Role of De Novo DNA Methyltransferases and Methyl C-binding Proteins in Gene Silencing in a Rat Hepatoma

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*Abbreviations used: Metallothionein, MT; 5-azacytidine, 5-AzaC; DNA methyltransferase, DNMT; Methyl-C Binding protein, MBD; Chromatin immunoprecipitation, ChIP; restriction landmark genome scanning with methylation-sensitive restriction enzymes, RLGS-M; Cytochrome c oxidase 1, COX-1.
Abstract

The expression of Metallothionein-I (MT-I), a known antioxidant, was suppressed in a transplanted rat hepatoma due to promoter methylation and was induced by heavy metal only after demethylation by 5-Azacytidine (5-AzaC). Treatment of the tumor-bearing rats with 5-AzaC resulted in significant regression of the hepatoma. When the inhibitor-treated tumor was allowed to grow in a new host MT-I promoter was remethylated, which suggested de novo methylation. The activities of both de novo (3-fold) and maintenance DNMT (5-fold) were higher in the hepatoma compared to the host liver. The mRNA levels of the de novo methyl transferases, DNMT3a and DNMT3b, were 3- and 6-fold higher, respectively in the tumor implicating transcriptional upregulation of these two genes in this tissue. Immunohistochemical analysis showed exclusive localization of DNMT3a in the nuclei of both the liver and hepatoma whereas DNMT3b was detected in the nuclei as well as cytoplasm. Immunoblot assay showed that the levels of DNMT1, DNMT3a and DNMT3b proteins in the hepatoma were 5-, 10- and 4-fold higher, respectively, than in the liver. The mRNA level of the major methyl C-binding protein (MeCP2) was 8-fold higher in the tumor compared to the liver. Immunohistochemical studies showed that MeCP2 is localized exclusively in the nuclei of both tissues. Chromatin immunoprecipitation (ChIP) assay demonstrated that MeCP2 was associated with the MT-I promoter in the hepatoma implicating its involvement in repressing the methylated promoter. Analysis of the DNA isolated from the liver and hepatoma by Restriction Landmark Genomic Scanning with methylation sensitive enzyme (RLGS-M) (NotI) showed that many genes in addition to the MT-I were methylated in the hepatoma. These data demonstrate suppression of the MT-I gene and probably other genes in a solid tumor by promoter methylation, and have provided potential molecular mechanisms for the altered methylation profile of the genes in this tumor.
Introduction

In the early studies, DNA methylation was considered a tool to control precisely the expression of a small portion of the entire genome amidst the overwhelming noise of repetitive elements, non-coding DNA, introns and potentially active transposable elements. Later on, its importance in the development of mammals became apparent (1,2). In the last decade, the silencing of tumor suppressor genes by promoter methylation in tumorigenesis emerged as an important alternative to gene silencing by classical mutation. There are several known tumor suppressor genes that are silenced due to promoter methylation in different tumors e.g. Rb, p16INK4a, BRCA-1, VHL, E-cadherin, MLH1 (3). Besides the well-established tumor suppressor genes, the list of hypermethylated genes in tumors that are not documented as tumor suppressors is growing in number. The list of these important genes includes p15INK4a (4), ER (5), GSTPi (6), TIMP3 (7), SOC (8), DAPK1 (9) and p73 (10). These genes are silenced in different tumors ranging from acute myeloid lymphoma to breast cancer. Metallothionein (MT) is one of the recently identified genes that is silenced in a rat hepatoma (11), human hepatocellular carcinoma and metastatic adenocarcinoma (12), colorectal carcinoma (13), and in human and murine hepatocellular tumor (14). The MT-gene can also be silenced in cells that over-express the p80 subunit of the Ku protein, an autoantigen (15), which is at least, in part, due to promoter methylation (16). We established the molecular mechanism for the downregulation of MT-I gene by demonstrating methylation of the CpG islands in its promoter (11,17).

Metallothioneins (MTs)* are a family of small cysteine-rich proteins that consists of four different isoforms, namely MT-I, MT-II, MT-III and MT-IV (18). Of these isoforms, MT-I and MT-II are ubiquitously expressed in all tissues from lower eukaryotes to vertebrates. The expression of MT-III is restricted to glutaminergic neurons of brain (19) and that of MT-IV is confined to squamous epithelial cells of skin, tongue and intestinal lining (20). MT-I and II are considered important mediators of cellular detoxification mechanism, scavenging heavy metals like Cd, Hg and Cu as well as free radicals. These are all strong inducers of these genes (21,22). MT gene expression is also upregulated in animals under stress (23), in response to viral infection (24) and in Cu, Zn-SOD null mice (25). By virtue of their heavy-metal binding capacity, MTs participate in maintaining cellular homeostasis of biologically essential metals like Zn$^{2+}$ and Cu$^{+}$. Many cellular proteins such as DNA and RNA polymerases along with several transcription factors (e.g. Sp1, TFIIA, and MTF1) require zinc for their optimum activity.
MTs can act both as zinc donor and acceptor and thus function in controlling the activities of these regulatory proteins (27). The silencing of the \textit{MT} gene in several human and rodent tumors implicates that the absence of metallothionein protein may facilitate tumor growth.

Methylation of the \textit{MT-I} promoter in the hepatoma but not in the liver of the same tumor bearing animal prompted us to compare the expression, abundance and importance of the DNA methylation machinery in the two tissues. The methylation-mediated silencing of genes involves an intricate interplay of many factors that methylate and assemble a repressor complex on the gene promoter resulting in heterochromatin formation (28). The key players involved in reactions ranging from initiation to maintenance of the silenced state of a gene include DNA methyl transferases (DNMT), methyl CpG binding proteins (MBDs) and the co-repressors that assist the MBDs in maintaining a closed chromatin structure.

Four different isoforms of DNMTs, encoded by different genes, have been identified and characterized. DNMT1, DNMT3a and DNMT3b are enzymatically active (2,29). DNMT1 is also called maintenance methylase, as it preferentially methylates hemimethylated substrates, whereas DNMT3a and 3b are characterized as \textit{de novo} methylases for their ability to methylate unmethylated DNA. The expression of DNMT1 and 3b appears to be cell cycle dependent, whereas DNMT3a can be expressed independent of cell cycle (30). Recent studies have shown that DNMTs not only methylate CpG islands but can also form repressor complex and facilitate the formation of heterochromatin. For example, DNMT1 is known to associate with the co-repressors HDAC2 and DMAP1, which in turn bind the transcriptional repressor TSG101 and form a repressor complex at the DNA replication foci (31). Likewise, DNMT3a can bind and form complexes with HDAC1 and RP58, a transcriptional repressor associated with the heterochromatin (32). Both DNMT3a and DNMT3b can repress transcription in a methylation-independent manner and co-localize with the heterochromatin protein HP-1\(\alpha\) at pericentromeric heterochromatin throughout the cell cycle (33). This diverse function of DNMTs makes it difficult to assign and understand the role of an individual protein or a complex in silencing a gene.

The silencing of methylated promoters requires binding of methyl CpG binding proteins to symmetrically methylated CpGs (28,34). Five such proteins that share a common methyl-binding domain (MBD) have been identified so far (35). Among these proteins, MBD1, MBD2 and MeCP2
repress the methylated promoters through recruitment of co-repressor complexes (36,37). Although mammalian MBD3, the DNA binding component of the chromatin remodeling complex NuRD, cannot bind to methylated CpG dinucleotides on its own, it is recruited to the methylated promoters through its interaction with MBD2 (38,39). These proteins are widely expressed, except in embryonic stem cells, where DNA methylation is dispensable (40). By virtue of their interaction with co-repressors like HDAC, Sin3A, and Mi-2, these MBDs can form corepressor complex on a methylated CpG island, resulting in chromatin compaction and gene silencing. MBD4 encodes a uracil DNA glycosylase that repairs CpG:TpG mismatches (41).

Previous study in our laboratory showed suppression of the \( MT-I \) gene in a solid rat hepatoma due to promoter methylation. The C-5 of cytosine in all the 21 CpG dinucleotides within the \( MT-I \) promoter spanning the region from -225 to +1 bp are methylated (16). None of the CpGs were methylated in the liver from the tumor-bearing rats, which is consistent with the robust MT-I induction in this tissue in response to heavy metals and free radicals (42). Genomic footprinting showed that the cis-acting elements within the promoter were inaccessible to the transcription factors as a result of methylation in these regions (16). Interestingly, the level of most transcription factors that include MTF-1, a key factor involved in the specific expression of the \( MT-I \) and \( MT-II \) genes (43), were either the same or higher in the tumor relative to the host liver. These data were similar to that generated earlier in our study where the \( MT-I \) promoter was shown to be silenced in mouse lymphosarcoma cells due to promoter methylation (17). The present study was undertaken to explore further the DNA methylation machinery in the hepatoma and liver, and to determine a potential relationship between the robust growth of the hepatoma in the host and the expression and activities of maintenance and \textit{de novo} DNA methyltransferases and methyl C-binding proteins.

**Methods and Materials**

**Maintenance of Morris Hepatoma in rat and 5-AzaC treatment**

Morris hepatoma 3924A was grown in ACI rats by transplanting a 0.5 X 2-3 mm slice of the solid tumor into their hind leg, as described previously (16). For 5-AzaC treatment, rats were injected intraperitonially with the drug (5 mg/kg body weight) dissolved in physiological saline or with saline
alone (control) after two weeks of tumor transplantation, when the tumor growth was visible. The animals were sacrificed when the control tumor grew to 15-20 g size (4-6 weeks). In 5-AzaC treated rats the tumor growth was significantly reduced. Thin slices of both 5-AzaC treated and the control tumor were re-transplanted in new donor rats and tumor growth followed until it attained is original size.

Bisulfite genomic sequencing
Preparation of genomic DNA, treatment with sodium bisulfite and amplification of the \textit{MT-I} promoter was done according to the protocol optimized in our lab (44). The amplified DNA was digested with Apo I and Tsp509 I to check complete conversion of unmethylated cytosines to uracils and sequenced with $^{33}$P-ddNTP using the Thermosequenase radiolabeled terminator cycle sequencing kit (USB).

Isolation of RNA and Northern blot analysis
Total RNA was isolated from the liver and hepatoma by the guanidinium thiocyanate-acid phenol method (45). Poly A$^+$RNA was isolated from the total RNA using the Poly A-Tract mRNA isolation kit (Promega). Five micrograms of poly A$^+$ RNA was fractionated by formaldehyde-agarose (1.2\%) gel electrophoresis and transferred to a nylon membrane. The membrane was then hybridized with $^{32}$P-labeled, mouse MBD2 or COX-1 cDNA fragments as probe in rapid hybridization buffer (Amersham Pharmacia Biotech) following the manufacturer’s protocol.

RT-PCR
For RT-PCR both total RNA and Poly A$^+$ selected RNA were used. Reverse transcription was carried out with random hexamers (Perkin Elmer) and M-MLV reverse transcriptase from 1\,$\mu$g total RNA or 25 ng Poly A$^+$ RNA following the protocol provided in the GeneAmp RNA PCR kit (Perkin Elmer). One fifth of the RT reaction was subsequently PCR amplified for each of the genes of interest with dNTP (Boehringer Mannheim) and Taq polymerase (Gibco) The gene specific primers used for amplification of the respective cDNA are as follows.

\begin{verbatim}
MBD1-F: 5'-CGGGATCTGCGACAAGCCCCAAATTTCG
MBD1-R: 5'-GGGGTACCCCAGTCTACTGCTTTCTAGCTCCAG
MeCP2-F: 5'-TACGCGGATCCCTTCTCAGGAGAGAGACAGAAACCA
\end{verbatim}
Isolation of the nuclei from the liver and hepatoma and preparation of nuclear extract

The liver and the hepatoma nuclei were isolated by homogenization in a high-density sucrose buffer following the protocol of Groski et al. (46) and Rose et al. (47), respectively. For DNA methyltransferase assay the isolated nuclei were extracted with buffer containing 0.35M KCl following the protocol of Wadzinski et al. (48). For Western blot analysis of methyl CpG binding proteins nuclei were resuspended in RIPA buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS) and sonicated (5X 5second pulses) at 4°C. After removal of the debris by centrifugation at 12,000Xg for 15minutes at 4°C, the nuclear proteins in the supernatant were separated by SDS-PAGE. The protein concentration in the nuclear extracts was measured with Bio-Rad reagent according to Bradford’s method with bovine serum albumin as standard.

Assay of de novo and maintenance DNA methyltransferase activities

Both de novo and maintenance DNA methyltransferase activities were measured as described (49). Briefly, nuclear extract (5-60μg) was incubated in assay buffer (10mM Hepes Buffer, pH7.9, 3mM MgCl$_2$, 100mM KCl and 5% glycerol) with 500ng of double-stranded oligonucleotide (unmethylated or hemimethylated) and 3μCi SAM (S-adenosyl-L-[methyl-3H] methionine, Amersham-Pharmacia, Piscataway, NJ) in a total volume of 100µl for 2hr at 37°C. The reaction was terminated by adding 300 µl of Stop solution (1.0% SDS, 2.0mM EDTA, 3.0% 4-amino salicylate, 5.0% Butanol, 0.25mg/ml calf thymus DNA, 1.0mg/ml proteinase K) and incubating at 37°C for 45min. The reaction mix was then spotted on a Whatman GF/C filter, dried and washed with 5% trichloroacetic acid, followed by 70% ethanol. The dried filters were then counted using Beckman liquid scintillation counter. All the reactions were done in triplicate. The oligonucleotide sequences are as follows:
Top: 5'-GGGGGCCAAGCGCGCGCCTGGCGCCCGGGCCGGCTCAAGCGCGCGCCTGGCGCCCGGATC), Bottom: 5'-GATCCGGGCGCCAGGCGCGCGCTTGAGCCGGCCCGGGCGCCAGGCGCGCGCTTGG). For hemimethylated substrate, the bottom strand oligonucleotide was synthesized with 5-methyl cytosine at all cytosines followed by guanines and annealed to the same unmethylated top strand.

**Antibody production against recombinant proteins**

To generate antibodies specific for DNMT3a and DNMT3b we expressed the fragments of the mouse proteins lacking N-terminal DNA binding domain as a histidine-tagged protein in *E. coli*. Similarly, rat MeCP2 and mouse MBD2 proteins with deleted N-terminal MBD (methyl CpG binding domain) were expressed as his-tagged proteins. DNMT3a, DNMT3b (generous gifts from Dr. En Li) and MeCP2 (a gift from Dr. Adrian Bird) partial cDNAs were amplified from the full-length cDNAs with gene-specific primers and cloned into BamH I and Kpn I sites of pQE 30 vector to express as histidine-tagged proteins (Qiagen). Mouse MBD2 partial cDNA was amplified from a lung cDNA library (Stratagene) using gene specific primers and subcloned into BamH I and Pst I sites of the same vector. The identity of the amplified products was confirmed by sequencing. Bacteria (M15) were treated with IPTG (2 mM) for induction of the proteins and the recombinant proteins were then purified through Ni-NTA resins (Qiagen) following manufacturer’s protocol. The purified proteins were separated on SDS-PAGE, and stained with 0.25M KCl to visualize the polypeptides, which was then excised out of the gel. The resulting product was then emulsified with Freund’s complete adjuvant and injected into a New Zealand male rabbit to raise antibodies. The following sets of primers were used to amplify the cDNA fragments. The production of antibodies was monitored by Western blot analysis.

1) DNMT3a F: 5'-CTACGCGGATCCGCGGAGCATGCCCTCCAGCGGCC
DNMT3a R: 5'-CTACGGGGTACCCCGACTCAGGCTCATCGTCGGCTGC
2) DNMT 3b: F: 5'-CTACGCGGATCCGCGATGAAGGGAGACAGCAGACATC
DNMT3b R: 5'-CTACGGGGTACCCCGATACTCTGTGCTGTCTCCATC
3) MeCP2 F: 5'-TACGCGGATCCCCCTTCCAGGAGAGACAGACAGACATC
MeCP2 R: 5'-TACGGGGTACCTGCAATCCGCTCTATGTAAAGTCA
4) MBD2 F: 5'-GGGGGTACCCGCTGTGTTGACCTTACGAGTTTTGAC
MBD2 R: 5'-AACTGCAAGTTAGCCTGCTCCTCAGCTGCTGCATCCT
Western blot analysis:
Nuclear extract (200-300µg) was separated on 7.5% SDS-PAGE, transferred to nylon membrane, blocked in 5% milk in Tris buffered saline at pH 7.5 (TBST) containing 0.1% Tween-20. For the DNMT3a antibody, a dilution of 1:6000 in TBST (0.1% Tween) was used. For the DNMT3b antibody, a dilution of 1:1000 in TBST (0.5% Tween) was used. For detection of MeCP2 and MBD2, a 1:5000 dilution in TBST (0.1% Tween) of the polyclonal antisera against the recombinant proteins was used. HRP - conjugated anti-rabbit IgG (Amersham) at a dilution of 1:5000 in TBST (0.1% Tween) was used as the secondary antibody. An ECL detection kit (Amersham) was used to detect the signal in the Western blots.

Immunohistochemistry studies
Tissue samples were harvested from the hepatoma and liver of the tumor bearing rats. The samples were frozen immediately on dry ice and cut on a cryostat to 15 micron thick sections. The sections were fixed in 4% formaldehyde for 15 min and then washed in PBS for 5 min. The sections were then treated in 0.3% triton in PBS for 15 min. After another wash in PBS, the non-specific binding sites were blocked by incubating the sections in 5% milk at room temperature. The endogenous peroxidase activity was quenched by incubating the sections in 0.3% H2O2 in methanol for 30 min. The sections were then incubated with the primary antibodies (1:2000 dilution in PBS) at 4°C overnight. After washing the section 3 times in PBS for 5 min, they were incubated with biotinylated anti-rabbit antibody (1:200) for 1 hour at room temperature, and visualized by the traditional horse radish peroxidase method. The images were acquired with a digital camera mounted on an Olympus microscope.

Chromatin immunoprecipitation (ChIP) assay
Intact nuclei were isolated from the liver and tumor without disturbing the internal macromolecular interaction as described above, and then washed with PBS at 1000Xg for 10 min. The pellet was resuspended in PBS (10^7 nuclei/ml) and treated with 1.0% formaldehyde at room temperature to cross link the DNA-binding proteins to cognate cis elements. The nuclei were harvested after 30 min, washed with PBS and solubilized in lysis buffer (50 mM Tris-HCl, pH 8.1; 1% SDS, 10 mM EDTA, 0.5 mM PMSF, 10 µg/ml aprotinin) at a density of 10^7 nuclei/ml. The lysate was sonicated to shear chromosomal DNA to a size of ~500 to 750 bp. The insoluble material was removed by centrifugation in a microfuge at maximum speed for 15 min at 4°C. The soluble supernatant (100µl) was then diluted 10 fold with
ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1; 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl) and chromatin immunoprecipitation was performed following the published protocol (50) with some modifications. The diluted chromatin was precleared by incubating for 3 hr with 5 µl of preimmune serum coupled protein A agarose beads saturated with BSA (1 mg/ml) and salmon sperm DNA (0.4 mg/ml). The mixture was spun at 2000 rpm for 2 min in a microfuge at 4°C and the supernatant was allowed to bind to 2 µl either preimmune sera or with antisera raised against recombinant MeCP2 overnight at 4°C. Immune complexes were pulled down by protein A-agarose beads and washed extensively with different buffers as described (50). DNA precipitated by MeCP2-antibody complex as eluted, de-crosslinked, purified DNA was dissolved in 20 µl of TE and 1 µl was used for semiquantitative PCR with gene-specific primers. DNA purified from the chromatin (input DNA) was used as the control for PCR. The primers used for amplification of rat MT-I promoter are 5'-TGTTCCACACGTCACACGG and 5'-GCGGACAGTCTGCTCTCTTTATAG. In the semiquantitative PCR, one of the primers (2 pmol/reaction) was labeled with (γ-32P)ATP catalyzed by polynucleotide kinase. Immunoprecipitated DNA (1 µl) as well as input DNA (1/100th of the total) was subjected to hot-start PCR under the following conditions: 94°C, 4 min; 25 cycles of 94°C, 30 sec, 55°C, 30 sec, 72°C, 30 sec and 72 for 5 min. The reaction products were separated on a polyacrylamide gel (5% acrylamide) with TBE as running buffer. The dried gel was subjected to autoradiography and quantitated by phosphoimager analysis.

**RLGS-M analysis:**

Genomic DNA was isolated from the liver and hepatoma of tumor bearing rats, which was then subjected to RLGS-M analysis following the protocol of Okazaki et.al. (51). Briefly, isolated DNA was first digested with Not I and a commonly occurring restriction enzyme, EcoR V, end labeled with (α-32P)dGTP and (α-32P)dCTP using Klenow and separated on a gel. A third digestion with Hinf I was performed in gel to fragment the DNA further and run in a second dimension. The two-dimensional gel was then exposed to autoradiogram and analyzed.
Results

MT-I promoter is re-methylated following re-transplantation of the 5-Azacytidine-treated tumors into new donor rats.

We have previously shown silencing of metallothionein-I (MT-I) gene due to promoter methylation in a rapidly growing rat hepatoma (Morris hepatoma 3924A) (see Methods for the tumor transplantation). The CpG dinucleotides in the rat MT-I promoter are depicted in Fig.1A. Treatment of the tumor bearing rats after 3 weeks of tumor transplantation (when tumor growth was visible) with 5-azacytidine, a potent inhibitor of DNA methyltransferases (DNMTs), resulted in demethylation of the MT-I promoter and MT induction by heavy-metals (16). Significant regression of the tumor was observed upon 5-AzaC treatment compared to the untreated control. We extended this study to see the consequence of re-transplanting the regressed tumor into new donor rats. Our particular interest was to study the state of MT-I promoter methylation upon re-transplantation and growing the tumor in absence of the inhibitor 5-AzaC.

It has been reported previously that de novo methylation of a gene promoter can be due to random methylation of an entire CpG island and maintenance there after, as was observed with many tumor suppressor genes (52). The other proposed mechanism is that the promoter methylation can be signaled through a specific chromatin structure or DNA sequence such as short or long interspersed nuclear elements (SINEs and LINEs) and thereafter the methylation spreads from the specific methylation hot spots (53). If methylation of the MT-I promoter is a random process, it is unlikely that remethylation of the promoter will occur upon growing the tumor in 5-AzaC-free medium. On the other hand, if there is any methylation hot spots on the MT-I promoter, remethylation should occur immediately after removal of 5-AzaC. To analyze the promoter remethylation event, a thin slice (~ 0.5 X 2mm) of the regressed tumor was transplanted to a new donor and allowed it to grow in absence of 5-AzaC. As a control, an untreated tumor slice of the same size was transplanted to another donor rat. Both tumors grew back to comparable size but the time required for the inhibitor-treated tumor to grow was twice (six weeks vs. twelve weeks) compared to that of the untreated control.
To determine the extent of MT-I promoter re-methylation, genomic DNA was isolated from the hepatoma before (-AzaC) and after 5-AzaC (+AzaC) treatment and following re-transplantation of the AzaC into new donors (± AzaC). The isolated DNAs were treated with sodium bisulfite reagent (16), and the MT-I immediate promoter (-304 to +148bp) was PCR amplified (see Fig.1A) with upper strand specific primers. After confirming the completion of bisulfite conversion by restriction enzyme digestion, the MT-I promoter was sequenced. Consistent with our previous results (16), exposure of the animals bearing the hepatoma to AzaC, resulted in demethylation of MT-I promoter (Fig.1B, compare panel c with b), as shown by conversion of demethylated C to T by bisulfite treatment. Re-methylation of the MT-I promoter at all CpG sites in the tumor occurred following re-transplantation (± AzaC) and growth in absence of 5-AzaC (Fig.1B, panel d). This data suggests that MT-I promoter methylation is not a stochastic event. Northern blot analysis showed that the suppression of MT-I expression and promoter re-methylation was concurrent (Fig.2). No significant MT-I transcript was detected in the tumor when the animal was treated with zinc (Fig.2, lane5), whereas treatment with 5AzaC resulted in MT-I gene transcription which increased 12-fold following treatment with zinc (lane 6). The MT-I mRNA was barely detectable even after zinc treatment when the 5-AzaC treated tumor was retransplanted and allowed to grow in absence of the nucleotide analog (Fig. 2, lanes 9 & 10). MT-I was, therefore, not inducible by zinc in the re-transplanted tumor. On the other hand, a low level of constitutive but very high level of zinc–induced MT-I expression was observed in the liver from the control rats, (lanes 1&2) and from re-transplanted rats (lanes 7&8).

Exploration of the potential molecular mechanisms of altered promoter methylation

a) The expression and activities of both de novo and maintenance DNA methyl transferases are higher in the rat hepatoma as compared to the liver

To get an insight into the potential mechanism of altered promoter methylation in the hepatoma; we determined the expression and/or activity of DNMT isoforms and MBDs in the liver and hepatoma. The levels of mRNA expression, the amount of DNMT proteins and both de novo and maintenance methyltransferase activities were compared between the liver and hepatoma of the same tumor-bearing rats. Northern blot analysis of poly A+ RNA isolated from both the liver and hepatoma using gene-specific probes showed a considerably higher expression level of all the three DNMTs (DNMT1,
DNMT3a and DNMT3b) in the hepatoma compared to the liver (Fig. 3A). Phosphorimager analysis revealed increase in the DNMT1 (3-fold), DNMT3a (2-3 fold) and DNMT3b (6-fold) in the hepatoma compared to the liver. Three different sizes of DNMT3a mRNAs were detected in the hepatoma, whereas only two were detected in the liver. We do not know whether alternative splicing generates all of these forms. Although other groups have shown expression of different spliced variants of human DNMT3b in both normal and tumor tissues (colon, pancreas, lung, spleen, placenta) by RT-PCR (29), we found predominant expression of only one species of DNMT3b in both liver and hepatoma by Northern blot analysis. It is likely that alternative forms are similar in size and could not be analyzed by Northern blotting. Western blot analysis of nuclear extract from hepatoma and liver revealed that DNMT1 (~190 kDa) protein was expressed at significantly higher level (5-fold) in the hepatoma compared to the liver (Fig. 3B). Similarly, the levels of DNMT3a and DNMT3b were also significantly higher (10-fold and 4-fold, respectively) in the hepatoma compared to the liver, which correlated with the Northern blot data. Antibodies against DNMT3a detected a ~130 kDa polypeptide in the hepatoma and a ~120 kDa polypeptide in the liver (Fig. 3C), the difference in size was likely due to post-translational modification. Two polypeptides of apparent molecular sizes of 103 kDa and 98 kDa were specifically detected with DNMT3b antibodies in both tissues. The levels of mRNA as well as proteins of all the three isoforms were significantly higher in the hepatoma relative to the liver.

To get further insight into the potential role of DNMTs in hypermethylation of certain promoters in tumor tissues, we performed immunohistochemical analysis of DNMT3a and DNMT3b in the liver and hepatoma. We analyzed 15-micron sections of both the liver and hepatoma that were probed with the respective immune sera with preimmune sera as the control. DNMT3a antisera detected the protein only in the nuclei of the hepatoma and the liver whereas the detection with preimmune sera was negligible (Fig. 4A). This data indicates that DNMT3a is localized only in the nuclei and that the antibody raised against DNMT3a is very specific. Comparison of the liver and hepatoma with respect to DNMT3a expression showed extensive staining in the tumor compared to the liver. This may also, in part be due to the larger nuclei of the hepatoma, where the ratio of nuclei to cell volume is significantly higher than that in the liver. Analysis of DNMT3b expression showed (Fig. 4B) that DNMT3b is also predominantly localized in the nucleus; it was, however, also detected in the cytoplasm of both tumor cells and the hepatocytes. DNMT3b antiserum was also very specific, as the staining by the corresponding preimmune serum was negligible.
Next, we determined the \textit{de novo} and maintenance DNA methyltransferase activities in the liver and hepatoma nuclear extracts using unmethylated and hemimethylated synthetic oligonucleotides as substrate and $^{3}$H-SAM as methyl group donor, respectively (Fig.4C). The \textit{de novo} activity was 3-fold higher and maintenance methyltransferases activity was 5-fold higher in the hepatoma compared to the liver. Because 5-AzaC is a known inhibitor of the DNMTs, it is very likely that both of the enzyme activities would be significantly reduced in the inhibitor treated hepatoma. This observation tends to suggest that a clear relationship exist between the growth of the hepatoma and the increased level of DNMT, both maintenance and \textit{de novo}, in this tissue.

\textbf{(b) The expression of different methyl-C binding proteins in the hepatoma is different from that in the liver}

The methyl CpG binding proteins (MBDs) are required for the assembly of repressor complex on a methylated promoter in order to silence its expression. We, therefore, compared the levels of expression of different MBDs in the hepatoma and liver of the tumor-bearing rats. We were particularly interested in identifying the MBD that is associated with the \textit{MT-I} promoter and plays a key role in silencing the gene in the tumor. This could provide a model to study the mechanism of methylation-mediated silencing of a promoter. To determine the mRNA levels of different MBDs, we performed RT-PCR with gene specific primers. To eliminate the possible amplification from the contaminating DNA, we included RNA samples without reverse transcription. The MBD2 message was analyzed by Northern blot analysis with poly(A)$^{+}$ RNA isolated from the two tissues. We used RT-PCR for other MBDs as their expression was too low to be detected by Northern blotting. Of the five MBDs identified so far, the expression levels of MBD1, MBD2 and MBD4 were comparable between the liver and the hepatoma (Fig.5AB&C). The expression of MeCP2 was 8-fold (Fig.5D), and that of MBD3 was 2-fold higher (Fig.5E) in the hepatoma than that in the liver.

The silencing of methylated promoter requires participation of methyl CpG binding proteins that do not show their sequence specificity \textit{in vitro}. The number of methylated CpGs required for binding varies among MBDs. For example, MeCP2 can bind to a single symmetrically methylated CpG whereas MBD2 requires 13 methylated CpGs for optimum binding \textit{in vitro}. The \textit{in vivo} targets of different
MBDs with very similar DNA binding domain is yet to be explored. To determine the MBD that is associated with methylated MT-I promoter, we used polyclonal antibodies against MeCP2 in a chromatin immunoprecipitation (ChIP) assay. We selected MeCP2 for its higher expression in the hepatoma and the ability of MeCP2 antibodies to immunoprecipitate the protein from cell extracts. Immunoblot analysis with specific antibodies showed that MeCP2 level was 2-3 fold higher in the hepatoma compared to the liver whereas that of MBD2 (DNA binding component of MeCP1 complex) was similar in the two tissues (see Fig.6A). In vivo DNA-protein complexes were cross-linked with formaldehyde in intact nuclei isolated from both liver and hepatoma. The protein bound chromatin was immunoprecipitated using the antibodies with the corresponding preimmune sera used as the control (Fig. 6B). Western blot analysis of the immunoprecipitated proteins with antibodies against MeCP2 showed specific pull down of a ~84 kDa polypeptide in the liver (lane2). In the hepatoma two polypeptides of ~84 kDa and 72 kDa were detected (lane4) which may be due to the occurrence of different forms of MeCP2 in the hepatoma or due to proteolytic cleavage during isolation of nuclei. After precipitation of the chromatin, the DNA-protein complex was de-crosslinked and isolated from the complex. MT-I promoter was then PCR amplified from the precipitated DNA with primers specific to the MT-I promoter (Fig.6C). The MT-I promoter could be amplified only from the DNA precipitated with MeCP2 antibodies from the hepatoma, but not from the liver (Fig.6C, lane 5). To show that the antibodies did not pull down DNA nonspecifically from the hepatoma chromatin we amplified a segment of cytochrome c oxidase I (COX-1) cDNA, which was amplified at a very low but equal level in all the immunoprecipitated DNA (lanes 1,2, 4 & 5). These results clearly demonstrated a strong association of MeCP2 with MT-I promoter in the hepatoma but not in the liver. Lack of association of MeCP2 with the MT-I promoter in the liver is consistent with the un-methylated status of the promoter. This data suggests that MeCP2, along with other co-repressors, participate in suppression of the methylated MT-I promoter in the hepatoma.

The strong association of the MT-I promoter with MeCP2 in the hepatoma along with the RT-PCR data suggesting higher level of MeCP2 expression in the hepatoma relative to the liver, prompted us to analyze its expression in these two tissues by immunohistochemical studies. Immunostaining of MeCP2 was localized predominantly in the nuclei of both the hepatoma and liver, where the staining in the hepatoma was significantly more pronounced than that in the liver (Fig.7). The preimmune sera failed to stain either the liver or the hepatoma section, indicating the specificity of the MeCP2 antibody. This
series of data further confirms the notion that MeCP2 plays an important role in silencing the methylated MT-I promoter in the rat hepatoma.

**RLGS-M analysis reveals methylation of other genes besides MT-I gene in the rat Hepatoma**

It is conceivable that additional genes could also be silenced in the hepatoma as aberrant hypermethylation of some gene promoters occurs during tumorigenesis along with global hypomethylation. It is known that de novo methylation of growth regulatory genes, particularly those of some tumor suppressor genes, and their consequent silencing contributes to tumorigenesis. To address this issue we explored the possibility of silencing a known tumor suppressor gene like Rb, p53 and p16 by Northern blot analysis. We found that all of them are expressed in the hepatoma to an extent comparable to the host liver (data not shown). We also analyzed the expression level of other growth regulatory genes in this hepatoma using a Gene Array blot. With the exception of cyclin E, which is equally expressed in both liver and hepatoma, all other genes were, expressed at much higher level in the hepatoma, because of its highly proliferating state (data not shown). To detect additional hypermethylated promoters, we performed RLGS-M analysis with Not I enzyme of DNA isolated from the liver and hepatoma of the same animal and DNA isolated from 5-AzaC-treated hepatoma. This relatively novel procedure has been used successfully to identify the genes that are hypermethylated in a variety of tumors (54-58). The two dimensional separation of the end labeled DNA fragments creates an array of spots when exposed to X-ray film, and this pattern is highly reproducible from sample to sample. If a Not I site is methylated in the experimental sample, Not I will fail to cleave the DNA at this site and the corresponding fragment will not be generated, resulting in the loss of the specific spot on the autoradiogram. The RLGS-M profile of DNA was very reproducible, and different samples can be compared to determine the spots lost or gained. Because Not I sites are present predominantly on the gene promoters, partial or complete loss of spots indicates methylation of the promoter and a gain of spots will be due to hypomethylation of the promoter. A representative RLGS-M pattern of the liver and hepatoma DNA isolated from three separate tumor bearing rats is depicted in Fig.8. Relative to the host liver, several spots were lost (indicated by red arrows) in the hepatoma, and a few spots were also gained (as indicated by blue arrows). Out of 1006 spots analyzed in the liver 181 were missing (total and partial) in hepatoma and 172 were regained after 5-AzaC treatment. Eighteen percent of the Not I
specific spots were lost, and 4.5% of the spots were gained in the hepatoma compared to the liver, whereas 70% of the spots lost in the hepatoma were regained after 5-AzaC treatment due to demethylation of the gene. This study demonstrates that many genes besides the *MT-I* gene are methylated in the hepatoma, some of which may have significant role in tumor progression. Identification and characterization of these genes from a Not I /EcoR V rat DNA library is in progress and is beyond the scope of this paper. The critical issue here is that *MT-I* is not the only gene that is methylated in the hepatoma.

**Discussion**

In the past decade, silencing of gene expression was considered the most important epigenetic alteration in tumorigenesis. The growing list of hypermethylated genes identified in the human cancer includes *Rb*, *p16*^INK4a*, *APC*, *BRCA1*, *E-cadherin*, *LKB1*, *MLH1*, and *VHL* (59). There has been considerable interest in deciphering the molecular mechanism(s) of this epigenetic alteration in cancer. In an effort to understand the alterations in the DNA methylation machinery, we selected a rat tumor model system that has been used in our laboratory for many years (60-62). The advantages of this system are as follows. First, the corresponding parental tissue is the liver of the tumor bearing animals; both control and test samples are, therefore, derived from the same animal. Second, the tumor is a rapidly growing hepatoma that yields as much as 20-30 g/rat following transplantation of a relatively small tumor slice into each hind leg. The high yield of the tumor is beneficial for obtaining the proteins or enzymes of interest in relatively large quantities that is required for extensive purification and characterization. Third, unlike random methylation of CpG islands that frequently occurs in cell culture, DNA/promoter methylation in the animals is physiologically significant. Further, most of the human tumors are solid tumors.

Previous study in our laboratory has shown that the *MT-I* gene is silenced in the rat hepatoma by promoter methylation (16). The present study addressed the following issues: First, will the MT-I promoter reactivated by demethylation with 5-AzaC treatment *in vivo* undergo re-methylation and silencing following re-transplantation into donor rats not treated with the inhibitor? Second, are there significant alterations in the DNA methylation machinery in the hepatoma relative to the host liver?
Third, does the altered methylation profile of the hepatoma relative to the liver require a distinct molecular mechanism(s)?

Retransplantation of the regressed tumor led to significantly slow but comparable growth of the tumor. The promoter was completely methylated after the tumor was allowed to grow in the absence of 5-AzaC. The bisulfite sequencing of the MT-I promoter indicates complete demethylation after treatment with the inhibitor. Consequently, remethylation of the promoter should be directed by de novo methyltransferase activity, as the maintenance DNMT utilizes only hemimethylated DNA as the substrate. This is the first report that demonstrates re-methylation of completely demethylated DNA in intact animals, and, therefore, suggests the physiological relevance of the de novo methylation of the MT-I promoter. This result is consistent with that obtained in a study with T24 cells in culture where p16 promoter demethylated by 5-AzaC treatment was remethylated to its original level after 21 population doubling (63). During 12 weeks of growth, the 5-AzaC-treated tumor had undergone at least 25 cell divisions facilitating de novo methylation of the demethylated promoters.

An inverse relationship between actively transcribing genes and promoter methylation has also been speculated by other laboratories. Brandies et al. (64) have shown that CpG islands in mouse and hamster aprt gene undergo de novo methylation when Sp1 site is mutated to block its binding to the cognate site. The binding of Sp1 to its cis element prevents promoter methylation and its spreading downstream (65). Bender et al. (63) attributed the preferential methylation of p16 exon 2 CpG islands over the promoter CpG islands to occupancy of the promoter by transcription factors. The basal level of MT-I gene expression is normally very low in all cells and tissues unless challenged with heavy metals or agents that generate free radicals. In the hepatoma where the gene is in the dormant state, the promoter is not accessible to transacting factors. We have previously shown that MT-I expression in the liver of tumor bearing animals is significantly higher than that of an age matched control rat due to stress caused by tumor load (16), as stress in animals is a known inducer of MT expression in the liver (23). It is logical to assume that the low but transcriptionally active state of the promoter coupled with the lesser abundance of DNA methylation machinery in the liver keeps the MT-I promoter free of methylation. Remethylation of the promoter upon removal of the drug suggests that methylation hot spot(s) might be present on the MT gene promoter, which facilitates the remethylation process. It is also possible that a minor fraction of cells in the regressed tumor harbors the methylated MT-I gene and that the
maintenance methyltransferase methylates the hemimethylated promoter. It is, however, evident that repression of genes by promoter methylation that negatively regulates cell proliferation will have clear advantage over the cells where the corresponding gene is demethylated and active.

It is conceivable that the relative lack of MT induction in the tumor could lead to an increased availability of functional zinc for a variety of zinc finger proteins that include transcriptional activators. Zinc is known to play a key role in cell proliferation and growth. Indeed, treatment of the animals bearing the hepatoma with 5-AzaC resulted in significant regression of the tumor in parallel with decreased methylation and reactivation of the MT-I promoter. A direct link between MT-I promoter silencing and tumorigenesis is yet to be established, and is beyond the scope of the present study. Admittedly, the expression of other genes that control tumor growth may also be suppressed by methylation. Nevertheless, the potential role of zinc in tumor growth and its retention in induced MT could play a key role in regulating tumor growth.

The aberrant de novo methylation in the hepatoma compared to the host liver and remethylation of the MT-I promoter provided us the impetus to study the status of the three catalytically active DNMT genes in these two tissues. As anticipated, the level of expression and activity of all the three DNMTs are significantly higher in the hepatoma compared to the liver. This result is consistent with the increased expression of the DNMT1 gene in fos-transformed cells compared to normal fibroblasts and the reversal of cell transformation following inhibition of DNMT1 expression (66). Significant elevation of DNMT1 expression has also been associated with tumorigenesis, particularly in colon cancer (30). Contrary to the relatively modest degree of DNMT1 expression reported in tumors compared to the normal counterpart (6,30), our study has revealed significant elevation (3-fold) of its activity and its expression in the rat hepatoma relative to the liver. It has also been observed that in B cell neoplasm (67) and neuroblastoma (68) the human chromosome 2p23 that houses the Dnmt3a gene is amplified. Overexpression of Dnmt3b has been more frequently associated with tumorigenesis (30).

Another important observation is in regard to the modification in the levels of methyl C-binding proteins (MBD) in the tumor, particularly that of MeCP2 and its interaction with the MT-I promoter. Based on the solution structure of the methyl C-binding domain, Ohki et. al. (69) and Wakefield et. al. (70) have proposed that the interaction between methyl CpG and MBD occur along the major groove of a standard
B-form DNA. Since no sequence specificity has been assigned for binding of these proteins to DNA, it remains uncertain as to which of these proteins will associate with a certain methylated promoter, and how this specific process is accomplished. The present study has addressed the first issue by performing a chromatin immunoprecipitation (ChIP) assay using antibodies against MeCP2. The data clearly shows that MeCP2 can associate robustly with the MT-I promoter. Because each MBD associates with a particular set or class of promoters destined for methylation and assembles a repressor complex recruiting a specific set of repressors, the co-repressors associated with MeCP2 will also have a role in the methylation and suppression of the gene. Identification of the co-repressors involved in this process is in progress.

Finally, the methylation of other genes in the hepatoma deserves comment. An important question is whether any other gene(s) besides the MT-I gene is (are) methylated. Interestingly, some of the genes for known tumor suppressor proteins that include Rb, p53 and p16 were not silenced, as shown by Northern Blot analysis (data not presented). On the other hand, the expression levels of most of the growth promoting genes e.g. cyclins and cdk5, were all increased in the hepatoma which correlates with its highly proliferating state (data not presented). We used a highly reproducible technique, namely RLGS-M, to identify other genes methylated in the hepatoma. It was evident from this analysis that other genes in addition to MT-I are also methylated along with some genes that were demethylated in the tumor relative to the liver. Since this analysis preferentially selects the methylated promoters (54), it is logical to assume that the majority of the spots lost represent the methylated genes and are confined to the promoter region. We have not identified these genes and studied their expression levels, which is beyond the scope of the present study. Nevertheless, this study has concluded that the methylation and the resultant suppression are not restricted to the MT-I gene in this tumor.

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Figure Legends

Fig.1. *MT-I* promoter in the rat hepatoma is completely demethylated after 5-AzaC treatment and is re-methylated when the re-transplanted tumor is grown in the absence of AzaC.

A. Schematic diagram of rat *MT-I* promoter depicting the methyl CpG \(^{m}CG\) dinucleotides and the relevant cis-elements.  
B. Bisulfite genomic sequencing of DNA isolated from the liver (a) hepatoma (b) 5AzaC-treated hepatoma (c) and retransplanted 5-AzaC-treated hepatoma (d). The upper strand of the *MT-I* promoter was amplified from the bisulfite-treated DNA of different samples and subjected to PCR sequencing. The arrows indicate the methylated CpGs in the hepatoma that were demethylated upon 5-AzaC treatment and remethylated back when the tumor was grown in absence of the analog.

Fig.2. *MT-I* gene is not induced by zinc in the re-transplanted 5-AzaC treated hepatoma. Total RNA (30µg) was isolated from the liver and hepatoma of the rats injected with saline or zinc (200µmole/kg body weight), and subjected to Northern blot analysis with \(^{32}\)P-labeled random primed, mouse MT-I cDNA or rat COX-1 cDNA as probe. *Lanes 1 to 3* represents RNA level in liver and lanes 4-6 represent RNA levels in the hepatoma of control or 5-AzaC treated rats as indicated. Lanes 4 to 7 show the RNA level in the liver and hepatoma of rats where 5-AzaC treated tumor was retransplanted (+/- AzaC) and grown in absence of the drug. Zinc treatment is as indicated in the figure.

Fig.3. The expression level of all the three DNMTs are higher in the hepatoma than in the liver.

A. Poly A+RNA (2.5µg) isolated from both the liver and the hepatoma was subjected to Northern blot analysis to compare the level of DNMT1, DNMT3a and DNMT3b mRNAs in the two tissues. COX1 cDNA was used to re-probe the membrane and the corresponding mRNA served as control for RNA integrity and equalization.  
B. Western blot analysis of DNMT1 and 
C. Western blot analysis of DNMT3a and DNMT3b. One hundred µg of nuclear extract from hepatoma-bearing rats were subjected to SDS-PAGE and blotted with rabbit polyclonal antibodies against DNMT1, DNMT3a or DNMT3b. LNE corresponds to liver nuclear extract and HNE corresponds to hepatoma nuclear extracts. Western blot with Ku-70 antibody served as protein loading and transfer control.

Fig.4. Immunohistochemical analysis shows that DNMT3a and 3b are predominantly localized in the nuclei. A. Rabbit polyclonal antibody against DNMT 3a was used to stain 15 micron sections of
frozen liver and hepatoma. Preimmune serum from the corresponding rabbit was used as control. 

**B.** Immunohistochemical analysis of hepatoma and the liver slices with DNMT3b antiserum and preimmune serum.

**Fig.5. The liver and hepatoma contain comparable levels of MBD1, MBD2 and MBD4 mRNAs.**

Expression of MBD1 mRNA in the liver (L) and hepatoma (H): poly A+ RNA prepared from the liver and hepatoma total RNA was used as template for cDNA synthesis. Gene-specific primers (see Methods and Materials) for MBD1 (lanes 1&2) and COX-1 (lanes 3&4) were used for PCR amplification from the cDNAs (25 cycles). RNA directly used for PCR without cDNA synthesis were the negative control (lane 6 &7). 

**B.** Northern blot analysis of MBD2: poly A+ RNA (2.5 µg) from the liver and hepatoma was subjected to Northern blot analysis and the same blot was probed with 32P-labeled MBD2 or COX-1 cDNA fragment. 

**C.** Expression of MBD4 in liver and hepatoma: RT-PCR analysis was the same as section A, lanes 1&2 –COX-1, lanes 3&4- MBD4 and lanes 5&6-no RT control. 

**D.** Expression of MeCP2 in liver and hepatoma: RT-PCR analysis was the same as section A, lanes 1,2 & 3,4 –MeCP2 from poly A+ RNA and total RNA respectively, lanes 5, 6- no RT control and lanes 7,8 & 9,10 – COX-1 from poly A+ RNA and total RNA respectively. 

**Fig.6. MeCP2 associates with the methylated MT-I promoter in the rat hepatoma.**

**A.** Western blot analysis of MeCP2 and MBD2 in the liver and hepatoma nuclear extracts. Identical amount (100 µg of protein) of liver and hepatoma nuclear extracts were separated by SDS-PAGE and subjected to immunoblot analysis with antibodies against MeCP2, MBD2 and p70 subunit of Ku (N3H10, Neomarker) respectively. 

**B.** Immunoprecipitation and western blot analysis with anti-MeCP2 antibodies. Identical amount (100 µg protein) of formaldehyde-crosslinked liver (LNE) and hepatoma nuclear extracts (HNE) in RIPA buffer were immunoprecipitated with 2 µl of preimmune sera (Pre-I) or immune sera (MeCP2) coupled to protein A beads after preclearing with protein A beads alone. Immunoprecipitated proteins were subjected to Western blot analysis with anti-MeCP2 antibodies (1:5000 dilution in TBST). 

**C.** Specific amplification of MT-I promoter from the hepatoma nuclei after ChIP with anti-MeCP2 antibodies. Formaldehyde-crosslinked chromatin (containing equal amount of DNA) isolated from the liver and hepatoma was immunoprecipitated as described in A and the co-
precipitated DNA was de-crosslinked, purified and amplified by semi-quantitative PCR $^{32}$P-labeled with MT-I and COX-1 gene-specific primers.

**Fig.7. Expression of MeCP2 protein is more pronounced in the hepatoma than the liver.**
Liver and hepatoma slices (15micron) were stained either with MeCP2 antibody (1:2000 dilution) or the corresponding pre-immune serum, followed by incubation with biotinylated anti-rabbit antibody (1:200) and visualized by traditional horse radish peroxidase method.

**Fig.8.** Several genes besides $MT-I$ gene are silenced due to methylation in the hepatoma.
Genomic DNA isolated from the livers of hepatoma-bearing rats, and from the hepatomas under different conditions of 5-AzaC treatment were subjected to Not I / EcoRV digestion, end-labeled and run on two dimensional gels (see Methods for details). Appearance or disappearance of individual spots was compared by analyzing the autoradiograms. A section of the autoradiogram displaying spots lost in hepatoma compared to the liver and subsequent reappearance upon 5-AzaC treatment is shown. Spots present in the liver but lost in the hepatoma are indicated by red arrows and the spots gained in the hepatoma compared to the liver are indicated by blue arrows.
Fig. 1A
| Liver | Hepatoma | 5-AzaC-treated Hepatoma | Retransplanted 5-AzaC-Hepatoma |
|-------|---------|-------------------------|-------------------------------|
| G A T C | G A T C | G A T C | G A T C |

Fig. 1B
Fig. 2
Fig. 3
Fig. 4A
Fig. 4B
Fig. 4c
Fig. 5
Fig. 5
Fig. 6
**Fig. 7**

**LIVER**

| Preimmune sera | Immune sera |
|----------------|-------------|
| ![Liver Preimmune Sera](image1) | ![Liver Immune Sera](image2) |

**HEPATOMA**

| Preimmune sera | Immune sera |
|----------------|-------------|
| ![Hepatoma Preimmune Sera](image3) | ![Hepatoma Immune Sera](image4) |
Fig. 8
Role of De novo DNA methyltransferases and methyl C-binding proteins in gene silencing in a rat hepatoma
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