants were separated on AUT PAGE as described.[16] H2A.1 and H2A.2 variants from complete gel were cropped, enlarged, and quantified by Image-J software (v1.42q, NIH) as shown in Figure 1. Individual spot intensities were normalized to total, H2A.1 and H2A.2 intensities, and alterations in band intensities as percentage difference were plotted using GraphPad Prism software.

Immunoblots detection

Histones (2 μg) were resolved on SDS–PAGE, transferred, and probed with global and site-specific H3 antibodies (pan acetyl, H3Lys9Ac, H3Lys14Ac, H3Lys9Me3, and H3Lys27Me3) according to the manufacturer's instructions (Abcam). The dilutions of primary and HRPO labeled secondary antibodies in 1% BSA were as follows: H3K9me3 (1:8000), H3K27me3 (1:5000), H3K9Ac (1:3000), H3K14Ac (1:3000), and anti-rabbit (1:8000). Band intensity was normalized with H3 intensity on transferred membrane and

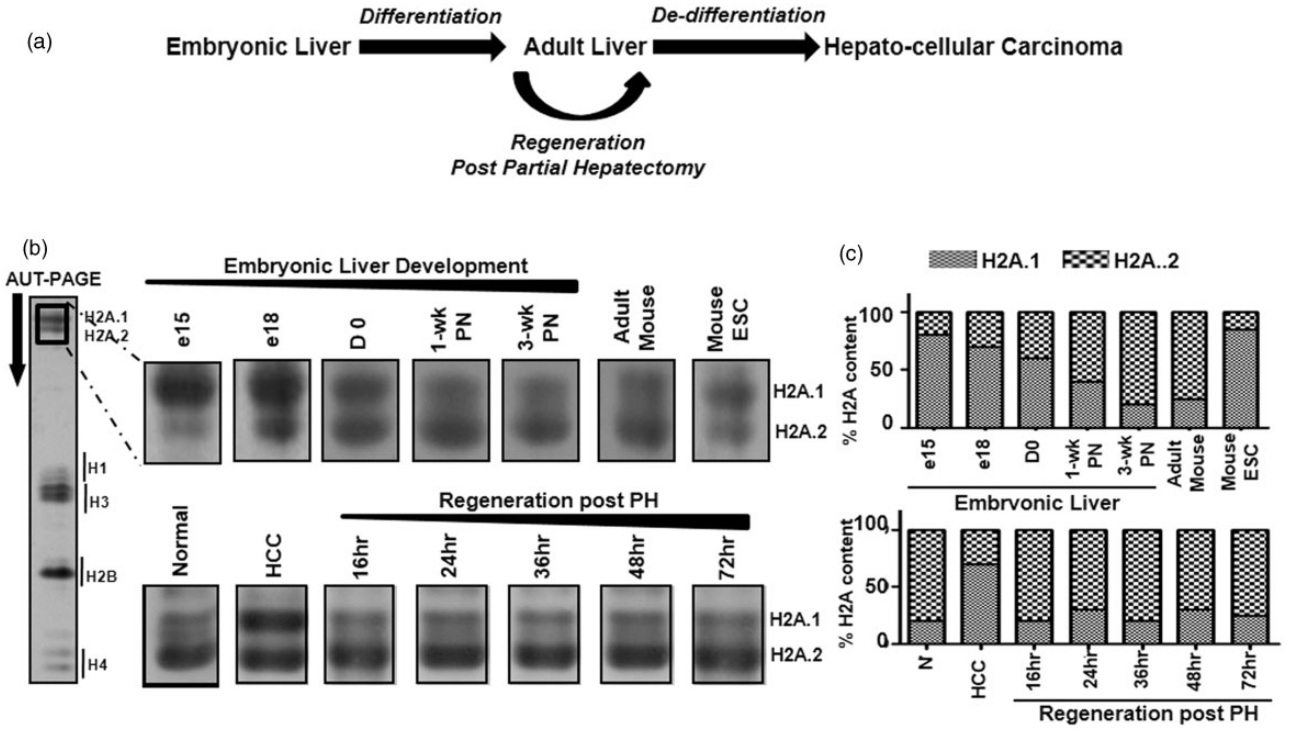

Figure 1 Profiling of H2A.1 and H2A.2 during step-wise liver regeneration and sequential embryonic liver development. (a) Schematic representation of different patho-physiological stages of liver and (b) histones (25 μg) that are resolved on AUT PAGE, silver-stained, and H2A.1/H2A.2 is represented in a square. The enlarged images of H2A.1 and H2A.2 are shown for e15 to 3-week-old adult rat, mouse ESC, mouse liver (*upper panel*), and normal adult liver, HCC, 16, 24, 36, 48, and 72 h regenerating liver post PH (*lower panel*). (c) Densitometric analysis of individual spot intensities was normalized to total, H2A.1 and H2A.2 intensities, and alterations in band intensities as percentage difference was plotted using GraphPad Prism software in different patho-physiological states of liver

analyzed by performing unpaired t-test within the respective groups, HCC, PH, and e15 with normal using Graph Pad prism software (version 5.0 for windows), and $p < 0.05$ is considered as significant values.

Semi-quantitative reverse transcriptase-polymerase chain reaction analysis

Total RNA (5 μg) prepared using TRIzol (Invitrogen, Cat#15596-026) was subjected to cDNA synthesis using random primers (Fermentas, Cat#K1632) as per the manufacturer's instructions. cDNA was subjected to PCR (NEB, Cat#M0271L) for the analysis of H2A.1 (216 bp, F:CTGTGCTGGAGTACCTGACG, R:TGTGGTGGCTCTC AGTCTTC), H2A.2 (220 bp, F:GAAGACGGAGAGCCAC CATA, R:GGAAGAGTAGGGCACACGAC), proliferating cell nuclear antigen (PCNA) (193 bp F: TCACAAAAG CCACTCCACTG, R: CATCTCAGAAGCGATCGTCA), SOX9 (88 bp F: AGTACCCGCATCTGCACAAC, R: ACG AAGGGTCTCTTCTCGCT), α-feto protein (AFP) (155 bp F: ACCTGACAGGGAAGATGGTG, R: GCAGTGGTTGAT ACCGGAGT), and 18SrRNA (219 bp F:CGCGGTTCTATT TTGTTGGT, R:AGTCGGCATCGTTTATGGTC). Band intensities of PCR products were normalized to 18 S rRNA and analyzed by performing unpaired t-test within the respective group, HCC, PH, and e15 with normal using Graph Pad prism software (version 5.0 for windows), and $p < 0.05$ is considered as significant values.

Results

In order to gain insight in relevant epigenetic overlapping mechanism involved in embryonic liver, regeneration, and cancer development (Figure 1(a)), we performed a comprehensive analysis of differentially expressing histone variants, H2A.1 and H2A.2.

H2A.1 loss is associated with liver differentiation and remains unaltered during liver regeneration

H2A variant expression during time -chase regeneration post PH and different stages of embryonic development was studied (Figure 1). The regenerating liver with higher rate of cellular proliferation and DNA replication at 16, 24, 36, 48, and 72-h post PH showed no alteration in the profile of H2A.1 and H2A.2 compared to normal liver (Figure 1(b) and (c)). The histopathological analysis of regenerating liver post PH showed normal liver architecture having maintained hexagonal structure with sinusoidal spaces though the nuclei look enlarged indicating toward high level of DNA replication coupled with cellular proliferation (Supplementary Figure 1(a)). The profiling of H2A variants during different stages of liver development, e15, e18, D0, 1 week, and 3 weeks, showed highest accumulation of upper band at e15 (Figure 1(b)). Differentially expressing bands (upper and lower) of H2A variants were identified as H2A.1 (upper band) and H2A.2 (lower band) by mass spectroscopy and further confirmed by MS–MS of identified peptides (Supplementary Figure 2 and Supplementary Table). The sequential decrease of H2A.1 was observed with progression of hepatoblast toward maturation; on the contrary, the level of H2A.2 increases with differentiation of embryonic liver to 3-week stage (Figure 1(b) and (c)). The e15 liver showed hyperplasia, hyperchromatic, dissimilar-sized hepatocytes with polyploidy, and a spongy appearance due to compactness of nuclei. The e20 stage had multinucleate giant cells with hexagonal hepatocyte having increased cytoplasm to nuclear ratio at D0. The histo-pathological analysis along with the development of new blood vessels confirmed the maturation of hepatoblast to hepatocyte during liver development (Supplementary Figure 1(b)). Thus, the higher expression of H2A.1 than H2A.2 variant is associated with cellular differentiation and not coupled with the regeneration of liver.

Expression profiling of H2A.1 and H2A.2 and their association with chromatin marks during liver regeneration, differentiation, and de-differentiation

The comparative analysis of mRNA expression was carried out within normal adult, embryonic (e15), HCC, and 24 h post-PH regenerating liver (PH) (Figure 2(a)). The expression of H2A.1 was significantly high in e15 and HCC compared to normal and PH. However, H2A.2 was higher in normal and PH. Along with histo-pathological analysis of different patho-physiological state of liver, the liver specific, and proliferation markers, AFP, a proto-onco gene, SOX9, and PCNA were also studied. AFP shows increased expression in e15 and HCC compared to adult and regenerating liver. SOX9 in consent with other report[17] showed overexpression only in HCC but not during normal cellular proliferation, neither in development nor post-PH. PCNA was significantly higher in e15 compared to the increase in HCC and PH. Collectively, the differential expression of markers, PCNA, SOX9, and AFP in coherence with stages confirms H2A.1 is associated with the process of cellular proliferation during differentiation or dedifferentiation but not with cellular proliferation during regeneration.

To understand whether change in H2A.1 and H2A.2 variant is associated with specific chromatin architecture, the active and inactive acetylation and methylation marks of histone H3 are studied by western blotting (Figure 2(b)). The pan-acetylation on histone H3 increases in all the patho-physiological states of liver compared to control but the increase was significant in regenerating liver. The active chromatin marks, H3Lys9Ac and H3Lys14Ac, showed an inverse correlation with different patho-physiological states of liver. H3Lys9Ac increases in HCC and decreases after PH, whereas H3Lys14Ac increases in PH and e15 liver compared to normal and HCC. The inactive marks, H3Lys9Me3 increases in PH, whereas in e15 liver and HCC, no significant change was observed compared to normal. The increased tri-methylation of H3Lys27 is associated with e15 liver and HCC, whereas the level decreases after PH is compared to normal liver.

Discussion

Earlier studies have shown broadly overlapping genetic mechanism leading to the biological changes during embryonic liver development and HCC.[18] Interestingly, our studies on H2A variants, H2A.1 and H2A.2, have shown the

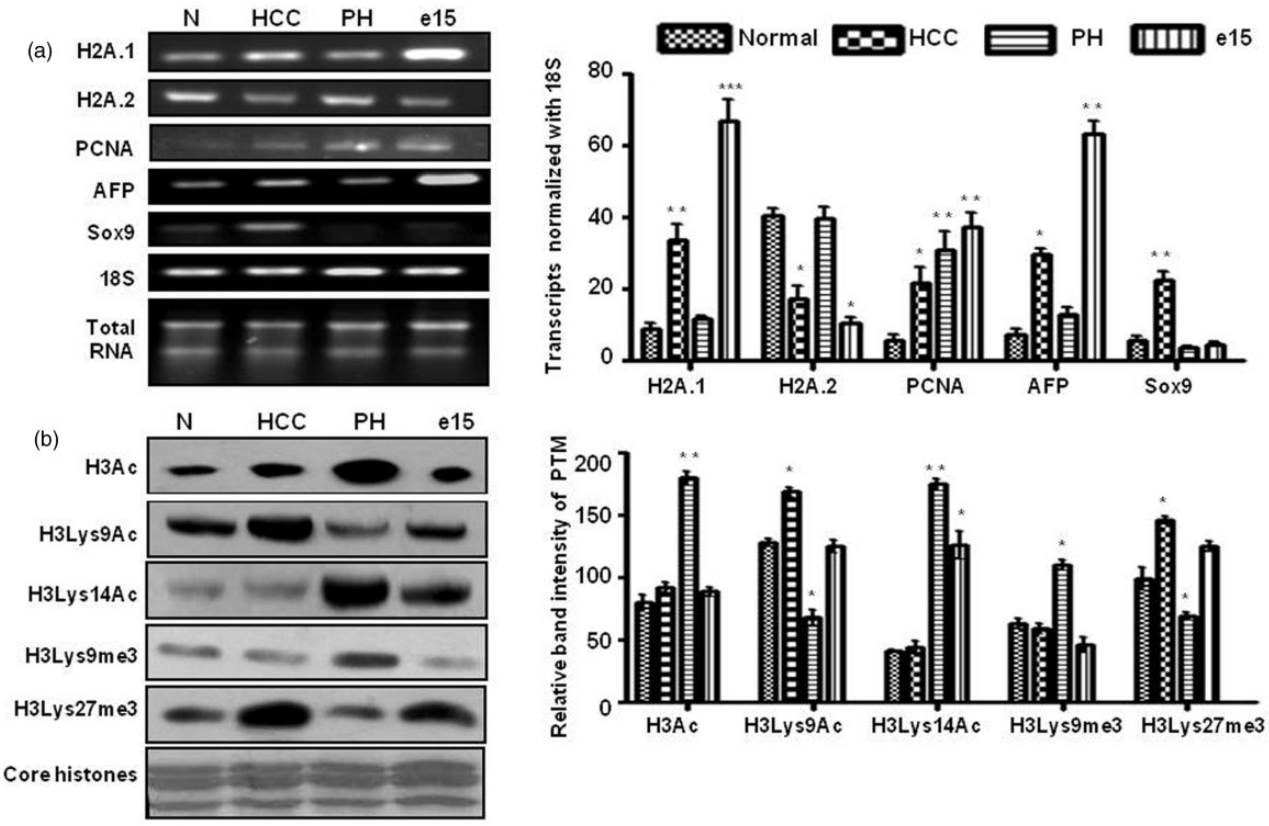

Figure 2 Stage-specific expression profiling of H2A.1 and H2A.2 and chromatin marks. Total RNA and histones purified from liver of normal adult, embryonic (e15), HCC, and 24 h post-PH regenerating liver (PH). (a) Semi quantitative RT-PCR of H2A.1, H2A.2, PCNA, AFP, and SOX9. 18 S is used as loading control and (b) immunoblots with antibodies against pan-Ac, H3Lys9Ac, H3Lys14Ac, H3Lys27me3, and H3Lys9me3. Densometric analysis of band intensity is represented graphically as an average of three independent experiments. Each histogram represents mean densitometry ± SEM of normal compared to HCC, PH, and e15. $p < 0.05$ was considered as significant

decrease in the expression of H2A.1 with simultaneous increase of H2A.2 during liver development and also confirmed our report of H2A.1 increase in HCC.[11] The level of H2AHISTIC, human H2A.1, is also reported to increase in tumor bladder cells compared to normal.[12] The liver development and de-differentiation of mature hepatocyte to HCC are in coherence with the expression profile of cell-proliferation marker, PCNA, and liver differentiation marker, AFP, whereas Sox9 remains minimal during differentiation and increases in HCC, which is in accordance with the earlier studies.[17,19] The observed higher level of H2A.1 expression in mouse embryonic stem cells (ESC) along with e15 and HCC strengthens that H2A.1 is associated with undifferentiated cells (Figure 1(b)). Furthermore, report on differentiation and cancer development has shown increase of macroH2A in maturation of new born to young adult liver,[20] though macroH2A decreases in cancers. The data have shown that altered transcription is contributing to change in histone variants, H2A.1 and H2A.2, that share 97.6% homology in their protein sequence but differ significantly in their promoter sequences (H2A.1:NCBI-*JX-661508* and H2A.2:NCBI-*JX-661509*). The transcriptional regulation in different patho-physiological states needs further investigation. Furthermore, our data have also shown increase in H3Ac suggesting open chromatin organization in different patho-physiological states. The alteration in active and inactive marks on H3 suggests similar pattern of modification(s) during early differentiation and dedifferentiation but further studies are required to understand their significance. The incorporation of different homomorphous histone variants of H2A and increase in global acetylation may have potential implication on chromatin architecture, downstream gene expression, and thereby maintaining high plasticity for genomic reprogramming during differentiation and dedifferentiation.

The DNA replication in liver regeneration is coupled with histone synthesis, modification(s) imprinting, and increase in PCNA for assembly of chromatin. Our studies on regenerating liver showed no alteration in the accumulation and expression of H2A.1 and H2A.2 variants. Also, the chromatin marks are different compared to e15 and HCC liver. The observed significant increase of H3Lys14Ac after PH is in accordance with the previous reports. Thus, increase in normal cell division for tissue maintenance or compensatory growth is not associated with change in expression of histone variant but only associated with different post-translational modification(s) to regulate change in gene expression.

The three amino acid differences at 16th, 51st, and 99th position make it difficult to generate highly specific

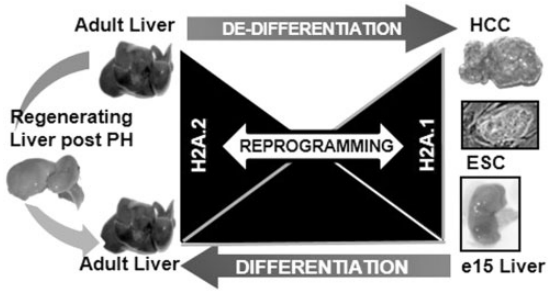

Figure 3 Proposed model for association of histone variants, H2A.1 and H2A.2 with regeneration, de-differentiation, and differentiation. Hepatocytes of adult and regenerating liver are H2A.2 enriched, whereas undifferentiated cells, e15 hepatoblasts, ESC, and de-differentiated hepatocytes of HCC are enriched with H2A.1. ESC: embryonic stem cell

antibodies against H2A.1 and H2A.2. Therefore, the limitation of the study is to understand and directly correlate the in vivo observation of change in histone variant profiles with the group of genes, expressing differentially or overlapping, during differentiation and de-differentiation. Based on our study, a model has been proposed (Figure 3) signifying H2A.1 and H2A.2. H2A.2 variants play a potential role in defining highly specialized biological mechanism, differentiation, and de-differentiation opposed to normal cellular proliferation during liver regeneration and suggest the overlapping functional relevance in epigenetic reprogramming of genome.

Authors' contributions

SG and MT conceived and designed the experiments. MT, BK, and SAK performed the experiments. MT, SAK, and SG analyzed the data and wrote the manuscript. AI performed histopathological analysis.

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